Impact of Zinc on Gelling and Film Forming Properties of Muscle Proteins from Yellow Stripe Trevally (*Selaroides leptolepis*)

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ABSTRACT

Gelling properties of surimi prepared from yellow stripe trevally (*Selaroides leptolepis*) were studied. Setting temperature showed significant impact on textural properties and cross-linking of myofibrillar proteins. Kamaboko gel with prior setting at 40 °C (K40) exhibited the highest breaking force with the lowest expressible moisture content ($P < 0.05$). The optimal temperature of yellow stripe trevally muscle transglutaminase (TGase) was found to be 40 °C. K40 sample had finer, denser and more ordered fibrillar structure than other gels.

Effect of zinc sulphate ($ZnSO_4$) and zinc chloride ($ZnCl_2$) on heat-induced aggregation of natural actomyosin (NAM) extracted from yellow stripe trevally was investigated. During heating from 20 to 75 °C, higher turbidity, surface hydrophobicity and disulphide bond formation were obtained in NAM added with $ZnSO_4$ or $ZnCl_2$ at temperature ranging from 40 to 75 °C, compared with the control. Nevertheless, the higher aggregation was found in NAM added with $ZnSO_4$, compared with $ZnCl_2$. To strengthen gel of yellow stripe trevally surimi, $ZnSO_4$ or $ZnCl_2$ (0-80 μmol/kg) was incorporated. Both kamaboko (40/90 °C) and modori (60/90 °C) gels added with $ZnSO_4$ or $ZnCl_2$ up to 60 μmol/kg had the increases in breaking force and deformation ($P < 0.05$), compared with the control. $ZnSO_4$ showed the higher gel strengthening effect than $ZnCl_2$ ($P < 0.05$). Whiteness of all gels increased with increasing levels of zinc salts ($P < 0.05$). Kamaboko gel had the compact structure when zinc salt, especially $ZnSO_4$ at 60 μmol/kg was incorporated.

To improve gel properties of yellow stripe trevally meat, protein isolate phosphorylated with sodium tripolyphosphate (STPP) (0.25 and 0.5%, w/w) was incorporated with $ZnSO_4$ (0–140 μmol/kg). Phosphorylated protein isolate

incorporated with $ZnSO_4$ yielded the gel with increased breaking force and deformation, compared with gel from surimi added with STPP and $ZnSO_4$ ($P < 0.05$). Phosphorylated protein isolate was more cross-linked by Zn^{2+} and the better gel properties could be obtained. Thus, gel with improved properties could be obtained from protein isolate phosphorylated with STPP in conjunction with addition of $ZnSO_4$ at an appropriate level.

To widen the utilization of yellow stripe trevally, blend films based on fish protein isolate (FPI) from yellow stripe trevally muscle and commercial fish skin gelatin (FSG) at different ratios prepared at pH 3 and 11 in the presence of glycerol at 30 and 50% (based on total protein) were characterized. At the same pH, tensile strength (TS) decreased, while elongation at break (EAB), water vapor permeability (WVP) and solubility increased as glycerol content increased ($P < 0.05$). FPI films generally had the lower TS, EAB and higher WVP than FSG films ($P < 0.05$). Both TS and EAB of blend films increased, while WVP decreased with increasing FSG content ($P < 0.05$), especially at pH 11. FPI/FSG blend films with higher FSG proportion had higher film solubility but lower b^*-values (yellowness) than FPI films ($P < 0.05$). Films prepared at pH 11 were less transparent than those prepared at pH 3 ($P < 0.05$). FTIR spectroscopic analysis revealed that hydrogen bonds in FPI/FSG blend film were dominant. Scanning electron microscopic study revealed no distinct phase separation in the matrix of FPI/FSG blend film. Thus, the incorporation of FSG into FPI film up to 50%, in conjunction with lowering glycerol content (30%) could improve the mechanical and water vapor barrier properties of resulting blend film.

When nanocomposite films based on FPI/FSG (pH 3 and 11) containing ZnO nanoparticles (ZnONP) at different levels (0-4% w/w of protein) were characterized. TS increased, whereas EAB and WVP decreased as ZnONP content increased, regardless of pH ($P < 0.05$). Incorporation of ZnONP yielded film with lower yellow discoloration and improved UV barrier property. Based on thermogravimetric analysis, ZnONP could improve thermal stability of the nanocomposites films. X-ray diffraction (XRD) analysis confirmed the crystalline nanocomposite films. FPI/FSG-ZnO nanocomposite films, especially those prepared

at pH 3, exhibited strong antibacterial activity against food pathogenic and spoilage bacteria.

Active films with antimicrobial properties, based on FPI/FSG blend were prepared by incorporating with 50 and 100% (w/w of protein) basil leaf essential oil (BEO) in the absence and presence of 3% (w/w of protein) ZnONP. TS decreased, while EAB increased as BEO level increased ($P < 0.05$). However, ZnONP addition resulted in higher TS but lower EAB ($P < 0.05$). The lowest WVP was observed for the film incorporated with 100% BEO and 3% ZnONP ($P < 0.05$). BEO and ZnONP incorporation decreased transparency of FPI/FSG films ($P < 0.05$). Both BEO and ZnONP had a marked impact on thermal stability of the films. FPI/FSG films incorporated with 100% BEO, especially in combination with ZnONP, exhibited strong antibacterial activity against food pathogenic and spoilage bacteria.

When active film incorporated with 3% ZnONP and 100% BEO (w/w, protein content) (FPI/FSG-ZnONP-BEO film) was used to wrap sea bass slices, the retarded growth of psychrophilic bacteria, lactic acid bacteria (LAB) and other spoilage microorganisms including pseudomonads, H_2S-producing bacteria and Enterobacteriaceae was observed throughout storage at 4 °C for 12 days in comparison with those wrapped with FPI/FSG-BEO, FPI/FSG-ZnONP, FPI/FSG film, polypropylene film (PP film) and the control (without wrapping), respectively ($P < 0.05$). Lowered increases in pH, total volatile base (TVB), peroxide value (PV) and TBARS value were also found in FPI/FSG-ZnO-BEO film wrapped samples, compared with others ($P < 0.05$). Sensory evaluation revealed that the shelf-life of sea bass slices was longest when wrapped with FPI/FSG-ZnONP-BEO film (12 days), whereas the shelf-life of control was 6 days. Therefore, microbial growth and lipid oxidation in sea bass slices could be retarded, thereby extending the shelf-life when wrapped with FPI/FSG-ZnONP-BEO film.

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CONTENTS

Page

Contents..... x

List of Tables..... xxii

List of Figures..... xxiv

Chapter

1. Introduction and Review of Literature

1.1 Introduction…..... 1

1.2 Review of literature……...… 4

1.2.1 Chemical composition of fish ……. 4

1.2.1.1 Sarcoplasmic protein……. 5

1.2.1.2 Myofibrillar proteins……. 6

1.2.1.2.1 Myosin…….. 6

1.2.1.2.2 Actin……. 7

1.2.1.2.3 Actomyosin……... 8

1.2.1.2.4 Troponin……..... 8

1.2.1.2.5 Tropomyosin……. 9

1.2.1.3 Stroma protein……... 9

1.2.2 Characteristics and compositions of dark fleshed fish…..…..……. 9

1.2.2.1 Surimi from dark fleshed fish……... 11

1.2.2.2 Surimi gelation…….. 12

1.2.3 Setting and weakening of surimi gel……... 15

1.2.3.1 Setting phenomenon...……. 15

1.2.3.2 Gel softening phenomenon…….. 17

1.2.4 Factors affecting gelling properties…..... 18

1.2.5 Improvement of surimi gel quality…….…….... 20

1.2.5.1 Use of food grade protein additives.....……..... 20

1.2.5.1.1 Egg white……..... 20

1.2.5.1.2 Whey protein concentrate..... 21

1.2.5.1.3 Plasma proteins..... 22

CONTENTS (Continued)

Chapter **Page**

1.2.5.2 Use of microbial transglutaminase (MTGase)…..……... 23
1.2.5.3 Use of divalent cations…...……... 24
1.2.6 Zinc and role in human nutrition and application…..…….. 27
1.2.6.1 Human nutrition……..…….. 27
1.2.6.2 Use of zinc as antimicrobial agent……..…….. 29
1.2.7 Biodegradable film……..……... 32
1.2.7.1 Principle and factors affecting the protein film formation... 32
1.2.7.2 Myofibrillar protein based films……...……..... 36
1.2.7.3 Fish protein isolate based films……..... 39
1.2.7.4 Gelatin based films……..……..... 41
1.2.7.5 Blend and nanocomposite biopolymer films……..... 45
1.2.7.5.1 Blend films……..... 45
1.2.7.5.2 Nanocomposite films……..... 46
1.2.8 Essential oil.……..……. 49
1.2.8.1 Antioxidative activity……...……... 50
1.2.8.2 Antimicrobial activity…..... 53
1.2.9 Protein based film incorporated with antimicrobial and antioxidants 55
1.3 Objectives ……. 61

2. Gelling characteristics of surimi from yellow stripe trevally (*Selaroides leptolepis*)

2.1 Abstract……..……. 62
2.2 Introduction……. 62
2.3 Material and methods…….. 64
2.3.1 Chemicals…… 64
2.3.2 Fish collection and mince preparation…… 64
2.3.3 Surimi and surimi gel preparation……..... 65
2.3.4 Determination of gel properties……... 66
2.3.4.1 Textural analysis……..... 66

CONTENTS (Continued)

Chapter **Page**

2.3.4.2 Determination of whiteness……..... 66
2.3.4.3 Determination of expressible moisture content……..... 66
2.3.4.4 Determination of TCA-soluble peptide content……..... 67
2.3.4.5 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) 67
2.3.4.6 Scanning electron microscopy (SEM)..... 68
2.3.5 Characterisation of endogenous TGase in yellow stripe trevally muscle..... 68
2.3.5.1 Preparation of TGase crude extract..... 68
2.3.5.2 Study on temperature profile of TGase..... 68
2.3.6 Statistical analysis..... 69
2.4 Results and discussion..... 69
2.4.1 Properties of gel from surimi of yellow stripe trevally prepared using different heating conditions..... 69
2.4.1.1 Textural property..... 69
2.4.1.2 Whiteness..... 72
2.4.1.3 Expressible moisture content..... 73
2.4.1.4 Protein Pattern..... 73
2.4.1.5 TCA soluble peptide content..... 76
2.4.1.6 Microstructure..... 77
2.4.2 Endogenous transglutaminase activity as affected by temperatures. 78
2.5 Conclusion..... 80

3. Impact of zinc salts on heat-induced aggregation of natural actomyosin from yellow stripe trevally

3.1 Abstract..... 81
3.2 Introduction..... 81
3.3 Material and methods..... 83
3.3.1 Chemicals..... 83

CONTENTS (Continued)

Chapter **Page**

3.3.2 Collection and preparation of fish..... 83
3.3.3 Preparation of natural actomyosin (NAM)..... 84
3.3.4 Study on the effect of $ZnSO_4$ and $ZnCl_2$ at different concentrations on heat-induced aggregation of NAM..... 84
3.3.4.1 Determination of thermal protein aggregation..... 84
3.3.4.2 Determination of surface hydrophobicity..... 85
3.3.4.3 Determination of total sulphydryl groups and disulphide bond contents..... 85
3.3.4.4 Determination of Ca^{2+}-ATPase activity..... 86
3.3.5 Study on the effect of $ZnSO_4$ and $ZnCl_2$ on thermal transition, charge, size and microstructure of NAM..... 86
3.3.5.1 Differential scanning calorimetry..... 86
3.3.5.2 Zeta potential and particle size..... 87
3.3.5.3 Transmission electron microscopy..... 87
3.3.6 Protein determination..... 87
3.3.7 Statistical analysis..... 87
3.4 Results and discussion..... 88
3.4.1 Effect of $ZnSO_4$ and $ZnCl_2$ at different concentrations on heat-induced aggregation of NAM..... 88
3.4.1.1 Thermal protein aggregation..... 88
3.4.1.2 Surface hydrophobicity..... 90
3.4.1.3 Total sulphydryl group and disulphide bond contents..... 91
3.4.1.4 Ca^{2+}- ATPase Activity..... 94
3.4.2 Effect of $ZnSO_4$ and $ZnCl_2$ on thermal transition, charge, size and microstructure of NAM..... 96
3.4.2.1 Thermal transition..... 96
3.4.2.2 Zeta potential and particle size..... 97
3.4.2.3 Microstructure..... 99
3.5 Conclusions..... 101

CONTENTS (Continued)

Chapter **Page**

4. Gel strengthening effect of zinc salts in surimi from yellow stripe trevally

4.1 Abstract..... 102
4.2 Introduction..... 102
4.3 Materials and methods..... 104
4.3.1 Chemicals..... 104
4.3.2 Fish collection and surimi preparation..... 104
4.3.3 Preparation of surimi gel containing zinc salt at different levels.... 105
4.3.4 Textural analysis..... 105
4.3.5 Determination of whiteness..... 106
4.3.6 Determination of expressible moisture content..... 106
4.3.7 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)..... 106
4.3.8 Scanning electron microscopy (SEM)..... 107
4.3.9. Protein determination..... 107
4.3.10 Statistical analysis..... 108
4.4 Results and discussion..... 108
4.4.1 Effect of $ZnSO_4$ or $ZnCl_2$ at different levels on textural properties of surimi gel..... 108
4.4.2 Effect of $ZnSO_4$ or $ZnCl_2$ on whiteness of surimi gels..... 112
4.4.3 Effect of $ZnSO_4$ or $ZnCl_2$ on expressible moisture content of surimi gels..... 113
4.4.4 Effect of $ZnSO_4$ or $ZnCl_2$ on protein pattern of surimi gels..... 114
4.4.5 Effect of $ZnSO_4$ or $ZnCl_2$ on microstructure of surimi gels.....….... 117
4.5 Conclusions..... 119

5. Effect of zinc sulphate on gelling properties of phosphorylated protein isolate from yellow stripe trevally

5.1 Abstract..... 120
5.2 Introduction..... 121
5.3 Materials and methods..... 123

CONTENTS (Continued)

Chapter **Page**

5.3.1 Chemicals..... 123
5.3.2 Preparation of fish mince..... 123
5.3.3 Preparation of surimi and fish protein isolate..... 123
5.3.4 Study on chemical compositions and Ca^{2+}-ATPase activity of surimi, protein isolate and phosphorylated protein isolate..…..... 124
5.3.4.1 Moisture and ash contents..... 125
5.3.4.2 Determination of lipid and phospholipid contents..... 125
5.3.4.3 Determination of phosphate content..... 125
5.3.4.4 Determination of Ca^{2+}-ATPase activity..... 126
5.3.5 Study on the impact of $ZnSO_4$ on properties of gel from surimi added with STPP and phosphorylated protein isolate..... 126
5.3.5.1 Gel preparation..... 126
5.3.5.2 Textural analysis..... 127
5.3.5.3 Determination of whiteness..... 127
5.3.5.4 Determination of expressible moisture content..... 127
5.3.5.5 SDS–polyacrylamide gel electrophoresis (SDS–PAGE).. 128
5.3.5.6 Microstructure..... 128
5.3.6 Statistical analysis..... 129
5.4 Results and discussion..... 129
5.4.1 Chemical compositions and Ca^{2+}-ATPase activity of surimi, protein isolate and phosphorylated protein isolate…….... 129
5.4.1.1 Moisture and ash contents.……..... 129
5.4.1.2 Lipid and phospholipid contents.……..... 130
5.4.1.3 Phosphate content..... 131
5.4.1.4 Ca^{2+}-ATPase activity..... 133
5.4.2 Effect of $ZnSO_4$ on properties of gel from surimi and protein isolates..... 133
5.4.2.1 Breaking force and deformation..... 133
5.4.2.2 Whiteness..... 137

CONTENTS (Continued)

Chapter **Page**

5.4.2.3 Expressible moisture content..... 139
5.4.2.4 Protein patterns..... 140
5.4.2.5 Microstructure..... 142
5.5 Conclusions..... 145

6. Development and characterisation of blend films based on fish protein isolate and fish skin gelatin

6.1 Abstract..... 146
6.2 Introduction..... 147
6.3 Materials and methods..... 148
6.3.1 Chemicals..... 148
6.3.2 Collection and preparation of fish Sample..... 148
6.3.3 Preparation of fish protein isolate..... 149
6.3.4 Preparation of film-forming solution (FFS) and film casting..... 149
6.3.5 Determination of film properties..... 150
6.3.5.1 Film thickness..... 150
6.3.5.2 Mechanical properties..... 151
6.3.5.3 Water vapor permeability (WVP)..... 151
6.3.5.4 Film solubility..... 151
6.3.5.5 Color..... 152
6.3.5.6 Light transmittance and transparency value..... 152
6.3.6 Characterisation of the selected films..... 153
6.3.6.1 Attenuated total reflectance-Fourier transforms infrared (ATR- FTIR) spectroscopy..... 153
6.3.6.2 Thermo-gravimetric analysis (TGA)..... 153
6.3.6.3 Microstructure..... 153
6.3.7 Statistical analysis..... 154

CONTENTS (Continued)

Chapter **Page**

6.4 Results and discussion..... 154
6.4.1 Properties of blend film based on FPI and FSG at different ratios. 154
6.4.3.1 Thickness..... 154
6.4.3.2 Mechanical properties..... 155
6.4.3.3 Water vapor permeability (WVP)..... 157
6.4.3.4 Film solubility..... 160
6.4.3.5 Color..... 161
6.4.3.6 Light transmittance and transparency value..... 163
6.4.2 Characteristics of blend films prepared from the selected FPI/FSG ratio..... 166
6.4.2.1 FTIR spectroscopy..... 166
6.4.2.2 Thermo-gravimetric analysis (TGA)..... 168
6.4.2.3 Microstructure..... 171
6.5 Conclusion…….. 173

7. Characteristics and antimicrobial properties of fish protein isolate/fish skin gelatin-zinc oxide (ZnO) nanocomposite films

7.1 Abstract..... 174
7.2 Introduction..... 174
7.3 Materials and methods..... 176
7.3.1 Chemicals..... 176
7.3.2 Collection and preparation of fish sample..... 176
7.3.3 Preparation of fish protein isolate..... 177
7.3.4 Preparation of FPI/FSG-ZnONP film-forming solution (FFS) and film casting..... 177
7.3.5 Determination of film properties..... 178
7.3.5.1 Film thickness..... 179
7.3.5.2 Mechanical properties..... 179
7.3.5.3 Water vapor permeability (WVP)..... 179

CONTENTS (Continued)

Chapter **Page**

7.3.5.4 Color..... 180
7.3.5.5 Light transmittance and transparency value..... 180
7.3.6 Characterisation of the selected films..... 181
7.3.6.1 Wide angle X-ray diffraction (WAXD) characterization.... 181
7.3.6.2 Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy..... 181
7.3.6.3 Thermo-gravimetric analysis (TGA)..... 181
7.3.6.4 Scanning electron microscopy (SEM)..... 182
7.3.7 Antimicrobial properties..... 182
7.3.8 Statistical analysis……..... 182
7.4 Results and Discussion…….. 183
7.4.1 Properties of FPI/FSG-ZnO nanocomposite films..... 183
7.4.1.1 Thickness..... 183
7.4.1.2 Mechanical properties..... 183
7.4.1.3 Water vapor permeability (WVP)..... 186
7.4.1.4 Color..... 187
7.4.1.5 Light transmittance and transparency value..... 188
7.4.2 Characteristics of FPI/FSG-ZnO nanocomposite films..... 191
7.4.2.1 Wide angle X-ray diffraction (WAXD) pattern..... 191
7.4.2.2 FTIR spectroscopy..... 193
7.4.2.3 Thermo-gravimetric analysis (TGA)..... 195
7.4.2.4 3.2.4. Scanning electron microscopy..... 198
7.4.3 Antimicrobial activity of FPI/FSG-ZnO nanocomposite films.... 200
7.5 Conclusions..... 202

8. Properties and antimicrobial activity of fish protein isolate/fish skin gelatin film containing basil leaf essential oil and zinc oxide nanoparticles

8.1 Abstract..... 203
8.2 Introduction..... 203

CONTENTS (Continued)

Chapter	Page
8.3 Materials and methods	205
8.3.1 Chemicals	205
8.3.2 Collection and preparation of fish sample	206
8.3.3 Preparation of fish protein isolate	206
8.3.4 Preparation of fish protein isolate/fish skin gelatin film added with BEO and ZnONP	207
8.3.5 Determination of properties and antimicrobial activity of films	208
8.3.5.1 Film thickness	208
8.3.5.2 Mechanical properties	208
8.3.5.3 Water vapor permeability (WVP)	209
8.3.5.4 Color	209
8.3.5.5 Light transmittance and transparency value	209
8.3.5.6 Antimicrobial properties	210
8.3.6 Characterisation of the selected films	210
8.3.6.1 Attenuated total reflectance-Fourier transforms infrared (ATR-FTIR) spectroscopy	210
8.3.6.2 Thermo-gravimetric analysis (TGA)	211
8.3.6.3 Scanning electron microscopy (SEM)	211
8.3.7 Statistical analysis	211
8.4 Results and discussion	212
8.4.1 Properties of FPI/FSG films incorporated with BEO and ZnONP.	212
8.4.1.1 Thickness	212
8.4.1.2 Mechanical properties	212
8.4.1.3 Water vapor permeability (WVP)	214
8.4.1.4 Color	216
8.4.1.5 Light transmittance and transparency value	217

CONTENTS (Continued)

Chapter **Page**

8.4.1.6 Antimicrobial activity of FPI/FSG films incorporated with BEO and ZnONP..... 220
8.4.2 Characteristics of FPI/FSG films incorporated with BEO and ZnONP..... 223
8.4.2.1 FTIR spectroscopy..... 223
8.4.2.2 Thermo-gravimetric analysis (TGA)..... 225
8.4.2.3 Film morphology..... 229
8.5 Conclusions..... 231

9. Shelf-life extension of refrigerated sea bass slices wrapped with fish protein isolate/fish skin gelatin-ZnO nanocomposite film incorporated with basil leaf essential oil

9.1 Abstract..... 232
9.2 Introduction..... 233
9.3 Materials and methods..... 234
9.3.1 Chemicals..... 234
9.3.2 Collection and preparation of fish sample..... 235
9.3.3 Preparation of fish protein isolate..... 235
9.3.4 Preparation of fish protein isolate/fish skin gelatin film containing ZnONP and BEO..... 236
9.3.5 Effect of FPI/FSG-ZnO nanocomposite film incorporated with BEO on quality changes of sea bass slices..... 237
9.3.6 Microbiological analyses..... 237
9.3.6.1 Total viable count and psychrophilic bacterial count..... 238
9.3.6.2 Pseudomonas count..... 238
9.3.6.3 Hydrogen sulphide (H_2S) producing bacterial count..... 238
9.3.6.4 Enterobacteriaceae count..... 238
9.3.6.5 Lactic acid bacteria count..... 238

CONTENTS (Continued)

Chapter **Page**

9.3.7 Chemical analyses..... 239
9.3.7.1 Determination of total volatile base (TVB) content..... 239
8.3.7.2 pH measurement..... 239
8.3.7.3 Determination of peroxide value (PV)..... 239
8.3.5.5 Determination of thiobarbituric acid reactive substances (TBARS)..... 240
9.3.8 Sensory evaluation..... 240
9.3.9 Statistical analysis..... 241
9.4 Results and discussion..... 241
9.4.1 Changes in microbial loads..... 241
9.4.2 Changes in chemical compositions..... 248
9.4.2.1 Total volatile base content..... 248
9.4.2.2 pH..... 249
9.4.2.3 Peroxide value (PV)..... 251
9.4.2.4 TBARS value..... 253
9.4.3 Sensory evaluation..... 254
9.5 Conclusion..... 256

10. Summary and future works

10.1 Summary..... 257
10.2 Future works..... 258

References..... 259
Vitae..... 321

LIST OF TABLES

Table		Page
1.	Conformational change occurring during the thermal denaturation of natural actomyosin…….....……..	14
2.	Factors influencing heat-induced gelation of muscle protein....……..	19
3.	Properties of muscle protein-based film from different fish species...	39
4.	Properties of gelatin-based film from different fish species.....	44
5.	Antimicrobials incorporated into various polymers used for food packaging.....	58
6.	Whiteness and expressible moisture content of gels of surimi from yellow stripe trevally using different heating conditions……	72
7.	Ca^{2+}- ATPase activity of NAM solutions (1 mg protein/ml) containing $ZnSO_4$ or $ZnCl_2$ at different concentrations after heating from 20 to 75 °C.....	95
8.	Thermal transition (T_{max}) and enthalpy change (ΔH) of NAM containing 100 µM $ZnSO_4$ or 100 µM $ZnCl_2$.....	97
9.	Zeta potential and particle size of NAM solutions (1 mg protein/ml) containing 100 µM $ZnSO_4$ or 100 µM $ZnCl_2$ after heating to 75 °C...	99
10.	Whiteness and expressible moisture content of kamaboko and modori gels from yellow stripe trevally surimi added with $ZnSO_4$ or $ZnCl_2$ at different levels….....……	113
11.	Chemical compositions and Ca^{2+}-ATPase activity of yellow stripe trevally surimi, protein isolate and phosphorylated protein isolates....	132
12.	Expressible moisture content and whiteness of gels from yellow stripe trevally surimi added with STPP at different levels and protein isolate without and with phosphorylation as influenced by zinc sulphate at different levels.....	138
13.	Thickness, mechanical properties, water vapor permeability and film solubility of FPI, FSG and FPI/FSG blend films as affected by blend composition, pH and glycerol content.....	159

LIST OF TABLES (Continued)

Table		Page
14.	Color of FPI, FSG and FPI/FSG blend films as affected by blend composition, pH and glycerol content.....	162
15.	Light transmittance and transparency values of FPI, FSG and FPI/FSG blend films as affected by blend composition, pH and glycerol content.....	165
16.	Thermal degradation temperature (T_d, °C) and weight loss (Δw, %) of FPI, FSG and FPI/FSG (5:5) blend films prepared at pH 3 and containing 30% glycerol.....	170
17.	Thickness, mechanical properties and water vapor permeability of FPI/FSG films as affected by pH and ZnONP content.....	185
18.	Color of FPI/FSG films as affected by pH and ZnONP content.....	188
19.	Light transmittance and transparency values of FPI/FSG films as affected by pH and ZnONP content…….....	190
20.	Thermal degradation temperature (T_d, °C) and weight loss (Δw, %) of FPI/FSG films prepared at pH 3 and 11 containing 0 and 3% ZnONP.....	197
21.	Thickness, mechanical properties and water vapor permeability of FPI/FSG films as affected by BEO and ZnONP incorporation.....	215
22.	Color of FPI/FSG films as affected by BEO and ZnONP incorporation.....	217
23.	Light transmittance and transparency values of FPI/FSG films as affected by BEO and ZnONP incorporation.....	219
24.	Thermal degradation temperature (T_d, °C) and weight loss (Δw, %) of FPI/FSG films as affected by 100% BEO and 3% ZnONP incorporation.....	228
25.	Sensory properties of sea bass slices wrapped without and with films (PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film) during storage at 4 °C for 12 days..	255

LIST OF FIGURES

Figure		Page
1.	Schematic diagram of fish muscle protein composition. [1] based on total proteins; [2] based on total muscle proteins; [3] based on total myofibrillar proteins ……...	4
2.	Schematic diagram of (a) the myosin molecule and (b) its subfragments prepared by proteolytic digestion …….	7
3.	Structure of actin, troponin and tropomyosin……..	8
4.	Reactions catalyzed by TGase (a) Acyl transfer. (b) Crosslinking of Gln and Lys residues in proteins or peptides. The resulting bridge is called an ε-(γ-glutamyl) lysine (GL) bond. (c) Deamidation……..	17
5.	Formation of calcium induced salt bridge between proteins ……..…	26
6.	Various mechanisms of antimicrobial activities exerted by nanomaterials.....	31
7.	Various ways for modifying the characteristics of edible films and coatings.....	34
8.	Scheme of pH shift process for production of fish protein isolate…….....…….....	41
9.	Hypothetical scheme depicting preparation of polymer blend….....	45
10.	Chemical structures of selected components of essential oils ……..	52
11	Breaking force (a) and deformation (b) of gels of surimi from yellow stripe trevally using different heating conditions.……..	71
12.	SDS-PAGE patterns (a) and TCA-soluble peptide content (b) of gels of surimi from yellow stripe trevally using different heating conditions.....	75
13.	Microstructure of gels of surimi from yellow stripe trevally using different heating conditions.....	78
14.	Temperature profile of crude TGase from yellow stripe trevally muscle.....	79

LIST OF FIGURES (Continued)

Figure **Page**

15. Turbidity of NAM solutions (1 mg protein/ml) containing $ZnSO_4$ or $ZnCl_2$ at different concentrations during heating from 20 to 75 °C.. 89
16. Surface hydrophobicity of NAM solutions (1 mg protein/ml) containing $ZnSO_4$ or $ZnCl_2$ at different concentrations during heating from 20 to 75 °C……..... 91
17. Total sulphydryl group content (A) and disulphide bond content (B) of NAM solutions (1 mg protein/ml) containing $ZnSO_4$ or $ZnCl_2$ at different concentrations during heating from 20 to 75 °C…….. 93
18. Transmission electron micrograph of NAM solutions containing 100 µM $ZnSO_4$ or 100 µM $ZnCl_2$ after heating at 0.65 °C/min to 75 °C..... 100
19. Breaking force (A) and deformation (B) of kamaboko and modori gels from yellow stripe trevally surimi added with $ZnSO_4$ or $ZnCl_2$ at different levels……. 111
20. SDS-PAGE pattern of kamaboko (A) and modori (B) gels from yellow stripe trevally surimi added with $ZnSO_4$ or $ZnCl_2$ at different levels..... 116
21. Microstructures of kamaboko gel from yellow stripe trevally surimi without and with $ZnSO_4$ or ZnCl2 at levels of 60 and 80 µmol/kg.... 118
22. Scheme for the cross-linking of surimi proteins induced by Zn^{2+}..... 119
23. Breaking force (A) and deformation (B) of gels from yellow stripe trevally surimi added with STPP at different levels and protein isolate without and with phosphorylation using 0.25 and 0.5 % STPP as influenced by $ZnSO_4$ at different levels (0-140 µmol/kg)…. 136
24. Protein pattern of gels from yellow stripe surimi added without and with STPP and protein isolate without and with phosphorylation as influenced by $ZnSO_4$ at different levels (0-140 µmol/kg)……..... 142

LIST OF FIGURES (Continued)

Figure **Page**

25. Electron microscopic image of gels from yellow stripe trevally surimi and protein isolate (surimi without additive (A), surimi with 0.5% STPP (B), surimi with 0.5% STPP and 60 μmol $ZnSO_4$/kg (C), surimi with 0.5% STPP and 140 μmol $ZnSO_4$/kg (D), protein isolate without additive (E), phosphorylated protein isolate (F), phosphorylated protein isolate with100 μmol $ZnSO_4$/kg (G), and phosphorylated protein isolate with 140 μmol $ZnSO_4$/kg)……. 144
26. FT-IR spectra of FPI, FSG and FPI/FSG (5:5) blend films prepared at pH 3 and 11 containing 30% glycerol..... 167
27. TGA curves of FPI, FSG and FPI/FSG (5:5) blend films prepared at pH 3 (A) and 11 (B) containing 30% glycerol..... 169
28. SEM micrographs of surface and freeze-fractured cross-section of FPI, FSG and FPI/FSG (5:5) blend films prepared at pH 3 and 11 containing 30% glycerol..... 172
29. WAXD patterns of pure ZnONP (A) and FPI/FSG films prepared at pH 3 and 11 without and with 3% (w/w) ZnONP (B)……..... 192
30. ATR-FTIR spectra of FPI/FSG films prepared at pH 3 and 11 without and with 3% (w/w) ZnONP ……..... 194
31. TGA curves of FPI/FSG films prepared at pH 3 and 11 without and with 3% (w/w) ZnONP..... 196
32. SEM micrographs of surface and freeze-fractured cross-section of FPI/FSG films prepared at pH 3 and 11 without and with 3% (w/w) ZnONP..... 199
33. Effects of ZnONP content on antimicrobial activity of FPI/FSG films prepared at pH 3 and 11 against *L. monocytogenes* (A) and *P. aeruginosa* (B)..... 201

LIST OF FIGURES (Continued)

Figure		Page
34.	Antimicrobial activity of FPI/FSG films against *L. monocytogenes* (A) and *P. aeruginosa* (B) as influenced by BEO and ZnONP.....	222
35.	ATR- FTIR spectra of FPI/FSG films as influenced by BEO and ZnONP incorporation.....	225
36.	TGA curves of FPI/FSG films as influenced by BEO and ZnONP incorporation.....	227
37.	SEM micrographs of surface and cross-section of FPI/FSG films as influenced by BEO and ZnONP incorporation.....	230
38.	Total viable bacterial count (a) and psychrophilic bacterial count (b) of sea bass slices wrapped without and with films (PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film) during storage at 4 °C for 12 days.....	243
39.	Pseudomonas (a), H_2S-producing bacteria (b), Enterobacteriaceae (c) and Lactic acid bacteria (d) counts of sea bass slices wrapped without and with films (PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film) during storage at 4 °C for 12 days.....	244
40.	TVB content (a) and pH (b) of sea bass slices wrapped without and with films (PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film) during storage at 4 °C for 12 days.....	250
41.	Peroxide value (a) and TBARS value (b) of sea bass slices wrapped without and with films (PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film) during storage at 4 °C for 12 days.....	252

CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Due to the over-exploitation of lean fish, which have been used for consumption and export, pelagic dark fleshed fish with the abundance and low price have been paid increasing attention as a potential alternative raw material for fish processing. However, products from dark fleshed fish generally are poorer in quality and acceptability. This is associated with high fat and pigments in muscle, which lead to the susceptibility to lipid oxidation and discoloration. Among fish products, surimi, a washed fish mince, can be used for gel production. However, surimi from pelagic dark fleshed fish has poorer gel forming ability, thereby limiting the utilization of those species (Chaijan *et al*., 2004; Haard *et al*., 1994). Various food grade ingredients have been used to increase the gel strength of surimi, but the addition of these ingredients poses the adverse effects on the surimi gel, particularly off-flavor or off-color (Rawdkuen and Benjakul, 2008). To improve the properties of surimi gel from dark fleshed fish, the novel and cheap additives capable of improving gel quality of surimi have gained increasing attention.

Divalent cations, e.g. calcium, magnesium and zinc are known to alter the functionality of protein during gelation (Mathew *et al*., 2009; Morales *et al*., 2001). Sorgentini *et al*. (1995) reported that suspension of native soy isolate had the highest turbidity when $MgCl_2$ at a level of 40 mM was present. At pH values sufficiently far from the isoelectric point of proteins, divalent ions such as Ca^{2+}, Zn^{2+}, Mg^{2+} induce protein cross-linking via the salt bridges between negatively charged carboxyl groups (Hongsprabhas and Barbut, 1997). Mathew *et al*. (2009) reported that the binding of zinc salts to actomyosin from oil sardine induced conformational changes with the exposure of functional groups such as sulfhydryl groups, which subsequently undergo oxidation to disulfide bond.

To widen the utilization of dark fleshed fish, their muscle has been used for film formation. The protein based films have increasingly attracted the

attention, mainly owing to biodegradability, edibility and the large variety of applications (Rouhi *et al*., 2013; Tongnuanchan *et al*., 2011a; Prodpran *et al*., 2007; Chinabhark *et al*., 2007; Iwata *et al*., 2000). Properties of fish muscle protein based films depend on various factors such as pH of protein solution, plasticizers, the preparation conditions and substances incorporated into film-forming solutions (Gerrard, 2002; Cuq *et al*., 1995a; Prodpran *et al*., 2007). Like other protein based films, fish myofibrillar protein films have inferior functional properties especially high water vapor permeability. The films are rather brittle and have relatively poor mechanical properties (Tongnuanchan *et al*., 2011a; Prodpran *et al*., 2007). Various attempts have been carried out to improve properties of those films including chemical treatment (Hernandez-Munoz *et al*., 2004), enzymatic treatment (Jiang *et al*., 2007) and thermal treatment (Lei *et al*., 2007). Another simple, inexpensive, effective and widely used approach to improve the properties of protein based films is polymer blend technique (Perez-Mateos *et al*., 2009). Gelatin is the polymer of choice being used to blend with various biopolymers due to their great compatibility (Cao *et al*., 2007). For film making, fish protein isolate (FPI) has been shown as the promising starting material, in which the discoloration was tackled and mechanical properties could be improved (Tongnuanchan *et al*., 2011a). The addition of gelatin into FPI could diminish brittleness and improve mechanical properties of FPI film. Moreover, addition of zinc nanoparticles (ZnONP) at an appropriate concentration could improve film forming property by filling and enhancing aggregation of proteins via salt bridges (Hongsprabhas and Barbut, 1997; Li *et al*., 2009a). ZnONP have the excellent ability for nano-scale dispersion and interfacial interactions in protein matrix due to their large specific surface area and high surface energy (Rouhi *et al*., 2013). ZnONP is an inexpensive inorganic compound generally recognized as safe (GRAS) material by the Food and Drug Administration (21CFR182.8991) and currently being investigated as an antibacterial and antifungal agent (Espitia et al., 2013; Eskandari *et al*., 2011). Essential oils from aromatic and medicinal plants have been known to be biologically active, mainly possessing antibacterial, antifungal and antioxidant properties (Hussain *et al*., 2008). Additionally, essential oils are hydrophobic in nature and the incorporation of essential oils improved the water vapor barrier property and imparted flexibility of gelatin films (Tongnuanchan *et al*., 2013a).

Moreover, the use of essential oil exhibited the increased antimicrobial activity, when combined together with various nanoparticles (Allahverdiyev *et al*., 2011).

Yellow stripe trevally (*Selaroides leptolepis*) is one of the abundant trash dark fleshed fish species in Southern Thailand. It can be used for production of surimi as well as for film preparation. Since the properties of gel and film from muscle proteins of dark-fleshed fish are generally inferior in mechanical properties, the use of divalent cations, especially zinc at an appropriate concentration could be a promising means to improve the gel strength and film forming property by enhancing aggregation of proteins via salt bridges and to enrich zinc in muscle protein product. Active film with antimicrobial activity can be obtained when the incorporation of ZnONP and essential oil is properly performed. The outcome of this research can be of great benefit for the surimi and biopolymer industries of Thailand and other countries. Moreover, the natural marine resources can be fully exploited and new products can be obtained for further commercialization.

1.2 Review of Literature

1.2.1 Chemical composition of fish

The main constituents of fresh fish are water (65-85 %), protein (15-24%), fat (0.1-22%), carbohydrate (1-3%) and inorganic substances (0.8-2%). The amount of fish meat varies according to the species, age, part of body, pre or post-spawning season and the feeding conditions (Suzuki, 1981). Protein is a major composition of fish muscle with the range of 15-20% (wet weight). Protein compositions of fish vary, depending upon muscle type, feeding period, and spawning, etc. The proteins of fish muscle can be categorized into the following three major groups: (1) the sarcoplasmic fraction which perform the biochemical tasks in the cells, (2) the myofibrillar proteins of the contractive system, and (3) the proteins of the connective tissues, responsible mainly for the integrity of the muscles (Moosavi-Nasab, 2003) (Figure 1).

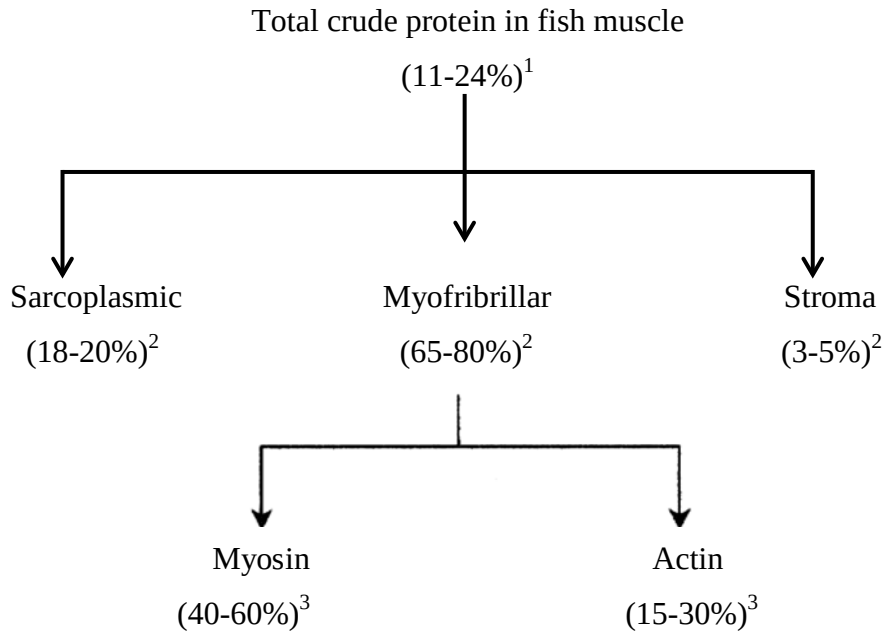


Figure 1. Schematic diagram of fish muscle protein composition. [1]based on total proteins; [2] based on total muscle proteins; [3] based on total myofibrillar proteins.
Source: Moosavi-Nasab (2003)

1.2.1.1 Sarcoplasmic proteins

Sarcoplasmic proteins contain many kinds of water soluble protein called myogen. It represents 20-35% of the total protein content in muscle (Mackie, 1994). It can be obtained simply by pressing fish meat, or by extracting with low ionic strength salt solution (Mackie, 1994). The content of sarcoplasmic protein in fish meat varies with fish species, but is generally higher in pelagic fish such as sardine and mackerel (Chaijan *et al*., 2004) and lower in demersal fish (Suzuki, 1981). Haard *et al.* (1994) suggested that the sarcoplasmic proteins from fish included myoglobin, enzymes and other albumins. The presence of sarcoplasmic proteins of dark muscle has often been cited as one of the reasons for the poorer gelation characteristics of dark muscle fish, compared with light muscle (Haard *et al*., 1994). Sarcoplasmic proteins bind to the myofibrillar proteins and thus interfere with the formation of gels (Haard *et al*., 1994). Hultin and Kelleher (2000a) and Haard *et al.* (1994) reported that small quantities of sarcoplasmic proteins had an adverse effect on the strength and deformability of myofibril protein gels. These proteins lowered myosin crosslinking during gel matrix formation because they did not form gels and had poorer water holding capacity (Chaijan *et al*., 2004; Hultin and Kelleher, 2000b; Haard *et al*., 1994). The presence of sarcoplasmic proteins may change the rheological properties of the fish gels (Sikorski, 1994). However, Benjakul *et al*. (2004a) reported that the sarcoplasmic fraction from bigeye snapper muscle possessed cross-linking activity towards myosin heavy chain (MHC). Sarcoplasmic fraction of fish flesh such as threadfin bream (*Nemipterus sp*.) and bigeye snapper (*Priacanthus tayenus*) contained transglutaminase (Piyadhammaviboon and Yongsawardigul, 2009; Benjakul *et al*., 2004a). Furthermore, Piyadhammaviboon and Yongsawatdigul (2009) revealed that addition of sarcoplasmic protein from threadfin bream in lizardfish surimi improved the textural properties of fish protein gels. Recently, Hemung *et al.* (2013) demonstrated that sarcoplasmic protein from threadfin bream is a potential functional protein ingredient whose properties can be manipulated through a proper pH adjustment.

1.2.1.2 Myofibrillar proteins

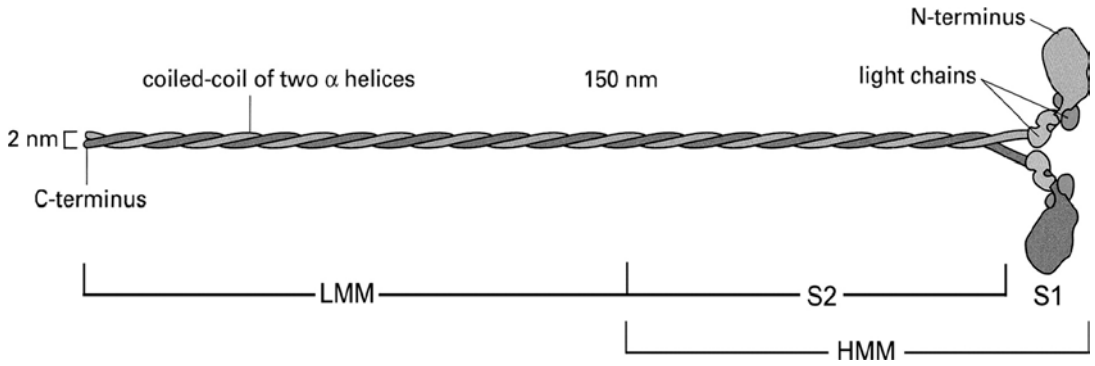
Myofibrillar proteins, the largest proportion of fish muscle proteins, account for 65 to 80% of total protein. They give the muscle its fibre-like structure (Hall and Ahmad, 1997). These proteins can be extracted from the comminuted fish with neutral salt solutions of ionic strength ranging from 0.3 to 1. Myofibrillar proteins can be further divided into two subgroups as contractile proteins (myosin, actin and actomyosin) and regulatory proteins (tropomyosin, and troponin) (Hall and Ahmad, 1997). The myofibrillar proteins participate in the postmortem stiffening of the tissues (rigor mortis) (Sikorski, 1994). During long-term frozen storage, they may cause toughening of the meat via their aggregation (Benjakul *et al*., 2005a; Leelapongwattana *et al*., 2005). The myofibrillar proteins are mainly responsible for the water-holding capacity of fish, for the characteristic texture of fish products, as well as for the functional properties of fish minces and homogenates, especially the gel forming ability (Zayas, 1997).

1.2.1.2.1 Myosin

Myosin with molecular weight of about 500 kDa makes up 40 to 60% of the myofibrillar fraction (Xiong, 1997). Myosin is a long, linear molecule made up of two identical heavy chains (MHC) with MW of 200 kDa and four light chains (MLC) with MW≈ 20 kDa each. The two heavy chains have a high degree of α - helical content and are wound around each other in a super-coiled helical conformation and form the rod portion (Xiong, 1997) (Figure 2A)**.** The globular regions are responsible for ATPase activity and contain actin-binding sites (Xiong, 1997). Myosin is a relatively large molecule that can be cleaved into several fragments. Myosin heavy chain can be cleaved by papain near the globular end of the rod, to produce fragment 1 (S-1) (Visessanguan *et al*., 2000). When myosin is digested with trypsin, two major fragments are obtained. The largest fragment is heavy meromyosin containing both myosin globular heads and a portion of the myosin rod. The other major fragment is light meromyosin, which is the helical rod portion of the myosin molecule. Further proteolytic digestion of heavy meromyosin

yields the S-1 fragment, the individual myosin head, and the S-2 fragment, the small portion of myosin rod (Rattrie and Regenstein, 1977) (Figure 2B).

(A)



(B)

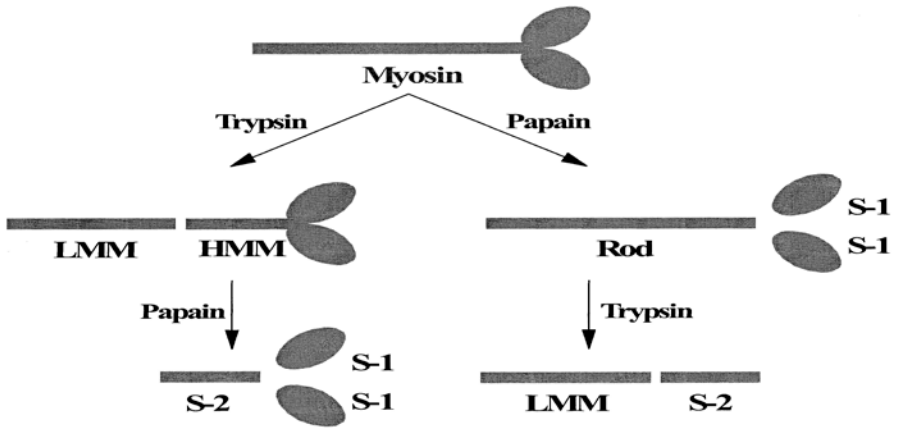


Figure 2. Schematic diagram of (A) the myosin molecule and (B) its subfragments prepared by proteolytic digestion.
Sources: Alberts *et al*. (2002); Visessanguan *et al*. (2000)

1.2.1.2.2 Actin

Actin constitutes about 22% of myofibrillar mass with a molecular weight of 42 kDa. Normally, actin in muscle tissue is associated with troponin and tropomyosin complex. It also contains a myosin binding site, which allows myosin to form temporary complex with it during muscle contraction or the permanent myosin-actin complex during rigor mortis in post-mortem (Xiong, 1997). Monomer form of actin is called G-actin and after polymerization, actin filaments are

formed and referred to as F-actin. Two F-actin in helix form is called super helix (Foegeding *et al*., 1996) (Figure 3).

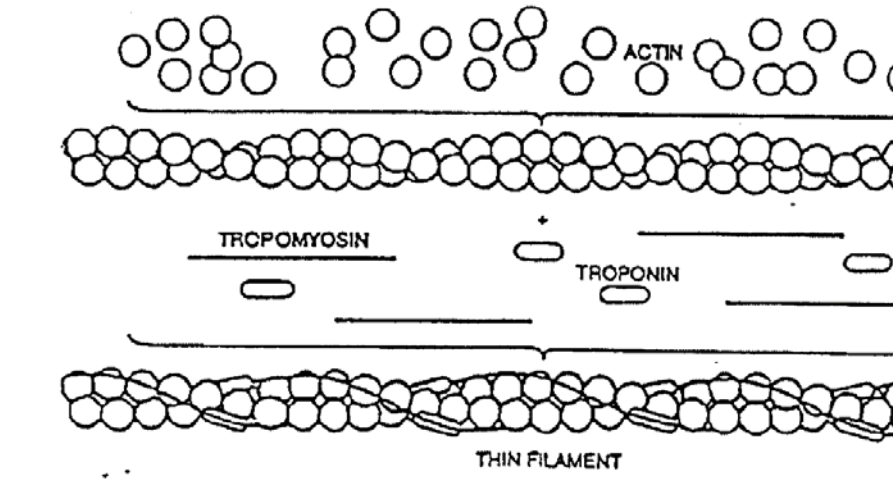


Figure 3. Structure of actin, troponin and tropomyosin.
Source: Foegeding *et al*. (1996)

1.2.1.2.3 Actomyosin

When actin and myosin are mixed *in vitro*, a complex, call actomysin, is formed. The complex is bound not by covalent, but by electrostatic bonding with the contribution of phosphate groups (Xiong, 1997). The reconstituted actomyosin produced from both component proteins demonstrates many biochemical and physicochemical features of myosin, however does not exhibit physicochemical and function features of F-actin (Kijowski, 2001). This complex can be dissociated by the addition of ATP or high ionic strengths (Xiong, 1997). Actomyosin is the main state of actin and myosin in postmortem muscle because ATP is depleted by postmortem metabolism (Ochai and Chow, 2000).

1.2.1.2.4 Troponin

Troponin and tropomyosin regulate muscle contraction. Troponin accounting for 8-10% of myofibrillar proteins. It consists of three subunits such as troponin C, which is a calcium binding protein and confers calcium regulation to the

contraction process via the thin filament; troponin I, which strongly inhibits ATPase activity of actomyosin and troponin T, which provides a strong association site for binding of tropomyosin (Foegeding *et al*., 1996).

1.2.1.2.5 Tropomyosin

Tropomyosin represents approximately 8-10% of myofibrillar protein. It has two subunit chains (Suzuki, 1981). In skeletal muscle, two polypeptides, α- and β-tropomyosin can combine to form a tropomyosin dimer. Tropomyosin aggregates end-to-end and binds to actin filaments along each groove of the actin double helix such that each molecule interacts with seven G-actin monomers (Foegeding *et al*., 1996).

1.2.1.3 Stroma protein

Stroma is the protein, which forms connective tissue, representing approximately 3% of total protein content of fish muscle. It cannot be extracted by water, acid, or alkali solution and neutral salt solution of 0.01-0.1 M concentration. The component of stroma is collagen, elastin or both (Suzuki, 1981). Elastin is very resistant to moist heat and cooking. Normally, it is a reflection of the different structural arrangements of muscle cells in fish, compared to mammals (Mackie, 1994). Collagen is almost totally insoluble in water or saline and does not participate in gel formation. Collagen can convert to gelatin when heated, depending on the structure of the collagen present. This soluble gelatin can interfere with the gelation of myofibrillar proteins (Park, 2000).

1.2.2 Characteristics and compositions of dark fleshed fish

Mackerel, sardine and yellow stripe trevally have high contents of dark muscles.Dark muscle contains the greater quantities of mitochondria, myoglobin, fats, glycogen and cytochromes and has a more abundant vascular supply. Mackerel and sardine have high contents of dark muscles and are reported to have high contents of lipid, and myoglobin (Chaijan *et al.*, 2004). Chaijan *et al.* (2007a) also found a high content of myoglobin in sardine (*Sardinella gibbosa*) dark muscle. Dark muscle fish

are often referred to as fatty fish. This is a reflection of their high lipid content. Dark muscle relies on the oxidative metabolism of lipid as its principal source of energy. This is the reason for the high content of oil in the muscle (Hultin and Kelleher, 2000b). The levels of PUFA, including 20:5 n-3 and 22:6 n-3 were higher in the dark muscle compared to the ordinary muscle in chub, mackerel and skipjack tuna (Bae *et al*., 2010; Sohn and Ohshima, 2010). PUFA are prone to oxidation thereby producing a large number of oxidation products such as aldehyde, ketone, alcohol, etc. Lipid oxidation is assoiated with the development of fishy odor in fish during the extended storage (Maqsood and Benjakul, 2011). The presence of haem pigments and trace amounts of metallic ions could induce lipid oxidation effectively in dark muscle fish (Maqsood and Benjakul, 2012). Tokur and Korkmaz (2007) demonstrated that iron-catalyzed oxidation systems were involved in oxidation of lipids and proteins in dark muscle fish, which may implicate functional and sensory quality changes of processed dark muscle fish.

Ordinary muscles are different in composition from dark muscle (Bae *et al*., 2011). Ordinary muscle showed significantly higher contents of moisture, crude protein and ash and lower contents of crude fat, compared to dark muscle ($P < 0.05$). It is known that variations in the proximate composition of marine fishes are closely related to nutrition, living area, fish size, catching season, seasonal and sexual variations as well as other environmental condition (Erkan and Ozden, 2007). Zlatanos and Laskaridis (2007) reported that fat and fatty acid contents of three common Mediterranean fish (sardine, anchovy and picarel) showed significant seasonal dependency. Dark muscle contained almost three times as much total lipid as ordinary muscle in herring (*Clupea harengus*) (Undeland *et al*., 1998). The stroma protein and myoglobin contents were around 5 and 10-40 times, respectively, higher in the dark than in the ordinary muscle of tuna (Kanoh *et al*., 1986). Hashimoto *et al*. (1979) determined the protein compositions of the dark and the white from sardine (*Sardinops melanosticta*) muscle. The dark muscle contained 23-29% sarcoplasmic protein, 62-66% myofibrillar protein, 6-9% alkali-soluble protein and 2-3% stromal protein. The white muscle comprised 33-37% sarcoplasmic protein, 59-61% myofibrillar protein, 1-5% of alkali-soluble protein and 1-2% stromal protein. Chaijan

et al. (2004) reported that lipid and myoglobin contents were higher in dark muscle than in ordinary muscle of both sardine and mackerel.

1.2.2.1 Surimi from dark fleshed fish

Surimi is stabilized myofibrillar proteins obtained from mechanically deboned fish flesh that is washed with water and blended with cryoprotectants (Park and Morrissey, 2000). The composition of dark muscle is the major problem when incorporated into surimi. Apart from its higher lipid content and susceptibility to oxidation, dark meat has greater proteolytic activity and higher concentrations of trimethylamine oxide and sarcoplasmic proteins (Shimizu *et al*., 1992; Eymard *et al*., 2005; Chaijan *et al*., 2010a). Myoglobin and haemoglobin are responsible for the red color of dark muscle but they also promote lipid oxidation (Eymard *et al*., 2005). Chaijan *et al*. (2004) found that myoglobin in sardine and mackerel muscle constitute 14.27 and 4.88 (% wet wt.), respectively. Nevertheless, the role of sarcoplasmic proteins such as myoglobin is controversial; some researchers state that they hamper myosin cross-linking during the gelation process, others consider that these proteins do not interfere with the myofibrillar proteins (Park and Park, 2007). Murakawa *et al*. (2003) reported that oxidized lipids can interact with proteins, induced denaturation, and polymerization, thereby bringing about an adverse effect on the quality of surimi products.

The gel-forming ability of dark muscle fish meat has been known to be lower than that of ordinary muscle. This apparently resulted from the difference in the unfolding abilities of the myosin between the muscles. Lo *et al*. (1991) found that the head portions of the heavy chain of myosin from the dark and ordinary muscles did not differ significantly in the thermostability, but the ordinary muscle myosin had two thermal transition points, at 36 and 57 °C, whereas that of the dark muscle had only one transition point, at 68 °C. Also, dark fleshed fish mince experiences a sharp pH drop after slaughter, often below 6, in comparison with 7, approximately, for Alaska pollock. This pH around the isoelectric point of the proteins increases their denaturation rate, affecting the gelling ability of pelagic species flesh kept in chilled storage for 1-2 days (Hultin *et al*., 2005). Additionally, surimi from pelagic dark

fleshed fish presents a higher and stronger proteolytic activity than surimi from light-fleshed species (Hamann and MacDonald, 1992; Shimizu *et al*., 1992; Hultin *et al*., 2005). This can cause modori, the gel weakening that occurs if the gel is held too long at a temperature around 50-60 °C (Sikorski, 1994). Chaijan *et al*. (2010a) suggested that gel forming ability of dark fleshed fish surimi depends on the type and species of fish.

1.2.2.2 Surimi gelation

Surimi shows gelling property, which varies with several factors, e.g species, muscle type, additives, etc. Prior to gelation, surimi is comminuted with salt and water. Addition of salt is needed to solubilize and destabilize the native structure of myofibrillar proteins (Park and Lanier, 1990). Gelation of fish protein generally involves denaturation and aggregation. Denaturation is a process, in which proteins undergo conformational changes, primarily unfolding without alteration of the amino acid sequence. Then, protein-protein interactions, known as association, aggregation and polymerization, take place and a three-dimensional network can be formed. Normally, gel is formed when partially unfolded proteins developed uncoiled polypeptide segments that interact at specific points to form a three dimensional cross-linked network (Zayas, 1997) and capable of holding water. Thermal unfolding of these proteins, especially of myosin and natural actomyosin (NAM), is important for gelation. Thermal denaturation of NAM begins at 30-35 °C and native tropomyosin and troponin is also dissociated from F-actin. Subsequently, helical structure of F-actin underwent a single chain at approximately 38 °C. Light chain (LC) subunits started to dissociate from the globular head of MHC when temperature reached at 40-45 °C and conformational changes of globular head also occurred (Sano *et al*., 1990). Actin-myosin complex started to dissociate from each other at 45-50 °C and helical regions of MHC then unfolded to the random coil structure (Chan *et al*., 1993). The denaturation of G-actin occurred at temperature greater than 70 °C. Thermal denaturation mechanism of NAM from carp was also proposed by Sano *et al*. (1994). The solubilized NAM began to unfold at temperature about 30 °C and aggregation started at this temperature. Aggregation of proteins was formed extensively around 40-60 °C. Myosin molecule dissociated from F-actin at

temperature greater than 40 °C. Tyska *et al*. (1999) reported that myosin gelation was initiated by interaction of HMM at 30-40 °C followed by thermal unfolding as well as interaction of LMM at 50 °C. In contrast, Sano *et al*. (1990) reported that gel formation of carp myosin was started to form through the interaction of LMM at temperature about 30-45 °C. Chan *et al*. (1993) suggested that myosin initially aggregated through the interaction of S2 at 30-40 °C. Further aggregation was from interaction of LMM at 40- 50 °C. The results of these studies indicated that the discrete regions of myosin sub-fragment play important role in gel network formation. The role of actin in gelation of myofibrillar proteins was also observed as synergistic effect. Table 1 gives a summary of changes, which may occur during the heat denaturation of natural actomyosin.

Several bondings are found to participate in gelation. Gel network formation of myosin was initiated by disulfide formation between myosin head (Visessanguan *et al*., 2000). Disulfide bond formation was noticed during incubation of herring myosin at 40 °C (Gill *et al*., 1992). Niwa (1992) reported that the disulfide bond formation of fish actomyosin and myosin occurred at 40 and 45 °C, respectively. Yarnpakdee *et al*. (2009) reported that disulfide bond played an important role in heat induced aggregation of NAM from goatfish at high temperatures. Yongsawatdigul and Park (1999) reported that disulfide bond formation in tilapia actomyosin required high temperature (above 50 °C). Benjakul *et al*. (2001a) reported that the hydrophobic interaction and disulfide bond formation determined the aggregation behavior of protein as well as gel properties of bigeye snapper natural actomyosin.

Table 1. Conformational change occurring during the thermal denaturation of natural actomyosin

Temperature (°C)	**Protein (s) or segment involved**	**Description of events**
30-35	Native tropomyosin	Thermal dissociation from the F-actin backbone
38	F-actin	Super helix dissociates into single chain
40-45	Myosin Head Hinge	Dissociates into light and heavy chains Possibly some conformational change Helix to random coil transformation
45-50	Actin, myosin	Actin-myosin complex dissociates
50-55	Light meromyosin	Helix to coil transformation and rapid aggregation
>70	Actin	Major conformational changes in the G-actin monomer

Source: Ziegler and Aton (1984)

Hydrogen bonds are weaker dipole bonds that, mainly by virtue of their great numbers rather than individual bond strength, can be important in the stabilization of bound water within the gel and improve gel strength during cooling and aging of surimi (Sánchez-González *et al*., 2008). Howe *et al*. (1994) reported that numerous hydrogen bonds between proteins were formed when the gel was colder. As a result, surimi gels became firmer at colder temperatures. Yoon *et al*. (2004) confirmed that aggregation of actomyosin or myosin was due to the development of cross-linking caused by formation of hydrogen bonds, covalent bonds and disulfide bonds. Hydrogen bonds between amino acids also stabilize the internal (secondary) structure of individual protein molecules in water. Ionic bonds and hydrogen bonds played important roles in maintaining the complex high-level structure of *Parabramis pekinensis* surimi (Zhi-he *et al*., 2010). The α-helix of native and partially denatured proteins and the β-structure that forms on heating and cooling were both stabilized by hydrogen bonds (Bouraoui *et al*., 1997). Sánchez-González *et al*. (2008) reported that

rearrangement of hydrogen bonds of proteins played the important roles in the formation of the protein network when the sol is heated.

In contrast to hydrogen bonds, which dissipate upon heating, hydrophobic interactions (which are effectively “bonds” between proteins in the aqueous environment of surimi gels) are strengthened by rising temperature, at least to near 60°C. The exposure of hydrophobic domains has been suggested as a prerequisite for the formation of large myosin aggregates via hydrophobic interaction (Chan *et al*., 1992a). Upon heating, the aromatic hydrophobic amino acid residues, i.e. phenylalanine and tryptophan, could be exposed to a greater extent (Visessanguan *et al*., 2000). The hydrophobic interaction of myosin from herring at 35-40 °C in the presence of NaCl was also suggested by Chan *et al*. (1995). Sano *et al*. (1994) suggested that hydrophobic interactions of carp NAM occurred extensively at 30-50°C. Yarnpakdee *et al*. (2009) reported that hydrophobic interactions play an important role in heat induced aggregation of NAM from goatfish at high temperatures. Thermal aggregation of cod myosin was also formed by hydrophobic interactions of the rod portion (Chan *et al*., 1993). In addition, tail region of cod myosin was involved in non-covalent interactions at about 40-50 °C.

1.2.3 Setting and weakening of surimi gel

1.2.3.1 Setting phenomenon

Setting is a phenomenon explaining the increased textural properties of surimi gels after pre-incubation at a certain temperature below 40 °C for a specific period of time prior to cooking (Lanier *et al*., 2000). A gel with higher rigidity and elasticity is obtained when setting is performed, followed by cooking (Roussel and Cheftel, 1990). This phenomenon involves gel network formation of muscle proteins triggered by protein unfolding. Setting temperature can be varied, depending on fish species, and setting phenomenon is related to habitat temperature of fish species. The optimum temperature for setting among species may be determined by the heat stability of myosin (Morales *et al*., 2001). Generally, setting can be carried out at low (0-4 °C), medium (25 °C), and high (40 °C) temperatures (Lanier, 1992). Low and

medium temperatures are applied for setting of surimi from cold and temperate habitats. Setting of Alaska pollock surimi from Bering sea was achieved at 4-5 °C (Kim *et al*., 1993). Nevertheless, Park (1994) reported that optimum setting temperature for Pacific whiting surimi was 25 °C instead of 5 °C. High temperature setting at 40 °C has been applied for surimi from tropical or warm water fish species such as Atlantic croaker, Mexican flounder, Northern kingfish, threadfin bream, bigeye snapper, barracuda, and white croaker (Lee and Park, 1998; Morales *et al*., 2001; Yongsawatdigul *et al*., 2002; Benjakul *et al*., 2004b).

Isopeptide bond catalyzed by transglutaminase (TGase) has been reported to be the major force involving polymerization of myosin during setting (Kamath *et al*., 1992). TGase is an enzyme that catalyzes acyl transfer reaction between the γ-carboxyl amide group of glutamine residues within protein to the side chain of lysine, resulting in protein cross-linking (Figure 4). Tissue or endogenous TGases require Ca^{2+} for catalytic reaction (Yongsawatdigul *et al*., 2002). Cross-linking of MHC was also the Ca^{2+}-dependent reaction in the setting and such cross-links play a crucial role in gel strengthening (Kumazawa *et al*., 1995). The effect of Ca^{2+} on setting at 25 °C (3 h) or 5 °C (20 h) in surimi from Pacific whiting and Alaska pollock was reported (Lee and Park, 1998). Addition of Ca^{2+} significantly improved textural properties of surimi from threadfin bream after setting at either low or high temperatures but breaking force value of gels set at 40 °C was higher than at 25 °C (Yongsawatdigul *et al*., 2002). Tsukamasa *et al*. (1993) found that the isopeptide bond formation of sardine sol was found at temperature below 30 °C. An increase in gel strength was correlated with the amount of isopeptide bond. Gel strength, cross-linking of MHC, and the content of isopeptide bond increased with setting time (Kumazawa *et al*., 1995). Takeda and Seki (1996) reported that complete suppression of myosin cross-linking of walleye pollock surimi gel was associated with the inhibition of indigenous TGase. Kumazawa *et al*. (1995) suggested that setting phenomenon was suppressed in the presence of TGase inhibitors, such as NH_4Cl and EDTA.

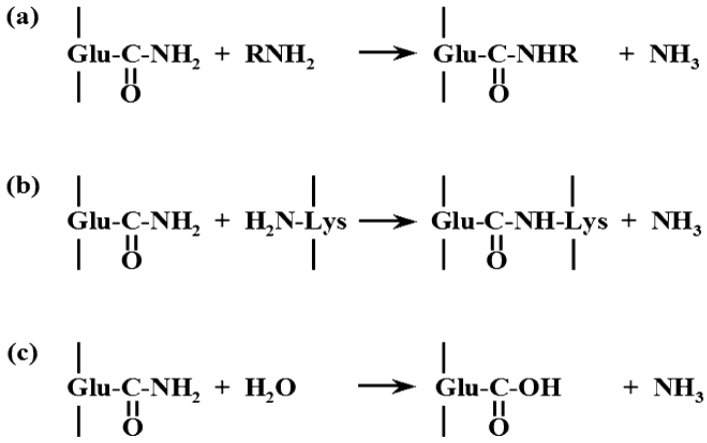


Figure 4. Reactions catalyzed by TGase (a) Acyl transfer (b) Crosslinking of Gln and Lys residues in proteins or peptides. The resulting bridge is called an ε-(γ-glutamyl) lysine (GL) bond. (c) Deamidation.
Source: Yokoyama *et al*. (2004)

1.2.3.2 Gel softening phenomenon

Gel softening or gel weakening termed "modori" is a problem found in surimi, especially from some fish species. This is associated with degradation of muscle proteins caused by the endogenous heat-activated proteinase (An *et al*., 1996; Benjakul *et al*., 1997; Jiang *et al*., 2000a). Proteolytic activity in muscle is high at temperature above 50°C and causes the rapid and severe degradation of myofibrillar proteins, particularly myosin (Wasson *et al*., 1992). Such proteolytic degradation of myofibrillar proteins has a detrimental effect on surimi quality, and substantially lowers the gel strength (Morrissey *et al*., 1993). Proteinases associated with gel weakening can be categorized into two major groups: cathepsin (Seymour *et al*., 1994) and heat-stable alkaline proteinase (Wasson *et al*., 1992). High level of cysteine proteinase activity mediated by cathepsin B, H, and L was found in Pacific whiting and arrowtooth flounder (An *et al*., 1994; Wasson *et al*., 1992), chum salmon and mackerel (Lee *et al*., 1993) during spawning migration (Yamashita and Konagaya, 1990). Softening of arrowtooth flounder gel is due to a cysteine proteinase that has the maximum autolytic activity at 50-60°C (Greene and Babbitt, 1990). When Pacific whiting muscle was incubated at 60°C for 30 min before cooking at 90°C, most of

MHC was degraded; the resultant surimi gel did not have measurable gel strength (Morrissey *et al*., 1993). Klomklao *et al*. (2008) reported the presence of proteinases in sardine (*Sardinop*s *melanosticus*). Cathepsins B and H are easily washed off during surimi processing, whereas cathepsin L remains in the muscle tissue (An et al., 1994; Park and Morrissey, 2000). Cathepsin L has an optimum temperature of 55°C and causes textural deterioration when the Pacific whiting surimi paste is slowly heated (An *et al*., 1994). Benjakul *et al*. (2003a) compared the autolysis from two species of bigeye snapper and found that *P macracanthus* had higher degradation of myosin heavy chain in both mince and washed mince than those from *P tayenus*, especially when the incubation time was increased. Autolysis of washed mince from both species was inhibited by soybean trypsin inhibitor, suggesting that myofibril-associated proteases were serine proteases, while *P tayenus* contained various proteases. The protease extracted from bigeye snapper muscle had an optimum pH and temperature of 8.5 and 60 ºC which was capable of hydrolyzing MHC effectively (Benjakul *et al*., 2003b).

During setting, proteolysis takes place to some degree and may affect the properties of resulting gels. Benjakul *et al*. (2004b) reported that suwari gel from some tropical fish, prepared by setting at 25°C showed lower degradation although the setting time increased up to 8 h, compared with setting at 40°C. Kamath *et al*. (1992) found that proteolysis in croaker paste increased with increasing temperature of setting, especially in the temperature range of 40-50°C. Proteolytic degradation of surimi gels is increased at temperatures above 40°C with the rapid and severe degradation of myofibrillar proteins, particularly myosin (Kudre and Benjakul, 2013).

1.2.4 Factors affecting gelling properties

In general, the characteristics of gel are governed by many factors including temperature (Sano *et al*., 1994), heating rate (Yongsawatdigul and Park, 1999), pH (Shikha *et al*., 2006) and type of actomyosin used (Lefevre *et al*., 2007). Muscle protein gel network is influenced by a number of factors as shown in Table 2.

Table 2. Factors influencing heat-induced gelation of muscle protein

Factors	Effects
Myosin	Myosin can form excellent gels.
Actin	F-actin cannot form gels; it becomes a curdy sol on heating. Combination of low levels of F-actin with myosin can enhance gel elasticity above that of myosin alone, but actin decreases gel elasticity at higher (6.1%) levels.
Types of muscles	White muscle generally forms stronger gels than red muscle.
Source of muscles	Gel forming ability of muscles from different species is complex and is influenced by different processing conditions.
Protein concentration	A "minimal gelation concentration" or critical protein concentration (CPC) is needed to form a gel. A myofibrillar protein content of about 0.5% is sufficient to produce a heat-induced gel. Regardless of protein source, gel hardness increases with increasing protein concentration.
pH	Gelation properties of myofibrillar protein are strongly pH-dependent. At the isoelectric point (pI) of myofibrillar protein (pH 5.3), either only poor gels are formed or gel formation is inhibited. At pH 6, the optimum pH value for heat-induced gelation of myosin is reached. Myofibrillar protein can form a gel at lower pH without heating.
Ionic strength	It is generally accepted that a high concentration (2% to 3%) of sodium chloride is required to solubilize the muscle protein to form a good gel. The microstructure of myofibrillar gels is also affected by ionic strength. At low ionic strength (0.25 M KCl), fine-stranded gel structures are formed; at high ionic strength (0.6 M KCl) coarsely aggregated gel structures are formed. The fine-stranded structure has higher rigidity than the coarsely aggregated structure.
Heating rate	A slow heating rate may allow more favorable protein-protein interactions to occur, producing a stronger, better-ordered 3-dimensional gel.
Fat content	Fat content has an impact on gelation properties of meat products, but the changes in gel hardness observed are inconsistent, due to different processing approaches.
Protein additives (egg white, whey protein, soy protein, blood globin, plasma, gluten hydrolysate)	Egg white, whey protein, soy protein, and sodium caseinate can enhance myofibrillar protein gel strength. However, egg white and whey protein weaken fish myofibrillar protein gels. Blood plasma and globin can increase or decrease myofibrillar protein gel strength, and this differs with different meat products.
Transglutaminase	Transglutaminase can significantly increase myofibrillar protein gel strength.

Source: Adapted from Sun and Holley (2011)

1.2.5 Improvement of surimi gel quality

Gelation of fish proteins is the most important step in forming desired texture in many seafood products, particularly those from surimi. To strengthen the gel, various additives as well as enzymes have been successfully used in surimi. Phosphates were added to increase the water-holding capacity (Park, 2005). Thickeners or gelling agents (hydrocolloids and polysaccharides) were used as filler (Perez-Mateos and Montero, 2000). Calcium compounds (Lee and Park, 1998), chitosan (Benjakul *et al*., 2003a), and oxidizing agents (Phatcharat *et al*., 2006) were used to strengthen protein gel networks.

1.2.5.1 Use of food grade protein additives

Proteolytic degradation of myofibrillar proteins has an adverse effect on the gel-forming properties of surimi. Gelation of fish proteins is the most important step in forming desired textures in many seafood products, particularly those from surimi. The gel strength of surimi can be enhanced by the addition of several ingredients. Various food-grade inhibitors, such as egg white, plasma proteins, potato extract, and whey protein concentrate, etc have been used to improve the physical properties and prevent the textural degradation, a modori phenomenon of surimi gels. However color and flavor may be altered (An *et al*., 1996; Benjakul *et al*., 2004c; Rawdkuen *et al*., 2007).

1.2.5.1.1 Egg white

Egg white is frequently used in surimi-derived products to improve gel texture (Park and Morrissey, 1994). It inhibited ‘modori’ (gel-softening) (Porter *et al*., 1993) and improved whiteness. Egg white makes the partially heat-set analog more elastic and stretchable. Aggregation of ovalbumin during heating results from hydrophobically driven protein–protein interactions. The amount of egg white added depends upon the fish species and the quality of the fish used. Campo-Deano and Tovar (2009) studied the influence of egg white protein at different proportions (1.5, 2 and 2.5%) on the viscoelastic properties of crab sticks made from two types of surimi (Alaska pollock or Pacific whiting surimi). Increasing protein content of the surimi

increased the gel strength of both types of crab sticks. The optimum egg albumen content was found to be about 1.5% for Alaska pollock and 2% for Pacific whiting surimi. Egg white added at 10–30 g/kg improved the mechanical properties of red tilapia surimi gels obtained by setting at 40 °C for 90 min followed by heating at 90 °C for 30 min, as well as for surimi gels from arabesque greenling (*P. azonus*) and walleye pollock (*T. chalcogramma*), incubated at 25 °C for up to 15 h before cooking at 90 °C for 30 min (Duangmal and Taluengphol, 2010). Egg whites added at 10% produced a gel from Alaska pollock surimi with high yield stress; gels containing up to 20% egg whites were softer, but there was a decrease in gel strength and the gel becomes brittle as the level was greater than 20% (Hui, 2006). Egg white contributes to the structure of surimi analog gels by filling interstitial spaces in the fish protein network. Benjakul *et al*. (2004c) reported that the addition of egg white up to 3% increased gelling properties of lizardfish surimi regardless of the heating conditions (40/90, 60/90 and 90°C). Adding egg white with cryoprotectants before freezing for 12 months was less efficient in improving the mechanical properties of surimi gels than adding 20–30 g/kg egg white during chopping of solubilized pastes (Hunt *et al*., 2009).

Nevertheless, egg white is an allergen and must therefore appear on the label of surimi analog products. Class II food recalls of analog products have been initiated in the United States because of the failure of companies to list egg whites on the ingredient statement (Hui, 2006). In addition, egg white must be used carefully because it often generates off-flavors (Park, 2005).

1.2.5.1.2 Whey protein concentrate

Whey protein concentrate (WPC) has commonly been used as a protein supplement, foam stabilizer, filler/water binder, thickening, emulsifying and gelling agents (Rawdkuen and Benjakul, 2008). It can be used to improve texture and nutritional value of a variety of foods such as sausages, meat balls and low-salt fish products (Ulu, 2004). Some studies have shown that WPC increases the shear strain of surimi gels prepared from Pacific whiting and Alaska pollock (Park, 1994; Weerasinghe *et al*., 1996). Rawdkuen and Benjakul (2008) reported that breaking

force and deformation of kamaboko gels of all surimi increased as the levels of WPC added increased (0-3%). However, WPC at 3% (w/w) significantly decreased the whiteness of gels. However, water-holding capacity of kamaboko gels was improved with increasing concentrations of WPC. The microstructure of surimi gels generally became denser with the addition of WPC. Benjakul *et al*. (2010) found that addition of WPC up to 3% (w/w) resulted in the increased breaking force and deformation with higher water-holding capacity of surimi from goatfish. Incorporation of $CaCl_2$ at 50 mmoles/kg in combination with 3% WPC effectively improved the gel strength of goatfish kamaboko gel (Benjakul *et al*., 2010).

1.2.5.1.3 Plasma proteins

Plasma proteins such as beef plasma protein (BPP), porcine plasma protein (PPP), chicken plasma protein (CPP), etc. have been used as a gelling agent and/or protease inhibitor in surimi gel (Tybor *et al*., 1975; Benjakul and Visessanguan, 2000; Benjakul *et al*., 2001b). Morrissey *et al*. (1993) reported that beef plasma protein showed the highest inhibitory activity in Pacific whiting surimi as compared to egg white and potato extract. Yada (2004) also reported that BPP exhibits a remarkable capability to inhibit modori, or gel weakening, during the cooking of surimi. The addition of BPP up to 3% increased gel strength of lizardfish surimi more effectively than egg white under a variety of heating conditions (40/90, 60/90 and 90 °C), but resulted in a lower whiteness in the finished product (Benjakul *et al*., 2004c). However, the use of BPP has been prohibited in the EU, Japan, Canada and the Unites States due to the outbreaks of BSE (bovine serum encephalopathy, or mad-cow disease) (Park, 2005).

Porcine plasma and chicken plasma proteins have been found to inhibit the autolysis associated with surimi gel weakening. Benjakul and Visessanguan (2000) found that porcine plasma protein showed the highest inhibitory activity in Pacific whiting surimi as compared to egg white and beef plasma protein. Proteinase inhibitor from pig plasma was found to have a molecular weight (MW) of 60-63 kDa determined by inhibitory activity staining with both papain and trypsin. Rawdkuen *et al*., (2004) studied the effects of chicken plasma protein (CPP)

on the hydrolysis of myofibrillar protein by endogenous proteinases in surimi made from bigeye snapper and lizardfish. The increased breaking force and deformation of surimi gel with higher water holding capacity were obtained when the CPP concentration increased, but the higher amount of CPP added resulted in the decrease in whiteness. Li *et al*. (2008a) found that the addition of rainbow trout plasma protein improved whiteness, gel texture, and even water-holding capacity of Alaska pollock surimi.

1.2.5.2 Use of microbial transglutaminase (MTGase)

Cross-linking enzymes such as a non-calcium-dependent microbial TGase (MTGase) can be added to improve the mechanical properties of surimi. MTGase is more stable at higher temperatures than the endogenous TGase (Lee *et al*., 1997; Gómez-Guillén *et al*., 2005) and shows a greater activity than fish TGase (Hemung *et al*., 2008). MTGase from *Streptoverticillium mobaraense* (Nonaka *et al*., 1997) or from *Streptoverticillium ladakanum* (Tsai *et al*., 1996) were shown to increase the gel strength of surimi (Benjakul and Visessanguan, 2003). Addition of MTGase to surimi significantly increases its gel strength, particularly when the surimi has lower natural setting ability, presumably due to lower indigenous TGase activity (Kumazawa *et al*., 1995; Lee and Park, 1998). Furthermore, MTGase has been demostrated to enhance the gel quality of lizardfish surimi, which was produced from fish stored even after 10 days in ice (Benjakul *et al*., 2008), and was also effective in improving gel quality of surimi from dark and fatty fish, such as sardines (*Sardina pilchardus*) (Karayannakidis *et al*., 2008). Breaking force and deformation of mackerel fish protein isolate gels markedly increased, especially as MTGase levels increased (Chanarat and Benjakul, 2013). An increase in non-disulfide polymerization and formation of ε-(γ-glutamyl) lysine isopeptides was found with increasing setting time and MTGase concentration (Tsukamasa *et al*., 1993). Jiang *et al*. (2000b) investigated the properties of surimi gels from threadfin bream and pollack surimi set at 30 or 45 °C with MTGase from *Streptoverticillium mobaraense*. The optimal amounts of MTGase and setting conditions were 0.3 unit/g surimi either at 30 °C for 90 min or at 45 °C for 20 min for threadfin bream surimi. For pollack surimi, MTGase at a level of 0.2 unit/g surimi with setting at 30 °C for 60 min was found to

be the optimum condition. MTGase catalyzed MHC cross-linking of both pollack and threadfin bream surimi and increased the gel forming ability of surimi. Benjakul *et al*. (2008) studied the effect of MTGase at different levels (0 to 0.8 units/g samples) on the properties of gels from lizardfish (*Saurida undosquamis*) mince set at 25 °C for 2 h or 40 °C for 30 min prior to heating at 90 °C for 20 min. MTGase showed the gel strengthening effect on lizardfish mince, particularly when high amounts of MTGase were used. For the gels added with MTGase at 0.8 units/g and set at 25 and 40 °C, the highest breaking force 93.1% and 90.7% was obtained, respectively. Karayannakidis *et al*. (2008) studied the effect of MTGase and Ca^{2+} ions on the textural characteristics of heat induced surimi gels from sardines (*Sardina pilchardus*). Incorporation of 2% MTGase (w/w) and 0.2 % Ca^{2+} ions (w/w) in surimi significantly affected the textural characteristics of heat-induced surimi gels. Fish gels with MTGase and $CaCl_2$ added were firmer and more cohesive, compared with the untreated gels. The former catalyzes the cross-linking reaction of myosin, while the latter activates indigenous TGase, which also leads to the formation of covalent non-disulfide cross-links. However, MTGase-containing fish gels exhibited a more elastic texture, compared with the untreated fish pastes and those containing $CaCl_2$.

1.2.5.3 Use of divalent cations

Apart from intrinsic factors, such as endogenous enzymes and thermal properties of proteins, the extrinsic conditions such as pH, ionic strength and type of salts have been reported to affect gel strength of surimi (Croguennec *et al*., 2002). Divalent cations, e.g. calcium, magnesium and zinc are known to alter the functionality of protein during gelation (Mathew *et al*., 2009; Morales *et al*., 2001). Gel formation induced either by divalent cations or action of heat or both is dependent on type and concentration of protein, processing conditions used to induce gel formation and pH and ionic environment (Schmidt, 1981). Sorgentini *et al*. (1995) reported that suspension of native soy isolate had the highest turbidity when $MgCl_2$ at a level of 40 mM was present. $ZnSO_4$ at concentrations above 0.1 mM decreased Ca^{2+}ATPase activity of actomyosin extracted from tilapia hybrid (*Tilapia nilotica* × *Tilapia aurea*) (MacDonald *et al*., 1996). Mathew *et al*. (2009) reported that the binding of zinc salts to actomyosin from oil sardine induced conformational changes

with the exposure of functional groups such as sulfhydryl groups, which subsequently undergo oxidation to disulfide bond. The role of zinc salts in combination with sucrose on the cryoprotection and aggregation of fish actomyosin has been studied by MacDonald *et al*. (1996). MacDonald *et al*. (1996) reported that increasing $ZnSO_4$ concentration above 0.1 mM decreased Ca^{2+}ATPase activity by inducing denaturation and consequent aggregation in these samples. Haque and Aryana (2002) found that bovine serum albumin added with $CuSO_4$, $FeSO_4$, and $ZnSO_4$ underwent aggregation, in which clustered proteins were formed.

At pH values sufficiently far from the isoelectric point of proteins, divalent ions such as Ca^{2+}, Mg^{2+} induce protein cross-linking via the salt bridges between negatively charged carboxyl groups (Hongsprabhas and Barbut, 1997). At pH values adequately far from the isoelectric point of the proteins and at low ion concentrations, the unfolded proteins tend to remain separate due to the electrostatic repulsive forces between them. Upon the addition of salt, these repulsive forces are screened, and the protein molecules can aggregate via salt bridge and form a gel (Doi, 1993; Hongsprabhas *et al*., 1999). Proteins aggregation is generally promoted by the electrostatic screening due to the action of monovalent and/or divalent ions. However, a concentration of divalent ions is much lower than monovalent one in order to cause aggregation. Divalent ions are much more effective at screening electrostatic interactions and have a greater ability to form salt bridges by cross-linking negatively charged carboxylic acid groups than monovalent ones (Hongsprabhas and Barbut, 1997, 1999; Remondetto and Subirade, 2003). A higher salt concentration change the distance distribution of the repulsive forces and thus the energy barriers. The loss of repulsive forces makes it possible for the charged protein molecules to get close enough to interact via noncovalent forces with a low potential energy (Remondetto and Subirade, 2003). Protein sites mainly involved in the coordination with positively charged metal ions are negatively charged sites, such as the carboxylic groups. The most relevant amino acid showing to have a great affinity in binding metal ions is aspartic acid, glutamic acid and histidine (Mesu *et al*., 2006). Divalent ions may act as inhibitors or promoters in aggregation processes depending on the divalent/protein ratio and the divalent ion binding mode; thus, they can differently affect the time

evolution of the aggregates (Garai *et al*., 2006). Salt bridges are especially abundant all along the myosin rod at position *e* and *g* of the seven-residue repeat of the coiled-coil rod. Ionic (electrostatic) interactions are thought to be the most important forces involved in the assembly of myosin thick filaments (Miroshinichenko *et al*., 2000). Haque and Aryana (2002) found that bovine serum albumin added with $CuSO_4$, and $ZnSO_4$ underwent ionic bond mediated aggregation. Myofibrillar proteins carry an overall net negative charge at the normal pH of surimi. Divalent cations like Ca^{2+}, Mg^{2+} and Zn^{2+} can thus form ionic linkages between negatively charged sites on two adjacent proteins (Figure 5). The addition of Ca^{2+}, Mg^{2+} and Zn^{2+} ions may contribute to the strengthening of surimi gels. Marangoni *et al*. (2000) investigated the structure of gels as a function of protein and salt concentration and found that particle size decreases as a function of increasing protein concentration and increases as a function of increasing salt concentrations. Moreover, the rate of aggregation formed in the presence of $CaCl_2$ was greater than that formed in the presence of NaCl, possibly due to the cross-linking of Ca^{2+} (Marangoni *et al*., 2000).

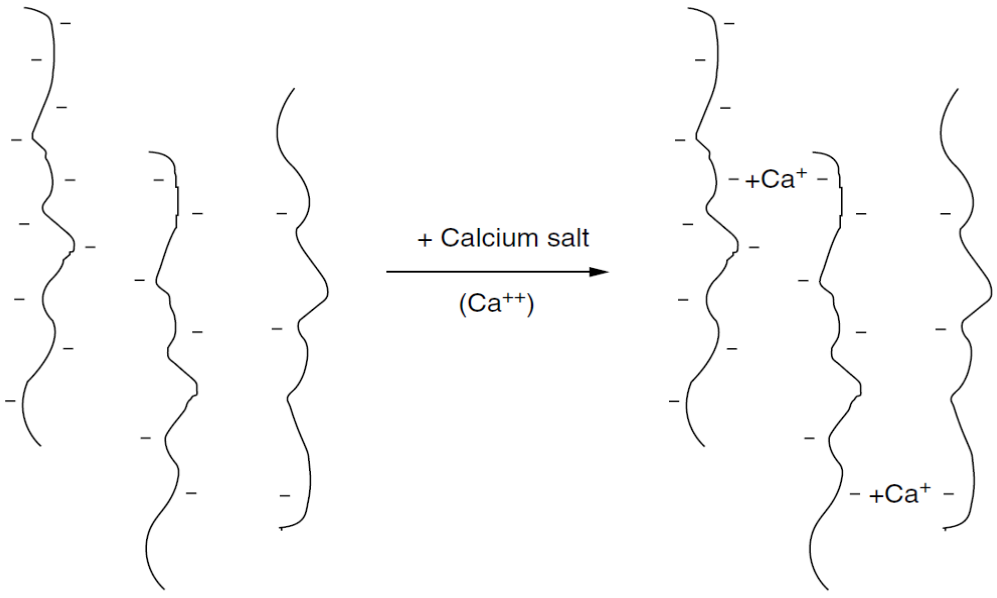


Figure 5. Formation of calcium induced salt bridge between proteins.
Source: Lanier *et al*. (2005)

1.2.6 Zinc and role in human nutrition and application

1.2.6.1 Human nutrition

Zinc is one of the essential trace elements and is one of the major subgroups of the micronutrients that have attained such prominence in human nutrition and health (Cole and Lifshitz, 2008; Maret and Sandstead, 2008). Over 300 proteins require zinc for their functions in microorganisms, plants and animals. The functions of zinc can be broadly divided into three categories: catalytic, structural, and regulatory (Russell *et al*., 2002). Tuerk and Fazel (2009) suggested that zinc is a critical component of the catalytic site of metalloenzymes, including pancreatic carboxypeptidases, carbonic anhydrase, alkaline phosphatase, RNA polymerases, and alcohol dehydrogenase. In its structural role, zinc is reported to coordinate with certain protein domains, facilitating protein folding and producing structures such as 'zinc fingers' (Bressler *et al*., 2007). In this manner, zinc is crucial to the production of biologically active molecules. Russell *et al*., (2002) confirmed zinc involvement in the structure and stabilization of some enzymes, such as superoxide dismutase, etc. Zinc is essential to the regulation of a number of biological processes, including gene regulation. Zinc regulates the expression of metallothionein. Metallothionein has multiple different functions, including intracellular zinc compartmentalization (Maret, 2003) and antioxidant function (Tuerk and Fazel, 2009). Zinc is especially important during periods of rapid growth, both pre and postnatally, and for tissues with rapid cellular differentiation and turnover, such as the immune system and the gastrointestinal tract (Sandstead *et al*., 2008). The results of multiple community-based intervention trials indicate that zinc supplementation decreases the incidence of diarrhea and pneumonia among young children (Hess *et al*., 2009), and clinical treatment studies have shown that zinc supplementation during diarrhea reduces the severity and duration of such illnesses (Hess *et al*., 2009). Zinc is essential for brain growth and its function (Sandstead *et al*., 2000). Capasso *et al*. (2005) reported that zinc homeostasis in the brain is closely associated with neurological diseases and is spatiotemporally altered in process of neurological diseases (Barnham and Bush, 2008).

The prevalence of zinc deficiency worldwide is estimated at more than 20% (Wuehler *et al*., 2005). It may affect over two billion people in the developing world. A study of 14,770 individuals aged 3–74 years estimated the prevalence of zinc deficiency in the USA at 1–3% (Tuerk and Fazel, 2009). Approximately 50% of the world population is at the risk of zinc deficiency (Brown *et al*., 2001) and 10% of the North American population consumes less than half the recommended daily allowance for zinc (King *et al*., 2000). Zinc deficiency in children is a nutritional and health problem in both developing and developed countries (Bryan *et al*., 2004).

Zinc nutrition is based on the quantity and bioavailability of zinc in food. Worldwide, pulses and cereals are the major sources of zinc for most people (Gibson, 1994a). Walsh *et al*. (1994) reported that pulses and cereals provide about 30%, meat about 50%, and dairy products about 20% of dietary zinc in the US. Usually, pulses are richer in zinc than refined cereals. Flesh foods are the most important dietary sources of readily bioavailable zinc. Red meat is the richest common source of zinc while fowl and fish provide less zinc (Gibson, 1994b). Maret and Sandstead (2006) reported that preference for poultry, fish and dairy products instead of red meat increases the risk of zinc deficiency.

The amount of zinc needed daily is relatively small, about 2–3 mg in adults, i.e. only 1/1000 of the total is renewed daily, in agreement with a biological half-life of zinc of about 280 days (Maret and Sandstead, 2006). Healthy adults have an absolute need for 2–3mg zinc per day to compensate for the relatively small loss of zinc in urine, stool, and sweat (Sandstead and Smith, 1996). The current RDA for men is 11 mg, and for women are 8 mg (Brown *et al*., 2010). These values are thought to be adequate for 97–98% of the US population. Noteworthy, the remaining 2–3% corresponds to 5–7.5 million Americans that could be at risk (Hambidge, 2001). In addition to gender, the recommendations are stratified for age and conditions of greater metabolic need such as pregnancy and lactation. For vegetarians, the requirement is at least 50% higher because zinc is not readily available from a vegetarian diet (Gibson, 1994b).

1.2.6.2 Use of zinc as antimicrobial agent

Nowadays zinc oxide (ZnO) nanoparticles have received considerable interests of both academia and industries owing to their attractive physicochemical properties. ZnO has been the focus of photocatalysts with UV irradiation because of its physical and chemical stability, need to the body, low cost, availability and nontoxicity (Yamamoto *et al*., 1998). ZnO has been widely used as functional filler in biopolymer films, in UV-absorbers for application in pharmaceutical materials, cosmetics, coating materials, and pigments (Kumar and Singh, 2008; Li *et al*., 2009b). ZnO has good photocatalytic activity, high stability, antibacterial property, and non-toxicity (Wang, 2004). It was also reported that ZnO was found not to be cytotoxic to cultured human dermal fibroblasts (Zaveri *et al*., 2009). ZnO nanostructures have many important applications in biomedical fields (Riggio *et al*., 2010), piezoelectric biosensors, food additives, and catalysis (Brunner *et al*., 2006).

ZnO is an inexpensive inorganic compound and is currently listed as a generally recognized as safe (GRAS) material by the Food and Drug Administration and is used as food additive. ZnO is currently being investigated as an antibacterial and antifungal agent in both microscale and nanoscale formulations (Espitia et al., 2013; Nair *et al*., 2009). ZnO nanoparticles (ZnO NPs) have higher antimicrobial effects on microorganism like *Staphylococcus aureus* than any other metal oxide nanoparticles (Jones *et al*., 2008). ZnO nanoparticles exert biocidal effects on bacterial, fungal, and viral species (Adams *et al*., 2006). ZnO NPs are effective for inhibiting both Gram-positive and Gram-negative bacteria. They even have antibacterial activity against spores that are high-temperature and high pressure resistant (Sawai *et al*., 1996). Smaller ZnO particles have a better antibacterial activity (Sawai *et al*., 1996; Makhluf *et al*., 2005). The antibacterial activity depends on the surface area and concentration, while the crystalline structure and particle shape have little effect. The higher the concentration and the larger the surface area, the better the antibacterial activity is (Yamamoto *et al*., 1998). High temperature treatment of ZnO particles has a significant effect on their antibacterial activity (Sawai *et al*., 1996). Tam *et al*. (2008) studied antibacterial activity of ZnO nanorods and found that ZnO exhibited good activity against *Escherichia coli* and *Bacillus atrophaeus*. ZnO NPs

possess antimicrobial activities against *Listeria monocytogenes* and *E. coli* O157:H7 in culture media (Jiang *et al*., 2009). ZnO NP could potentially be used as an effective antibacterial agent to protect food related bacteria *Bacillus subtilis, Escherichia coli* and *Pseudomonas fluorescens* (Jiang *et al*., 2009). There are also other studies confirming the strong antimicrobial activity of ZnO nanoparticles wherein the nanoparticles could completely lyse the food-borne bacteria *Salmonella typhimurium* and *Staphylococcus aureus* (Liu *et al*., 2009). ZnO NPs (12 nm) inhibited the growth of *E. coli* by disintegrating the cell membrane and increasing the membrane permeability (Jin *et al*., 2009). Polyurethane films incorporated with ZnO (27 nm) had antimicrobial activity against *E. coli* and *B. subtilis*. *E. coli* was more sensitive to the developed nanocomposite material (Li *et al*., 2009a). A strong affinity of the nanoparticles with *E. coli* cells contributes to antibacterial activity of ZnO. Chitosan and polyvinyl alcohol (PVA) films incorporated with ZnO NPs (25–30 nm) showed antibacterial activity against *S. aureus* (Vicentini *et al*., 2010).

Although metal oxide NPs has been widely studied for their antibacterial properties, there are almost rare studies regarding their antifungal activities. Sawai and Yoshikawa (2004) found that ZnO powder (at the microscale) exhibits a very weak antifungal activity against *C. albicans*. Though growth inhibition was observed, conductivity change was observed only at concentration above 100 mg ml^{-1}. Fang *et al*. (2006) found that ZnO in a 'whisker' form inhibit the growth of *C. albicans.* There are relatively few agents that can be used to treat fungal infections (Bolard, 1996). ZnO nanorods (30 nm in diameter and 500 nm in length) deposited onto film surface showed antifungal activity against *C. albicans* (Eskandari *et al*., 2011). The advantage of using ZnO nanoparticles is that they strongly inhibit the action of pathogenic fungi when used in small concentrations. Moreover these are durable and show great selectivity and heat resistance (Sharma *et al*., 2010a). The toxic effects of ZnO NPs towards the pathogenic species of bacteria and fungi are enhanced on prolonged contact between the microbial cell membrane and the nanoparticles. The bacterium and fungal lipid bilayer gets ruptured due to cytotoxic behavior of ZnO nanoparticles resulting in the drainage of the cytoplasmic contents (Sharma *et al*., 2010a).

The mechanisms regarding the antimicrobial activity of ZnO particles are not well understood but the generation of hydrogen peroxide is a main factor of the antibacterial activity (Sawai *et al*., 1998). Stoimenov *et al*. (2002) indicated that the binding of the particles on the bacteria surface due to the electrostatic forces could be a mechanism. Recently Eskandari *et al*. (2011) suggested the formation of reactive oxygen species (ROS), especially H_2O_2, on the surface of ZnO as responsible for its antimicrobial activity. Moreover, it has been suggested that the zinc ion release, membrane disfunction, and nanoparticles internalization are the main cause of cell swelling (Nair *et al*., 2009). The summarized mechanism of antimicrobial activities of nanoparticles is shown in Figure 6.

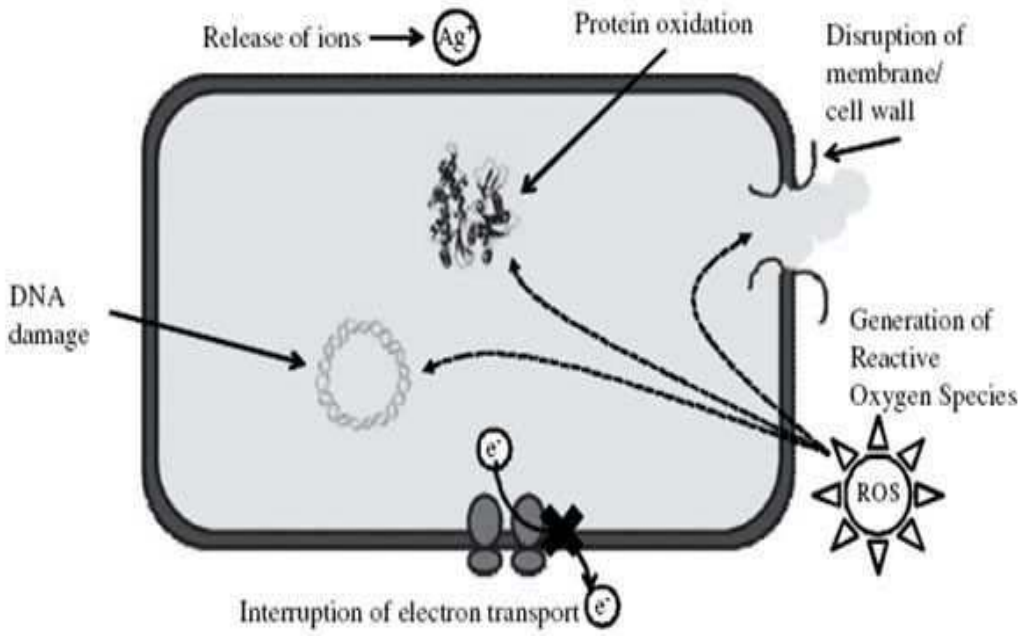


Figure 6. Various mechanisms of antimicrobial activities exerted by nanomaterials. Source: Li *et al*. (2008b)

1.2.7 Biodegradable film

Biodegradable film and coating have been received the increasing attention owing to their biocompatibility and they can serve as alternative packaging to synthetic polymers or plastics. Almost food packagings are generally made from plastics, which are non-biodegradable synthetic polymers and have the negative impact on environment (De Mulder-Johnston, 1997; Rhim and Ng, 2007; Kanmani and Rhim, 2014). Biodegradable or compostable packaging is preferable to recyclable packaging because recyclable packaging, though better than non-recyclable packaging, still requires external energy to be provided to bring about the recycling process. Nevertheless, biodegradable or compostable packaging is difficult to be recycled (Cuq *et al*., 1995a; Guilbert and Gontard, 1995; Guilbert, 2002). Over the last decade, there has been a widespread interest in films made from renewable and natural polymers which can degrade naturally and more rapidly than petroleum-based plastics. Among all biopolymers, proteins have been paid increasing attention as a potential material for biodegradable films and coating. Biopolymeric materials used for biodegradable films can be divided into 4 categories: biopolymer hydrocoilloids (proteins and polysaccarides), lipids, resins and composites (Krochta *et al*., 1994). Physical and chemical characteristics of the biopolymers greatly influence the properties of resulting films and coatings (Sothornvit and Krochta, 2000). In general, plasticizers are required to increase the flexibility of film by lowering the interaction between polymers. Films can be incorporated with other additives for different purposes.

1.2.7.1 Principle and factors affecting the protein film formation

Proteins are thermoplastic heteropolymers containing 20 amino acids. They are macromolecules with specific amino acid sequences and there are limitless number of sequential arrangements with a wide range of interactions and chemical reactions (Pommet *et al*., 2003). All structures of proteins can be easily modified by heat, pressure, irradiation, mechanical treatment, acids, alkalines, metal ions, salts, chemical hydrolysis, enzymatic treatment and chemical cross-linking (Han *et al*., 2005; Krochta, 2002). Proteins are commonly used as film-forming materials. The

most distinctive characteristics of proteins compared to other film-forming materials are conformational denaturation, electrostatic charges and amphiphilic nature. Many factors can affect the conformation, charge density and hydrophilic-hydrophobic balance of proteins, thereby influencing the physical and mechanical properties of prepared films and coatings (Bajpai *et al*., 2008). In addition, properties of protein based-films depend on various factors such as the source of protein, pH of protein solution, plasticizers, film thickness, preparation conditions, formation process and additives incorporated into the film forming solutions (Limpan *et al*., 2010; Cuq *et al*., 1996; Hoque *et al*., 2011a; Sobral *et al*., 2005). Proteins used as film-forming materials are derived from both animal and plant sources, such as animal tissues, milks, eggs, grains and oilseeds (Krochta, 2002).

Protein based-film can be formed in three steps (Marquie and Guilbert, 2002):

1. Break intermolecular bonds (non-covalent and covalent bonds) that stabilize polymers in their native forms by using chemical or physical rupturing agents (by solubilization or thermal treatment). Polymer chains become mobile.

2. Arrange and orient mobile polymer chains in the desired shape.

3. Allow the formation of new intermolecular bonds and interactions to stabilize the three-dimensional network. The shape obtained in step 2 is maintained by eliminating agents used in step 1 (e.g., solvent removal or cooling).

Based on these three steps, solvent process is based on dispersing and solubilizing the proteins in various solvents and then casting, spraying or dipping, followed by drying. This process has been extensively studied and applied to produce films from various proteins (Cuq *et al*., 1995a).

Figure 7 describes potential chemical and physical approaches to the modification of film forming mechanisms by altering film-forming raw materials, varying film-forming processing conditions and applying treatments on formed films. Potential chemical methods of modifying the film-forming mechanisms of protein

based films include pH changes, salt addition, heat denaturation, solvent changes, chemical modification of the side chains of peptides, cross-linking and hydrolysis of peptides (Yildirim and Hettiarachchy, 1997; Were *et al*., 1999), and the addition of foreign proteins (Denavi *et al*., 2009).

Physical modifications of edible films and coatings include lamination, formation of composites, addition of particles or emulsions, perforation, over-coating, annealing heat curing (Micard *et al*., 2000; Miller *et al*., 1997), orientation, radiation (Gennadios *et al*., 1998; Micard *et al*., 2000) and ultrasound treatment (Banejee *et al*., 1996).

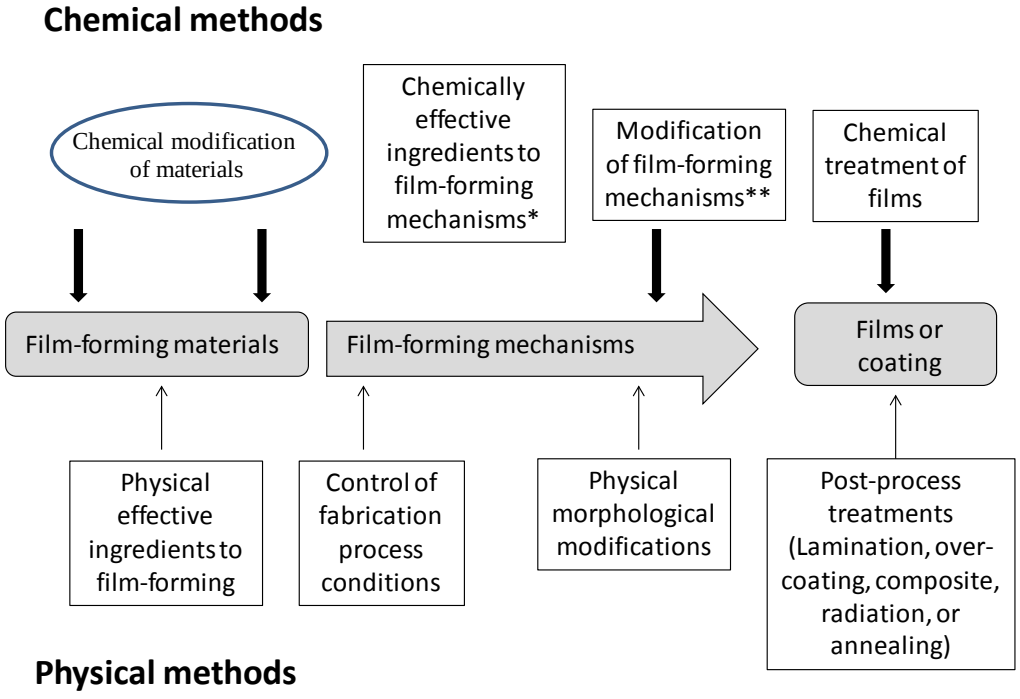


Figure 7. Various ways for modifying the characteristics of edible films and coatings.
* indicates the addition of chemically or physically active ingredients, which may enhance or interfere with the film-forming mechanisms.
** includes any chemical cross-linking, chemical substitution of side chains to create hydrophobic interactions or electrostatic interactions and other extra mechanisms caused by chemical modifications.
Source: Han *et al*. (2005)

Physico-chemical properties of proteins determine the behavior of proteins during preparation, processing, storage and consumption. The determination of most physical and mechanical characteristics of film structures is related to physical chemistry parameters, which include mechanical strength, elasticity, moisture and gas permeation, cohesion of polymers, film adhesion onto food surfaces, surface energy, surface roughness/smoothness, light transmittance, color (opaque/gloss), viscosity, thermoplastic characteristics and others (Sothornvit and Krochta, 2000). Cohesion of film-forming materials is a very important parameter that influences the mechanical strength of films, especially homogeneously continuous film structures (Guilbert *et al*., 1996). Cohesion is the attractive force between molecules of the same substance (Anonymous, 1992). If the film-forming materials contain heterogeneous ingredients that are not compatible with the main biopolymers, the cohesion of the film forming materials decreases and the film strength weakens. When the use of new biopolymers or additives is investigated, the compatibility of all film-forming ingredients should be maintained to obtain strong cohesion (Han *et al*., 2005).

Plasticizers are the agents reducing the cohesion of film-forming polymers (Guilbert *et al*., 1996). The addition of a plasticizing agent to edible films is required to overcome film brittleness caused by intensive intermolecular forces. The increase in mobility of polymer chains can improve the flexibility and extensibility of the films (Limpan *et al*., 2010; Gontard *et al*., 1993). A variety of common plasticizers used in edible films include glycerol, polyethylene glycol (PEG), sorbitol, propylene glycol (PG) and ethylene glycol (EG), monosaccharide, disaccharide or oligosaccharide, lipids and their derivatives (Yang and Paulson, 2000). The addition of plasticizers affects not only the elastic modulus and other mechanical properties, but also the resistance of edible films and coatings to permeation of vapors and gases (Paschoalick *et al*., 2003; Sothornvit and Krochta, 2001). For protein films, plasticizers disrupt inter- and intra-molecular hydrogen bonds, increase the distance between polymer molecules, and reduce the proportion of crystalline to amorphous region (Krochta, 2002). The addition of hydrophilic chemical plasticizers to films can reduce water loss through dehydration, increase the amount of bound water, and

maintain a high water activity. Sobral *et al*. (2005) studied the effect of plasticizer concentrations (15-65% based on protein) in FFS (pH 2.7) on the physical properties of edible films based on muscle protein of Thai Tilapia.

1.2.7.2 Myofibrillar protein based films

Fish myofibrillar proteins have been used as film-forming materials (Chinabhark *et al*., 2007; Shiku *et al*., 2003; Tongnuanchan *et al*., 2011b). The edible films or biodegradable films based on fish myofibrillar protein have been developed by solution casting process (Sobral *et al*., 2005; Cuq *et al*., 1995b). Cuq *et al*. (1995b) found that the pH and protein concentration had strong interactive effects on viscosity of film forming solution (FFS) from Atlantic sardines myofibrillar protein. The functional properties of the resulting film were slightly better than other protein based films (such as whey protein, soy protein, wheat protein and corn zein films), with tensile strength close to those of low density polyethylene films. Inter and intra-interaction between protein molecules, such as hydrogen bonds, ionic-ionic interactions, hydrophobic interactions and covalent bonds, could be formed during drying condition (Chinabhark *et al*., 2007; Iwata *et al*., 2000). Properties of muscle protein-based film from different fish species is shown in Table 3.

Generally, acid or alkaline solubilization is required for preparation of film forming-solution. Films prepared from myofibrillar proteins are flexible and semi transparent and their mechanical properties are altered with pH used for solubilization (Chinabhark *et al*., 2007; Hamaguchi *et al*., 2007; Tongnuanchan *et al*., 2011a). The removal of undesirable components and increasing concentration of myofibrillar proteins by washing could improve the properties of films (Artharn *et al*., 2007). Protein film formation is pH dependent. Limpan *et al*. (2010) reported that pH of film-forming solutions had an effect on film formation of films based on fish myofibrillar proteins. Myofibrillar protein-based films were formed between pH range of 2-3 and 7-12, whereas films were not formed between pH 4 and 6 because of the poor protein dispersion around the isoelectric point (Shiku *et al*., 2003). TS of the films was higher whereas EAB was almost constant irrespective of pH. WVP of myofibrillar protein films was slightly lower than that of other protein-based films and

was higher than that of synthetic films. The myofibrillar protein films prepared at acidic and basic conditions had strong protein networks and their transparency was similar to that of synthetic films. Tongnuanchan *et al*. (2011b) reported that TS of red tilapia muscle protein films prepared at pH 3 was higher than that of films prepared at pH 11 for both of washed and unwashed mince ($P < 0.05$). Cuq *et al*. (1995b) examined the effects of protein concentration, pH, temperature and storage time before casting film. The optimal condition for preparing film-forming solution based on Atlantic sardine myofibrillar protein was at pH of 3, 2 g protein/100g protein solution, 25 ºC and 6 h storage time. The TS of the film was close to that of low density polyethylene films.

Protein concentration in filmogenic solution can also influence the formation of protein matrix. Sobral *et al*. (2005) reported that the effect of protein concentration was observed mainly upon the mechanical properties of Thai tilapia muscle protein films. The films prepared with 2 g of protein/100g of FFS had more force resistance than those with 1 g of protein/100g of FFS. Chinabhark *et al*. (2007) reported that protein concentration and pH affected the mechanical properties and color of myofibrillar protein film from bigeye snapper surimi. Protein content influenced the mechanical properties and color of films. Film with greater protein content (2%) prepared at acidic condition exhibited higher tensile strength. Similar elongation at break was found between film with 1% and 2% protein content at the same pH used. The film with 2% protein content had more yellowness but lower lightness than those having 1% protein content and the film was more transparent when the lower protein was used.

Protein based-films have excellent oxygen, carbon dioxide, and volatile compounds barrier properties in comparison with synthetic film under low relative humidity condition (Limpan *et al*., 2010). Barrier property of protein films decrease with increasing film thickness. However, barrier property can be varied with the source of protein, which can be associated with amino acid composition (Miller *et al*., 1997). Like other protein-based films, fish myofibrillar protein films have inferior functional properties to synthetic counterparts. They possess high water absorptivity and water vapor permeability, owing to hydrophilicity of amino acids in protein

molecules and to the significant amounts of hydrophilic plasticizers, such as glycerol and sorbitol, incorporated into the films to impart adequate film flexibility (Tongnuanchan *et al*., 2011a; Prodpran *et al*., 2007; McHugh *et al*., 1994). Moreover, as compared to the synthetic films, myofibrillar protein films have relatively poor mechanical properties as fish myofibrillar protein (FMP) by itself forms brittle film, due to the presence of strong covalent bonding especially disulfide bond, which is useless in application (Limpan *et al*., 2010). The usual approach to increase flexibility of FMP film is to add a hydrophilic plasticizer, which is a low-molecular-weight nonvolatile substance, into the film to reduce protein–protein interaction. However, plasticizer added also decreases the strength and water vapor barrier property of films (Hoque *et al*., 2011a; Cuq *et al*., 1995b).

Fish muscle protein-based films are susceptible to yellow discoloration during the extended storage (Artharn *et al*., 2009; Tongnuanchan *et al*., 2011b, c). Yellow discoloration of fish muscle protein-based film was mainly caused by non-enzymatic browning reaction or Maillard reaction (Artharn *et al*., 2009; Limpan *et al*., 2010; Tongnuanchan *et al*., 2011b). Tongnuanchan *et al*. (2011b) reported that lipid oxidation played an essential role in yellow discoloration of fish muscle protein film by providing the carbonyl groups involved in Maillard reaction.

Table 3. Properties of muscle protein-based film from different fish species

Fish Species	Protein Conc.	Mechanical property TS (MPa)	EAB (%)	WVP ($x10^{-11}$g m^{-1} s^{-1} Pa^{-1})	References
Round scad (*Decapterus maruadsi*)[1]	2% (w/v) (pH= 3)	2.0 - 7.85	138.49 - 159.43	0.89 - 1.22	Artharn *et al*., 2007
Red tilapia (*Oreochromis niloticus*)[2]	2% (w/v) (pH= 3)	6.98 - 14.46	42.79 - 74.15	3.15 - 4.46	Tongnuanchan *et al*., 2011b
	(pH= 11)	6.34 - 12.28	49.01 - 80.39	3.31 - 4.58	
Bigeye snapper (*Priacanthus tayenus*)[3]	2% (w/v) (pH=3)	6.97	32.95	9.95	Limpan *et al*., 2012
Blue marlin (*Makaira mazara*)	2% (w/v) (pH 2 –12)	8.0 - 16.7	27.6 - 32.4	6.6 - 8.6	Shiku *et al*., 2003
Cape hake (*Merluccius capensis*)[4]	2% (w/v) (pH=11)	4.13 - 6.16	111.2 - 147.9	3.8 - 5.92	Pires *et al*., 2011

[1] FFS from different muscle types (ordinary, dark and whole); [2] FFS from washed mince and FPI prepared at pH 3 and 11; [3]Muscle protein solution with or without different concentration of poly (vinyl alcohol); [4]FFS prepared by adding 0.025, 0.05, 0.1and 0.25 ml thyme oil/g protein.

1.2.7.3 Fish protein isolate based films

The production of protein isolates with improved stability and functionality from fish by-products and low value underutilized fish species are of great interest in the fish industry. The pH-shift process is a one method for making functional protein isolate from underutilized muscle protein resources (Hultin and Kelleher, 2000a) (Figure 8). An acid or alkaline solubilization process is applied to solubilize muscle proteins at either high or low pH. The solubilization of proteins makes it possible to remove unwanted high-density components, such as bones, scales, connective tissues, cell membranes, low-density components, such as neutral lipids, by centrifugation. The solubilized muscle proteins are then collected and recovered by precipitating them at their isoelectric point (Kristinsson and Hultin, 2003; Kristinsson *et al*., 2005; Undeland *et al*., 2002). Although different amount of

membrane lipids can be removed during acid and alkali-aided protein isolation, the residual membrane phospholipids still readily undergo oxidation in the presence of strong pro-oxidants like Hb (Nolsøe and Undeland, 2009). The use of neutral antioxidants, may offer a novel approach to enhance the oxidative stability of fish protein isolates. Marmon and Undeland (2010) reported that protein isolates from herring (*Clupea harengus*) prepared by both acid and alkaline pH-shift processes had a significantly whiter color and higher protein and lower in ash, Ca, Mg and lipid contents than the starting material. The removal of pigments such as melanin and heme proteins during pH-shift processing would thus be of great interest to increase the possibilities to utilize protein isolates originating from dark muscle fish species (Marmon and Undeland, 2010). Higher protein yields, and greater lipid and pigment reductions of protein isolate from tilapia muscle were achieved with the acid and alkaline aided processes than with the conventional washing process (Rawdkuen *et al*., 2009). Chaijan *et al*. (2010b) reported that gel of short-bodied mackerel (*Rastrelliger brachysoma*) protein isolate prepared using alkaline-aided process showed the higher breaking force than conventional surimi, possibly due to the partial denaturation of protein after alkaline treatment. Some sulfhydryl groups exposed underwent oxidation during heat treatment and disulfide bond was formed in gel network. On the other hand, proteins from some fish species were extremely denatured during pH-shift process, particularly in acidic condition (Choi and Han, 2002; Rawdkuen *et al*., 2009), resulting in poor gelation.

For film making, protein isolates has been shown as the promising starting material, in which the discoloration was tackled and mechanical properties could be improved. Tongnuanchan *et al*. (2011a) reported that films from FPI of red tilapia muscle had higher tensile strength (TS) and elongation at break (EAB) than those from washed mince for both pH (3 and 11) used for film preparation ($P < 0.05$). Tongnuanchan *et al*. (2011a) found that FPI from red tilapia muscle had the marked decreases in lipid, heme, and non-heme iron contents. At the same pH used for film preparation (3 or 11), films from FPI showed the lower TBARS values than those from washed mince ($P < 0.05$). With lower contents of lipid and prooxidants, yellow

discoloration of fish myofibrillar protein-based film could be lowered during extended storage (Tongnuanchan *et al*., 2011a).

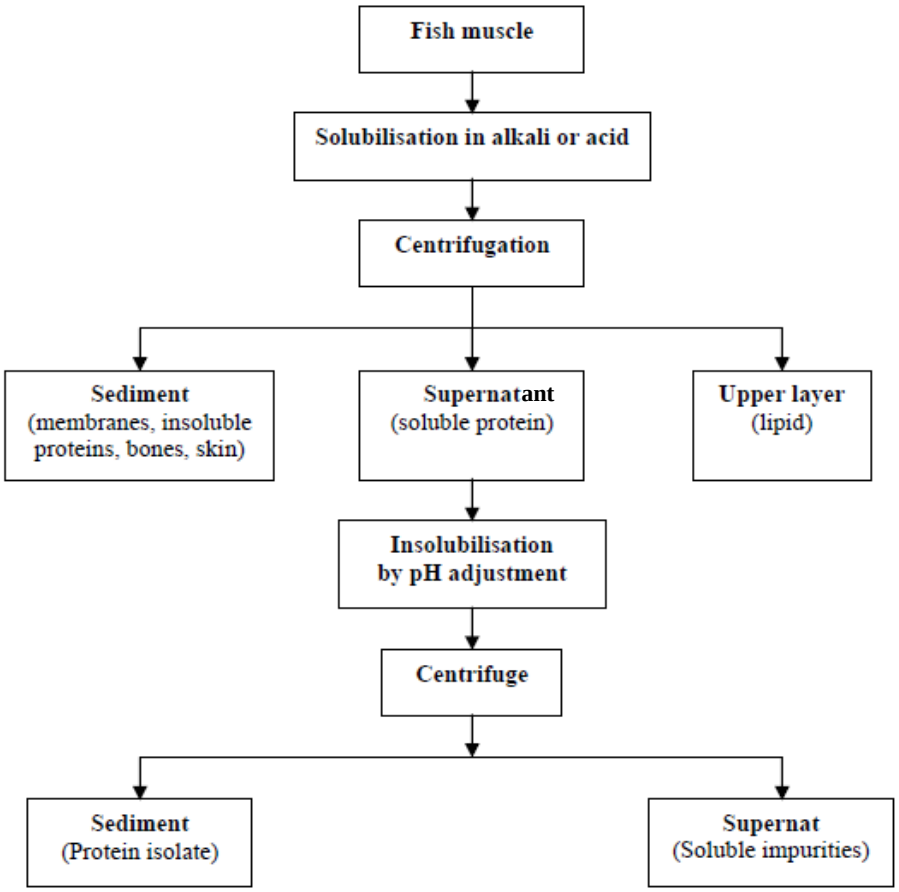


Figure 8. Scheme of pH shift process for production of fish protein isolate.
Source: (Hultin *et al*. 2005)

1.2.7.4 Gelatin based films

Gelatin is a denatured protein derived from collagen by thermo-hydrolysis and has a rheological property of thermo-reversible transformation between sol and gel (Cho *et al*., 2005). Gelatin is obtained by thermal denaturation or physical and chemical degradation of collagen. The process involves the disruption of non-covalent bonds and it is partially reversible in agreement with the gelling properties of gelatin (Bigi *et al*., 1998). During the collagen-to-gelatin transition, many covalent bonds are broken along with some covalent inter- and intra-molecular

bonds (Shiff-base and aldo condensation bonds). This results in conversion of the helical collagen structure to a more amorphous form, known as gelatin. These changes lead to the denaturation of the collagen molecule but not to the point of a completely unstructured product (Foegeding *et al*., 1996).

Gelatin can be extracted from skin and bone of animal origins. Nowadays, fish gelatin has gained increasing attention. Gelatin from different fish species, including bigeye snapper (Benjakul *et al*., 2009), unicorn leatherjacket (Ahmad and Benjakul, 2012a), yellowfin tuna (Rahman *et al*., 2008), shark (Cho *et al*., 2004) and grass carp (Kasankala *et al*., 2007) etc. have been produced and showed varying molecular and functional properties. On a dry weight basis, gelatin consists of 98 to 99% protein. The molecular weight of these large proteins typically ranges between 20,000 and 250,000 Da (Kennan, 1994). The predominant amino acid sequence is Gly-Pro-Hyp (Poppe, 1997). Gelatin contains relatively high levels of following amino acids: glycine (Gly) 26-34%; proline (Pro) 10-18%; and hydroxyproline (Hyp) 7-15% (Poppe, 1997). Other significant amino acids include alanine (Ala) 8-11%; arginine (Arg) 8-9%; aspartic acid (Asp) 6-7%; and glutamic acid (Glu) 10-12% (Poppe, 1997). Gelatin is not a nutritionally complete protein. It contains no tryptophan and is deficient in isoleucine, threonine, and methionine (Potter and Hotchkiss, 1998). Sulfur-containing amino acids, cysteine and cystine, are also deficient in gelatin. Gelation is an important functional property of gelatin, more likely governed by amino acid composition and chain length (Gómez-Guillén, *et al*., 2002). Hydrolysis of collagen produces gelatin with molecular weights from 3 to 200 kDa depending on the raw material used and the extraction conditions (Lacroix and Cooksey, 2005). The strength of the gel formed is proportional to the square of the concentration of gelatin and directly proportional to molecular weight (Burghagen, 1999). Circular dichroism analysis reveals that gelling involves a refolding of denatured collagen chains into the typical triple helix conformation and conversely unfolding upon reheating. The folding process seems to be directly related in the stabilization of the gels without disregarding its role in triggering the gelation process (Gómez-Guillén, *et al*., 2002).

Gelatin has attracted the attention for the developments of edible films due to its abundance and biodegradability (Hoque *et al*., 2011b; Jongjareonrak *et al*., 2006; Sobral *et al*., 2001). Gelatin films have been used in various fields such as an ideal delivery system for a wide range of medicine, food industry as a sausage casing component or coating materials with antioxidants. Gelatin-based films with various additives have good potential for applications in a number of integrated optics devices such as holographic recording materials (Arvanitoyannis *et al*., 1998a; Iwamoto *et al*., 1999).

Fish skin gelatin can be used as film forming material, but properties of film vary depending on the source of gelatin, plasticizer and other factors (Table 4). Nevertheless, heat activated metallo- and/or serine-proteinases/collagenases were present in fish skin gelatin and decreased the mechanical properties of gelatin film via the hydrolysis of the peptide or protein chain, particularly during preparation process (Jongjareonrak *et al*., 2006). Various attempts have been carried out to improve protein film properties. Chemical and physical treatments can be applied to modify the polymer network through cross-linking of the polymer chains to improve protein film functionality. The chemical agents used for cross-linking agents include aldehyde, gossypol, different types of phenolic acid and others (Cao *et al*., 2007). Various physical methods have been developed to improve the mechanical properties of film including a dehydrothermal treatment, ultraviolate, and gamma irradiation (Bigi *et al*., 1998; Chambi and Grosso, 2006).

Table 4. Properties of gelatin-based film from different fish species

Fish Species	Protein Conc.	Mechanical property TS (MPa)	EAB (%)	WVP ($x10^{-10}$g m^{-1} s^{-1} Pa^{-1})	References
Bigeye snapper (*Priacanthus tayenus*) [1]	2% (w/v)	10.04 - 11.43	12.11 - 5.98	0.89 - 1.28	Rattaya *et al*., 2009
Blue shark (*Prionace glauca*)	2% (w/v)	23.30 - 45.90	1.57 - 80.40	0.71- 1.05	Limpisophon *et al*., 2009
Bigeye snapper (*Priacanthus marcracanthus*)	3% (w/v)	7.97 - 57.34	3.04 - 50.30	1.31- 2.73	Jongjareonrak *et al*., 2006
Cuttlefish (*Sepia pharaonis*) [2]	3% (w/v)	4.99 - 9.66	15.56 - 51.89	0.92 - 1.30	Hoque *et al*., 2010
Tilapia fish [3]	Gelatin 5 g/100 ml	101.23 - 109.76	5.08 - 6.81	1.75 - 2.4	Pranoto *et al*., 2007
Alaska pollock (*Theragra chalcogramma*[4]	Gelatin 5% (w/w)	45.9 - 50.1	3.23 - 3.44	0.73 - 0.86	Chiou *et al*., 2008
Alaska pink salmon (*Oncorhynchus gorbuscha*)[5]	Gelatin 5% (w/w)	49.7 - 60.0	3.36 - 3.8	0.85 - 1.08	Chiou *et al*., 2008
Tuna-fish (*Thunnus tynnus*) [6]	Gelatin 2 g/100ml	2.75 - 5.91	3.56 - 13.77	1.83 - 2.87	Gómez Guillén *et al*., 2007
Unicorn leatherjacket (*A. monoceros*) [7]	Gelatin 3% (w/v)	21.21-43.82	3.06-8.76	1.0-1.94	Ahmad et al., 2012a

[1]FFS with 6% seaweed extract at pH 6 and 9; [2] FFS with different heating temperatures (40 – 90° C); [3] Gelatin solution added with gellan and *k*-carrageenan (1 and 2 g/100) of gelatin; [4,5] FFS prepared by adding 0.25%, 0.50%, and 0.75% (w/w) glutaraldehyde; [6]FFS added with murta extracts; [7]FFS added with various ratios of bergamot and lemongrass essential oils.

1.2.7.5 Blend and nanocomposite biopolymer films

1.2.7.5. 1 Blend films

Polymer blend technique is a mixing of two or more polymers together to produce blend, for achieving a specified portfolio of physical properties without the need to synthesize specialized polymer system (Debeaufort *et al*., 1998; Were *et al*., 1999; Yildirim and Hettiarachchy, 1997). Polymer blend is one of the effective methods for providing new desirable polymeric materials for a variety of applications. Biodegradable blend films, which contain mixture of biopolymer and other biodegradable polymers, either natural or synthetic, have been developed to improve the properties of blend films. Poly(vinyl alcohol) (PVA), has been used for the implementation of the mechanical properties of films based on polysaccharides and proteins, since its hydrophilic and filming character allows for some degree of compatibility with functional natural polymeric materials (Limpan *et al*., 2010; Kim *et al*., 2002). Various biopolymers can be mixed together to form a film with unique properties that combine the most desirable attributes of each component (Wu *et al*., 2002). A hypothetical scheme showing the formation of a binary blend from its constituent polymers is shown in Figure 9. Solution blending, the common method used to prepare protein blend films (Bajpai and Shrivastava, 2004) can be carried out by dissolving the two components in a common solvent and precipitating out the blend by addition of a suitable precipitant or by removal of the solvent by evaporation, for example, in cast films.

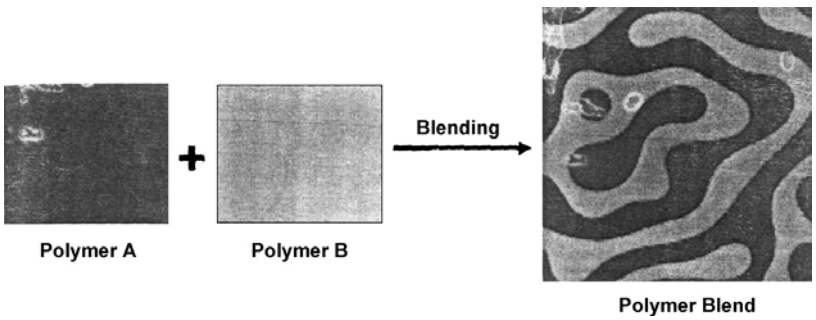


Figure 9. Hypothetical scheme depicting preparation of polymer blend.
Source: Bajpai *et al*. (2008)

Protein based edible film is rather brittle, and has relatively poor mechanical properties. The properties of fish protein isolate films may be improved by blending with gelatin, starch, sodium alginate, whey protein isolation, etc. (Cao *et al*., 2007; Sabato *et al*., 2001; Tang *et al*., 2003). Gelatin is the polymer of choice being used to blend with various biopolymers due to their great compatibility (Cao *et al*., 2007). With increasing gelatin ratio in the soy protein isolate/gelatin blend films, tensile strength (TS), elongation to break (EB), elastic modulus (EM) and swelling property were increased (Cao *et al*., 2007). In addition, the films became more transparent, and easier to handle. The blend film of konjac glucomannan and gelatin was prepared successfully by using the solvent-casting technique with different blending ratios of the two polymers. The blend film had the best miscibility, a good tensile strength, heat-seal and the least water–vapor transmission ratio at the same ratio. All blend films showed high water solubility (Li *et al*., 2006). The incorporation of increasing amounts of mesquite gum (MG) in whey protein isolate (WPI) and MG blend film resulted in improved flexibility with significantly lower tensile strength and higher elongation at break. Therefore, MG can be used as an alternative of using larger amounts of low molecular weight plasticizers (Osés *et al*., 2009). Physical properties of biodegradable films based on blends of gelatin and PVA were improved where PVA hydrolysis degree and concentration have an important effect for the elongation at break, puncture deformation and solubility in water (Carvalho *et al*., 2009).

1.2.7.5.2 Nanocomposite film

Nanomaterials have attracted an increasing attention owing to their potential role in a wide range of industries and packaging markets (Liang *et al*., 2006; Rhim and Ng, 2007). Moreover, nanosized material fillers improve polymer properties when compared with single polymer or micro-scale composites. Nanofillers have excellent interfacial interactions on polymer branches due to their large specific surface area and high surface energy, thus significantly enhancing polymer properties (Rouhi *et al*., 2013; Kovacevic *et al*., 2008). The potential improvements include enhanced mechanical strength, weight reduction, increased heat resistance and improved barrier properties (Kanmani and Rhim, 2014; Ray and Okamoto, 2003).

Biodegradable nanocomposites were successfully fabricated from corn starch and montmorillonite (MMT) nano-clay by melt extrusion processing (Tang *et al*., 2008). Sothornvit *et al*. (2009) reported the effect of nano-clay types (Cloisite Na+, Cloisite 20A and Cloisite 30B) on the properties of whey protein isolate (WPI)/clay composite films. The nanocomposite films exhibited an opaque appearance and haze, and degree of this effect depended on type of nano-clays added. The type of nano-clay used significantly influenced the tensile and water vapor barrier properties. Tensile properties (tensile strength, tensile modulus and elongation at break) of WPI/Cloisite Na^+ or 30B composite films did not significantly decrease as compared with those of WPI pure film. Bae *et al*. (2009) investigated the influences of clay content, speed of homogenization, pH and ultrasonication on the mechanical and barrier properties of fish gelatin/montmorillonite nanocomposite films. The addition of 5% nano-clay (w/w) increased tensile strength and oxygen and water barrier properties of films. The ultrasonically treated nanocomposite films exhibited an exfoliated type structure with improved tensile strength and barrier properties, and the films produced were uniform in thickness and relatively transparent. Rhim *et al*. (2005) studied the mechanical and water barrier properties of composite films of SPI with various clay minerals. The tensile strength of SPI-layered nano films increased by 30% whereas the water vapor permeability decreased by 52%. Chen and Zhang (2006) prepared highly exfoliated and intercalated SPI–MMT nanocomposites by using the solution exfoliation method in a neutral aqueous medium and investigated the correlation between the microstructure and mechanical properties. Vicentini *et al*. (2010) reported that chitosan poly (vinyl alcohol) films containing ZnO nanoparticles **(**CS/PVA–ZnO–T80) films had the increase in the tensile strength for both dried and wet films, while the elongation decreased for the dried films and increased for the wet films by approximately 100%.

Antimicrobial packaging based on nanotechnology is one of the most promising active packaging systems, quite effective in killing or inhibiting spoilage and pathogenic micro-organisms contaminated in foods (Emamifar *et al*., 2011). Antimicrobial films are usually made from proteins, lipids, and polysaccharides which are non-toxic, bio-compatible, and biodegradable (Reinose *et al*., 2008).

Antimicrobial films are being used to control microbial growth in food ingredients. These films contain antimicrobial agents (Dutta *et al*., 2009) which belong to a wide range of organic/inorganic compounds (Eswaranandam *et al*., 2004), essential oils (Chaibi *et al*., 1997), enzymes (Gucbilmez *et al*., 2007), fruit extracts (Conte *et al*., 2007), etc. However, due to the development of new resistant strains of microbes to current antimicrobials (Singh *et al*., 2008), these conventional antimicrobial agents have been losing their effectiveness. Thus, the current research is focused to search new potential antimicrobials to reduce the harmful effects of microorganism. With the emergence of nanotechnology, the search has now focused on the development of nanostructure of coinage metals like silver, copper, zinc and gold as biocidal agents (Sondi and Salopek-Sondi, 2004).

Nanocomposite film or packaging has been shown to prolong the shelf-life of several food commodities. Fresh asparagus spears coated with AgNP/polyvinylpyrrolidone nanocomposite films had their shelf-life extended to 25 days when stored at 2 °C and had less microorganism (psychotrophic bacteria, yeast and mold) growth during storage (An *et al*., 2008). Li *et al*. (2009b) reported that Chinese jujube fruit stored in food storage bags composed of AgNP/nanoparticulate TiO_2/polyethylene films were firmer, heavier, and had less microbial contamination over a period of 12 days than those stored in control materials. Emamifar *et al*. (2010) confirmed that application of active packaging based on low-density polyethylene (LDPE) containing ZnO nanoparticles prolonged the shelf-life of fresh orange juice up to 28 days by reducing the rate of microbial growth. In another study, LDPE nanocomposite packaging containing Ag and ZnO causes reduction of *Lactobacillus plantarum* growth in orange juice (Emamifar *et al*., 2011). Fernandez *et al*. (2010a) reported that cellulose pads containing AgNPs reduced the microbial levels of exudates from beef meat stored in modified atmosphere packaging. Fresh cut melon stored on AgNP-containing cellulose pads had lower microbial counts (mesophiles, psychrophiles and yeasts) and longer microbial growth lag times (Fernandez *et al*., 2010b). Chawengkijwanich and Hayata (2008) developed a TiO_2 powder-coated oriented-polypropylene (OPP) packaging film and verified its ability to reduce *E. coli* contamination on meat surfaces. The cell number of *E. coli* obtained from the meat

packed in a TiO_2-coated oriented polypropylene film bag decreased from 6.4 to 4.9 log CFU/g after 1 day of storage while that obtained from meat packed in an uncoated film bag only decreased from 6.4 to 6.1 log CFU/g, confirming that the film can be used for shelf-life extension of meat products. ZnO nanoparticle exhibits antimicrobial effects against *L.monocytogenes* and *S.enteritidis* in egg (Jin *et al*., 2009). Bodaghi *et al*. (2013) reported that cell loads of mesophilic bacteria from the pear fruit packaged in a TiO_2 nanocomposite carbohydrate film decreased from 3.14 to less than 2 log CFU/g for a total storage period of 17 days.

1.2.8 Essential oil

Essential oils are volatile oils, which constitute the aroma and flavor components of organic material (Suppakul *et al*., 2003; Greaves *et al*., 2005). In total, approximately 3000 essential oils are known, and approximately 300 of them are considered to be commercially important, in particular for the pharmaceutical, agronomic, food, sanitary, cosmetic, and perfume industries (Bakkali *et al*., 2008). The market for these oils demands a consistently high quality and reliable supplies at competitive prices. Essential oils from aromatic and medicinal plants have been known to be biologically active, mainly possessing antibacterial, antifungal and antioxidant properties (Politeo *et al*., 2007). The main components can represent up to 85% of the total, while the remainder is present as traces. The concentration of the specific compound in the total mix of plant oils can be very variable, depending on factors such as the origin, species and plant organ, climatic conditions and growth, extraction and storage conditions. Essential oils consist mainly of volatile terpenoids, linked isoprene units in structures of 10 carbons (monoterpenoids) and 15 carbons (sesquiterpenoids). The oil is composed of at least 100 different chemical compounds classified as aldehydes, phenols, oxides, esters, ketones, alcohols and terpenes (Fasseas *et al*., 2007). Essential oils are very complex natural mixtures which contain about 100 components at different concentrations. They are characterized by two or three major components at fairly high concentrations (20-70 %) compared to other components present in trace amounts. For example, carvacrol (30 %) and thymol (27 %) are the major components of the *Origanum compactum* essential oil, linalool (68 %) of the *Coriandrum sativum* essential oil, α- and β-thuyone (57 %) and camphor

(24 %) of the *Artemisia herba-alba* essential oil, 1,8-cineole (50 %) of the *Cinnamomum camphora* essential oil, α-phellandrene (36 %) and limonene (31 %) of leaf and carvone (58 %) and limonene (37 %) of seed *Anethum graveolens* essential oil, menthol (59 %) and menthone (19 %) of *Mentha piperita* (= *Mentha* × *piperita*) essential oil. Generally, these major components determine the biological properties of the essential oils. The components include two groups of distinct biosynthetical origin (Croteau *et al*., 2000). The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, which are characterized by low molecular weight (Figure 10).

1.2.8.1 Antioxidative activity

The antioxidant property of essential oils and components has been frequently confirmed by in vitro physical–chemical methods (Hussain *et al*., 2008; Bakkali *et al*., 2008). Due to their hydrophobicity in nature, essential oils can be dissolved in oils or lipids and function as antioxidant, preventing lipid oxidation (Hussain *et al*., 2008). The antioxidant activities of essential oils from savory, thyme and basil (Lee and Shibamoto, 2002), cloves and nutmeg (Dorman *et al*., 2000), and rosemary and cinnamon (Dang *et al*., 2001) are similar to those of synthetic antioxidants. Dorman *et al*. (1995) screened *Pelargonium* sp., *Monarda citriodora var. citriodora*, *Myristica fragrans*, *Origanum vulgare ssp. hirtum* and *Thymus vulgaris* for their antioxidative effect using a thiobarbituric acid (TBA) assay. The oils showed active antioxidant capacities at extremely low levels of dilution. Rosemary has long been recognized as having antioxidative activity and these have been identified as carnosic acid, carnasol, carsolic acid, rosmaridiphenol and rosmarinic acid, found in ethanol-soluble fraction (Nahak and Sahu, 2011). Antioxidant properties are also found in the volatile oil fraction. The biological activities of essential oils depend on their major components. Due to synergistic and antagonistic effects, minor components may play an important role in this respect (Voitkevich, 1999). The antioxidant properties of 98 individual terpenoids and phenol derivatives present as components of essential oils were compared (Ruberto and Baratta, 2000). The antioxidant activities of eugenol, thymol, carvacrol and methoxyphenol were similar to that of α-tocopherol. Cyclic monoterpene hydrocarbons with two double

chains (γ- and α-terpinenes, α-terpinolene and sabinene) were shown to have very high activity (80-98 %) (Ruberto and Baratta, 2000). The presence of these compounds in essential oils determines their antioxidant properties (Nahak and Sahu, 2011). Nevertheless, antioxidative activity of essential oils can be affected by several factors including processing and storage conditions (Hussain *et al*., 2008; Sivropoulou, 1996). Essential oils from sage (*Salvia sclarea* L.), cloves (*Caryophyllus aromaticus* L.), cardamom (*Elettaria cardamomum* Maton), coriander (*Coriandrum sativum* L.), and marjoram (*Majorana hortensis* M.), undergo significant changes throughout storage, largely related to autoxidation (Sivropoulou, 1996).

Essential oil of nutmeg was able to suppress lipid peroxidation in chicken tissue homogenates and egg yolk fat (Dorman *et al*., 1995). Antioxidant of extracted essential oils and phenolic compounds in individual fractions of oregano was studied (Tsimogiannis *et al*., 2006). Oregano extract could stabilize lard and vegetable oils against oxidation (Vekiari *et al*., 1993). Oregano diethyl ether fraction (500 ppm) could decrease the rate of peroxide formation in refined cotton seed oils (Tsimogiannis *et al*., 2006). Oregano has also demonstrated synergism with ascorbyl palmitate on the antirancidity and preservation of α-tocopherol in sunflower oil at 95 °C (Beddows *et al*., 2001). The activity of oregano extracts against 2´2´-azobis dihydrochloride (Zheng and Wang, 2001) and DPPH (Exarchou *et al*., 2002) radicals was also remarkable. In addition, inhibition of lipid oxidation during long-term frozen storage of chicken meat was observed when oregano essential oil was used as a dietary supplement (Botsoglou *et al*., 2003).

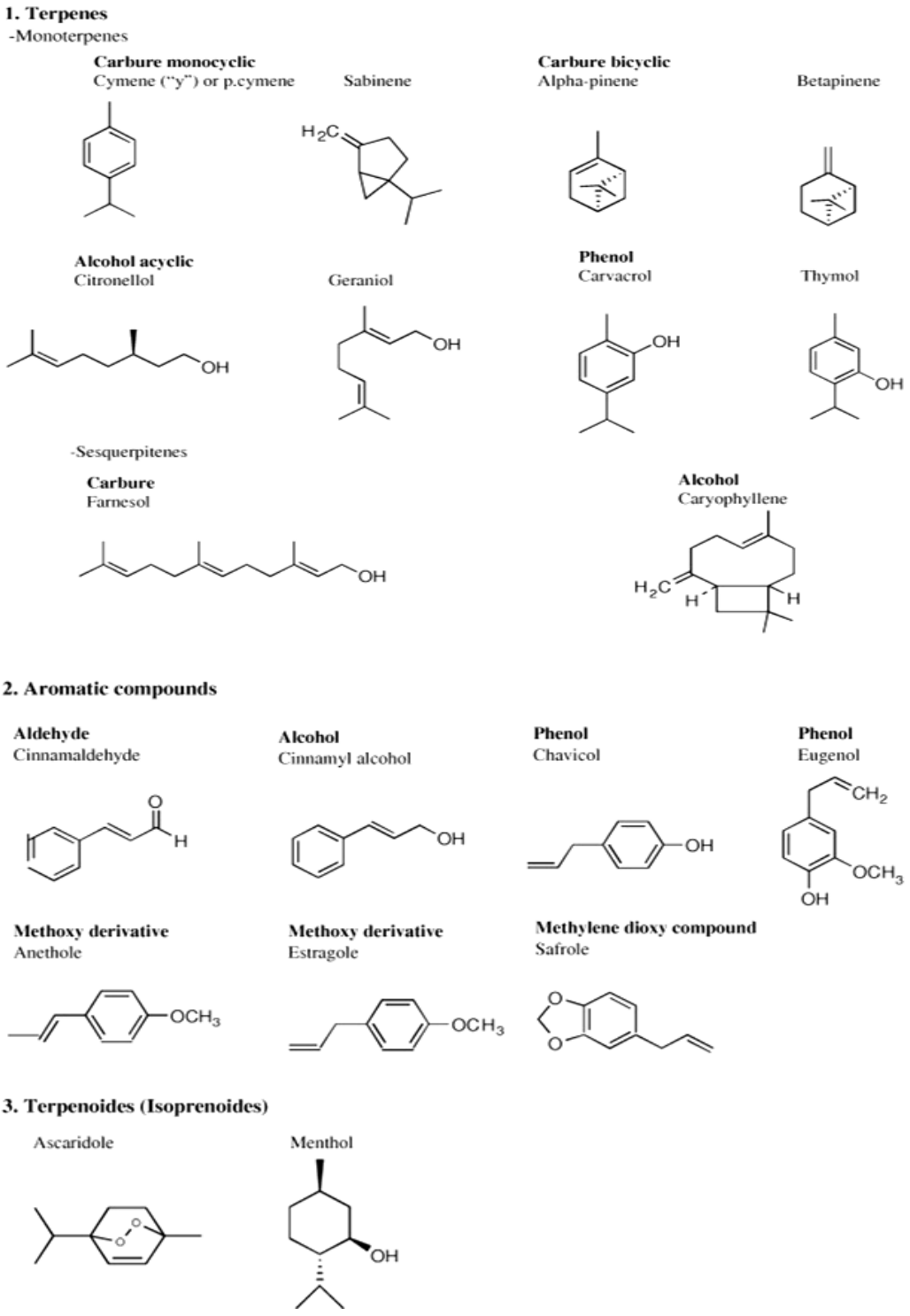


Figure 10. Chemical structures of selected components of essential oils.

Source: Bakkali *et al*. (2008)

1.2.8.2 Antimicrobial activity

Antimicrobial agents can be extracted from various sources such as spices and their essential oils (Beuchat, 1994), food plants (Walker, 1994), and antimicrobial peptides produced by bacteria (Hill, 1995). Plant secondary metabolites, such as essential oils and plant extracts had antimicrobial activities (Tepe *et al*., 2004) and most essential oils derived from plants are known to possess insecticidal, antifungal, antibacterial and cytotoxic activities (Faleiro *et al*., 1999). The antimicrobial activities of the essential oils against the tested bacteria differed, depending on location and seasonal variations (Celiktas *et al*., 2005). Kabuki *et al*. (2000) reported that mango seed kernel ethanol extract was composed of 79.5 % polyphenol and had a broad spectrum antimicrobial activity. The extract was more active against Gram-positive than Gram-negative bacteria including some food borne pathogen. The essential oil of *Lippia origanoides* inhibits the growth of several microorganisms (bacteria; *Staphylococcus* spp., *Lactobacillus casei*, *Streptococcus mutans* and fungi; *Candida* spp., *Cryptococcus neoformans*) (Kabuki *et al*., 2000). The inhibitory effect of several terpenoids on microbial oxygen uptake and oxidative phosphorylation has also been demonstrated (Kabuki *et al*., 2000). Phenolic and non-phenolic alcohols in *L. origanoides* exhibited the strongest inhibitory effects, followed by aldehydes and ketones. The monoterpene hydrocarbons were less active and this behavior depends on the free hydroxyl group from the alcohols (Griffin *et al.*, 1999). Moreover, these spices and herbs, as well as essential oils have been well known to have inhibitory effects against a variety of bacteria including Gram-negative bacteria (Suppakul *et al*., 2003). Carom seed, ginger, Japanese pepper, sage, spearmint and turmeric were additionally found to exhibit antibacterial activities against *V. parahaemolyticus*. Carvacrol and thymol were found to act differently against Gram-positive and Gram-negative species (Dorman and Deans, 2000). The significance of the phenolic ring itself (destabilized electrons) is demonstrated by the lack of activity of menthol compared to carvacrol (Ultee *et al.*, 2002). The addition of an acetate moiety to the molecule appeared to increase the antibacterial activity; geranyl acetate was more active against a range of Gram-positive and negative species than geraniol (Dorman and Deans, 2000). For non-phenolic components of essential oils, the type

of alkyl group has been found to influence activity (alkenyl > alkyl). Components of essential oil also appear to act on cell proteins embedded in the cytoplasmic membrane (Knobloch *et al.*, 1986). Enzymes such as ATPases are known to be located in the cytoplasmic membrane and to be bordered by lipid molecules. Two possible mechanisms have been suggested whereby cyclic hydrocarbons could act on these. Lipophilic hydrocarbon molecules could accumulate in the lipid bilayer and distort the lipid-protein interaction. Alternatively, direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible (Sikkema *et al.*, 1995). Some essential oils have been found to stimulate the growth of pseudomycelia (a series of cells adhering end-to-end as a result of incomplete separation of newly formed cells) in certain yeasts. This could be an indication that essential oils act on the enzymes involved in the energy regulation or synthesis of structural components (Conner and Beuchat, 1984). Cinnamon oil and its components have been shown to inhibit amino acid decarboxylases in *Enterobacter aerogenes*. The mechanism of action was thought to be the binding of proteins (Wendakoon and Sakaguchi, 1995). In general, essential oils are slightly more active against Gram-positive than Gram-negative bacteria (Harpaz *et al*., 2003). Those Gram-negative organisms are less susceptible to the action of antibacterials, since they possess an outer membrane surrounding the cell wall (Ratledge and Wilkinson, 1988), which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Vaara, 1992). Essential oil achieved a greater reduction in the viable count of *S. enteritidis* than for *L. monocytogenes* when added to the Greek appetizers taramosalata and tzatziki (Tassou *et al*., 1995). A study testing 50 commercially available essential oils against 25 genera found no evidence for a difference in sensitivity between Gram-negative and Gram-positive organisms (Deans and Ritchie, 1987). However, a later study using the same test method and the same bacterial isolates but apparently using freshly distilled essential oils, revealed that Gram-positive bacteria were indeed more susceptible to two of the essential oils tested and equally sensitive to four other essential oils than were Gram-negative species (Dorman and Deans, 2000). Individual components of essential oils exhibit different degrees of activity against Gram-positives and Gram-negatives (Dorman and Deans, 2000) and it is known that the chemical composition of essential oils from a particular plant species can vary

according to the geographical origin and harvesting period. Of the Gram-negative bacteria, Pseudomonads, and in particular *P. aeruginosa*, appear to be least sensitive to the action of essential oils (Wilkinson *et al*., 2003).

Essential oils have been included in edible film prepared from various film-forming polymers such as chitosan (Zivanovic *et al*., 2005), milk proteins (Oussalah *et al*., 2004), whey proteins (Fernández-Pan *et al*., 2013) and fish skin gelatin (Ahmad *et al*., 2012a) and have shown promising sources of antimicrobials for food packaging.

1.2.9 Protein based film incorporated with antimicrobial and antioxidants

Active packaging is an innovative concept that can be defined as a type of packaging that changes the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food (Salgado *et al*., 2013; Vermeiren *et al*., 1999). Active packaging, coatings and films prepared from proteins have a variety of advantages such as biodegradability, edibility, biocompatibility, appearance and barrier properties. Protein-based films have been used to improve or maintain the quality of food products (Emiroglu *et al*., 2010; Ahmad *et al*., 2012b). Active packaging technologies includes gas absorbing or emitting packaging (Vermeiren *et al*., 1999), selective permeable films (Yam and Lee, 1995), microwave susceptors (Waite, 2003), etc. Furthermore, enriching these films with functional additives allows nutritional and aesthetic quality aspects to be enhanced without affecting the integrity of the food product. The packaging can serve as a carrier for antimicrobial and antioxidant compounds in order to keep high concentration of preservatives on the food surfaces. Their presence could prevent moisture loss during storage, reduce the rate of rancidity caused by lipid oxidation and brown coloration, reduce the load of spoilage and pathogen microorganism on the surface of foods and restrict the volatile flavor loss. In this connection, a number of recent studies have dealt with extending the functional properties of biodegradable films by adding natural substances with antioxidant or antimicrobial activities in order to yield an active packaging biomaterial (Fernandez-Pan *et al*., 2013). Controlled release packaging is a group of technologies that uses a packaging as a delivery

system to release active compounds such as antimicrobial, antioxidant, enzyme, flavor, and nutraceuticals. Most attention in this group has been focused on antimicrobial packaging (Ahmad *et al*., 2012b) and antioxidant packaging (Wessling *et al*., 2000). Recently, protein-based film was applied to incorporate with several antimicrobial agents to protect microbial spoilage and enhance food quality (Table 5). Whey protein isolate (WPI) films (pH 5.2) containing 0.5-1.5 % sorbic acid (SA) or *p*-aminobenzoic acid (PABA) was able to inhibit *Listeria monocytogenes, E. coli* O157:H7 and *Salmonella* Typhimurium DT104 (Cagri *et al.*, 2004). WPI-based film containing SA or PABA could reduce *L. monocytogenes, E. coli* O157:H7 and *S.* Typhimurium DT104 inoculated on slices of commercially produced bologna and summer sausage after 21 d of storage at 4 °C (Cagri *et al*., 2004). WPI-based edible films (pH 5.2) containing PABA were heat-sealed to form casings. When hotdog was made using the casing containing PABA, *L. monocytogenes* remained relatively unchanged. However, hotdog prepared using casing without PABA had the increase by 2.5 logs (Cagri *et al*., 2004). Whey protein film incorporated with clove essential oil reduced the growth of spoilage microorganisms on chicken breast muscle during cold storage (Fernández-Pan *et al*., 2013). Gómez-Estaca, *et al*., (2010) prepared films from a commercial catfish gelatin mixed with chitosan incorporated with clove essential oil. The films showed the good *in vitro* antimicrobial activity against *Pseudomonas fluorescens, Lactobacillus acidophilus, Listeria innocua* and *Escherichia coli*. The film also delayed the total bacterial counts by 2 log cycles when used for storing raw sliced salmon chilled at 2 °C for 11 days. Edible films with antimicrobial properties have been made from cold-water fish-skin gelatins added with lysozyme, a food-safe antimicrobial enzyme (Bower *et al*., 2006). The lysozyme-enhanced films appeared to exhibit a slight increase in water vapor permeability compared with control films, and they proved effective against Gram-positive bacteria including *Bacillus subtilis* and *Streptococcus cremoris*. However, these films did not inhibit the growth of *E. coli*, because lysozyme cannot penetrate through the lipopolysaccharide layer of Gram-negative bacteria (Masschalck and Michiels, 2003). Rodrigues and Han (2000) incorporated lysozyme, nisin and ethylenediamine tetracetic acid (EDTA) in whey protein isolate (WPI) films. Such lysozyme or nisin-containing films were effective in inhibiting *Brochothrix thermosphacta* but failed to

suppress *L. monocytogenes*. Oussalah *et al*. (2004) studied the antimicrobial and antioxidant effects of milk protein based film containing 1.0 % (w/v) oregano, 1.0 % (w/v) pimento or 1.0 % oregano-pimento (1:1) essentials oils for the preservation of beef muscle and to control the growth of pathogenic bacteria during storage at 4 °C. Film containing oregano was the most effective against *E. coli* O157:H7 and *Pseudomonas* spp, whereas film containing pimento oil was the least effective against these two bacteria. Films containing oregano extracts showed at the end of storage, a 0.95 log reduction of *Pseudomonas* spp level as compared to samples without film. A 1.12 log reduction of *E. coli* O157:H7 level was observed in samples coated with oregano-based films. Ahmad *et al*. (2012b) reported that sea bass slices wrapped with gelatin film incorporated with lemongrass essential oil had the retarded growth of lactic acid bacteria, psychrophilic bacteria and spoilage microorganisms including H_2S-producing bacteria and Enterobacteriaceae throughout storage of 12 days in comparison with the control and those wrapped with gelatin film without LEO. Sunflower protein films incorporated with clove essential oil retarded lipid oxidation and delayed the growth of total mesophiles in refrigerated sardine patties, thus extending shelf-life of fish patties by several days (Salgado *et al*., 2013).

Table 5. Antimicrobials incorporated into protein based films used for food packaging

Antimicrobial agents	Edible packaging materials	Target microorganisms	References
Organic acids			
Sorbic acid, Benzoic acid	Argentine anchovy film	*L. monocytogenes Escherichia coli*	Rocha *et al*. (2014)
Potassium sorbate	Wheat gluten	*Aspergillus niger* , *Fusarium incarnatum*	Türe *et al*. (2012)
Potassium sorbate	Whey protein isolate	*E. coli* O157:H7	Pérez *et al*. (2011)
p-aminobenzoic, sorbic acids	Whey protein isolate	*L. monocytogenes Escherichia coli, Salmonella Typhimurium*	Cagri *et al*. (2001)
Sorbic acid	Wheat gluten	*L. monocytogenes, Escherichia coli*	Redl *et al*. (1996)
Enzyme			
Catechin-lysozyme	Fish skin gelatin	*L. monocytogenes, S. aureus, E. coli*	Rawdkuen *et al*. (2012)
Lysozyme	Whey protein isolate	*L. monocytogenes*	Min *et al*. (2008)
Lactoperoxidase system	Whey protein isolate	*E. coli* O157:H7	Min *et al*. (2007)
Lactoperoxidase system	Whey protein isolate	*L. monocytogenes*	Min *et al*. (2005a)
Lactoperoxidase system	Whey protein isolate	*Salmonella enterica, E. coli* O157:H7	Min *et al*. (2005b)
Lactoferrin, Lactoferrin hydrolyzate, lactoperoxidase system	Whey protein isolate	*Polytrichum commune*	Min and Krochta (2005)
Lysozyme, nisin, EDTA	Soy protein, corn zein	*E. coli, Lactobacillus plantarum*	Padgett *et al*. (1998)

Table 5. (Continued)

Bacteriocins			
Nisaplin and Guardian CS1-50	Gelatin film	*L. monocytogenes*	Min *et al*. (2010)
Nisin	Corn zein, wheat gluten	*L. plantarum*	Dawson *et al*. (2003)
Nisin, lauric acid, EDTA	Corn zein	*L. monocytogenes, Salmonella enteritidis*	Hoffman *et al*. (2001)
Nisin, lauric acid	Soy protein	*L. monocytogenes*	Dawson *et al*. (2002)
Nisin, grape seed extract, malic acid, EDTA	Whey protein isolate	*L. monocytogenes, E. coli* O157:H7, *S.* Typhimurium	Gadang *et al*. (2008)
Pediocin	Corn zein, whey protein isolate	*Listeria innocua*	Quintero-Salazar *et al*. (2005)
Natural extracts			
Oregano and Clove essential oils	Whey protein	Spoilage and pathogenic bacteria	Fernandez-Pan et al. (2013)
Lemongrass essential oil	Gelatin	*Food spoilage and pathogenic bacteria*	Ahmad et al., (2012a)
Clove essential oil	Sunflower protein	*Food spoilage bacteria*	Salgado *et al*. 2013
Grape seed extracts	Pea starch films	*Brochothrix thermosphacta*	Corrales *et al*. (2009)
Oregano, pimento, essential oils	Whey protein isolate	*E. coli* O157:H7	Oussalah *et al*. (2004)
Thyme oil, trans-cinnamaldehyde	Whey protein isolate, soy protein	*P. putida*	Ouattara *et al*. (2001)
oregano oil, lemongrass oil	Alginate-apple puree	*E. coli* O157:H7	Rojas-Grau *et al*. 2007

Antioxidant releasing packaging is a kind of food preservation system, in which an antioxidant or a mixture of antioxidants is incorporated into the package. Antioxidant released in a controlled manner to the food contributes to the shelf-life extension. Since the oxidation is commonly initiated at the food surface, antioxidant-releasing packaging is a promising means to protect the food surface from rancidity. The slow release mechanism also provides a continuous replenishment of antioxidant to the food (Rooney and Yam, 2004). Therefore, a small amount of additive is required (Guilbert *et al*., 1996). Jongjareonrak *et al*. (2008) characterized films with antioxidant properties made from bigeye snapper and brownstripe red snapper-skin gelatin incorporated with α-tocopherol or BHT (butylated-hydroxy-toluene). For identical additive concentrations (200 ppm), the films containing α-tocopherol displayed appreciably higher radical scavenging capacity (DPPH method) than the films containing BHT. Gómez-Estaca *et al.* (2009) reported that films prepared from tuna-skin gelatin, added with oregano and rosemary extracts had the increased antioxidant activity of the films as measured by the FRAP method. Oregano extract showed 1.7-fold higher antioxidant activity than the rosemary extract. As a consequence, the potential antioxidant activity of the films containing the rosemary extract was lower than that of the films containing the oregano extract (Gómez-Estaca *et al*., 2009). Ahmad *et al*. (2012b) reported lower TBARS value in Sea bass samples wrapped with gelatin film incorporated with lemon grass essential oil, compared with those wrapped with control film (without essential oil). The effect of active packaging film based on whey protein containing rosemary extract on the quality of smoked rainbow trout was investigated by Coban and Can (2013). The peroxide value, free fatty acid and thiobarbituric acid content of smoked rainbow trout were significantly higher in the control samples than in samples wrapped with active film during storage. Round scad protein-based film incorporated with palm oil and chitosan has been used as a packaging material to prevent lipid oxidation of dried fish powder (Artharn *et al*., 2009). Salgado *et al*. (2013) reported lower lipid oxidation in fish patties wrapped with sunflower protein films incorporated with clove essential oil.

1.3 Objectives

1. To study gelling characteristics of surimi from yellow stripe trevally (*Selaroides leptolepis*).

2. To elucidate the impact of zinc salts on heat-induced gelation and gel properties of surimi from yellow stripe trevally.

3. To investigate gel strengthening effect of zinc sulphate in phosphorylated protein isolate from yellow stripe trevally.

4. To prepare and characterize blend films based on fish protein isolate and fish skin gelatin.

5. To study the characteristics and antimicrobial properties of fish protein isolate/fish skin gelatin-zinc oxide (ZnO) nanocomposite films.

6. To prepare antimicrobial fish protein isolate/fish skin gelatin film containing basil leaf essential oil and zinc oxide nanoparticles and to use for shelf- life extension of refrigerated sea bass slices.

CHAPTER 2

Gelling characteristics of surimi from yellow stripe trevally (*Selaroides leptolepis*)

2.1 Abstract

Gelling properties of surimi prepared from yellow stripe trevally (*Selaroides leptolepis*) were studied. Setting temperature shows significant effect on textural properties and cross-linking of myofibrillar proteins. Kamaboko gel with prior setting at 40 °C (K40) exhibited the highest breaking force, followed by another kamaboko gel having setting temperature of 25 °C (K25), directly heated gel and modori gel, respectively ($P < 0.05$). For deformation, both kamaboko gels showed the lower values than both directly heated gel and modori gel ($P < 0.05$). The lowest expressible moisture content and whiteness were found in K40 sample ($P < 0.05$). The optimal temperature of yellow stripe trevally muscle transglutaminase (TGase) was found to be 40 °C when determined using monodansyl cadaverine incorporation method. Thus, the enhanced TGase activity at 40 °C contributed to the maximized setting of K40 sample. The highest TCA-soluble peptide content with decreased myosin heavy chain (MHC) was found in modori surimi gel ($P < 0.05$). Nevertheless, some degradation was found in kamaboko gels. Since polymerisation occurred to a much higher extent, a strengthened gel matrix could be developed in both kamaboko gels. K40 sample had finer and more ordered fibrillar structure with smaller voids than other gels. Therefore, setting at 40 °C for an appropriate time should be a promising means to improve gelling properties of surimi produced from yellow stripe trevally.

2.2 Introduction

Thailand is one of the largest surimi producers in Southeast Asia. About 16 surimi factories are located in Thailand, with a total production of 96,500-1, 13,500 metric tons per year of which 80% is exported to Japan and Korea and the remainder to Singapore and other countries (Hong and Eong, 2005). In general, lean

fish have been used for surimi production. Due to the limited fish resources, especially lean fish, pelagic dark fleshed fish have been paid more attention as a potential alternative raw material for surimi production (Chaijan *et al*., 2004). Dark fleshed fish make up 40–50% of the total fish catch in the world (Hultin and Kelleher, 2000) and the catch of those species in the Gulf of Thailand was approximately 844.2 metric tons in 2006 (Department of Fisheries, 2006). Due to the abundance and lower price, these pelagic fish can be used for surimi production. However, those pelagic fish have high content of dark flesh associated with the high lipid and myoglobin contents (Chaijan *et al*., 2004). Those components contribute to the difficulties in making high-quality surimi (Chen, 2002; Ochiai *et al*., 2001). The presence of sarcoplasmic proteins of dark muscle also contributes to the poorer gelation (Haard *et al*., 1994). Sarcoplasmic proteins are able to bind with myofibrillar proteins, thus interfering the formation of strong gel network. In addition, lipid oxidation seems to be a distinct problem in surimi made from some dark fleshed fish (Lanier, 2000; Wu *et al*., 2000).

Setting temperature has been reported to play an essential role in the formation of protein cross-links mediated by endogenous transglutaminase (TGase) (Kumazawa *et al*., 1995; Seki *et al*., 1990). Benjakul and Visessanguan (2003) reported that optimal setting temperatures varied with species. Surimi from *Priacanthus tayenus and Priacanthus macracanthus* showed the maximized setting at 40 °C and 25 °C, respectively. Setting response can be varied, depending on fish species (Shimizu *et al*., 1981; Araki and Seki, 1993). The setting phenomenon is related to habitat temperature of fish species (Morales *et al*., 2001). Setting at different temperatures may lead to different gel characteristics, especially with different fish species. However, the incubation of surimi paste at 60-65 °C mainly resulted in gel weakening or ‘modori’ phenomenon (Benjakul *et al*., 2003b). This phenomenon is caused by endogenous heat-activated proteases (An *et al*., 1996; Benjakul *et al*., 1997). Generally, gel weakening is varying with fish species. Dark fleshed fish also had a high proteolytic activity, resulting in high susceptibility to gel weakening (Shimizu *et al*., 1992).

Yellow stripe trevally (*Selaroides leptolepis*) is one of the abundant dark fleshed fish species caught in Southern Thailand. Therefore, the use of this pelagic fish for surimi production is one of challenges in transforming the underutilized fish into value-added products, particularly surimi. This species has been used for protein hydrolysate production with antioxidative activity (Klompong *et al*., 2007). However, no information regarding the gel properties of surimi from this species have been reported. Therefore, the objective of this study was to investigate gel-forming ability of surimi from yellow stripe trevally (*Selaroides leptolepis*) prepared with different heating conditions.

2.3 Materials and methods

2.3.1 Chemicals

Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, *N*,*N*,*N'*,*N'* – tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA). Trichloroacetic acid was purchased from Merck (Darmstadt, Germany). Monodansyl cadaverine (MDC), *N*,*N'*-dimethylated casein (DMC) and dithiothreitol (DTT) were obtained from Sigma Chemical Co., Ltd (St. Louis, MO., USA). All chemicals were of analytical grade.

2.3.2 Fish collection and mince preparation

Yellow stripe trevally (*Selaroides leptolepis*) with an average weight of 65–75 g were caught from Songkhla Coast along the Gulf of Thailand during June-July 2011. The fish, off-loaded approximately 12 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 30 min. The fish were immediately washed, gutted, filleted and de-skinned. Fish flesh was minced to uniformity using a mincer with a hole diameter of 5 mm. Fish and mince were kept in ice during preparation.

2.3.3 Surimi and surimi gel preparation

Surimi was prepared according to the method of Benjakul and Visessanguan (2003) with slight modifications. Fish mince was washed with cold water (4 °C) at a water/mince ratio of 3:1 (v/w). The mixture was stirred gently for 4 min and washed mince was filtered with a layer of nylon screen. The washing process was repeated twice. For the third washing, cold 0.2% NaCl solution was used. Finally, the washed mince was subjected to centrifugation using a Model CE 21K basket centrifuge (Grandiumpiant, Belluno, Italy) pre-cooled with ice at a speed of 700 x g for 10 min. The temperature of dewatered mince was approximately 8-10 °C. To the dewatered mince, 4% sucrose and 4% sorbitol were added and mixed uniformly using a kneader (Crypto Peerless Ltd., Birmingham, England). Prior to mixing, the kneader bowl was kept at -20 °C for 1 h, in which the temperature of surimi was maintained below 10 °C throughout the mixing process. The mixture (500 g) was packed in a polyethylene bag and frozen using an air-blast freezer (-20 °C). Frozen surimi was stored at -20 ºC for not longer than 1 month.

To prepare the gels, the frozen surimi was tempered at 4 ºC for 3–4 h until the core temperature reached 0 ºC. The sample was then cut into small pieces and the moisture content was adjusted to 80% by the addition of iced water. The mixture was added with 2.5% (w/w) NaCl and chopped for 5 min in a walk-in cold room at 4 ºC to obtain the homogeneous sol. The sol was then stuffed into a polyvinylidine casing with a diameter of 2.5 cm and both ends of the casing were sealed tightly (Benjakul *et al*., 2003a). The sol was then incubated at 40 ºC for 30 min, followed by heating at 90 ºC for 20 min in a temperature-controlled water bath (Memmert Gmbh Co. KG, Schwabach, Germany). The obtained gel was referred to as ‘kamaboko gel-40 ºC; K40’.The gel prepared by incubating the sol at 25 ºC for 3 h, followed by heating at 90 ºC for 20 min was referred to as ‘kamaboko gel-25 ºC; K25’. Modori gel was prepared by incubating the sol at 60 ºC for 30 min, followed by heating at 90 ºC for 20 min (Benjakul *et al*., 2010). A directly heated gel was prepared by heating the sol at 90 ºC for 20 min. After heating, all gels were immediately cooled in iced water for 30 min and stored for 24 h at 4 ºC prior to analysis.

2.3.4 Determination of gel properties

2.3.4.1 Textural analysis

Textural analysis of gel samples was performed using a Model TA-XT2i texture analyser (Stable Micro Systems, Godalming, Surrey, UK). Gels were equilibrated and evaluated at room temperature (28–30 °C). Five cylinder-shaped samples with a length of 2.5 cm were prepared and subjected to determination. Breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyser equipped with a spherical plunger (diameter 5 mm, depression speed of 60 mm/min).

2.3.4.2 Determination of whiteness

All gels were subjected to whiteness measurement using a Hunterlab (ColorFlex, Hunter Associates Laboratory, Reston, VA). Illuminant C was used as the light source of measurement. *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were measured and whiteness was calculated as described by Park (1994) as follows:

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{\frac{1}{2}}$$

2.3.4.3 Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al*. (2003c) with slight modifications. A gel sample with a thickness of 0.5 cm was weighed (X g) and placed between two pieces of Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, England) at the top and three pieces of the same type of filter paper at the bottom. The standard weight (5 kg) was placed on the top of the sample and maintained for 2 min. The sample was then removed and weighed again (Y g). Expressible drip was calculated and expressed as percentage of sample weight as follows:

$$\text{Expressible moisture (\%)} = \left[\frac{(X-Y)}{X}\right] \times 100$$

2.3.4.4 Determination of TCA-soluble peptide content

TCA-soluble peptide content was determined according to the method of Benjakul *et al*. (2010). To 3 g of finely chopped gel samples, 27 ml of cold 5% (w/v) TCA were added and homogenized for 2 min using an IKA homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was allowed to stand in ice for 1 h and centrifuged at 8000 x g for 10 min. TCA-soluble peptides in the supernatant were measured according to the Lowry method (Lowry *et al*., 1951) and expressed as µmole tyrosine/g sample (Morrissey *et al*., 1993).

2.3.4.5 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of surimi and surimi gels were determined by SDS–PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) hot SDS (85°C) solution were added to the sample (3 g). The mixture was then homogenized at speed of 11,000 rpm for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The sample was centrifuged at 8000 x g for 20 min at room temperature (26–28 °C) using a centrifuge (Model MIKRO20, Hettich ZENTRIFUGEN, Tuttlingen, Germany). Protein concentration in the supernatant was determined as per the method of Lowry *et al*. (1951). Solubilized samples were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol and 10% βME) and boiled for 3 min. Samples (15 µg protein) were loaded onto polyacrylamide gels comprising a 10% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein III unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.02% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid. A protein standard (Bio-Rad Laboratories, Inc., Richmond, CA, USA) containing myosin (206 kDa), β-galactosidase (116 kDa),

phosphorylase B (97.4 kDa), serum albumin (66.2 kDa) and ovalbumin (45 kDa) was used to estimate the molecular weight of the proteins.

2.3.4.6 Scanning electron microscopy (SEM)

Microstructure of surimi gels prepared with different heating conditions was determined using a scanning electron microscope (JE0L JSM-5800 LV, Tokyo, Japan). Samples with a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h. The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with serial concentrations of 50, 70, 80, 90 and 100% (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater, SPI-Module, West Chester, PA, USA). The specimens were visualized with a SEM at an acceleration voltage of 15 kV.

2.3.5 Characterisation of endogenous TGase in yellow stripe trevally muscle

2.3.5.1 Preparation of TGase crude extract

Fish flesh was homogenized with four volumes of extraction buffer (10 mM NaCl, 2 mM DTT, 10 mM Tris–HCl, pH 7.5). The homogenate was centrifuged at 16,000 x g using a refrigerated centrifuge (Beckman Coulter, Allegra 25R, Palo Alto, CA, USA) for 20 min at 4 °C. Subsequently, the supernatant obtained was centrifuged at 18,000 x g for 60 min at 4 °C. The supernatant was used as ‘crude TGase extract’.

2.3.5.2 Study on temperature profile of TGase

TGase activity was measured in terms of the incorporation of MDC into DMC according to the procedure of Takagi *et al*. (1986) with a slight modification. To study the temperature profile, the assay was performed at various temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C) for 30 min. The reaction mixture containing 100 µl of 2 mg/ml DMC, 100 µl of 0.5 mM MDC, 100 µl of 0.2 M DTT, 0.4 ml of 0.1 M $CaCl_2$ and 2.4 ml of 0.1M Tris–HCl (pH 7.5) was prepared. The reaction was initiated by the addition of 0.2 ml of crude TGase extract and the

mixture was incubated at different temperatures for 30 min. The reaction was terminated by the addition of 0.2 ml of 1.0 M ammonium sulphate. Fluorescence intensity of MDC incorporated into DMC was measured with a spectrofluorophotometer (RF-1501, Shimadzu, Kyoto, Japan) at excitation and emission wavelengths of 350 and 480 nm, respectively. Blanks were prepared in the same manner, except ammonium sulphate was added prior to the addition of crude extract. TGase activity was calculated after blank subtraction and expressed as the increase in fluorescence intensity upon the incorporation of MDC into DMC.

2.3.6 Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, U.S.A.).

2.4 Results and discussion

2.4.1 Properties of gel from surimi of yellow stripe trevally prepared using different heating conditions

2.4.1.1 Textural property

Breaking force and deformation of surimi gels with various heating conditions are shown in (Figure 11). Different heating conditions rendered gels with different properties. Among all gels tested, kamaboko gel with setting at 40 °C (K40) had the highest breaking force, followed by another kamaboko gel (K25) ($P < 0.05$). Breaking force of K40 was 37.96, 134.99 and 118.2 % higher than that of K25, modori and directly heated samples, respectively. Breaking force of directly heated gel was lower than that of kamaboko gel, but was higher than that of modori gel ($P < 0.05$). The results indicated that protein–protein interactions stabilized by strong bonds were established in kamaboko gels. For deformation, it was noted that kamaboko gels, either with prior setting at 25 or 40 °C, showed the lower values than

both directly heated gel and modori gel ($P < 0.05$). The result suggested that kamaboko gels had slightly lower elasticity than other two gels. The decreased deformation of kamaboko gels was concomitant with their higher breaking force. The rigid and hard gel governed by the strong network generally losses its elasticity (Park *et al*., 2005). It was noted that gel of surimi from yellow stripe trevally showed the high deformation (16.33-19.10 mm), compared with those reported in surimi from other fish species including bigeye snapper (*Priacanthus macracanthus)* (8-10 mm) (Benjakul *et al*., 2002a) frigate mackerel (6-10 mm), Indian mackerel (9-10 mm) (Chaijan *et al*., 2010).

Setting played an important role in cross-linking of gel network, especially by non-disulphide covalent bonds induced by endogenous TGase (Kumazawa *et al*., 1995; Seki *et al*., 1990). The differences in setting response at different temperatures were presumed to be due to the differences in protein and TGase stability. TGase was found to mediate myosin cross-linking via the formation of ε-(γ-glutamyl) lysine linkage (Seki *et al*., 1990). When comparing the setting at high temperature (40 °C) and medium temperature (25 °C), the superior gel was obtained in the former. Protein molecules of yellow stripe trevally might undergo unfolding at 40°C at a greater extent. As a result, more exposure of those reactive residues favored TGase mediated reaction. Tsukamasa and Shimizu (1991) found that heat susceptibility of myosin heavy chain (MHC) was a factor affecting TGase associated setting phenomenon in fish muscle. Additionally, at sufficiently high temperature during setting, hydrophobic interaction might be more enhanced (Niwa, 1992). Without setting, protein cross-linking induced by endogenous TGase might not be enhanced. Furthermore, gradual alignment and aggregation via some weak bonds occurred to some degree. This was reflected by the lower breaking force in directly heated gel in comparison with kamaboko gels. For modori gel, the lowest breaking force was obtained ($P < 0.05$). This result was in agreement with several reports for surimi from other fish species including sardine (Chaijan *et al*., 2004) and Indian mackerel (Chaijan *et al*., 2010). Gel weakening or modori phenomenon has been known to be caused by heat-activated proteases, which are active in temperature range of 60-65 °C (An *et al*., 1996; Benjakul *et al*., 1997). The lower breaking force of

modori gel indicated that endogenous heat-activated proteases were involved in degradation of surimi proteins at 60 °C used in this study.

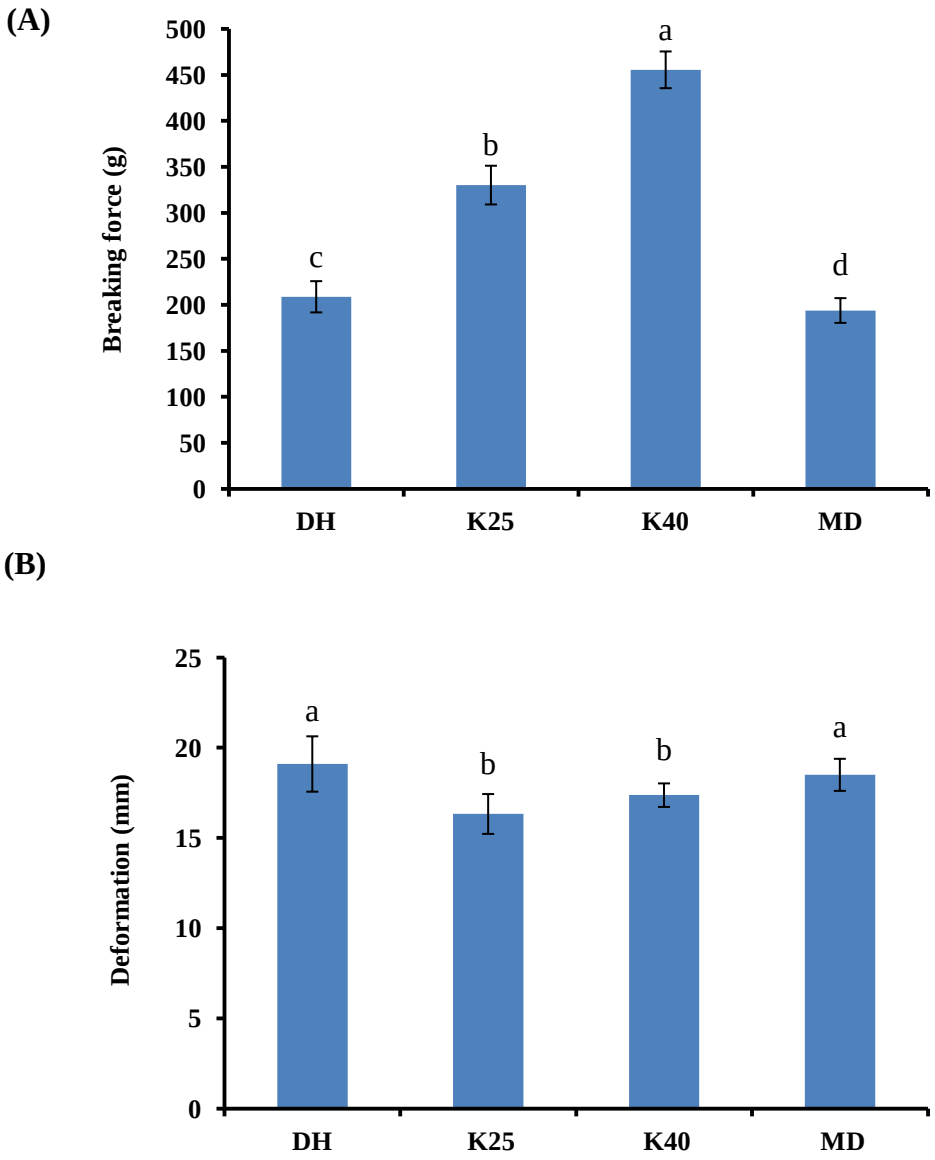


Figure 11. Breaking force (A) and deformation (B) of gels of surimi from yellow stripe trevally using different heating conditions. DH: directly heated gel; MD: modori gel; K25 and K40: Kamaboko gels with setting at 25 and 40 °C, respectively. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant differences ($P < 0.05$).

2.4.1.2 Whiteness

Whiteness of gels prepared from yellow stripe trevally surimi with different heating conditions was in the range of 73.18-74.41 (Table 6). Slight differences in whiteness were observed in different gels ($P < 0.05$). Directly heated gel exhibited the highest whiteness, compared with those prepared by two-step heating ($P < 0.05$). This could be due to non-enzymatic browning, which might take place at higher extent with a longer exposure time used for two-step heating. During heating, not only metmyoglobin formation may cause a decreased whiteness of gels but the Maillard browning reaction may also affect the color of gels (Whistler and Daniel, 1985). Yellow stripe trevally is a dark fleshed pelagic fish and has high lipid content. Lipid oxidation products including aldehydes formed during gelation could be participated in the Maillard reaction (Chaijan *et al*., 2007b). Nevertheless, gels of yellow stripe trevally surimi showed the higher whiteness than those from surimi of other dark fleshed fish including sardines (Chaijan *et al*., 2004) and Indian mackerel (Chaijan *et al*., 2010). Whiteness is an important factor determining the quality and acceptability of surimi gels (Yoon *et al*., 1997).

Table 6. Whiteness and expressible moisture content of gels of surimi from yellow stripe trevally using different heating conditions

Sample	Whiteness	Expressible moisture content (%)
Directly heated gel	74.41±0.08^{*a}	5.30±0.01^{ab}
Kamaboko (K25) gel	73.65±0.11^{b}	4.78±0.01^{bc}
Kamaboko (K40) gel	73.19±0.15^{c}	4.43±0.01^{c}
Modori gel	73.18±0.14^{c}	5.77±0.02^{a}

* Mean ± S.D (n=3). Different superscripts in the same column indicate significant differences ($P < 0.05$).

2.4.1.3 Expressible moisture content

The lowest expressible drip was found in kamaboko gels, both K40 and K25 ($P < 0.05$), indicating the highest water holding capacity (Table 6). Modori gel and directly heated gel had the highest expressible drip ($P < 0.05$), suggesting that protein network of the gel was lower in water-binding capacity (Niwa, 1992). Generally the lowered expressible moisture content was in accordance with the increased breaking force (Figure 11A). During direct heating, rapid unfolding of proteins possibly resulted in more intense coagulation. More water was released from the gel network and the protein dispersion becomes very uneven (Niwa, 1992). Benjakul *et al*. (2010) also reported that kamaboko gel from goatfish surimi showed the higher water holding capacity than modori gel. Therefore, improved water holding capacity of yellow stripe trevally surimi gel could be achieved via appropriate setting prior to heating.

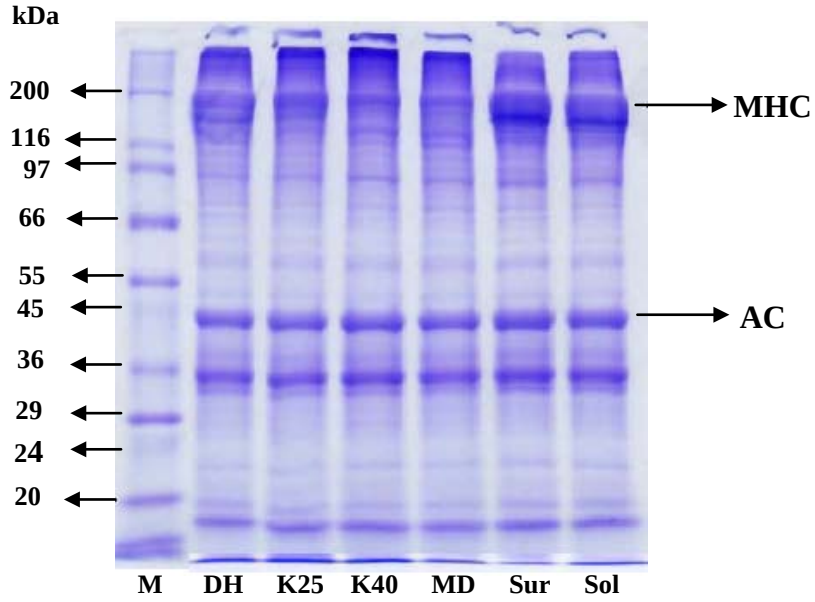
2.4.1.4 Protein Pattern

SDS–PAGE protein patterns of surimi, surimi sol and surimi gels with different heating conditions under reducing conditions are shown in (Figure 12A). MHC constituted as the major protein in surimi and surimi sol as indicated by the highest band intensity. The decrease in MHC was noticeable in all gels. However MHC was more retained in directly heated gel. The result indicated that MHC might undergo either polymerisation or degradation to a lowest extent, compared with other gels. The decrease in MHC after heating was generally due to the polymerisation or degradation (Benjakul and Visessanguan, 2003).

Setting at 25°C or 40 °C prior to heating at 90 °C might allow the protein cross-linking mediated by endogenous TGase to take place effectively, especially when setting was performed at 40 °C. It was noted that protein bands with MW of 120-130 kDa were formed in K40 sample, suggesting that the degradation of MHC occurred to some extent. Therefore, polymerisation and degradation of MHC occurred simultaneously, especially during the setting at 40 °C. However, polymerisation more likely took place to a higher degree than degradation. The ratio

of polymerisation to degradation, which varied among the fish species, directly determined the final gel quality. MHC was most susceptible to cross-linking during setting (Benjakul and Visessanguan, 2003). Benjakul and Visessanguan (2003) reported the decrease in MHC of surimi gel from bigeye snapper, particularly when the setting at high temperature was implemented. Benjakul *et al*. (1997) reported that MHC was also more prone to proteolytic degradation than other muscle proteins, including actin, troponin, and tropomyosin. In modori gel, the disappearance of MHC with the occurrence of new protein bands of MW approximately 110-120 kDa suggested the degradation of MHC during incubation at 60°C. The degradation of MHC in the modori gel was coincidental with the decreased breaking force (Figure 11A). High degradation of MHC was reported in surimi gel from dark fleshed fish including mackerel (Chaijan *et al*., 2010). Alkaline proteases have been reported to contribute to the protein degradation in surimi gels (Boye and Lanier, 1988; Toyohara *et al*., 1987).

(A)



(B)

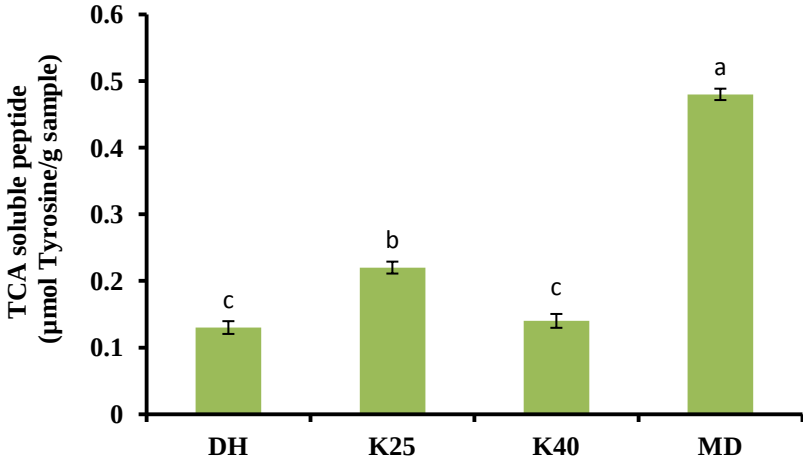


Figure 12. SDS-PAGE patterns (A) and TCA-soluble peptide content (B) of gels of surimi from yellow stripe trevally using different heating conditions. DH: directly heated gel; MD: modori gel; K25 and K40: Kamaboko gels with setting at 25 and 40 °C, respectively. M: marker; Sur: surimi; Sol: surimi sol; MHC: myosin heavy chain; AC: actin. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant differences ($P < 0.05$).

2.4.1.5 TCA soluble peptide content

TCA-soluble peptide content of surimi gels prepared by different heating conditions is shown in (Figure 12B). The highest content of TCA-soluble peptides was found in modori gel ($P < 0.05$), which was in agreement with the highest degradation of proteins as shown in SDS-PAGE (Figure 12A). This indicated that the proteolysis was maximized in modori gel. The highest degradation of muscle proteins led to the marked weakening of the gel as indicated by the lowest breaking force of this gel (Figure 11A). Dark fleshed fish had a high proteolytic activity, resulting in poorer gelation characteristics and high susceptibility to modori (Shimizu *et al*., 1992). Shimizu *et al*. (1992) also reported that the poor gel-forming properties of muscle from dark fleshed species is caused by the presence of heat-stable proteases, which are active in degrading myosin during heating at temperature ranges of 50–70 °C. The directly heated gel had the lower TCA-soluble peptide content than modori gel ($P < 0.05$). Lower TCA- soluble peptide content was also found in kamaboko gels in comparison with modori gel. Bigeye snapper surimi gel, prepared by setting at 40 °C, also contained the degradation products (Benjakul *et al*., 2004b). Takeda and Seki (1996) also found some proteolysis in walleye pollack paste during setting at 25 °C. Polymerized proteins found during setting might be less susceptible to degradation by endogenous proteases. As a consequence, the lower degradation took place in kamaboko gel with prior setting. K25 samples had the higher TCA-soluble peptide content than K40 samples ($P < 0.05$). Polymerized proteins might make gel network more resistant to hydrolysis by proteases. Without incubation or setting, the low TCA-soluble peptide content was observed. This result suggested that slow heating at temperature enhancing proteolytic activity might cause the protein degradation and loss in gel property. Since increased breaking force and deformation were obtained with setting at 25 °C and 40 °C, it was suggested that cross-linking occurred to a greater extent than proteolysis. As a consequence, properties of surimi produced from the yellow stripe trevally could be improved by prior setting.

2.4.1.6 Microstructure

The microstructures of surimi gels prepared by heating at different conditions are illustrated in (Figure 13). Kamaboko gel, particularly K40, showed the higher interconnected three dimensional protein networks. More compact and denser gel network with finer strand observed in K40 sample was coincidental with the higher breaking force (Figure 11) and higher water holding capacity (Table 6). K25 sample showed similar microstructure, but exhibited lower interconnected three-dimensional protein network with larger voids. However, K25 exhibited denser protein network than directly heated gel and modori gels. The directly heated and modori gels had loose protein network with larger voids. This was in agreement with the lower breaking force with less water holding capacity of directly heated and modori gels. . The finer and ordered gel network with smaller voids was observed in gels with highest gel strength as compared with the very loose network with larger voids observed in the gels with lower gel strength (Balange and Benjakul, 2009a). Rawdkuen *et al*. (2008) reported that kamaboko gel from bigeye snapper with high breaking force and deformation was finer and denser than others, while the gel structure of lizardfish with poorer gel property consisted of a large number of pores and loose structure. Jafarpour *et al*. (2008) reported changes in polygonal structures of kamaboko gel from threadfin bream surimi as a result of cross-linking between sarcoplasmic protein and myofibrillar protein. However, no association between the area or the number of polygonal structures/mm^2 and gel strength of the resultant kamaboko was found.

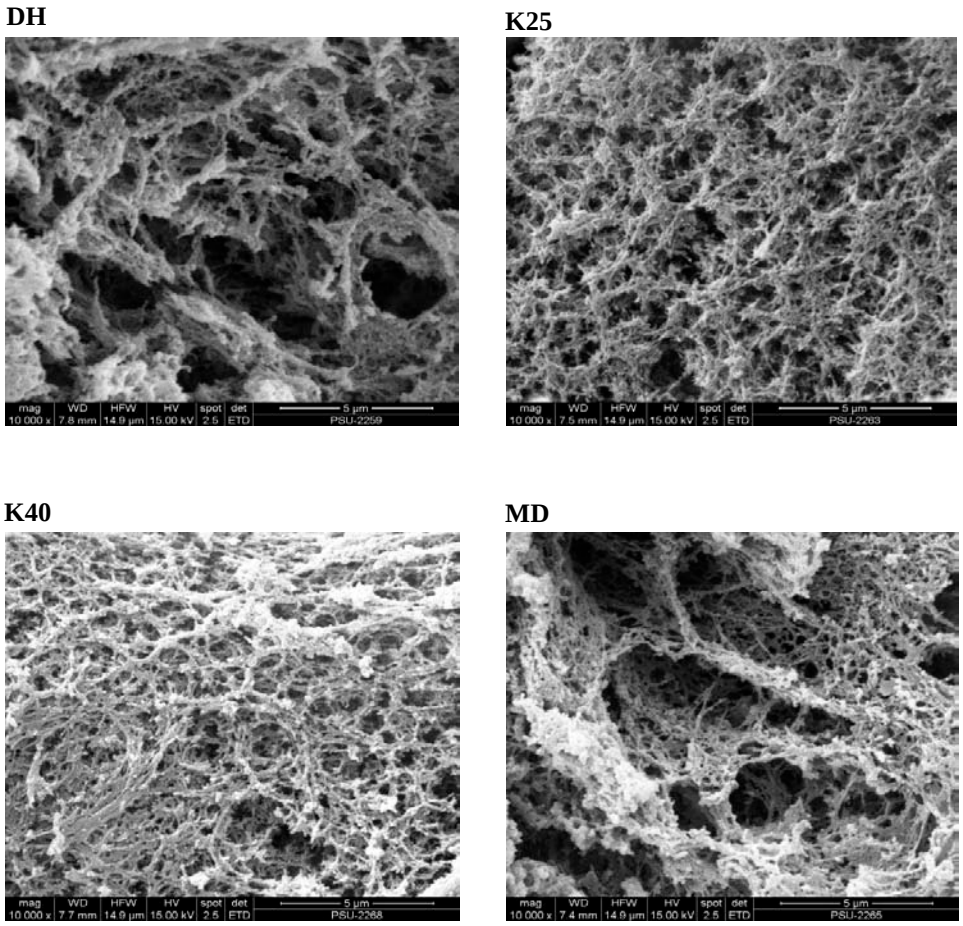


Figure 13. Microstructure of gels of surimi from yellow stripe trevally using different heating conditions. DH: directly heated gel; MD: modori gel; K25 and K40: Kamaboko gels with setting at 25 and 40 °C, respectively.
Magnification: x 10,000

2.4.2 Endogenous transglutaminase activity as affected by temperatures

Activity of endogenous TGase from yellow stripe trevally muscle as a function of temperature determined by MDC incorporation method is shown in (Figure 14). The activity increased as the temperature increased and reached the maximum at 40 °C ($P < 0.05$). The decrease in activity was observed at temperature higher than 40 °C. The loss in activity at higher temperature was caused by the thermal denaturation of TGase. Benjakul and Visessanguan (2003) reported that TGase from *P. tayenus* muscle showed the highest activity at 40 °C, whereas that from *P. macracanthus* muscle had the optimum temperature at 25 °C. The optimum

temperature for TGase activity varies with fish species. Red sea bream liver TGase had an optimum temperature of 55 °C (Yasueda *et al*., 1994), whereas walleye pollack liver TGase showed the maximum activity at 50 °C (Kumazawa *et al*., 1996). Therefore, temperature was crucial to maximize the setting phenomenon. Temperature of 40 °C used for prior setting of K40 sample in this study was coincidental with the optimum temperature for endogenous TGase. As a result, yellow stripe trevally TGase remaining in surimi catalysed the cross-linking of muscle proteins to a greater extent at 40 °C than at a lower temperature (25 °C), leading to higher breaking force of K40 sample.

The activity of endogenous TGase at 60 °C was found to be very low as compared to that found at 40 °C. The loss in activity at higher temperature was caused by the thermal denaturation of TGase, which consequently resulted in lowered cross-linking of muscle proteins. Additionally, the degradation at 60 °C was pronounced. As a consequence, the lowest breaking force of modori gel was obtained. However, the rate of TGase mediated cross-linking of MHC was primarily dependent on the conformation of substrate at a given temperature rather than on the optimum temperature of TGase (Araki and Seki, 1993; Kamath *et al*., 1992). Thus TGase activity played a role in cross-linking of protein and gel property.

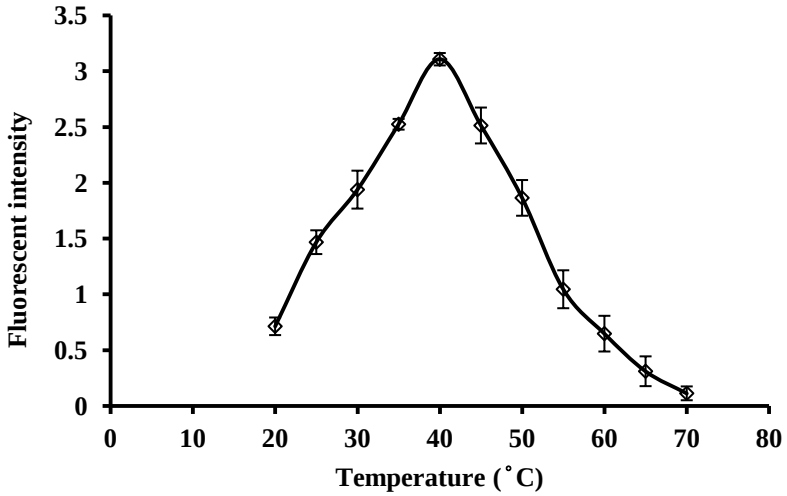


Figure 14. Temperature profile of crude TGase from yellow stripe trevally muscle. Bars represent the standard deviation (n=3).

2.5 Conclusion

Yellow stripe trevally could be used as the new raw material for surimi which yielded the gel with high elasticity. Setting temperature showed significant effect on textural properties and cross-linking of myofibrillar proteins. Kamaboko (K40) gel exhibited the highest breaking force with the lowest expressible moisture content ($P < 0.05$), corresponding to the optimum temperature of TGase activity. Therefore setting at 40 °C is a promising means to improve gel quality of surimi from yellow stripe trevally. Conversely, the incubation of sol at temperature close to 60 °C should be avoided to prevent gel weakening.

CHAPTER 3

Impact of zinc salts on heat-induced aggregation of natural actomyosin from yellow stripe trevally

3.1 Abstract

Impact of zinc sulphate ($ZnSO_4$) and zinc chloride ($ZnCl_2$) on heat-induced aggregation of natural actomyosin (NAM) extracted from yellow stripe trevally (*Selaroides leptolepis*) was investigated. In the presence of $ZnSO_4$ or $ZnCl_2$, transition temperature (T_{max}) of myosin shifted from 47.83±0.30 °C to 46.05±0.36 and 46.49±0.49 °C, with the coincidental decreases in ΔH from 1.07±0.03 J/g to 0.63±0.02 and 0.67±0.04 J/g, respectively ($P < 0.05$). Additionally, Ca^{2+}-ATPase activity of NAM decreased with increasing concentrations of $ZnSO_4$ or $ZnCl_2$ during heating up to 40 °C. During heating from 20 to 75 °C, higher turbidity, surface hydrophobicity and disulphide bond formation were obtained in NAM added with $ZnSO_4$ or $ZnCl_2$ at temperature ranging from 40 to 75 °C, compared with the control. Nevertheless, the higher aggregation was found in NAM added with $ZnSO_4$, compared with $ZnCl_2$. Zeta potential (ζ) analysis suggested that the surface of NAM added with $ZnSO_4$ became less negatively charged, compared with that of $ZnCl_2$ counterpart. Transmission electron microscopy showed that the structure of NAM was highly interconnected, finer and denser when zinc salts, especially $ZnSO_4$ were incorporated. Therefore, $ZnSO_4$ could be used to induce aggregation of fish muscle proteins, thereby improving gelling property of fish mince or surimi.

3.2 Introduction

Surimi is the concentrated myofibrillar proteins, prepared from fish mince subjected to thorough washing, in which undesirable components including sarcoplasmic proteins, lipids, heme pigments, etc. are removed. Lean fish have been widely used as the raw material for surimi production owing to the good gel forming ability and whiteness. Due to the limited fish resources, especially lean fish, pelagic dark fleshed fish have been paid more attention as a potential alternative raw material

for surimi production (Chaijan *et al*., 2004). However, poor gel forming ability of dark fleshed fish is generally recognized and limits the utilisation of this species. To improve the properties of surimi gel from dark fleshed fish, the novel and cheap additives capable of improving gel quality of mince or surimi have gained increasing attention.

Divalent cations like calcium, magnesium and zinc are known to alter the functionality of proteins during gelation (Mathew *et al*., 2009; Morales *et al*., 2001). Sorgentini *et al*. (1995) reported that aqueous dilutions of native soy isolate had the highest turbidity when $MgCl_2$ at a level of 40 mM was present. The role of zinc salts in combination with sucrose on the cryoprotection and aggregation of fish actomyosin has been studied by MacDonald *et al*. (1996). MacDonald *et al*. (1996) reported that increasing $ZnSO_4$ concentration above 0.1 mM decreased Ca^{2+}-ATPase activity by inducing denaturation and consequent aggregation in these samples. Haque and Aryana (2002) found that gels with $CuSO_4$, $FeSO_4$, and $ZnSO_4$ had aggregates that appeared clustered and more fused together than the control. At pH values sufficiently far from the isoelectric point of proteins, divalent ions such as Ca^{2+}, Zn^{2+}, Mg^{2+} induce protein cross-linking via the salt bridges between negatively charged carboxyl groups (Hongsprabhas and Barbut, 1997). Mathew *et al*. (2009) reported that the binding of zinc salts to actomyosin from oil sardine induced conformational changes with the exposure of functional groups such as sulphydryl groups, which subsequently undergo oxidation to disulphide bond.

Yellow stripe trevally (*Selaroides leptolepis*) is one of the abundant dark fleshed fish species in Southern Thailand. Surimi from this species had low gel forming ability, compared with surimi from lean fish (Arfat and Benjakul, 2012a). Addition of divalent cations, especially zinc at an appropriate concentration could be a promising means to improve the gel property by enhancing aggregation of proteins via salt bridges. From our previous study, the addition of $ZnSO_4$ (1-5 mmol/kg) into the surimi from threadfin bream surimi was able to increase the breaking force and deformation (unpublished data). However, the role of zinc salts on physicochemical changes and heat-induced aggregation of fish myofibrillar proteins, which play an essential role in gelation, has not yet been elucidated. The present study was

undertaken to elucidate the impact of two different zinc salts on thermal stability, physicochemical changes and heat-induced aggregation of natural actomyosin from yellow stripe trevally (*Selaroides leptolepis*).

3.3 Materials and methods

3.3.1 Chemicals

Adenosine-5′-triphosphate (ATP), 8-anilino-1-naphthalenesulphonic acid (ANS), guanidine thiocyanate, sodium hydrogen sulphite, β-mercaptoethanol (*β*-ME) and Tris-maleate were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Zinc sulphate ($ZnSO_4$) and zinc chloride ($ZnCl_2$) were purchased from Ajax Finechem. Pty. Ltd. (Taren Point, NSW, Australia). Potassium chloride, sodium chloride, calcium chloride, trichloroacetic acid, potassium dihydrogen phosphate and ammonium molybdate were procured from Merck (Darmstadt, Germany). 5,5′-Dithiobis (2- nitrobenzoic acid) (DTNB) was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Bovine serum albumin (BSA) was obtained from Fluka (Buchs, Switzerland).

3.3.2 Collection and preparation of fish

Yellow stripe trevally (*Selaroides leptolepis*) with an average weight of 65–75 g were caught from Songkhla coast along the Gulf of Thailand during January and February, 2012. The fish, off-loaded approximately 12 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 45 min. The fish were headed, gutted and washed with water. The flesh was separated manually from skin and bone and kept on ice prior to natural actomyosin (NAM) preparation. The storage time was not longer than 2 h.

3.3.3 Preparation of natural actomyosin (NAM)

NAM was prepared according to the method of Balange and Benjakul (2010) with a slight modification. Yellow stripe trevally muscle was homogenized in chilled 0.6 M KCl, pH 7.0 at a ratio of 1:10 (w/v) using a homogenizer (IKA, Labortechnik, Selangor, Malaysia). To avoid overheating, the sample was placed in ice and homogenized for 20 s, followed by a 20 s rest interval for a total extraction time of 4 min. The extract was centrifuged at 5000 × g for 30 min at 4 °C using a refrigerated centrifuge (Avanti J-E Centrifuge; Beckman Coulter, Fullerton, CA, USA). Three volumes of chilled deionized water (0-2 °C) were added to precipitate NAM. The NAM was collected by centrifuging at 5000 × g for 20 min at 4 °C. NAM pellet was dissolved in chilled 0.6 M KCl, pH 7.0 for 30 min at 4 °C and then centrifuged at 5000 × g for 20 min at 4 °C. The supernatant was collected and used as NAM.

3.3.4 Study on the effect of $ZnSO_4$ and $ZnCl_2$ at different concentrations on heat-induced aggregation of NAM

NAM extracted from yellow stripe trevally was diluted to 1 mg/ml with chilled 0.6 M KCl (pH 7.0). NAM solutions containing $ZnSO_4$ or $ZnCl_2$ at different concentrations (25, 50, 100 µM) were heated at a heating rate of 0.65 °C /min from 20 to 75 °C using a digital thermoregulator (TH5/150, Ratek, Boronia, Victoria, Australia). NAM without the addition of zinc salt was used as the control. The samples were taken every 5 °C of temperature increment. At temperature designated, the samples were cooled immediately with iced water. The samples obtained were subjected to analyses.

3.3.4.1 Determination of thermal protein aggregation

Different NAM solutions (1 mg protein/ml) were placed in cuvette (light path length of 1 cm). Degree of protein aggregation was estimated by measuring the turbidity of NAM solutions after being heated to the designated temperatures at a wavelength of 660 nm (Benjakul *et al*., 2001a) using a UV–visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan).

3.3.4.2 Determination of surface hydrophobicity

Surface hydrophobicity was measured as per the method of Li-Chan *et al*. (1985) as modified by Benjakul *et al*. (2001a) using 8-anilo-1-naphthalenesulphonic acid (ANS) as a probe. Heated NAM solutions were diluted to 0.125, 0.25, 0.5 and 1 mg/ml using the same buffer. To 2.0 ml of diluted NAM solution, 10 µl of 10 mM ANS dissolved in 50 mM potassium phosphate buffer (pH 7.0) was added and the mixtures were mixed thoroughly. Sample blanks of each protein concentration were prepared in the same manner, except the same volume of 50 mM potassium phosphate buffer (pH 7.0) was used instead of ANS solution. Fluorescence intensity was measured using a RF-1501 spectrofluorometer (Shimadzu, Kyoto, Japan) at the excitation and emission wavelength of 374 and 485 nm, respectively. Surface hydrophobicity was calculated from initial slope of plot of fluorescence intensity against protein concentration using a linear regression analysis. The initial slope was referred to as S_0ANS.

3.3.4.3 Determination of total sulphydryl groups and disulphide bond contents

Determination of total sulphydryl group content was done using 5, 5-dithio-bis (2-nitrobenzoic acid) (DTNB) following the method of Ellman (1959) as modified by Benjakul *et al*. (2001a). To 1.0 ml of sample solutions, 9 ml of 0.2 M Tris–HCl buffer, pH 7.0, containing 8 M urea, 2% SDS and 10 mM ethylenediaminetetraacetic acid (EDTA) were added. To 4 ml of the mixture, 0.4 ml of 0.1% DTNB was added and incubated at 40 °C for 25 min. Absorbance at 412 nm was then measured. A sample blank of each sample was conducted in the same manner except that distilled water was used instead of DTNB. The sulphydryl group content was calculated using the extinction coefficient of 13,600 M^{-1} cm^{-1}. Determination of disulphide bond content was carried out by using 2-nitro-5-thiosulphobenzoate (NTSB) (Thannhauser *et al*., 1987). To 0.5 ml of sample solution, 3.0 ml of freshly prepared NTSB assay solution was added. The mixture was incubated in the dark at room temperature (26–28 °C) for 25 min. A sample blank of each sample was prepared in the same manner but the distilled water was used instead

of NTSB assay solution. Absorbance at 412 nm was measured. Disulphide bond content was calculated using the extinction coefficient of 13,900 M^{-1} cm^{-1}.

3.3.4.4 Determination of Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity of NAM samples was determined according to the method of Jiang *et al*. (1988) as modified by Benjakul *et al*. (1997). To 1 ml of different NAM solutions (1mg protein/ml) incubated at different temperatures (20, 30, 40, 50, 60 and 70 °C), 0.6 ml of 0.5 M Tris-maleate, pH 7.0, was added. The mixture was added with $CaCl_2$ solution to obtain a final concentration of 10 mM $CaCl_2$ with the total volume of 9.5 ml. To initiate the reaction, 0.5 ml of 20 mM ATP was added. The reaction was conducted for exactly 10 min at 25 °C and was terminated by adding 5 ml of chilled 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3500 × g for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). Ca^{2+}-ATPase activity was expressed as µmoles inorganic phosphate released/mg protein/min. A blank solution was prepared by adding chilled trichloroacetic acid prior to addition of ATP.

3.3.5 Study on the effect of $ZnSO_4$ and $ZnCl_2$ on thermal transition, charge, size and microstructure of NAM

3.3.5.1 Differential scanning calorimetry

Thermal transition of NAM from yellow stripe trevally was determined using the differential scanning calorimetry (DSC) (Perkin-Elmer, Model DSCM, Norwalk, CT, USA). NAM pellet samples (15–20 mg wet weight) added with $ZnSO_4$ or $ZnCl_2$ with a final concentration of 100 µM were placed in the DSC hermetic pans, assuring a good contact between the sample and the pan bottom. Sample without addition of zinc salt was used as the control. An empty hermetic pan was used as a reference. The samples were scanned at 10 °C/min over the range of 20–100 °C. The maximum transition temperature (T_{max}) was measured and enthalpy change (ΔH) was estimated by measuring the area under the DSC transition curve. The system was calibrated using indium.

3.3.5.2 Zeta potential and particle size

NAM solutions (1 mg protein/ml) with different treatments heated to reach the final temperature of 75 °C were stirred gently for 6 h in ice. Thereafter, the mixtures were allowed to stand at 4 °C for 30 min prior to analysis. The zeta (ζ) potential and particle size of NAM solutions were measured using a ZetaPlus zeta potential analyser (Brookhaven Instruments Corporation, Holtsville, NY) at room temperature.

3.3.5.3 Transmission electron microscopy

NAM solutions (1 mg protein/ml) with different treatments heated to reach the final temperature of 75 °C, followed by cooling, were diluted to 0.2 mg/ml with 50 mM potassium phosphate buffer containing 0.6 M KCl (pH 7). A drop of sample was fixed for 5 min on a carbon-coated grid, negatively stained with 4% uranyl acetate for 5 min and washed with distilled water until the grid was cleaned. The specimens were visualized using a JEOL JEM-2010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) (60,000×) at an accelerating voltage of 160 kV.

3.3.6 Protein determination

Protein content was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as the standard.

3.3.7 Statistical analysis

The experiments were run in triplicate with three lots of sample. Analysis of variance (ANOVA) was performed and the mean comparisons were carried out by Duncan’s multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 17.0 for windows: SPSS Inc., Chicago, IL, USA).

3.4 Results and discussion

3.4.1 Effect of $ZnSO_4$ and $ZnCl_2$ at different concentrations on heat-induced aggregation of NAM

3.4.1.1 Thermal protein aggregation

Changes in turbidity of NAM solutions in the absence and in the presence of $ZnSO_4$ or $ZnCl_2$ at different concentrations during heating from 20 to 75 °C were monitored (Figure 15). Turbidity represents a rough estimate of the degree of aggregation and is affected by environmental conditions (pH and/or ionic strength) (Hermansson, 1986). A marked increase in turbidity of all NAM samples was observed above 35 °C ($P < 0.05$). The higher rate in turbidity development was found with increasing concentrations of zinc salts ($P < 0.05$). At the same concentration used, $ZnSO_4$ resulted in the higher turbidity at all temperatures, compared with $ZnCl_2$ and the control, respectively. Nevertheless, no difference in turbidity was found between NAM added with $ZnSO_4$ and $ZnCl_2$ at a level of 25 µM ($P > 0.05$). The increased turbidity of NAM was therefore induced by $ZnSO_4$ and $ZnCl_2$. This was more likely governed by 'salt bridge' between proteins. Zn^{2+} could act as the bridge between negatively charged residues, particularly COO^{-}. As a result a large aggregate could be formed. Furthermore, protein molecules also underwent aggregation via hydrophobic interaction, electrostatic and hydrogen bonds, Van de Waals interaction and disulphide bond during heat induced gelation process (Chawla *et al*., 1996; Xiong, 1997). Divalent metal ions might cause changes in protein conformation and interact with negative charges on the polypeptide chains of proteins, thereby inducing cross-linking via salt bridges (Ding *et al*., 2011).

At temperature above 35 °C, unfolding of some protein molecules occurred, more likely due to the instability of hydrogen bonds. T_{onset} of myosin in all samples was around 40ºC (data not shown), where the alteration of protein molecules more likely take place. As a consequence, the greater numbers of reactive groups and hydrophobic portions were exposed (Niwa, 1992). In the presence of Zn^{2+}, this divalent cation might induce cross-linking of protein molecules via the exposed

carboxyl group. As a result, the higher aggregation via salt bridge could be enhanced, along with other bondings including hydrophobic interaction and disulphide bond. When higher levels of zinc salt were present, the higher aggregation was noticeable. Thus, cross-linking activity was determined by the amount of zinc ions. When comparing the cross-linking activity between $ZnSO_4$ and $ZnCl_2$ towards NAM, $ZnSO_4$ exhibited higher capacity of forming the protein aggregate than $ZnCl_2$. The greater screening effect of $ZnSO_4$ on the negatively charged carboxyl groups of proteins was reported in comparison with $ZnCl_2$ (Twomey *et al*., 1997). Formation of large aggregates is presumably a prerequisite to the formation of a good elastic gel (Chan *et al*., 1992a). Chan *et al*. (1992a) reported that the poorer aggregating ability of herring actomyosin reflected the inferior gelling properties of surimi. Therefore, the aggregation of NAM from yellow stripe trevally was enhanced by the addition of zinc salt, especially $ZnSO_4$.

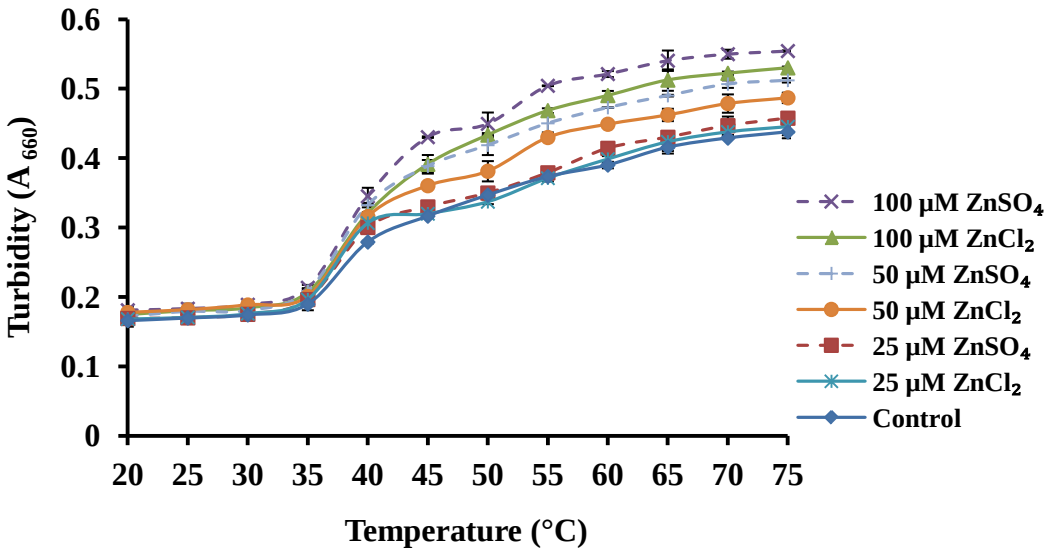


Figure 15. Turbidity of NAM solutions (1 mg protein/ml) containing $ZnSO_4$ or $ZnCl_2$ at different concentrations during heating from 20 to 75 °C. Heating rate was 0.65 °C/min. Bars represent the standard deviation (n = 3).

3.4.1.2 Surface hydrophobicity

Surface hydrophobicity (S_0ANS) of different NAM solutions in the absence and presence of zinc salts ($ZnSO_4$ or $ZnCl_2$) at different concentrations heated

from 20 to 75 °C is illustrated in Figure 16. S_0ANS of control NAM increased continuously after heating at the temperature above 35 °C up to 70 °C ($P < 0.05$). The increase in S_0ANS indicated the exposure of hydrophobic domains of NAM during heating. Addition of either $ZnSO_4$ or $ZnCl_2$ at 50 and 100 µM further increased S_0ANS, particularly when temperature were above 35 °C ($P < 0.05$). This confirmed that Zn^{2+} in solution could induce the conformational change of NAM to a higher extent, particularly when more exposure of reactive group was favored at higher temperatures. At temperature above 70 °C, slight decreases in S_0ANS were observed for all samples, suggesting that the hydrophobic interaction between proteins molecules most likely took place. ANS, a fluorescence probe, has been found to bind to hydrophobic amino acids containing an aromatic ring, such as phenylalanine and tryptophan, and can be used to indicate the conformational changes occurring in the proteins (Benjakul *et al*., 1997). The increase in ANS binding could be due to either the presence of exposed hydrophobic sites on unfolded, not aggregated proteins, or unfolded and aggregated proteins. The exposure of hydrophobic domains has been suggested as a prerequisite for formation of large myosin aggregates via hydrophobic interaction (Chan *et al*., 1992b). Upon heating, the aromatic hydrophobic amino acid residues could be exposed to a greater extent (Visessanguan *et al*., 2000). In the presence of zinc salts at higher concentration, the attachment of Zn^{2+} with protein molecules could be augmented. As a result, the alteration of protein conformation could be increased as indicated by the higher S_0ANS ($P < 0.05$). The rate of the increase in S_0ANS of NAM added with $ZnSO_4$ was higher than those added with $ZnCl_2$, when the same concentration was used. This might be due to the higher reactivity of $ZnSO_4$ on binding or interacting with proteins, in which the exposure of hydrophobic domain was favored. The effect of other divalent cations such as Ca^{2+} on surface hydrophobicity was also reported in other proteins. Addition of 1–15 mM $CaCl_2$ induced structural changes in β-lactoglobulin, resulting in an increased hydrophobicity (Jeyarajah and Allen, 1994). Thus, the aggregation of NAM by both ionic and hydrophobic interaction could be more induced when $ZnSO_4$ or $ZnCl_2$ was incorporated. The result was in agreement with the higher turbidity of NAM when zinc salts were added (Figure 15). The result suggested that Zn divalent was able to

improve the gel strength of surimi via enhancing hydrophobic interaction, apart from ionic interaction or salt bridges.

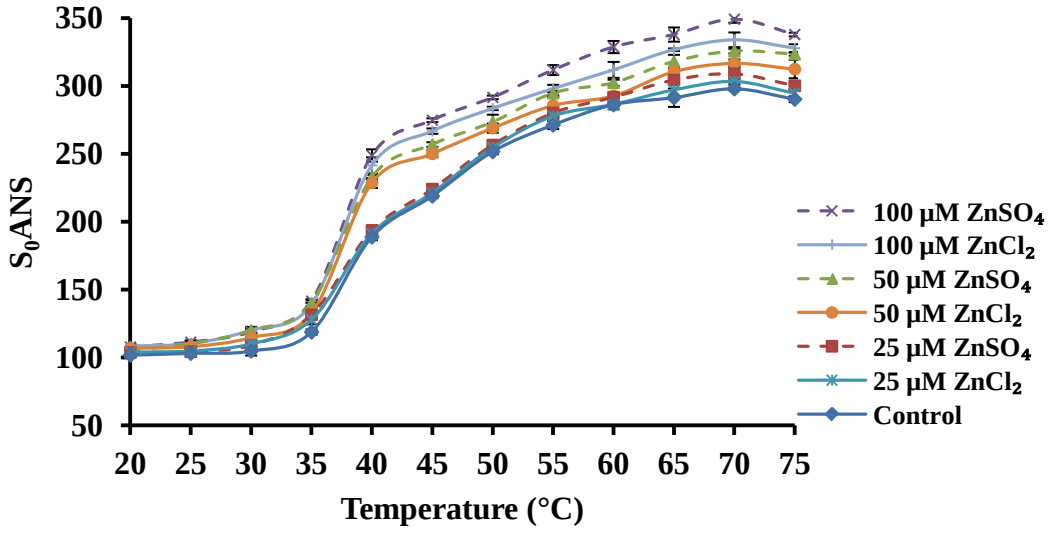


Figure 16. Surface hydrophobicity of NAM solutions (1 mg protein/ml) containing $ZnSO_4$ or $ZnCl_2$ at different concentrations during heating from 20 to 75 °C. Heating rate was 0.65 °C/min. Bars represent the standard deviation (n = 3).

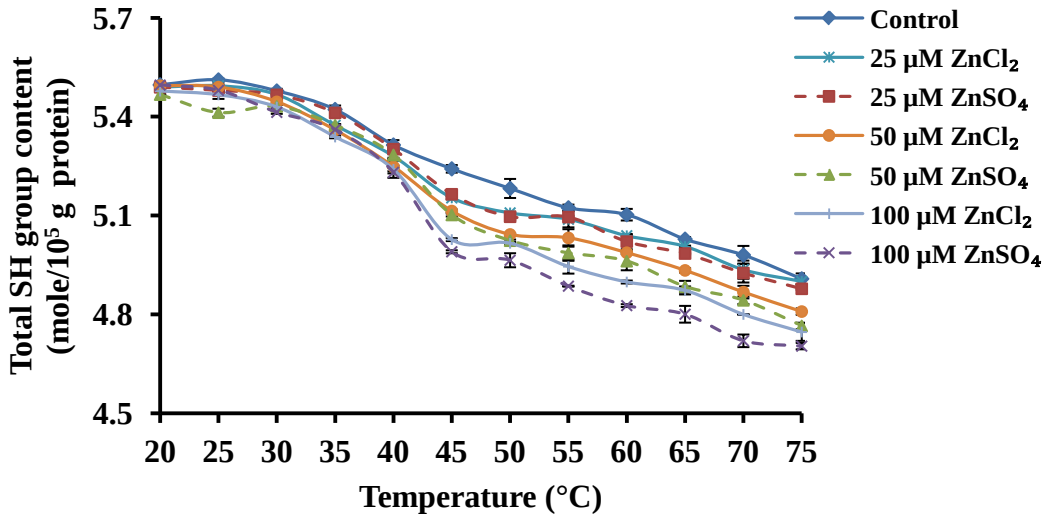
3.4.1.3 Total sulphydryl group and disulphide bond contents

Total sulphydryl (SH) group content and disulphide bond formation of NAM without and with the addition of $ZnSO_4$ or $ZnCl_2$ at various concentrations during heating from 20 to 75 °C are shown in Figure 17. Total SH content of control NAM decreased continuously after heating at the temperature above 35 °C. In the presence of $ZnSO_4$ or $ZnCl_2$ at all concentrations, higher decrease in total SH content were observed, especially at temperature above 35 °C ($P < 0.05$). However, the rate of decrease in total SH content was higher in NAM added with $ZnSO_4$, compared with those added with $ZnCl_2$, when the same concentration was used. The total SH content of the NAM solutions decreased with concomitant increase in disulphide bond formation, particularly at temperature above 40 °C (Figure 17B). However, no differences in total SH group content and disulphide bond content were found in NAM added with either $ZnSO_4$ or $ZnCl_2$ at a level of 25 µM, compared with those of the control ($P > 0.05$). Elevated temperature most likely resulted in the enhanced

oxidation of sulphydryl groups with the accompanied disulphide bond formation. Disulphide bond formation of fish actomyosin and myosin occurred at 40 and 45 °C, respectively (Niwa, 1992). Yarnpakdee *et al*. (2009) reported that the disulphide bond formation in goatfish actomyosin required temperature above 40 °C.

Zinc salts might induce the conformation of NAM, in which sulphydryl groups might be exposed to a higher extent and favored oxidation process. This was evidenced by the decrease in total SH group content with concomitant increase in disulphide bond formation of NAM as the concentration of zinc salts increased ($P < 0.05$). Mathews *et al*. (2009) reported that the addition of zinc led to the decrease in free SH group content of actomyosin. A decrease in total SH group content was reported to be due to the formation of disulphide bond through oxidation of SH group or disulphide interchanges (Hayakawa and Nakai, 1985). An inter-molecular disulphide bond is formed by the oxidation of two cysteine molecules on adjacent protein chains (Lanier, 2000). At the same concentration, $ZnSO_4$ addition resulted in the higher formation of disulphide bond of NAM. The result was in accordance with the higher decrease in SH group content. The formation of disulphide bond correlated well with those of turbidity of NAM solution (Figure 15). The result suggested that the stronger gel of surimi could be obtained via the increased formation of disulfide bond in the presence of zinc divalent. The change of actomyosin conformation correlates with the exposure of functional groups such as sulfhydryl groups. Subsequently, those groups most likely undergo disulfide bond formation (Benjakul *et al*., 2001a).

A)



B)

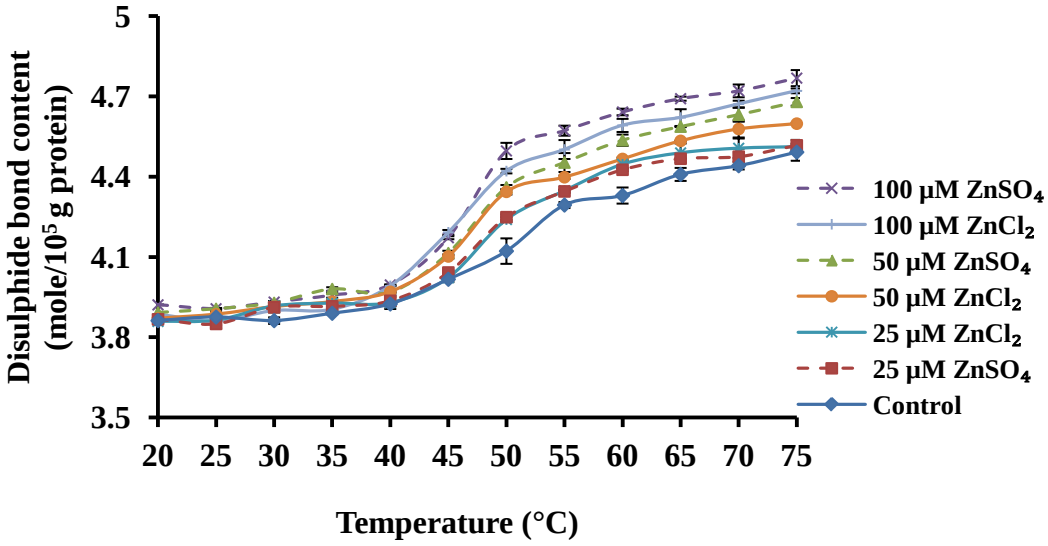


Figure 17. Total sulphydryl group content (A) and disulphide bond content (B) of NAM solutions (1 mg protein/ml) containing $ZnSO_4$ or $ZnCl_2$ at different concentrations during heating from 20 to 75 °C. Heating rate was 0.65 °C/min. Bars represent the standard deviation (n = 3).

3.4.1.4. Ca^{2+}-ATPase Activity

The effect of $ZnSO_4$ and $ZnCl_2$ at various concentrations on Ca^{2+}-ATPase activity of NAM as a function of heating temperature is shown in Table 7. No Ca^{2+} -ATPase activity of NAM was detected when heated at temperature above 40 °C. Ca^{2+}-ATPase activity of NAM decreased continuously as the temperature increased from 20 to 40 °C ($P < 0.05$). In the presence of $ZnSO_4$ or $ZnCl_2$, the decrease in Ca^{2+} -ATPase activity was more pronounced with increasing concentrations of $ZnSO_4$ or $ZnCl_2$. At 50 and 100 µM, both $ZnSO_4$ and $ZnCl_2$ caused the marked loss in Ca^{2+}-ATPase activity. The inhibitory effect of 100 µM zinc salts was more profound when the temperatures were 30 and 40 °C ($P < 0.05$). Foegeding *et al*. (1996) reported that Ca^{2+}-ATPase activity is a sensitive indicator of myosin denaturation. Ca^{2+}-ATPase is also used as a good indicator of the integrity of myosin molecule (Benjakul *et al*., 1997; Benjakul *et al*., 2011). Mathew *et al*. (2009) reported the complete inhibition of ATPase activity of actomyosin by zinc salt at a concentration of 1 mM. Prevalent Zn^{2+} might induce denaturation of Ca^{2+}-ATPase by inducing the conformational change of myosin, especially at head portion. SH groups located in the head portion (SH1 and SH2) play an essential role in Ca^{2+} -ATPase activity (Kielley and Bradley, 1956). The result indicated that zinc salts at higher concentrations were effective in altering conformation of myosin. Conformational changes and aggregation between myosin, particularly at head portions, induced by Zn^{2+} was postulated to cause the decrease in Ca^{2+} -ATPase activity. Therefore, zinc in conjunction with increasing temperature could induce the denaturation of NAM and the denatured proteins could further undergo aggregation.

Table 7. Ca^{2+}- ATPase activity of NAM solutions (1 mg protein/ml) containing $ZnSO_4$ or $ZnCl_2$ at different concentrations after heating from 20 to 75 °C

Temperature	**Control**	**$ZnSO_4$ (µM)**			**$ZnCl_2$ (µM)**		
(°C)		**25**	**50**	**100**	**25**	**50**	**100**
20	0.19±0.02aA*	0.18±0.01aAB	0.15±0.02aBC	0.13±0.01aC	0.18±0.02aAB	0.15±0.02aBC	0.12±0.01aC
30	0.13±0.03bA	0.12±0.02bA	0.10±0.03bAB	0.07±0.01bC	0.12±0.02bA	0.10±0.01bAB	0.08±0.03bC
40	0.06±0.02cA	0.05±0.01cAB	0.04±0.01cB	0.01±0.01cC	0.05±0.02cAB	0.04±0.01cB	0.01±0.03cC
50	ND†	ND	ND	ND	ND	ND	ND
60	ND	ND	ND	ND	ND	ND	ND
70	ND	ND	ND	ND	ND	ND	ND

Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

Different uppercase letters in the same row indicate significant differences ($P < 0.05$).

* Values are given as mean±SD ($n = 3$).

† ND: Not detectable.

3.4.2 Effect of $ZnSO_4$ and $ZnCl_2$ on thermal transition, charge, size and microstructure of NAM

3.4.2.1 Thermal transition

Transition temperature (T_{max}) and enthalpy (ΔH) of NAM from yellow stripe trevally in the presence of $ZnSO_4$ or $ZnCl_2$ at a concentration of 100 µM and the control NAM are shown in Table 8. The control NAM showed two major endothermic peaks with T_{max} of 47.83 and 64.15 °C, respectively. The first and second peaks were postulated to be the transitions of myosin and actin, respectively (Hastings *et al*., 1985). The observed T_{max} of myosin and actin were in the temperature range reported for various fish species. Yarnpakdee *et al*. (2009) reported that NAM from goatfish had two endothermic transitions with T_{max} of myosin and actin of 47.4 and 63.5 °C, respectively. Fresh bigeye snapper (*Priacanthus tayenus*) muscle proteins had two major endothermic peaks with T_{max} at 47.7 and 70.6 °C and ΔH values of 1.42 and 0.69 J/g, respectively (Benjakul *et al*., 2002b). Myosin and actin of Japanese stingfish had T_{max} of 40.9 and 61.1 °C, respectively (Nagai *et al*., 1999). Whole cod muscle showed two maximal transitions on DSC thermogram with T_{max} at about 45 and 75 °C (Hasting *et al*., 1985) and whole muscle of fresh hake also showed two endothermic transitions with T_{max} values of 46 and 75 °C (Beas *et al*., 1990). In the presence of $ZnSO_4$ or $ZnCl_2$, T_{max} of myosin shifted to the lower temperatures 46.05 and 46.49 °C, respectively. The coincidental decreases in ΔH were observed for both peaks ($P < 0.05$). Lower T_{max} and ΔH indicated that myosin became less thermally stable in the presence of $ZnSO_4$ or $ZnCl_2$. However, T_{max} and ΔH of actin were not affected by both $ZnSO_4$ and $ZnCl_2$ ($P > 0.05$). The result suggested that myosin might undergo conformational change when $ZnSO_4$ or $ZnCl_2$ was incorporated, while those salts have no impact on actin structure. Generally, myosin was unstable and easily irreversibly denatured during both handling and processing. Actin, on the other hand, was more stable and its denaturation was largely reversible (Hastings *et al*., 1985). Zn^{2+} more likely interacted with negatively charged residues of myosin molecules. As a consequence, the configurational change could be induced. This resulted in the ease of thermal denaturation of myosin with subsequent enhanced aggregation. It was presumed that myosin with less thermal stability could undergo the denaturation,

followed by aggregation with ease. As a consequence, the protein network could be formed more effectively, especially in the presence of Zn divalent. Thermodynamic stability, charge distribution and hydrophobic characteristics are important to the structure function relationship of proteins (Damodaran, 1994). Different conformational changes and protein-protein interactions of myofibrillar proteins occurred during gelation (Chawla *et al*., 1996).

Table 8. Thermal transition (T_{max}) and enthalpy change (ΔH) of NAM containing 100 µM $ZnSO_4$ or 100 µM $ZnCl_2$

Treatment	Peak I		Peak II	
	Tmax(°C)	ΔH (J/g protein)	Tmax(°C)	ΔH (J/g protein)
Control	47.83±0.30*a	1.07±0.03a	64.15±0.36a	0.23±0.02a
ZC	46.49±0.49b	0.67±0.04b	63.71±0.44a	0.19±0.03a
ZS	46.05±0.36b	0.63±0.02b	63.60±0.35a	0.20±0.04a

* Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column indicate significant differences (P < 0.05).

3.4.2.2 Zeta potential and particle size

Zeta potential (ζ) representing the surface charge of NAM added with $ZnSO_4$ or $ZnCl_2$ at a level of 100 µM after heating to 75 °C in comparison with the control NAM was determined (Table 9). The highest negative charge was found in the control (P < 0.05). NAM solutions became less negatively charged when $ZnSO_4$ or $ZnCl_2$ at 100 µM was incorporated (P < 0.05). The negative charge of the proteins was governed by the acidic amino acids like aspartic acid and glutamic acid in the NAM at the neutral pH. When NAM was heated, proteins were more unfolded, exposing the charged amino acids. Zn^{2+} ion might neutralize the negative charge of protein during heating, as indicated by the lowered negative charge as $ZnSO_4$ or $ZnCl_2$ was added (Table 9). This behavior could be attributed to ionic interactions between protein molecules and Zn^{2+} ions. Ding *et al*. (2011) reported that divalent metal ions might cause changes in protein conformation and interact with negative

charges on the polypeptide chains of proteins and induce cross-linking by the formation of salt bridges. However, the higher decrease in negative charge was observed in NAM added with $ZnSO_4$, compared with $ZnCl_2$ ($P < 0.05$). The higher drop in negative charge by $ZnSO_4$ was likely due to the greater screening effect of $ZnSO_4$ on the negatively charged carboxyl groups of proteins (Twomey *et al*., 1997). With the addition of zinc salts, progressive decreases in electronegativity of protein molecules with the lowered electrostatic repulsion between protein molecules could occur. This resulted in the induced protein aggregation as evidenced by the increased turbidity of NAM incorporated with both zinc salts (Figure 15).

Average particle sizes of control NAM solution, and NAM solution containing $ZnSO_4$ or $ZnCl_2$ at a level of 100 μM after heating to 75 °C are shown in Table 9. Particle size of NAM increased from 710.78 nm for the control to 1242.37 and 1558.89 nm in the presence of $ZnCl_2$ and $ZnSO_4$, respectively ($P < 0.05$). The increase in particle sizes of NAM was in accordance with the increase in turbidity (Figure 15). The aggregation of heated protein molecules not only affected the size of particulates formed, but also had an impact on the surface charge of the aggregates. These results suggested that Zn^{2+} played a role in modification of protein charge. This more likely determined the physicochemical changes of protein molecules, in which the aggregation of proteins was enhanced. With the sufficient heat, proteins underwent unfolding. Simultaneously, Zn^{2+} was able to bind with protein molecules. As a consequence, the aggregation was enhanced as indicated by the increase in particle size of protein aggregate.

Table 9. Zeta potential and particle size of NAM solutions (1 mg protein/ml) containing 100 µM $ZnSO_4$ or 100 µM $ZnCl_2$ after heating to 75 °C

Treatment	Zeta potential (mV)	Particle size (nm)
Control	-14.41±0.64c*	710.78±25.68c
ZC	-2.67±0.32b	1242.37±34.78b
ZS	-0.92±0.47a	1558.89±33.62a

* Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column indicate significant differences (P < 0.05).

3.4.2.3 Microstructure

Microstructure of control NAM solution, NAM solutions containing $ZnSO_4$ or $ZnCl_2$ at 100 µM after heating to 75 °C revealed that the aggregation of NAM occurred differently as depicted in Figure 18. A highly interconnected and denser network was developed in NAM added with $ZnSO_4$, followed by NAM added with $ZnCl_2$ and the control, respectively. This was concomitant with the turbidity development, the increase in hydrophobicity and disulphide bond formation. It was noted that looser protein network was formed in the control (Figure 18). Van den Berg *et al*. (2009) reported that aggregated protein constituents were engaged in network formation. The alignment of myofibrillar proteins as well as aggregation pattern induced by $ZnSO_4$ was different from those mediated by $ZnCl_2$. This might be due to the different reactivity of NAM towards both zinc salts. Gelation is the result of protein denaturation, followed by the aggregation via inter-molecular covalent bonds and non-covalent interactions, including disulphide bonds and hydrophobic interactions (Lee and Lanier, 1995). The heat treatment might provide the sufficient energy for unfolding protein molecules, which allowed Zn^{2+} to interact easily and large aggregates were formed among actomyosin molecules by various bonds. Zn^{2+} also served as the contributor in bridging between negatively charged groups on adjacent unfolded protein molecules. The result indicated that Zn^{2+} was able to induce the aggregation of NAM, but the degree of aggregation varied, depending on zinc salt.

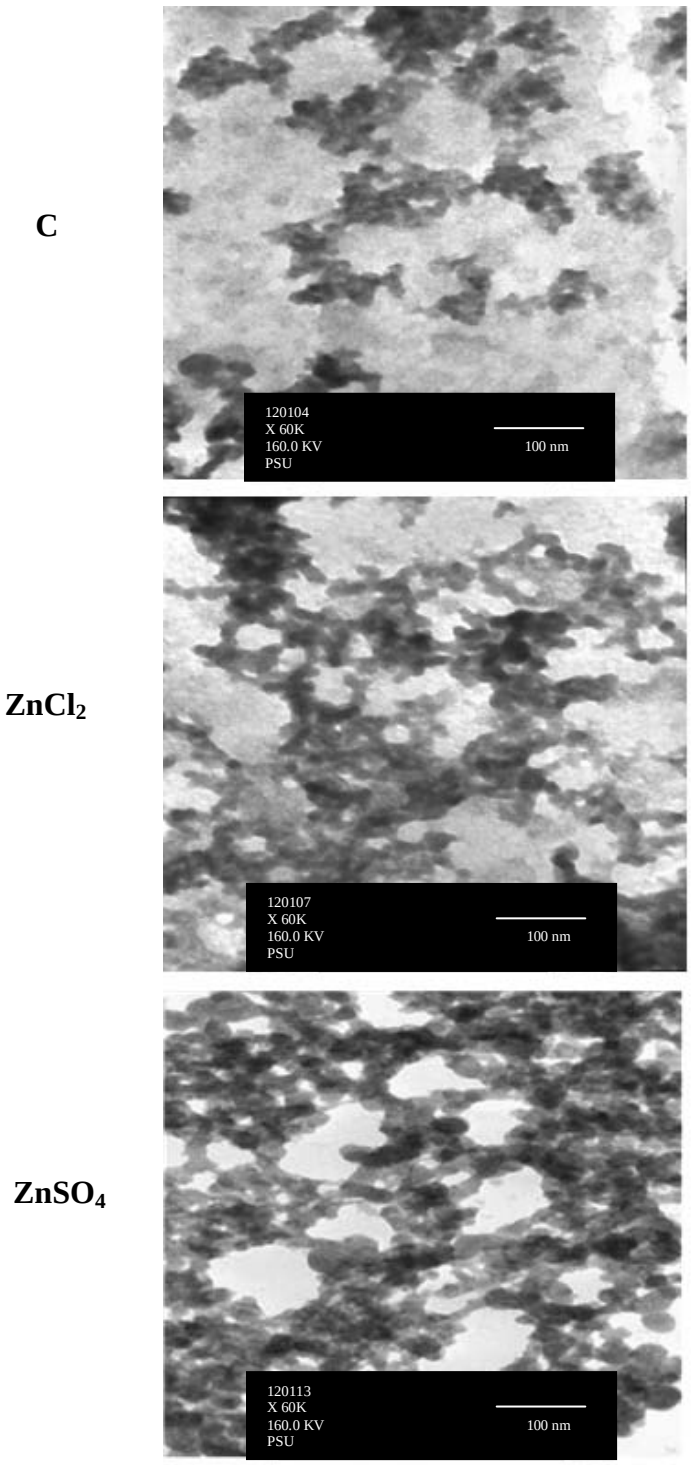


Figure 18. Transmission electron micrograph of NAM solutions containing 100 µM $ZnSO_4$ or 100 µM $ZnCl_2$ after heating at 0.65 °C/min to 75 °C. C: control NAM solution (without zinc salt). Heated NAM was cooled rapidly in iced water prior to TEM analysis. Magnification: 60,000×.

3.5 Conclusion

Heat-induced aggregation of NAM solution was governed by heating temperature, type and concentration of zinc salts. Addition of $ZnSO_4$ was associated with the higher aggregation of NAM, compared with $ZnCl_2$ counterpart, mainly by induction of higher hydrophobic interaction, disulphide bond and ionic interaction. Salts bridge, mediated by Zn^{2+} play a role in the aggregation of NAM. Therefore, zinc salts particularly $ZnSO_4$ can serve as promising additive to improve aggregation of muscle proteins, thereby strengthening surimi gel.

CHAPTER 4

Gel strengthening effect of zinc salts in surimi from yellow stripe trevally

4.1 Abstract

Effects of zinc sulphate ($ZnSO_4$) and zinc chloride ($ZnCl_2$) at various levels (0-80 µmol/kg) on the properties of gels from yellow stripe trevally (*Selaroides leptolepis*) surimi were investigated. Both kamaboko (40/90 °C) and modori (60/90 °C) gels added with $ZnSO_4$ or $ZnCl_2$ up to 60 µmol/kg had the increases in breaking force and deformation ($P < 0.05$), compared with the control. The higher breaking force and deformation were generally found in kamaboko gels added with $ZnSO_4$, compared with $ZnCl_2$ ($P < 0.05$). Whiteness of both kamaboko and modori gels increased with increasing levels of zinc salts ($P < 0.05$). Salt bridge and ionic interactions played a major role in cross-linking of proteins. Kamaboko gel had highly interconnected, denser and finer network with smaller voids when zinc salts, especially $ZnSO_4$ at 60 µmol/kg, was incorporated. Therefore, $ZnSO_4$ at an appropriate level could improve gel strength and whiteness of surimi from dark fleshed fish.

4.2 Introduction

Gelation is an important functional property of fish muscle proteins, especially surimi. The process involves denaturation or conformational alteration of native proteins, followed by aggregation of denatured proteins to orient themselves and interact at specific points, resulting in a three dimensional network (Ko *et al*., 2007). Gel strength, a quality index of surimi, is governed by several factors. Apart from intrinsic factors, such as endogenous enzymes and thermal properties of proteins, the extrinsic conditions such as pH, ionic strength and type of salts have been reported to affect gel strength of surimi (Croguennec *et al*., 2002). Due to the over-exploitation of lean fish, which have been used for surimi production, dark fleshed fish have been paid increasing attention as a potential alternative raw material for surimi production, due to its abundance and low price (Chaijan *et al*., 2004). However, surimi from dark fleshed fish generally has poorer gel forming ability,

thereby limiting the utilisation of those species (Arfat and Benjakul, 2012a; Chaijan *et al*., 2004). Various food grade ingredients have been used to increase the gel strength of surimi, but the addition of these ingredients poses the adverse effects on the surimi gel, particularly off-flavor or off-color (Rawdkuen and Benjakul, 2008). To improve the properties of surimi gel from dark fleshed fish, the novel and cheap additives capable of improving gel quality of surimi have gained increasing attention.

Divalent cations, e.g. calcium, magnesium and zinc are known to alter the functionality of protein during gelation (Mathew *et al*., 2009; Morales *et al*., 2001). Sorgentini *et al*. (1995) reported that suspension of native soy isolate had the highest turbidity when $MgCl_2$ at a level of 40 mM was present. $ZnSO_4$ at concentrations above 0.1 mM decreased Ca^{2+}-ATPase activity of actomyosin extracted from tilapia hybrid (*Tilapia nilotica* × *Tilapia aurea*) (MacDonald *et al*., 1996). At pH values sufficiently far from the isoelectric point of proteins, divalent ions such as Ca^{2+}, Zn^{2+}, Mg^{2+} induce protein cross-linking via the salt bridges between negatively charged carboxyl groups (Hongsprabhas and Barbut, 1997). Haque and Aryana (2002) found that bovine serum albumin added with $CuSO_4$, $FeSO_4$, and $ZnSO_4$ underwent aggregation, in which clustered proteins were formed.

Recently, Arfat and Benjakul (2012b) have reported that the addition of zinc salts, especially zinc sulphate, induced the aggregation of natural actomyosin, mainly by the induction of higher hydrophobic interaction, salt bridge and ionic interaction. Therefore, the addition of divalent cations, especially zinc at an appropriate concentration, could be a promising means to improve the gel property by introducing salt bridges in gel network. Moreover, zinc is indispensable for numerous physiological processes in humans, including growth, development, immune system, endocrinal functions and has a very crucial role in neurogenesis and neurotransmission (Prasad, 1979; Sandstead *et al*., 1998). The recommended dietary allowances (RDAs) of zinc for adults range from 7 to 19 mg (Brown *et al*., 2004). However, the role of zinc salts on the gel properties of surimi has not yet been elucidated. Thus, this study aimed to investigate the impact of two different zinc salts, $ZnSO_4$ and $ZnCl_2$ on the gel properties of surimi from yellow stripe trevally, an abundant dark fleshed fish in the Southern Thailand.

4.3 Materials and methods

4.3.1 Chemicals

Zinc sulphate ($ZnSO_4$) and zinc chloride ($ZnCl_2$) were purchased from Ajax Finechem, Pty. Ltd. (Taren Point, NSW, Australia). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, *N*,*N*,*N'*,*N'* –tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA).

4.3.2 Fish collection and surimi preparation

Yellow stripe trevally (*Selaroides leptolepis*) with an average weight of 65-75 g were caught from Songkhla coast along the Gulf of Thailand during April and May, 2012. The fish, off-loaded approximately 12 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 30 min. The fish were immediately washed, gutted, filleted and de-skinned. Fish flesh was minced to uniformity using a mincer with a hole diameter of 5 mm. Fish and mince were kept in ice during preparation.

Surimi was prepared according to the method of Benjakul and Visessanguan (2003) with slight modifications. Fish mince was washed with cold water (4 °C) at a water/mince ratio of 3:1 (v/w). The mixture was stirred gently for 4 min and washed mince was filtered with a layer of nylon screen. The washing process was repeated twice. For the third washing, cold 0.2% NaCl solution was used. Finally, the washed mince was subjected to centrifugation using a Model CE 21K basket centrifuge (Grandiumpiant, Belluno, Italy) pre-cooled with ice at a speed of 700 × g for 10 min. The temperature of dewatered mince was approximately 8-10 °C. To the dewatered mince, 4% sucrose and 4% sorbitol were added and mixed uniformly using a kneader (Crypto Peerless Ltd., Birmingham, England). Prior to mixing, the kneader bowl was kept at -20 °C for 1 h, in which the temperature of surimi was maintained below 10 °C throughout the mixing process. The mixture (500 g) was packed in a

polyethylene bag and frozen using an air-blast freezer (-20 °C). Frozen surimi was stored at -20 ºC for not longer than 1 month.

4.3.3 Preparation of surimi gel containing zinc salt at different levels

To prepare the gels, the frozen surimi was tempered at 4 ºC for 3–4 h until the core temperature reached 0–2 °C. The surimi was then cut into small pieces with an approximate thickness of 1 cm. The surimi was placed in a mixer (National Model MK-K77, Tokyo, Japan). The moisture was adjusted to 80% and 2.5% salt was added. The mixture was chopped for 1 min until the viscous paste was obtained. $ZnSO_4$ or $ZnCl_2$ was added into the paste to obtain the various final concentrations (0, 20, 40, 60 and 80 µmol/kg paste). The mixture was chopped for another 4 min. All processes were performed in a walk-in cold room (4 °C).

The sol was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Sols were incubated at 40 °C for 30 min, followed by heating at 90 °C for 20 min (Benjakul *et al*., 2004b).The obtained gel was referred to as ‘kamaboko gel’. Modori gel was prepared by incubating the sol at 60 ºC for 30 min, followed by heating at 90 ºC for 20 min (Benjakul *et al*., 2010). After heating, all gels were immediately cooled in iced water for 30 min and stored for 24 h at 4 ºC prior to analyses.

4.3.4 Textural analysis

Textural analysis of gel samples was performed using a Model TA-XT2i texture analyser (Stable Micro Systems, Surrey, England). Gels were equilibrated and evaluated at room temperature (28–30 °C). Three cylinder-shaped samples of 2.5 cm in length were prepared and subjected to determination. Breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyser equipped with a spherical plunger (diameter 5 mm, depression speed of 60 mm/min).

4.3.5 Determination of whiteness

All gels were subjected to whiteness measurement using a Hunterlab (ColorFlex, Hunter Associates Laboratory, Reston, VA). Illuminant C was used as the light source of measurement. *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were measured and whiteness was calculated as described by NFI (1991) as follows:

$$\text{Whiteness} = 100 - \left[\left(100 - L^*\right)^2 + a^{*2} + b^{*2} \right]^{\frac{1}{2}}$$

4.3.6 Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al*. (2001b) with slight modifications. A gel sample with a thickness of 0.5 cm was weighed (X g) and placed between two pieces of Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, England) at the top and three pieces of the same type of filter paper at the bottom. The standard weight (5 kg) was placed on the top of the sample and maintained for 2 min. The sample was then removed and weighed again (Y g). Expressible moisture content was calculated and expressed as percentage of sample weight as follows:

$$\text{Expressible moisture content (\%)} = \left[\frac{(X-Y)}{X}\right] \times 100$$

4.3.7 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of surimi gels were analysed by SDS–PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85 °C were added to the sample (3 g). The mixture was then homogenized at a speed of 11,000 rpm using a homogenizer (IKA Labortechnik, Selangor, Malaysia) for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 8000 × g for 20 min at room temperature (26–28 °C) using a centrifuge (Model MIKRO20, Hettich ZENTRIFUGEN, Tuttlingen, Germany). Protein concentration in the supernatant was

determined as per the method of Lowry *et al*. (1951). Solubilized samples were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol and 10% *β*ME) and boiled for 3 min. Samples (15 µg protein) were loaded onto polyacrylamide gels comprising a 10% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein III unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.02% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid. A protein standard (Bio-Rad Laboratories, Inc., Richmond, CA, USA) containing myosin (200 kDa), *β*-galactosidase (116 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kDa) and ovalbumin (45 kDa) was used to estimate the molecular weight of the proteins.

4.3.8 Scanning electron microscopy (SEM)

Microstructure of surimi gels was determined using SEM (JEOL JSM-5800 LV, Tokyo, Japan). The control kamaboko gel (without $ZnSO_4$ or $ZnCl_2$) and those containing $ZnSO_4$ or $ZnCl_2$ at levels of 60 and 80 µmol/kg with a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h. The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with serial concentrations of 50, 70, 80, 90 and 100% (v/v). Dried samples were mounted on a bronze stub, and sputter-coated with gold (Sputter coater SPI-Module, PA, USA). The specimens were observed with a scanning electron microscope at an acceleration voltage of 20 kV.

4.3.9 Protein determination

Protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

4.3.10 Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, U.S.A.).

4.4 Results and discussion

4.4.1 Effect of $ZnSO_4$ or $ZnCl_2$ at different levels on textural properties of surimi gel

Breaking force and deformation of both kamaboko and modori gels without and with the addition of $ZnSO_4$ or $ZnCl_2$ at various levels are shown in Figure 19. Both kamaboko and modori gels added with $ZnSO_4$ or $ZnCl_2$ had the increases in breaking force and deformation with increasing levels of $ZnSO_4$ or $ZnCl_2$ up to 60 µmol/kg ($P < 0.05$). However, at the concentration above 60 µmol/kg, the sharp decreases in both breaking force and deformation were noticeable ($P < 0.05$). Excessive amount of zinc salts (80 µmol/kg $ZnSO_4$ or $ZnCl_2$) more likely led to coagulation or precipitation of proteins, in which a particulate gel was formed. The concentration of salt is of the major factor affecting structure and spatial organization of protein aggregates (Hongsprabhas *et al.*, 1999). It was noted that the appropriate concentration of zinc salts used in surimi was 60 µmol/kg, which was quite lower than the optimal concentration used in actomyosin solution (100 µM) reported by Arfat and Benjakul (2012b). Since surimi contained a higher protein concentration in comparison with natural actomyosin (1 mg/mL), most protein molecules could align themselves closely and were prone to be cross-linked by divalent, e.g. zinc. As a consequence, the lower amount of zinc was required for cross-linking of surimi protein. Also, the excessive amount of zinc salt showed the negative effect on gel property, more likely due to protein coagulation (Figure 19). Breaking force of kamaboko and modori gels added with 60 µmol/kg $ZnSO_4$ increased by 34.0 and 34.9%, respectively, and the deformation increased by 21.2 and 15.5%, respectively,

compared with the control (without $ZnSO_4$ or $ZnCl_2$ addition). For surimi gels added with $ZnCl_2$, a similar gel enhancing effect was observed. However, the addition of $ZnCl_2$ resulted in the lower increase in breaking force and deformation, compared with the incorporation of $ZnSO_4$ ($P < 0.05$). Gel strengthening effect of both zinc salts was likely due to the increased salt bridges formed between Zn^{2+} and negatively charged residues, mainly COO^- of protein side chains or C- termini. The higher gel enhancing property of $ZnSO_4$ in comparison with $ZnCl_2$ was plausibly owing to the greater screening effect of $ZnSO_4$ on the negatively charged carboxyl groups of proteins (Twomey *et al*., 1997). With addition of 60 μmol/kg $ZnCl_2$, breaking force of kamaboko and modori gels increased by 24.7 and 29.7%, respectively, and deformation increased by 15.2 and 14.4%, respectively, compared with the control. Divalent metal ions might cause changes in protein conformation and interact with negatively charged residues on the polypeptide chains of proteins, thereby inducing cross-linking via salt bridges (Ding *et al*., 2011). Moreover, Zn^{2+} was able to induce configurational change in myosin heavy chain, resulting in the ease of thermal denaturation with subsequent enhanced aggregation via salt bridge, hydrophobic interaction and ionic interactions (Arfat and Benjakul, 2012b). Additionally, disulphide bonds could be involved in the network formation of surimi gel (Visessanguan *et al*., 2000). Zinc was reported to induce disulphide bond formation in natural actomyosin during thermal process (Arfat and Benjakul, 2012b). This, disulphide bonds might contribute to gel strengthening of surimi added with zinc to some degree. Arfat and Benjakul (2012b) reported that yellow stripe trevally natural actomyosin solutions became less negatively charged in the presence of $ZnSO_4$ or $ZnCl_2$. This behavior could be attributed to ionic interactions between protein molecules and Zn^{2+} ions. The gelation of proteins is also induced by reducing the electrostatic repulsion between the aggregates by increasing the salt concentration (Hongsprabhas and Barbut, 1997). Haque and Aryana (2002) reported that bovine serum albumin gels added with $ZnSO_4$ had higher clustered aggregates than the control. Additionally, divalent cations can improve the hardness and WHC of gels by accelerating protein–protein interaction from three effects: electrostatic shielding, ion-specific hydrophobic interaction and cross-linking of adjacent anionic molecules by

forming protein-divalent cation-protein bridges (Havea *et al*., 2002; Nayak *et al*., 1998).

Kamaboko gels showed higher breaking force and deformation than modori gels, regardless of zinc salt addition. This was due to the enhanced setting phenomenon mediated by endogenous transglutaminase in the former (Arfat and Benjakul, 2012a). Furthermore, the higher degradation induced by heat-activated proteases took place to a higher extent in the latter (Arfat and Benjakul, 2012a). It was noted that the addition of $ZnSO_4$ or $ZnCl_2$ was able to improve the gel strength of modori gel to some degree. Nevertheless, the marked improvement of kamaboko gel could be achieved via setting in conjunction with Zn^{2+} addition, in which non-disulfide bonds and salt bridges could be formed, respectively. This resulted in the stronger gel network with higher elasticity as evidenced by the increases in both breaking force and deformation.

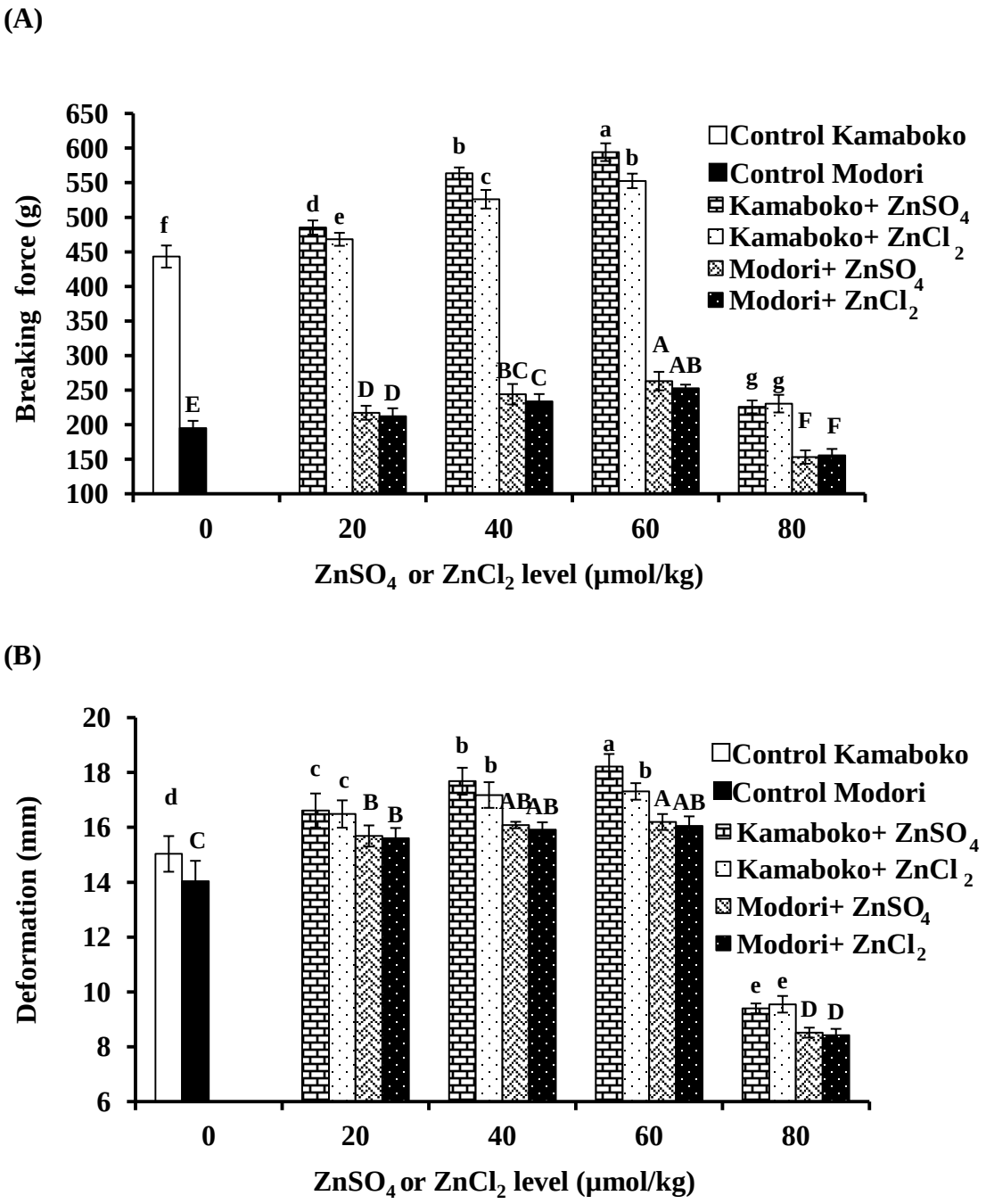


Figure 19. Breaking force (A) and deformation (B) of kamaboko and modori gels from yellow stripe trevally surimi added with $ZnSO_4$ or $ZnCl_2$ at different levels. Bars represent the standard deviation (n=3). Different lowercase letters or uppercase letters on the bars within the same gel indicate significant differences ($P < 0.05$).

4.4.2 Effect of $ZnSO_4$ or $ZnCl_2$ on whiteness of surimi gels

Whiteness of kamaboko and modori gels added with $ZnSO_4$ or $ZnCl_2$ at different levels is shown in Table 10. The increase in whiteness was observed in both gels, as $ZnSO_4$ or $ZnCl_2$ levels were above 40 µmol/kg ($P < 0.05$). There was no difference in whiteness between gels added with either $ZnSO_4$ or $ZnCl_2$ at levels of 60 and 80 µmol/kg ($P > 0.05$). Zinc salts considerably increased whiteness of both kamaboko and modori gels, probably due to light scattering by zinc ions or zinc-protein complex. Benjakul *et al*. (2004d) reported that increase in whiteness of surimi gels was associated with the light scattering effect of insoluble calcium carbonate in surimi gel. Divalent cation induced cold gelation of WPI and affected the color property of gel (Barbut, 1995; Hongsprabhas and Barbut, 1997). At an excessive level (80 µmol/kg), $ZnSO_4$ or $ZnCl_2$ more likely caused the rapid and pronounced coagulation or precipitation of proteins. Those large coagulums formed eventually led to the increased light scattering effect. Doi (1993) confirmed that gel whiteness is related with particle size. Generally, modori gels had a slightly higher whiteness, compared with kamaboko gel regardless of $ZnSO_4$ or $ZnCl_2$ levels used. Incubation of sol at 60 °C might cause the higher denaturation of proteins in modori gel, compared with the setting at lower temperature (40 °C) implemented in kamaboko gel, prior to heating at 90 °C. As a consequence, more turbidity or opaqueness was found in modori gels, as indicated by higher whiteness. Thus, zinc salts could improve whiteness of surimi gel, apart from gel enhancement.

Table 10. Whiteness and expressible moisture content of kamaboko and modori gels from yellow stripe trevally surimi added with $ZnSO_4$ or $ZnCl_2$ at different levels

Parameters	Zinc salt concentration (µmol/kg)	Kamaboko gel		Modori gel	
		$ZnSO_4$	$ZnCl_2$	$ZnSO_4$	$ZnCl_2$
Whiteness	0	72.79±0.31^{b}	72.79±0.31^{b}	73.39±0.35^{c}	73.39±0.35^{c}
	20	72.91±0.46^{b}	72.96±0.49^{b}	73.47±0.40^{bc}	73.52±0.36^{bc}
	40	73.18±0.2^{ab}	73.23±0.37^{ab}	73.76±0.35^{abc}	73.84±0.44^{abc}
	60	73.56±0.23^{a}	73.53±0.16^{a}	74.14±0.31^{ab}	74.1±0.30^{ab}
	80	73.73±0.10^{a}	73.71±0.12^{a}	74.40±0.27^{a}	74.43±0.35^{a}
Expressible moisture content (%)	0	4.87±0.03^{b}	4.87±0.03^{b}	6.14±0.11^{b}	6.14±0.11^{b}
	20	4.61±0.09^{b}	4.74±0.10^{b}	5.98±0.11^{bc}	6.06±0.05^{bc}
	40	3.86±0.17^{d}	4.28±0.09^{c}	5.47±0.16^{c}	5.67±0.06^{bc}
	60	3.24±0.13^{e}	4.03±0.10^{cd}	4.68±0.08^{d}	5.46±0.17^{c}
	80	14.50±0.41^{a}	14.42±0.31^{a}	17.35±0.54^{a}	17.46±0.75^{a}

Mean ± S.D (n=3).

Different superscripts within the same column under the same parameter indicate significant differences ($P < 0.05$).

4.4.3 Effect of $ZnSO_4$ or $ZnCl_2$ on expressible moisture content of surimi gels

Expressible moisture content of kamaboko and modori gels without and with addition of $ZnSO_4$ or $ZnCl_2$ at different levels is presented in Table 10. Control kamaboko gel showed the lower expressible moisture content than that of the control modori gel, indicating that kamaboko gel had higher water holding capacity than the modori gel. During setting at 40 °C, proteins underwent some denaturation and aligned themselves gradually to form the network, which could imbibe water (Benjakul and Visessanguan, 2003). Stronger network developed during setting mediated by endogenous transglutaminase has been reported to possess the higher water holding capacity (Benjakul *et al*., 2010). On the other hand, degraded proteins caused by heat activated proteases could not form the strong gel with capacity of

holding water (Benjakul *et al*., 2010). The expressible moisture content of all gels decreased as the levels of $ZnSO_4$ or $ZnCl_2$ increased up to 60 µmol/kg ($P < 0.05$). Expressible moisture content of kamaboko gels decreased by 33.5 and 17.2%, when 60 µmol/kg $ZnSO_4$ and $ZnCl_2$ were added, respectively (Table 10). When $ZnSO_4$ and $ZnCl_2$ at a level of 60 µmol/kg was incorporated, expressible moisture content of modori gel decreased by 23.8 and 11.1%, respectively. At a level of 60 µmol/kg, kamaboko and modori gel containing $ZnSO_4$ had lower expressible moisture content than those added with $ZnCl_2$ ($P < 0.05$). The lowered expressible moisture content was in accordance with the increased breaking force and deformation of corresponding kamaboko and modori gels (Figure 19). When an appropriate level of zinc salt was added, the cross-linking of proteins could be enhanced in the ordered fashion, resulting in the formation of stronger and fine network with greater water holding capacity. Remondetto *et al*. (2002) reported that gels with high textural properties retained more water due to a denser network with greater capillary forces. However, further increase in concentration of $ZnSO_4$ or $ZnCl_2$ up to 80 µmol/kg increased the expressible moisture content of both kamaboko and modori gels ($P < 0.05$), indicating a poor gel matrix with low water holding capacity. This was more likely associated with the excessive coagulation or precipitation of proteins at high salt concentration. Barbut (1994) reported that disproportionate addition of divalent cation resulted in high levels of protein coagulation, leading to lower water holding capacity. Therefore, the addition of zinc salts, especially $ZnSO_4$, at an optimal level resulted in the formation of protein network with superior water holding capacity.

4.4.4 Effect of $ZnSO_4$ or $ZnCl_2$ on protein pattern of surimi gels

Protein patterns of both kamaboko and modori gels added with $ZnSO_4$ or $ZnCl_2$ at different levels are depicted in Figure 20. The decrease in MHC band intensity was noticeable in all gels in comparison with that found in surimi. The result indicated that MHC might undergo either polymerisation or degradation. The decrease in MHC in kamaboko gel was generally due to the polymerisation mediated by endogenous transglutaminase (Benjakul and Visessanguan, 2003), whilst the degradation was dominant in modori gel (Arfat and Benjakul, 2012a). No differences in MHC band intensity were observed in gels added with $ZnSO_4$ or $ZnCl_2$ at levels up

to 60 µmol /kg. Nevertheless, at 80 µmol /kg concentration, MHC was much more retained in both kamaboko and modori gels. Protein cross-links via salt bridge and ionic interactions mediated by Zn^{2+} were destroyed by SDS used for electrophoresis. As a result, those cross-links could not be detected under these conditions (Hall and Struhl, 2002). Rapid and excessive coagulation or precipitation of proteins in the presence of $ZnSO_4$ or $ZnCl_2$ at 80 µmol /kg more likely limited protein unfolding and ordered network formation. Furthermore, zinc salt at high concentration (80 µmol/kg) might inactivate endogenous transglutaminase, thus preventing polymerization via non-disulfide covalent bonds. Worratao and Yongsawatdigul (2005) reported that tilapia TGase was inhibited by Zn^{2+} at 10mM concentration. Additionally, the coagulated proteins induced by excessive Zn^{2+} might not have the reactive groups, lysine and glutamine, available for transglutaminase. This might result in the lower setting, which was concomitant with poor gel properties as indicated by low breaking force and deformation (Figure 19). High zinc salt content possibly inactivated endogenous proteases at some degree, resulting in low degradation of MHC as evidenced by the higher MHC band intensity retained. Zn^{2+} has been reported as a potent inhibitor of several proteases, e.g. calpains. $ZnCl_2$ infusion blocked postmortem proteolysis and tenderization of ovine carcasses because Zn^{2+} inhibited both calpains and cathepsins B and L activities (Koohmaraie, 1990).

In modori gel, the disappearance of MHC with the coincidental increase in trichloroacetic acid (TCA) soluble peptide content (data not shown) suggested the degradation of MHC during incubation at 60 °C. The degradation of MHC in the modori gel was coincidental with the decreased breaking force (Figure 19A). High degradation of MHC was reported in surimi gel from dark-fleshed fish including mackerel (Benjakul *et al*., 2002a). Alkaline proteases have been reported to contribute to the protein degradation in surimi gels from dark flesh fish such as sardine and mackerel (Chaijan *et al*., 2004). Modori gel added with 80 µmol/kg $ZnSO_4$ or $ZnCl_2$ also had the high MHC band intensity, suggesting that Zn^{2+} might inactivate both proteases and transglutaminase in modori gel. Thus, Zn^{2+} concentration determined both protein polymerisation and degradation in surimi gel from yellow stripe trevally.

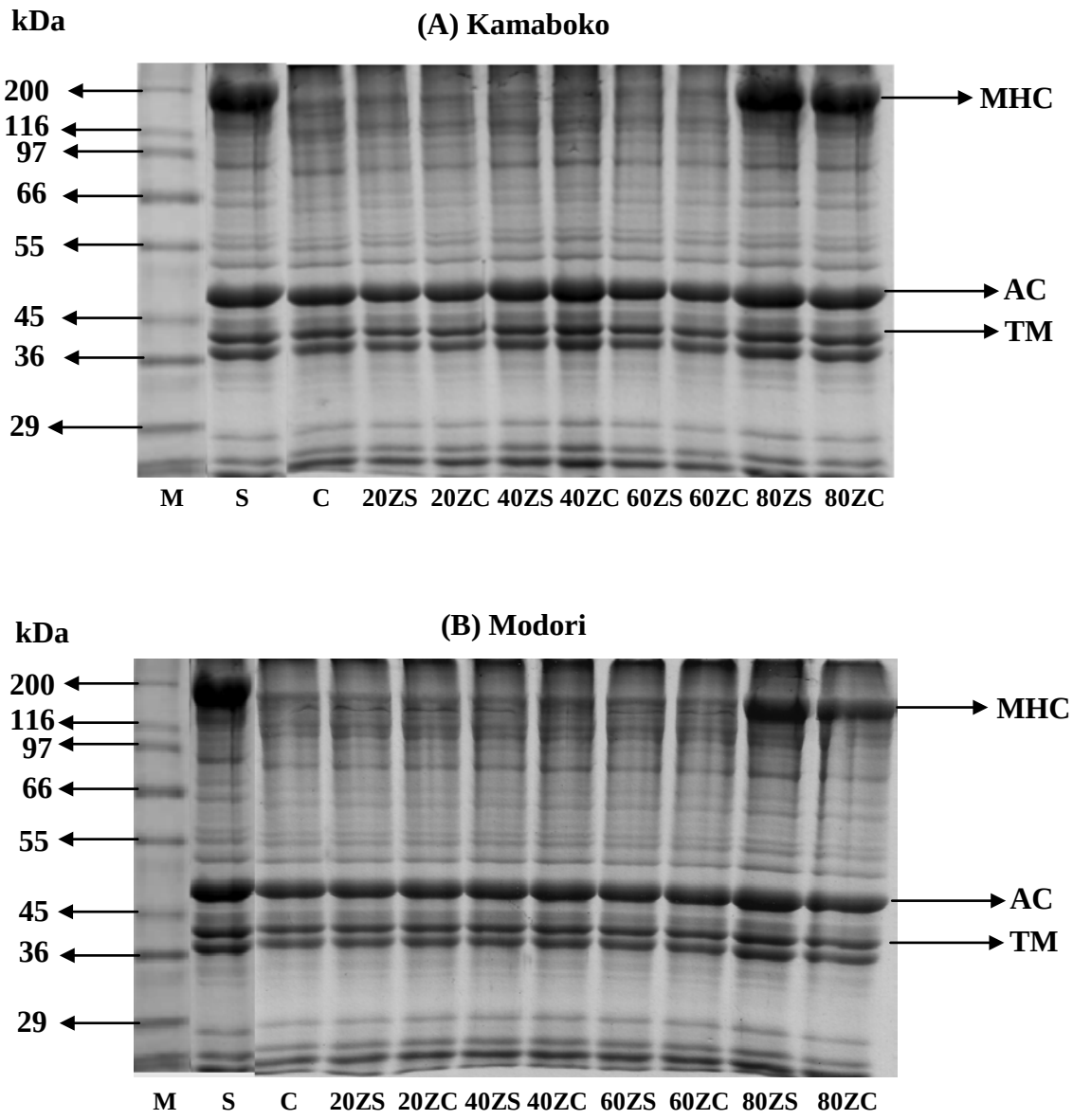
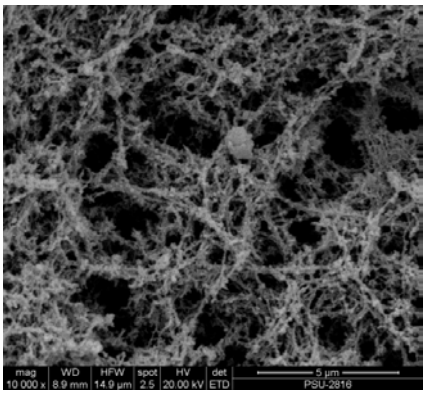


Figure 20. SDS-PAGE pattern of kamaboko (A) and modori (B) gels from yellow stripe trevally surimi added with $ZnSO_4$ or $ZnCl_2$ at different levels. M: marker; S: surimi; C: Control; ZS: gel added with $ZnSO_4$; ZC: gel added with $ZnCl_2$; MHC: myosin heavy chain; AC: actin; TM: tropomyosin. Numbers designate the level of $ZnSO_4$ or $ZnCl_2$ added (µmol/kg).

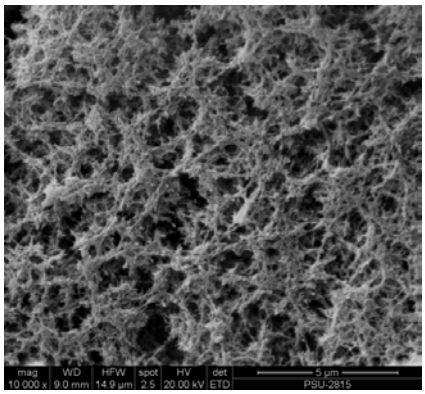
4.4.5 Effect of $ZnSO_4$ or $ZnCl_2$ on microstructure of surimi gels

Microstructures of kamaboko gels from yellow stripe trevally surimi without and with 60 and 80 µmol/kg $ZnSO_4$ or $ZnCl_2$ addition are illustrated in Figure 21. Kamaboko control gels (without $ZnSO_4$ or $ZnCl_2$) displayed a relatively loose protein gel matrix with larger voids, compared with gels added with $ZnSO_4$ or $ZnCl_2$ at a level of 60 µmol/kg. Gels incorporated with 60 µmol/kg $ZnSO_4$ had slightly more compact, finer and higher interconnected protein network, compared with that containing $ZnCl_2$ at the same concentration. This was in agreement with the higher breaking force with superior water holding capacity of the former gel. The finer and ordered gel network with smaller voids was observed in gels with the higher gel strength, whilst the looser network with larger voids was formed in the gels with lower gel strength (Balange and Benjakul, 2009b). When $ZnSO_4$ or $ZnCl_2$ at a level of 80 µmol/kg was incorporated, a coarser particulate network containing coagulated or precipitated proteins arranged in random clusters was developed (Figure 22). This was due to the rapid coagulation and precipitation of proteins at high zinc concentration. This was associated with the poor gel property with low water holding capacity, as evidenced by very low breaking force and deformation along with very high expressible moisture content. Thus, the addition of zinc salts, especially $ZnSO_4$, at 60 µmol/kg concentration resulted in the formation of fine and ordered filamentous gel network (Figure 22), thus improving gel strength and water holding capacity.

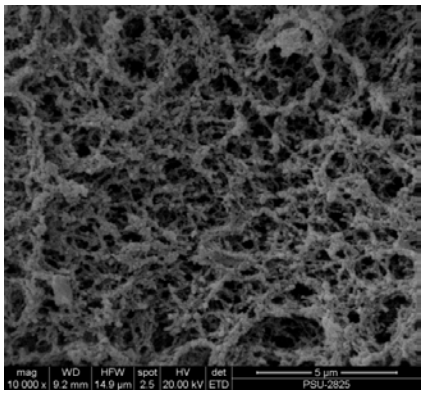
Control



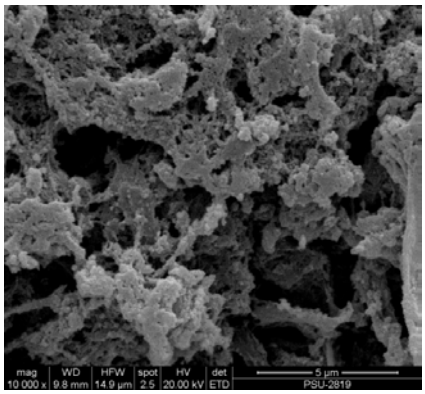
60 µmol/kg $ZnSO_4$



60 µmol/kg $ZnCl_2$



80 µmol/kg $ZnSO_4$



80 µmol/kg $ZnCl_2$

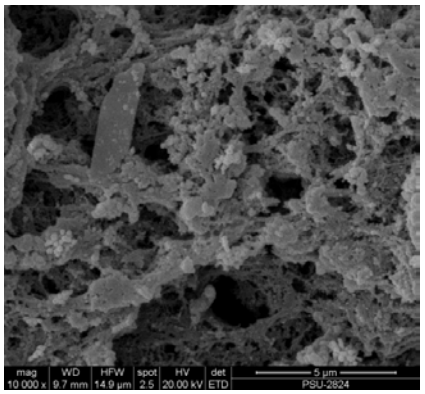


Figure 21. Microstructures of kamaboko gel from yellow stripe trevally surimi without and with $ZnSO_4$ or $ZnCl_2$ at levels of 60 and 80 µmol/kg. Magnification: × 10,000.

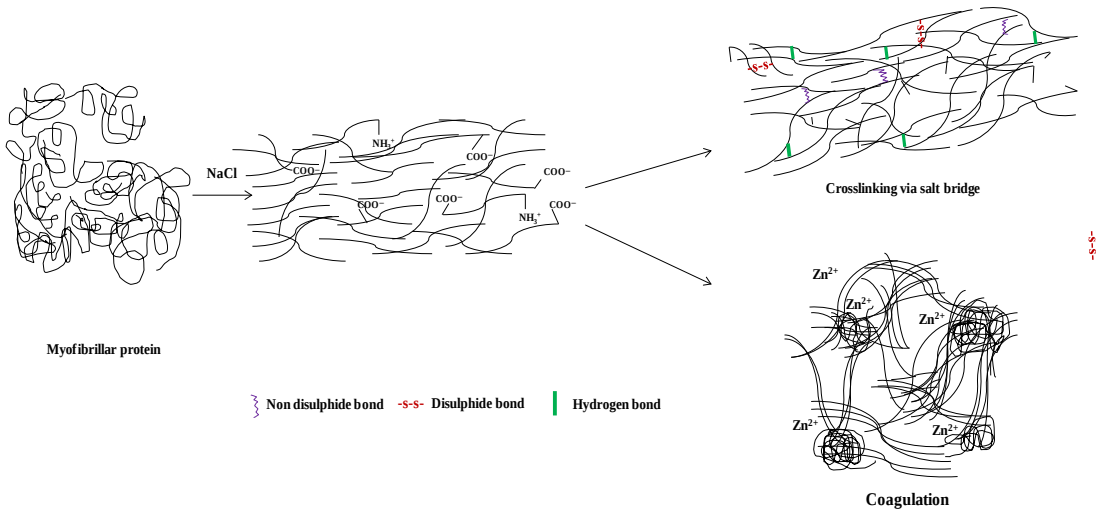


Figure 22. Scheme for the cross-linking of surimi proteins induced by Zn^{2+}.

4.5 Conclusion

Gel properties of surimi from yellow stripe trevally were governed by type and concentration of zinc salts. Kamaboko gel incorporated with 60 µmol/kg $ZnSO_4$ yielded the gel with increased breaking force, deformation and whiteness. Zn^{2+} mainly induced the formation of salt bridge and ionic interaction in gel network. Therefore, $ZnSO_4$ at an appropriate level could serve as promising additive to improve the strength and water holding capacity of gel from surimi manufactured from dark fleshed fish.

CHAPTER 5

Effect of zinc sulphate on gelling properties of phosphorylated protein isolate from yellow stripe trevally

5.1 Abstract

Impacts of zinc sulphate ($ZnSO_4$) (0–140 μmol/kg) on gel properties of yellow stripe trevally surimi added with sodium tripolyphosphate (STPP) (0.25 and 0.5%, w/w) and protein isolate phosphorylated with STPP at 0.25 and 0.5% (w/w) were studied. Gels from surimi added with 60 μmol $ZnSO_4$/kg in the absence and presence of 0.5% STPP had the increases in breaking force and deformation by 20.9% and 33.3%, and 11.6% and 18.6%, respectively, compared with the control surimi gel (without additives). Gel of protein isolate phosphorylated with 0.5% STPP containing 100 μmol $ZnSO_4$/kg had the increases in breaking force and deformation by 14.87% and 5.6%, respectively, compared with the gel from non-phosphorylated protein isolate at the same $ZnSO_4$ level, suggesting that the phosphorylated protein isolate was more crosslinked by Zn^{2+}. The addition of $ZnSO_4$ at the suitable level lowered the expressible moisture content, but increased whiteness of surimi or protein isolate gels ($P < 0.05$). Non-covalent bonds, more likely salt bridge and ionic interactions, played a major role in cross-linking of proteins in both surimi and protein isolate added with $ZnSO_4$, regardless of phosphates incorporated. Microstructure study revealed that a gel having highly interconnected and denser network with smaller voids was formed when protein isolate phosphorylated with 0.5% STPP was added with $ZnSO_4$ at a level of 100 μmol/kg. Thus, gel with improved properties could be obtained from protein isolate from yellow stripe trevally phosphorylated with STPP in conjunction with addition of $ZnSO_4$ at an appropriate level.

5.2 Introduction

Due to the over-exploitation of lean fish, which have been used for surimi production, dark fleshed fish have been paid increasing attention as a potential alternative raw material for surimi production, due to its abundance and low price (Chaijan *et al*., 2004). However, it is difficult to obtain high quality surimi from those species due to the high content of dark muscle, which contains a large amount of lipids and myoglobin. To overcome this problem, a pH-shift process, developed by Hultin and Kelleher (2000), has been used to both increase yield and improve gel property (Kristinsson and Hultin, 2003). The extraction mechanism of this process is to solubilize the muscle protein at low or high pH to separate soluble proteins from bone, skin, connective tissue, cellular membranes, and neutral storage lipids through the centrifugation (Nolsøe and Undeland, 2009). The solubilized proteins are recovered by isoelectric precipitation to give a highly functional and stable protein isolate (Kristinsson and Ingadottir, 2006). The major advantages of this process include economical feasibility, high recovery yield and improved functionalities. In some cases, the better gel properties were obtained, compared with surimi prepared by the conventional process (Kristinsson *et al*., 2005; Undeland *et al*., 2002). Gels prepared from rockfish and Atlantic croaker from proteins solubilized at alkaline pH exhibited better gel quality than those prepared from the acid-aided process (Perez-Mateos *et al*., 2004).

Divalent cations, e.g. calcium, magnesium and zinc are known to affect the functionality of proteins during gelation (Mathew *et al*., 2009; Morales *et al*., 2001). At pH values sufficiently far from the isoelectric point of proteins, divalent ions such as Ca^{2+}, Zn^{2+}, Mg^{2+} induce protein cross-linking via the salt bridges between negatively charged carboxyl groups (Hongsprabhas and Barbut, 1997). Recently, Arfat and Benjakul (2012b) have reported that the addition of zinc salts, especially zinc sulphate, induced the aggregation of natural actomyosin, mainly by the induction of higher hydrophobic interaction, salt bridge and ionic interaction.

Phosphates have been reported to dissociate protein complexes and are widely accepted as potential additives in fish and seafood to improve the functional

properties of those products (Chang and Regenstein, 1997). Phosphates were used to improve gelling property of surimi from bigeye snapper (Julavittayanukul *et al*., 2006). Therefore, the addition of sodium tripolyphosphate in combination with zinc salt might be an approach to strengthen gel network of surimi via introducing the negatively charged phosphate, which can be subsequently served for 'salt bridge' induced by Zn^{2+}.

During pH-shift process, the proteins undergo dissociation via repulsion, thereby becoming solubilized. Phosphate compounds could react with –OH or $–NH_2$ groups on the side chains of proteins, particularly at alkaline condition (Ferrel Sung, 1982). For the new process, the simultaneous incorporation of phosphates during solubilization under alkaline condition could not only improve the effectiveness of phosphorylation, but also has the potential to produce quality protein isolate with dominant negative charged side chains that could serve as a better substrate for crosslinking mediated by Zn^{2+} via salt bridge mechanism. As a consequence, the superior gel properties of fish muscle proteins can be obtained. Additionally, the excessive phosphates could be removed by the precipitation of proteins at their isoelectric point. Nevertheless, the use of divalent cations on gelling properties of phosphorylated fish protein isolate has not been reported previously. Therefore, the objective of this study was to investigate the effect of $ZnSO_4$ at different levels on the gel properties of surimi added with STPP and protein isolates from yellow stripe trevally phosphorylated with STPP.

5.3 Materials and methods

5.3.1 Chemicals

All chemicals were of analytical grade. Sodium dodecyl sulphate (SDS), β-mercaptoethanol (β-ME), glycerol, sodium tripolyphosphate and glutaraldehyde were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide, hydrochloric acid, *N*,*N*,*N'*,*N'*–tetramethyl ethylene diamine (TEMED), acrylamide, and bisacrylamide were procured from Fluka (Buchs, Switzerland). Zinc sulphate ($ZnSO_4$) was purchased from Ajax Finechem, Pty. Ltd. (Taren Point, NSW, Australia).

5.3.2 Preparation of fish mince

Fresh yellow stripe trevally (*Selaroides leptolepis*) with an average weight of 80–90 g/fish were caught from Songkhla coast along the Gulf of Thailand during December, 2012 and January, 2013. The fish, off-loaded approximately 12 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 1 h. The fish were immediately washed, gutted, cleaned and filleted. Fillets were subjected to mincing using a mincer with the hole diameter of 5 mm. Mince obtained was placed in polyethylene bag and imbedded in ice until use.

5.3.3 Preparation of surimi and fish protein isolate

To prepare surimi by the conventional washing process, fish mince was washed with cold water (4 °C) using a water/mince ratio of 3:1 (w/w). The mixture was stirred gently for 10 min in a cold room (4 °C) and the washed mince was filtered with a layer of nylon screen. Washing was performed three times. Finally, the washed mince was centrifuged at 700 × g for 15 min using a basket centrifuge (Model CE 21 K, Grandiumpiant, Belluno, Italy). The resulting pellet was referred to as ‘surimi’.

To prepare the protein isolate, the alkaline solubilization process was used following the method of Undeland *et al*. (2002) with a slight modification. The

mince was prewashed with 3 cycles of cold water using a water/mince ratio of 3:1 (w/w). The mince (250 g) was homogenized for 1 min with 2.25 l of cold distilled water (4 °C) using an IKA homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was adjusted to the pH of 11 using 2 N NaOH. Thereafter, the solubilized proteins were added with 0.25 or 0.5% (w/w) STPP and stirred continuously for 1 h at 4 °C. For the control protein isolate, no STPP was incorporated. The mixtures were then centrifuged at 10,000 × g for 20 min using a refrigerated centrifuge (Avanti-JE Centrifuge, Beckman 163 Coulter Inc., Fullerton, CA, USA) at 4 °C to remove the insoluble materials. The soluble proteins were then precipitated by adjusting the pH to 5.5 using 2 N HCl. Precipitated proteins were collected by centrifuging at 10,000 × g for 20 min at 4 °C. The pH of obtained pellet was adjusted to 7.0 using 2 N NaOH. The recovered proteins were referred to as 'phosphorylated protein isolate'.

One set of surimi, protein isolate, and phosphorylated protein isolate were subjected to analyses. Another set of those samples was added with cryoprotectants including 4% sucrose and 4% sorbitol, mixed well and frozen using an airblast freezer (Patkol Co., Ltd, Bangkok, Thailand). The frozen samples were kept at -18 °C until used for gel preparation. The storage time was not more than 1 month.

5.3.4 Study on chemical compositions and Ca^{2+}-ATPase activity of surimi, protein isolate and phosphorylated protein isolate

Surimi, protein isolate and phosphorylated protein isolate without cryoprotectants were determined for chemical composition and Ca^{2+}-ATPase activity.

5.3.4.1 Moisture and ash contents

The samples were analysed for moisture and ash contents according to the method of AOAC (2000) with the analytical No. of 950.46 and 920.153, respectively.

5.3.4.2 Determination of lipid and phospholipid contents

Lipid content was determined by the Soxhlet apparatus according to the method of AOAC (2000) with the analytical No. of 920.39B. Phospholipid content was measured based on the direct spectrophotometeric measurement of complex formation between phospholipids and ammonium ferrothiocyanate as described by Stewart (1980). Lipids were firstly extracted by the Bligh and Dyer method (Bligh and Dyer, 1959). Thereafter, lipids (20 µl) were dissolved in chloroform to obtain a final volume of 2 ml. One millilitre of thiocyanate reagent (a mixture of 0.10 M ferric chloride hexahydrate and 0.40 M ammonium thiocyanate) was added. After thorough mixing for 1 min, the lower layer was removed and the absorbance at 488 nm was measured. A standard curve was prepared using phosphatidylcholine (0–50 ppm). The phospholipid content was expressed as mg/100 g dry sample.

5.3.4.3 Determination of phosphate content

The phosphate content was determined according to the method of Suzuki and Suyama (1985) with a slight modification. To the samples (0.6–0.8 g), 20 ml of 4 M NaOH were added and mixed vigorously. The samples were heated in a boiling water bath (90-95 °C) for 30 min and then cooled at room temperature for 1 h. The mixture was mixed with 20 ml of 4 M HCl for neutralisation. The supernatant (0.2 ml) was mixed with 2 ml of phosphate reagent (4.2% ammonium molybdate solution: 0.045% malachite green, 1:3 v/v). The mixture was then incubated at room temperature for 30 min. The absorbance was measured at 620 nm. The phosphate content was calculated from a phosphate standard curve. Disodium hydrogen phosphate solutions with concentrations of 0 to 15 µg/ml were used as standard. Phosphate content was expressed as mg/100g sample.

5.3.4.4 Determination of Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity of natural actomyosin (NAM) was determined according to the method of Benjakul *et al*. (1997). NAM prepared as described by Benjakul *et al*. (1997) was diluted to 5 mg/ml with 0.6 M KCl (pH 7.0). To the diluted

NAM solution (1 ml), 0.6 ml of 0.5 M Tris–maleate (pH 7.0) and 1 ml of 0.1 M $CaCl_2$ were added. Deionized water was added to make up a total volume of 9.5 ml. To the mixture, 0.5 ml of 20 mM adenosine 5-triphosphate (ATP) solution was added to initiate the reaction. The reaction was conducted for 10 min at 25 °C and terminated by adding 5 ml of chilled 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3500 × g for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). The Ca^{2+}-ATPase activity was expressed as micromoles inorganic phosphate released/mg protein/min. A blank was prepared in the same manner, except that chilled trichloroacetic acid was added prior to addition of ATP.

5.3.5 Study on the impact of $ZnSO_4$ on properties of gel from surimi added with STPP and phosphorylated protein isolate

5.3.5.1 Gel preparation

Frozen samples were partially thawed at 4 °C for 6 h. The samples were cut into small pieces. The samples were ground for 2 min using a Moulinex Masterchef 350 mixer (Paris, France). NaCl (2.5%, w/w) was then added and the mixture was chopped for 1 min. The surimi paste was added with STPP (0.25 and 0.5%, w/w) and $ZnSO_4$ (0, 60, 100 and 140 µmol/kg paste). For protein isolate and phosphorylated protein isolate, $ZnSO_4$ (0, 60, 100 and 140 µmol/kg paste) was added after mixing with (2.5%, w/w) NaCl for 1 min. Final moisture content was adjusted to 80% using cold water (1-2 °C). All mixtures were chopped for another 4 min at 4 °C to obtain the homogenous paste. The paste was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a temperature controlled water bath (Memmert, Schwabach, Germany). The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analyses.

5.3.5.2 Textural analysis

Textural analysis of gel samples was performed using a Model TA-XT2i texture analyser (Stable Micro Systems, Surrey, England). Gels were equilibrated and evaluated at room temperature (28–30 °C). Cylinder-shaped samples of 2.5 cm in length were prepared and subjected to determination. Breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyzer equipped with a spherical plunger (diameter 5 mm, depression speed of 60 mm/min).

5.3.5.3 Determination of whiteness

All gels were subjected to whiteness measurement using a Hunterlab (ColorFlex, Hunter Associates Laboratory, Reston, VA). Illuminant C was used as the light source of measurement. *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were measured and whiteness was calculated as described by NFI (1991) as follows:

$$\text{Whiteness} = 100 - \left[\left(100 - L^*\right)^2 + a^{*2} + b^{*2} \right]^{\frac{1}{2}}$$

5.3.5.4 Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al*. (2001b) with slight modifications. A gel sample with a thickness of 0.5 cm was weighed (X g) and placed between two pieces of Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, England) at the top and three pieces of the same type of filter paper at the bottom. The standard weight (5 kg) was placed on the top of the sample and maintained for 2 min. The sample was then removed and weighed again (Y g). Expressible moisture content was calculated and expressed as percentage of sample weight as follows:

$$\text{Expressible moisture content (\%)} = \left[\frac{(X-Y)}{X}\right] \times 100$$

5.3.5.5 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of gels were analysed by SDS–PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85 °C were added to the sample (3 g). The mixture was then homogenized at a speed of 11,000 rpm using a homogenizer for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 8,000 × g for 20 min at room temperature (26–28 °C) using a centrifuge (Model MIKRO20, Hettich ZENTRIFUGEN, Tuttlingen, Germany). Protein concentration in the supernatant was determined as per the method of Lowry *et al*. (1951). Solubilized samples were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol and 10% β-ME) and boiled for 3 min. Samples (15 µg protein) were loaded onto polyacrylamide gels comprising a 10% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein III unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.02% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid.

5.3.5.6 Microstructure

Microstructure of gels was determined using a scanning electron microscope (SEM). Gels were cut into small pieces (0.25 × 0.25 × 0.25 cm^3) and fixed with 2.5 % (v/v) glutaraldehyde in 0.2 M phosphate buffer, pH 7.2 for 2 h at room temperature. The fixed samples were rinsed twice with distilled water. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50%, 70%, 80%, 90%, and 100%. Samples were critical point dried (Balzers mod. CPD 030, Liechtenstein, Switzerland) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA) and examined on a JSM 5800 scanning electron microscope (JEOL, Ltd., Tokyo, Japan) at an acceleration voltage of 20 kV.

5.3.6 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, U.S.A.).

5.4 Results and discussion

5.4.1 Chemical compositions and Ca^{2+}-ATPase activity of surimi, proteins isolate and phosphorylated protein isolate

5.4.1.1 Moisture and ash contents

Moisture content of surimi, protein isolate and protein isolate phosphorylated with 0.25 and 0.5% STPP was 76.64, 84.53, 84.23 and 84.11%, respectively (Table 11). The moisture content of protein isolate samples was higher ($P < 0.05$) than that of surimi. However, there was no marked difference in moisture content between protein isolate without and with phosphorylation ($P > 0.05$).

Ash content of surimi was slightly higher than that of protein isolates. Surimi produced in the present study was not refined. As a result, small scales or pin bones could be retained to some extent. Nevertheless, those materials were removed by centrifugation during preparation of protein isolate as indicated by low ash content. Marmon and Undeland (2010) reported that ash content in the protein isolates was drastically lowered to < 20% of that found in the herring mince. It was noted that ash content of phosphorylated protein isolate was non-significantly higher than that found in non-phosphorylated counterpart. It implied that some phosphates were attached to muscle proteins via phosphorylation.

5.4.1.2 Lipid and phospholipid contents

Lipid content of surimi, protein isolate and protein isolate phosphorylated with 0.25 and 0.5% STPP was 3.93, 1.21, 1.17 and 1.27% (dry basis), respectively (Table 11). Lipid content of protein isolate was decreased by 69.2%, whilst that of protein isolate phosphorylated with 0.25 and 0.5% STPP lipid content was reduced by 70.2 and 67.7%, respectively, compared with that found in surimi. During alkaline solublisation, proteins were more likely dissociated. As a result, lipids could be liberated from proteins. After solubilization, these components were separated on the basis of density and solubility differences. The higher lipid content of surimi was noticeable because membrane lipids were still retained. Furthermore, a portion of storage lipids might co-aggregate with the proteins during the process. The higher lipid removal by alkaline solubilization process might be due to the higher emulsification ability of proteins at alkaline pH (Kristinsson *et al*., 2005). Hultin and Kelleher (2000) also suggested that the first centrifugation step causes a portion of the membrane phospholipids to sediment at the bottom, and also causes significant separation of neutral lipids to the top. Batista *et al*. (2007) reported the decrease in fat content in sardine muscle by 65.3% and 51.0% after alkaline and acid solubilization, respectively. Rawdkuen *et al*. (2009) reported that the lipid reduction of 85.2 and 86.6% was achieved for protein isolate recovered using acid and alkaline solubilization processes, respectively.

For phospholipid content, it was in accordance with lipid content, in which the lowest phospholipid content was obtained in protein isolates (Table 11). However, there was no difference in phospholipid content between protein isolate and protein isolate phosphorylated with 0.25 and 0.5% STPP ($P > 0.05$). Protein isolate without and with phosphorylation using 0.25 and 0.5% STPP had the decreases in phospholipid by 69.4%, 68.1% and 69.3%, respectively, compared with that found in surimi. In muscle tissue, phospholipid membranes are connected with cytoskeletal proteins through electrostatic attraction between the phospholipids of membranes and the basic amino acid residues of the cytoskeletal proteins (Haleva *et al*., 2004). Undeland *et al*. (2002) found that phospholipid to protein ratio from herring light muscle was reduced from 0.13 g lipid/g protein to 0.02 g/g protein by alkaline

solubilization process. During alkaline solubilization, proteins became dissociated, in which phospholipids could be more exposed to hydrophilic environment. As a result, they were leached out potentially from the proteins. Therefore, the removal of lipid and phospholipid from yellow stripe trevally could be achieved by using alkaline solubilization process.

5.4.1.3 Phosphate content

Phosphate content of surimi, protein isolate and protein isolate phosphorylated with 0.25 and 0.5% STPP is shown in Table 11. Phosphate content was higher in surimi (298.97 mg/100g), compared to that of protein isolate (96.63 mg/100g) ($P < 0.05$). The removal of phospholipids during alkaline solubilization more likely lowered phosphate in protein isolate. Phospholipids contain phosphate group at *sn*-3 position (Vance and Vance, 2008). On the other hand, the phosphate content of protein isolate increased as the levels of STPP used for phosphorylation increased ($P < 0.05$), indicating the attachment of phosphates to proteins at alkaline pH. Polyphosphates was reported to bind with protein, particularly at alkaline pH (Sung *et al*. 1983). Furthermore, Wang and Smith (1994) reported binding of phosphate to the myosin molecules, mainly the S1 subunit of the myosin heavy chain.

Table 11. Chemical compositions and Ca^{2+}-ATPase activity of yellow stripe trevally surimi, protein isolate and phosphorylated protein isolates

Parameters	Surimi	Protein isolate	Protein isolate phosphorylated with 0.25% STPP	Protein isolate phosphorylated with 0.5% STPP
Moisture content (% wet wt. basis)	76.64±0.19b*	84.53±0.13a	84.23±0.37a	84.11±0.53a
Ash content (% wet wt. basis)	1.79±0.16a	0.47±0.10b	0.59±0.10b	0.66±0.08b
Lipid (% dry wt. basis)	3.93±0.17a	1.21±0.16b	1.17±0.11b	1.27±0.13b
Phospholipid (mg/100 g dry sample)	488.72±16.43a	149.67±15.37b	155.91±14.15b	150.15±11.85b
Phosphate content (mg/100 g dry sample)	298.97±12.83a	96.63±8.95c	254.21±11.30b	320.17±15.17a
Ca^{2+}-ATPase activity (micromoles inorganic phosphate/mg protein/min)	0.142±0.003a	0.021±0.001b	0.019±0.002b	0.018±0.002b

Values are given as mean ± SD (n = 3).

*Different letters within the same row indicate the significant differences ($P < 0.05$).

5.4.1.4 Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity of NAM extracted from surimi, protein isolate and protein isolate phosphorylated with 0.25 and 0.5% STPP is shown in Table 11. The highest activity of Ca^{2+}-ATPase was found in NAM extracted from surimi. Loss in Ca^{2+}-ATPase activity is an indicator of myosin denaturation (Benjakul *et al*., 1997; Benjakul *et al*., 2011). For protein isolates prepared by the alkaline solubilization process, a marked decrease in Ca^{2+}-ATPase activity was observed ($P < 0.05$). The results suggested that the denaturation of myosin was pronounced by alkaline solubilization process. These results were in agreement with Chaijan *et al*. (2006) who reported that myosin of sardine (*Sardinella gibbosa*) and mackerel (*R. kanagurta*) underwent denaturation by alkaline solubilization. When muscle proteins were adjusted to pH above pI, the protein molecule became negatively charged. As a consequence, the repulsion became dominated, leading to the dissociation of protein as well as unfolding of myosin head with ATPase activity. No differences in Ca^{2+}-ATPase activity was observed between protein isolates without and with phosphorylation ($P > 0.05$).

5.4.2 Effect of $ZnSO_4$ on properties of gel from surimi and protein isolates

5.4.2.1 Breaking force and deformation

Breaking force and deformation of gels from yellow stripe trevally surimi without and with STPP at 0.25 and 0.5% and protein isolate without and with phosphorylation using 0.25 and 0.5% STPP in the presence of $ZnSO_4$ at different levels are depicted in Figure 23. Without $ZnSO_4$ and STPP incorporation, gels prepared from protein isolate showed the higher breaking force and deformation ($P < 0.05$) than surimi gel. Generally, breaking force of the gel was positively correlated with gel strength, whilst the deformation represented the elasticity of the gels. The increases in breaking force and deformation of gel from protein isolate were plausibly due to the partial denaturation of protein after alkaline treatment, leading to the exposure of reactive groups that subsequently underwent interaction during thermal gelation (Chaijan *et al*., 2010b). Moreover, the reduction of lipid also resulted in

higher ability of proteins to interact with each other, thereby increasing breaking force of gels. The presence of lipids may interfere with myosin cross-linking during gel matrix formation because they do not form gels and have poor water holding capacity (Chanarat and Benjakul, 2013). Kristinsson and Ingadottir (2006) reported that washed mince, and protein isolates had different protein compositions, which contributed to the different gel forming ability.

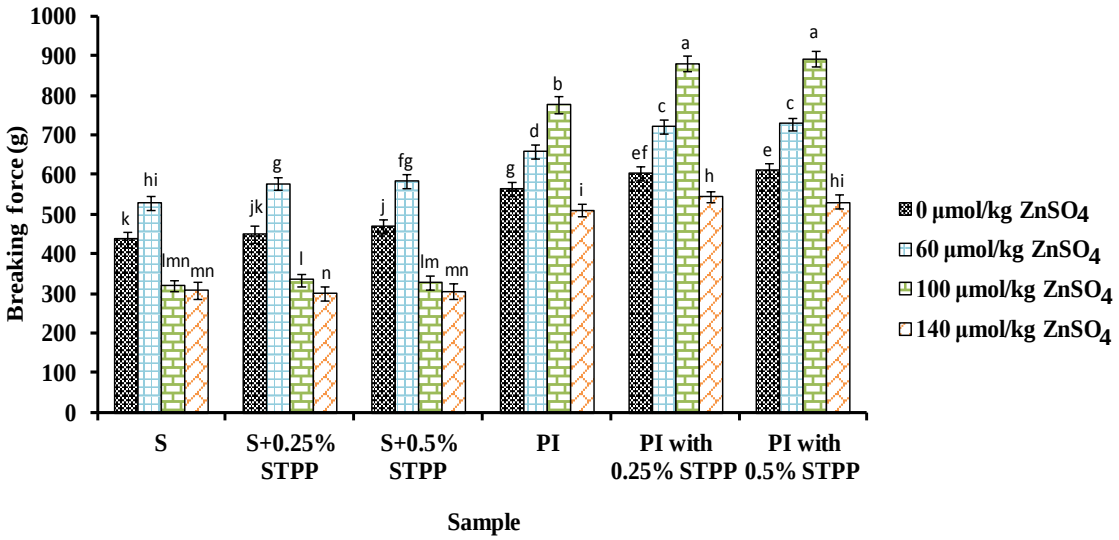
For surimi gel, breaking force and deformation increased when STPP was added. STPP has been known to improve gel property of surimi gel. STPP can be dissociated to pyrophosphate, which induced the dissociation of actomyosin complex. As a result, the monomer was able to form the ordered and firm network (Julavittayanukul *et al*., 2006). Julavittayanukul *et al*. (2006) also found the increase in breaking force and deformation when sodium pyrophosphate at levels of 0.05% (w/w) was added into surimi from bigeye snapper. When $ZnSO_4$ was added, it was found that $ZnSO_4$ at 60 µmol/kg yielded the gel with increased breaking force and deformation ($P < 0.05$). Breaking force of surimi gels without and with 0.25 and 0.5% STPP added with 60 µmol/kg $ZnSO_4$ increased by 20.9, 32.2 and 33.3%, respectively, and the deformation increased by 11.6, 17.3 and 18.6%, respectively, compared with the control (without additives). However, gels from surimi added with higher level of $ZnSO_4$ had the marked decreases in breaking force and deformation, as compared with surimi gel and gel added with 60 µmol/kg $ZnSO_4$, regardless of STPP levels used ($P < 0.05$). Excessive amount of zinc salts more likely led to coagulation of proteins, in which a particulate gel was formed. The concentration of salt is of the major factor affecting structure and spatial organization of protein aggregates (Hongsprabhas *et al*., 1999). It was noted that STPP addition had no synergistic effect with $ZnSO_4$ on gel strengthening.

For protein isolate, phosphorylation resulted in the increases in breaking force and deformation. Phosphates attached to muscle proteins plausibly provided negatively charged domains for interaction via ionic bonding. When $ZnSO_4$ at various levels was added into both protein isolate and phosphorylated protein isolates, the increases in breaking force and deformation were obtained with increasing $ZnSO_4$ levels up to 100 µmol/kg ($P < 0.05$). At 140 µmol/kg, the sharp

decreases in both breaking force and deformation were observed ($P < 0.05$). With addition of 100 µmol/kg $ZnSO_4$, breaking force of protein isolate gels and gels of protein isolate phosphorylated with 0.25 and 0.5% STPP increased by 37.1, 55.6 and 57.5%, respectively, and deformation increased by 20.7, 26.7 and 27.5%, respectively, compared with the control protein isolate gel (without additives). Gels from phosphorylated protein isolate showed higher breaking force and deformation than non-phosphorylated protein isolate gels, at all levels of $ZnSO_4$ addition ($P < 0.05$). However, no differences in breaking force and deformation between both phosphorylated protein isolate gels were found ($P > 0.05$). Gel strengthening effect of $ZnSO_4$ was likely due to the salt bridges formation between Zn^{2+} and negatively charged residues, mainly COO^{-} of protein side chains or C- termini. Divalent metal ions might cause changes in protein conformation and interact with negatively charged residues on the polypeptide chains of proteins, thereby inducing cross-linking via salt bridges (Ding *et al*., 2011). Zn^{2+} was able to induce configurational change in myosin heavy chain, resulting in the ease of thermal denaturation with subsequent enhanced aggregation via salt bridge, hydrophobic interaction and ionic interactions (Arfat and Benjakul, 2012b). Haque and Aryana (2002) reported that bovine serum albumin gels added with $ZnSO_4$ had higher clustered aggregates than the control. Additionally, divalent cations can improve the hardness and WHC of gels by accelerating protein–protein interaction from three effects: electrostatic shielding, ion-specific hydrophobic interaction and cross-linking of adjacent anionic molecules by forming protein-divalent cation-protein bridges (Havea *et al*., 2002; Nayak *et al*., 1998). The higher gel enhancing effect of $ZnSO_4$ observed in phosphorylated protein isolate was plausibly due to the increased salt bridge formation between Zn^{2+} and negatively charged domains caused by phosphate incorporation. A prerequisite for protein cross-linking induced by Zn^{2+} is the availability of substrates, which undergo salt bridge cross-linking (Remondetto *et al*., 2002). Moreover, alkaline solubilization more likely induced the dissociation of protein complexes, in which protein monomers with the higher exposure of reactive groups, especially aspartic acid and glutamic acid, could be formed. As a result, the cross-linking of proteins could be enhanced in the presence of Zn^{2+}, between carboxylic groups (COO^{-}) of side chains.

The results suggested that gel properties of phosphorylated protein isolate could be much improved by $ZnSO_4$ additions.

A)



B)

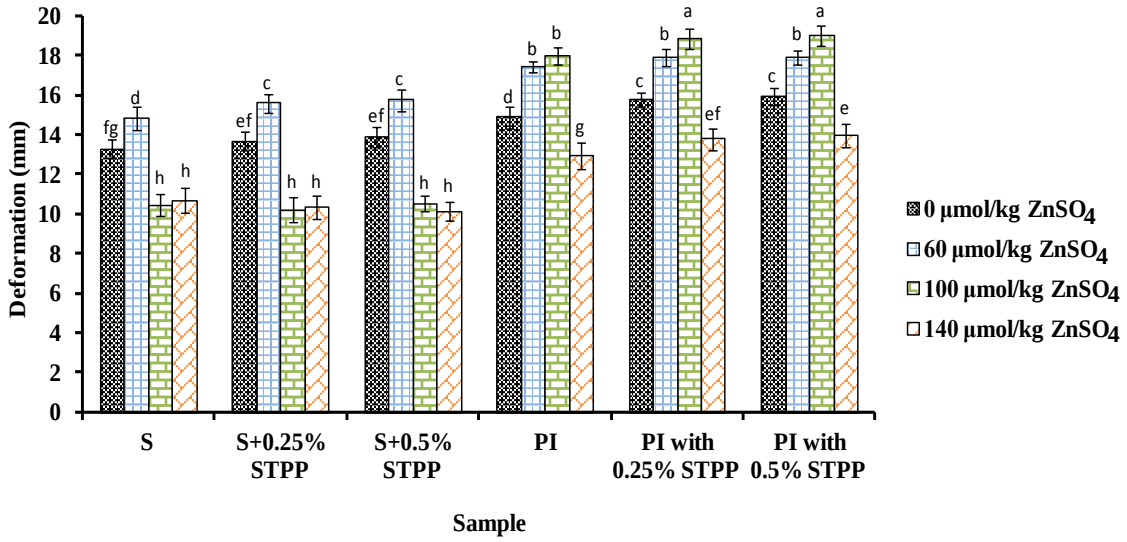


Figure 23. Breaking force (A) and deformation (B) of gels from yellow stripe trevally surimi added with STPP at different levels and protein isolate without and with phosphorylation using 0.25 and 0.5% STPP as influenced by $ZnSO_4$ at different levels (0-140 µmol/kg). Bars represent the standard deviation (n=3). Different letters on the bars indicate significant differences ($P < 0.05$).

5.4.2.2 Whiteness

Whiteness of gels from surimi added without and with STPP at 0.25 and 0.5% and gels of protein isolate without and with phosphorylation using 0.25 and 0.5% STPP in the presence of $ZnSO_4$ at various levels is shown in Table 12. Whiteness of surimi gel was higher than that of protein isolate gel ($P < 0.05$). Yongsawatdigul and Park (2004) also reported that the highest whiteness was found in conventionally processed surimi from rockfish, followed by alkaline-produced protein isolate and crude mince. Gels of conventional surimi from Atlantic menhaden exhibited a higher whiteness than those of the alkaline-aided protein (Perez-Mateos and Lanier, 2006). The lower whiteness of gels of protein isolate, compared to that of surimi, was possibly due to the oxidation of haem proteins in the recovered proteins, which was induced by the alkaline pH. Chaijan *et al*. (2007a) reported that alkaline and acid conditions accelerated autoxidation of sardine myoglobin. Addition of STPP in surimi gel led to slight increases in whiteness. Phosphorylation of protein isolate using 0.5% STPP also resulted in the slight increase in whiteness ($P < 0.05$). Julavittayanukul *et al*. (2006) reported the increase in whiteness of surimi gels as the phosphate concentrations increased.

When $ZnSO_4$ at different levels was incorporated, the increase in whiteness of all gels was observed ($P < 0.05$). In general, whiteness of all gels increased with increasing $ZnSO_4$ levels. $ZnSO_4$ considerably increased whiteness of both surimi and protein isolate gels, probably due to light scattering by zinc ions or zinc-protein complex. Benjakul *et al*. (2004d) reported that increase in whiteness of surimi gels was associated with the light scattering effect of insoluble calcium carbonate in surimi gel. Light scattering in meat is caused by structural, myofibrillar proteins and size of scattering particle (Kang *et al*., 2007). Thus, the addition of both $ZnSO_4$ and STPP affected whiteness of surimi and protein isolate gels.

Table 12. Expressible moisture content and whiteness of gels from yellow stripe trevally surimi added with STPP at different levels and protein isolate without and with phosphorylation as influenced by zinc sulphate at different levels

Parameters	$ZnSO_4$ levels	Surimi	Surimi+0.25STPP	Surimi+0.5STPP	Protein isolate	Protein isolate Phosphorylated with 0.25% STPP	Protein isolate Phosphorylated with 0.5% STPP
Expressible	0	4.74±0.17b[*]C[**]	4.40±0.27bCD	4.21±0.38bD	6.09±0.17bA	5.95±0.10bA	5.51±0.20bB
moisture	60	3.19±0.12cC	3.08±0.12cC	2.95±0.27cC	5.74±0.16bA	5.54±0.08bAB	5.30±0.20bcB
content (%)	100	13.37±0.46aA	13.21±0.31aA	13.37±0.50aA	5.43±0.18bB	5.30±0.16bBC	4.78±0.08cC
	140	13.93±0.63aB	13.71±0.48aB	13.54±0.52aB	15.03±0.75aA	15.15±0.72aA	15.02±0.33aA
Whiteness	0	72.41±0.08cB	72.96±0.22cA	73.21±0.31cA	70.68±0.28cD	71.03±0.22cCD	71.43±0.23dC
	60	73.19±0.30bB	73.61±0.36bB	74.24±0.11bA	71.29±0.27bD	71.71±0.25bCD	72.02±0.17cC
	100	73.58±0.11abB	74.19±0.44abA	74.53±0.23abA	71.72±0.19abD	72.27±0.23aC	72.69±0.08bC
	140	73.76±0.31aB	74.48±0.14aA	74.85±0.10aA	72.19±0.47aE	72.65±0.17aD	73.16±0.10aC

Values are given as mean ± SD (n = 3).

[*]Different letters within the same column under the same parameter tested indicate the significant differences ($P < 0.05$).

[**]Different capital letters within the same row indicate the significant differences ($P < 0.05$).

5.4.2.3 Expressible moisture content

Expressible moisture content of gels from surimi containing STPP at various levels and protein isolate without and with phosphorylation as influenced by levels of $ZnSO_4$ is shown in Table 12. Different gels showed varying expressible moisture content ($P < 0.05$). Surimi gel had the lower expressible moisture content than protein isolate gel ($P < 0.05$), suggesting the highest water holding capacity of the former. Chaijan *et al*. (2006) also reported that higher expressible moisture content was found in the gels of sardine and mackerel protein isolate prepared by alkaline solubilization process, compared with those from the conventional washing method. It was noted that water holding capacity of surimi increased with increasing concentration of STPP ($P < 0.05$). This was evidenced by the lowered expressible moisture content. The lower expressible moisture content in kamaboko gels of bigeye snapper surimi added with 0.5% polyphosphate was also reported by Julavittayanukul *et al*. (2006).

When $ZnSO_4$ at levels up to 60 μmol/kg for all surimi samples and 100 μmol/kg for all protein isolate samples was added, the increase in water holding capacity of gels was noticeable as evidenced by the lowered expressible moisture content ($P < 0.05$). At 60 μmol/kg, expressible moisture content of surimi gels without and with 0.25 and 0.5% STPP decreased by 32.1, 35.0 and 37.8%, respectively, as compared with control (without additives) (Table 12). When $ZnSO_4$ at a level of 100 μmol/kg was incorporated, expressible moisture content of protein isolate gels without and with phosphorylation using 0.25 and 0.5% STPP decreased by 10.8, 12.9 and 21.5%, respectively, as compared with control (without additives). The lowered expressible moisture content was in accordance with the increased breaking force and deformation of corresponding gels (Figure 23). The greater interaction between protein molecules was associated with the denser network and higher water holding capacity. When an appropriate level of $ZnSO_4$ was added, the cross-linking of proteins could be enhanced in the ordered fashion, resulting in the formation of stronger and fine network with greater water holding capacity. Remondetto *et al*. (2002) reported that gels with high textural properties retained more water, due to a denser network with greater capillary forces. However, further

increase in concentration of $ZnSO_4$ increased the expressible moisture content of both surimi and protein isolate gels, regardless of STPP addition or phosphorylation ($P < 0.05$), indicating a poor gel matrix with low water holding capacity. This was more likely associated with the pronounced coagulation of proteins at high salt concentration. Barbut (1994) reported that disproportionate addition of divalent cation resulted in high levels of protein coagulation, leading to lower water holding capacity. The result suggested that water holding capacity of gel network was determined by protein substrates and level of $ZnSO_4$ and STPP incorporated, both by direct addition or via phosphorylation.

5.4.2.4 Protein patterns

Protein patterns of surimi and their gels without and with 0.5% STPP addition as well as protein isolate without and with phosphorylation using 0.5% STPP and their gels as affected by $ZnSO_4$ at different levels are depicted in Figure 24. Both surimi and protein isolate pastes had myosin heavy chain as the major protein. Actin was also present at a high extent. Kristinsson and Ingadottir (2006) reported that more actin was found at high pH, compared with low pH.

The decrease in MHC band intensity was noticeable in all gels in comparison with that found in surimi and protein isolate. The decrease in MHC was generally due to the polymerisation mediated by various bonds (Benjakul and Visessanguan, 2003). The gels from protein isolates had slightly lower MHC band retained in comparision with surimi gel. This was coincidental with the higher breaking force and deformation of protein isolate gel (Figure 23). It was postulated that dissociated proteins could undergo interaction via exposed reactive groups, which were abundant in protein isolates. Yongsawatdigul and Park (2004) reported that disulphide linkage plays an important role in gelation of alkaline produced gel. Since the SDS-PAGE was carried out using reducing conditions, the disappearance of most of proteins more likely resulted from the cross-linking via non-disulfide covalent bonds. Surimi gels contained non-disulphide covalent bonds induced by endogenous transglutaminase (Benjakul and Visessanguan, 2003).

No differences in MHC band intensity were observed in surimi gels added with $ZnSO_4$ at 0 and 60 µmol/kg in the absence and presence of STPP. Nevertheless, at 140 µmol /kg concentration, MHC was much more retained in surimi gels. No differences in MHC band intensity were observed for protein isolate and phosphorylated protein isolate gels added with $ZnSO_4$ at 0 and 100 µmol /kg. When $ZnSO_4$ at 140 µmol/kg was added, MHC was much more retained in gels of protein isolate, regardless of phosphorylation. Protein cross-links via salt bridge and ionic interactions mediated by Zn^{2+} were destroyed by SDS used for solubilization and electrophoresis. As a result, those cross-links could not be detected under these conditions (Hall and Struhl, 2002). Rapid and excessive coagulation or precipitation of proteins in the presence of $ZnSO_4$ at higher concentration limited protein unfolding and ordered network formation. Furthermore, zinc salt at high concentration might inactivate endogenous transglutaminase, thus preventing polymerisation via non-disulphide covalent bonds. Additionally, the coagulated proteins induced by an excessive Zn^{2+} might not have the reactive groups, lysine and glutamine, available for transglutaminase. This might result in the lower setting, which was concomitant with poor gel properties as indicated by low breaking force and deformation (Figure 23). Thus, level of $ZnSO_4$ played an essential role in cross-linking of proteins, which determined gelling property of both surimi and protein isolate.

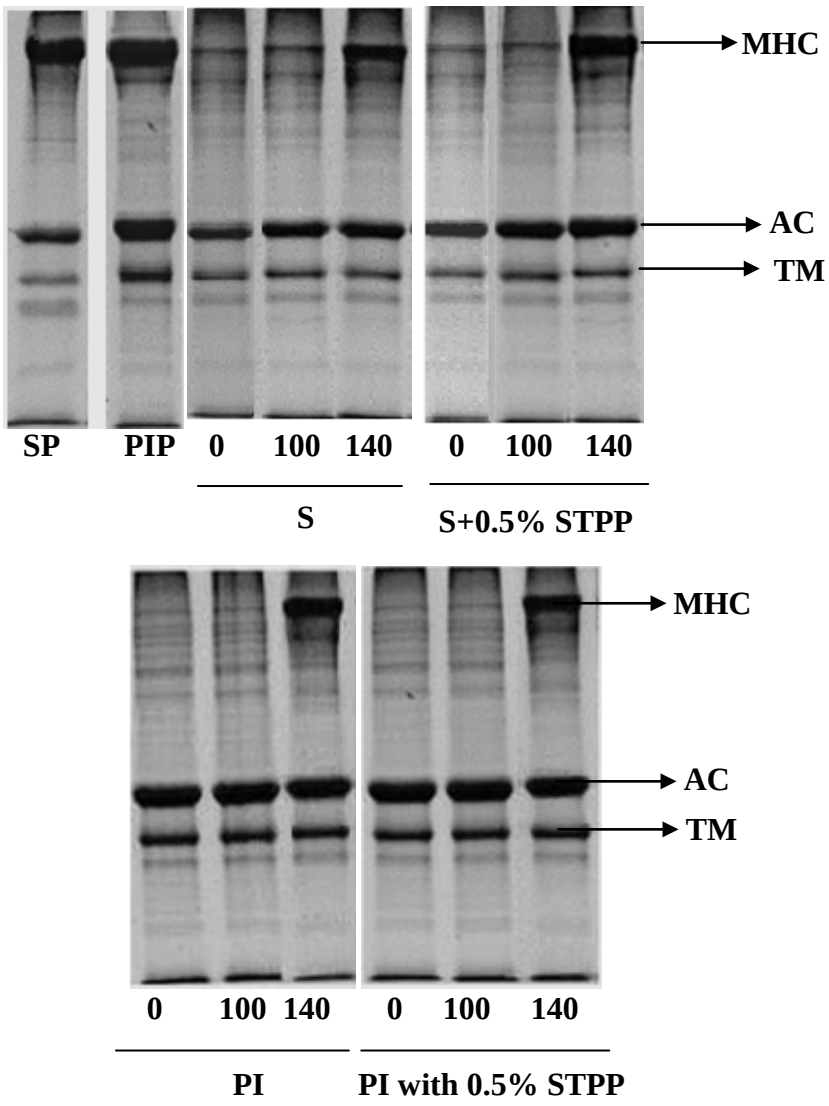


Figure 24. Protein pattern of gels from yellow stripe surimi added without and with STPP and protein isolate without and with phosphorylation as influenced by $ZnSO_4$ at different levels (0-140 µmol/kg). MHC: myosin heavy chain; AC: actin; TM: tropomyosin; SP: surimi paste; PIP: protein isolate paste; S: surimi gel; PI: protein isolate gel; STPP: sodium tripolyphosphate. Numbers designate the level of $ZnSO_4$ added (µmol/kg).

5.4.2.5 Microstructure

Microstructures of gels from surimi, surimi added with 0.5% STPP, surimi with 0.5% STPP and 60 or 140 µmol/kg $ZnSO_4$ and gel from protein isolate, protein isolate phosphorylated using 0.5% STPP containing $ZnSO_4$ at 100 or 140

μmol/kg are illustrated in Figure 25. Surimi control gels (without $ZnSO_4$ and STPP) displayed a relatively loose protein gel matrix with larger voids, compared with gels added with 0.5% STPP or 0.5%STPP in combination with 60 μmol/kg $ZnSO_4$. However, with the addition of 140 μmol/kg $ZnSO_4$, surimi gel containing 0.5% STPP had disconnected structure with very large voids.

Compared to surimi gel, protein isolate gel had slightly larger strands of network. Aggregated protein induced by alkaline solubilization more likely provided those strands in the gel network. Control protein isolate gels (without $ZnSO_4$ and STPP) had a loose protein gel matrix with larger voids, compared with gels of phosphorylated protein isolate. Phosphorylated protein isolate gels added with 100 μmol/kg $ZnSO_4$ had more compact and higher interconnected protein network, compared with gels from protein isolate or phosphorylated protein isolate. In the presence of $ZnSO_4$, the increased negative charge of protein isolate via phosphorylation could favor the formation of salt bridges. This was in agreement with the higher breaking force and deformation of gel (Figure 23). The compact and ordered gel network with smaller voids was observed in gels with the higher gel strength, whilst the looser network with larger voids was formed in the gels with lower gel strength (Balange and Benjakul, 2009b). When $ZnSO_4$ at a level of 140 μmol/kg was incorporated into phosphorylated protein isolate, a coarser particulate network containing coagulated proteins arranged in random clusters was developed. This was due to the rapid coagulation of proteins at high zinc concentration. This was associated with the poor gel property with low water holding capacity, as evidenced by very low breaking force and deformation along with very high expressible moisture content. Thus, $ZnSO_4$ at appropriate level resulted in the formation of fine, denser and ordered filamentous gel network from surimi and protein isolate, especially when phosphate was present via direct addition for surimi or phosphorylation for protein isolate.

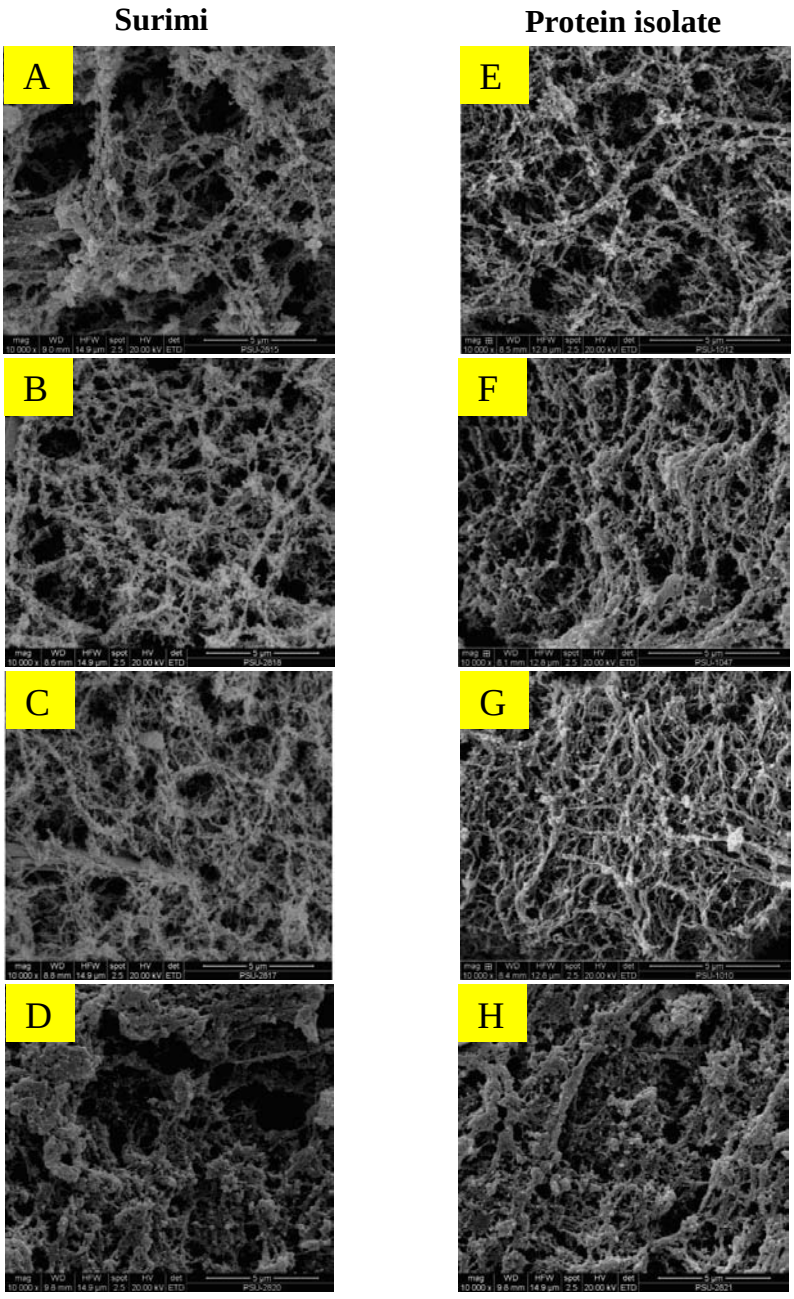


Figure 25. Electron microscopic image of gels from yellow stripe trevally surimi and protein isolate (surimi without additive (A), surimi with 0.5% STPP (B), surimi with 0.5% STPP and 60 μmol $ZnSO_4$/kg (C), surimi with 0.5% STPP and 140 μmol $ZnSO_4$/kg (D), protein isolate without additive (E), phosphorylated protein isolate (F), phosphorylated protein isolate with100 μmol $ZnSO_4$/kg (G) and phosphorylated protein isolate with 140 μmol $ZnSO_4$/kg). (Magnification: × 10,000).

5.5 Conclusion

The alkaline solubilization process induced the denaturation of the muscle proteins as evidenced by the decrease in the Ca^{2+}-ATPase activity. Moreover, this process could lower lipid and phospholipid from mince, leading to the better properties of gels. Phosphorylation of protein isolate can be a modification process to yield protein with negatively charged side chains. Phosphorylated protein isolate incorporated with $ZnSO_4$ yielded the gel with increased breaking force and deformation, compared with gel from surimi added with STPP ($P < 0.05$). Phosphorylated protein isolates were better cross-linked by Zn^{2+}. Thus, the use of Zn^{2+} at an optimal level as cross-linker via salt bridge of proteins, especially those with negative charge could improve gel properties of yellow stripe trevally effectively.

CHAPTER 6

Development and characterization of blend films based on fish protein isolate and fish skin gelatin

6.1 Abstract

Blend films based on fish protein isolate (FPI) from yellow stripe trevally muscle and commercial fish skin gelatin (FSG) at different blend ratios (FPI/FSG = 10:0, 8:2, 6:4, 5:5 and 0:10, w/w) prepared at pH 3 and 11 in the presence of glycerol at 30 and 50% (based on total protein) were characterized. At the same pH, tensile strength (TS) decreased, whilst elongation at break (EAB), water vapor permeability (WVP) and solubility increased as glycerol content increased ($P < 0.05$). However, glycerol content had no marked impact on color and transparency of resulting films ($P > 0.05$). FPI films generally had the lower TS, EAB and higher WVP than FSG films ($P < 0.05$). Both TS and EAB of blend films increased with increasing FSG content ($P < 0.05$), especially at pH 11. Decreased WVP was obtained for blend films having the increasing proportion of FSG ($P < 0.05$). FPI/FSG blend films with higher FSG proportion had higher film solubility, L^*-values (lightness), a^*- values (redness) but lower b^*-values (yellowness) andΔE^*-values (total color difference) than FPI films ($P < 0.05$). At all FPI/FSG ratios, films prepared at pH 11 were less transparent than those prepared at pH 3 ($P < 0.05$). FTIR spectroscopic analysis revealed that hydrogen bonds in FPI/FSG blend film were dominant. Thermogravimetric analysis indicated that films prepared at pH 11 showed higher thermal stability than those prepared at pH 3. Scanning electron microscopic study revealed no distinct phase separation in the matrix of FPI/FSG blend film. Thus, the incorporation of FSG into FPI film up to 50%, in conjunction with lowering glycerol content (30%) could improve the mechanical and water vapor barrier properties of resulting blend film.

6.2 Introduction

Biopolymers have become the alternative to synthetic packaging materials, which are non-biodegradable and have the negative impact on environment. Biopolymer films can improve shelf-life and food quality by serving as the selective barriers to moisture transfer, oxygen uptake, lipid oxidation, and losses of volatile aroma compounds (Campos *et al*., 2011). Amongst biopolymer materials, proteins from different sources have been impressively used for the development of biodegradable films due to their relative abundance and good film-forming ability. Specific structure of proteins provides a wider range of potential functionalities resulting in various intermolecular bondings (Ou *et al*., 2005; Prodpran *et al*., 2007).

Fish proteins including myofibrillar and sarcoplasmic proteins have been used as film-forming materials (Chinabhark *et al*., 2007; Iwata *et al*., 2000). Fish protein isolate (FPI) prepared by prior washing followed by alkaline solubilisation has been shown as the promising starting material with lower haem protein and lipid contents, leading to the improved mechanical and physical properties of film with negligible discoloration (Tongnuanchan *et al*., 2011a). However, FPI based film is rigid, due to the presence of strong covalent bonding, especially disulphide bond (Rocha *et al*., 2013). Additionally, it shows the poor water vapor barrier property, owing to hydrophilicity of amino acids in protein molecules and to the significant amounts of hydrophilic plasticizers added, such as glycerol and sorbitol, to impart the adequate film flexibility (Prodpran *et al*., 2007; McHugh *et al*., 1994). To prepare sufficiently flexible FPI film, addition of high amount (40-60%) of plasticizer, especially glycerol, is generally required (Chinabhark *et al*., 2007; Prodpran *et al*., 2007). Various attempts have been carried out to improve the properties of protein films including chemical treatment (Hernandez-Munoz *et al*., 2004), enzymatic treatment (Jiang *et al*., 2007) and thermal treatment (Lei *et al*., 2007). Blending of FPI with other miscible biopolymers, in the way which renders the improved strength and flexibility and requires the lower amount of plasticizer, would be an inexpensive and effective alternative approach to improve those properties of FPI films. Basically, polymer blends are expected to produce materials with unique properties that combine the most desirable attributes of each component (Wu *et al*., 2002).

Gelatin, a natural biopolymer obtained by thermal degradation of collagen, is the polymer of choice, which can blend with various biopolymers due to their great compatibility (Cao *et al*., 2007). The addition of gelatin into FPI could diminish brittleness and improve mechanical properties of FPI film. Gelatin based films generally require glycerol as plasticizer at lower level, as compared to FPI film (Tongnuanchan *et al*., 2011a; Hoque *et al*., 2011c). Due to hydrophilicity of glycerol, its use at the lower level in the blend film could be a promising means to improve water vapor barrier property. However, the impact of gelatin on properties of FPI film as affected by processing parameters has never been reported. Therefore, the objective of this study was to determine the properties of blend film from FPI and gelatin as influenced by blend composition, plasticizer concentration and pH.

6.3 Materials and methods

6.3.1 Chemicals

Glycerol and sodium azide were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Merck (Darmstadt, Germany). Commercial fish skin gelatin (FSG) from tilapia (~240 bloom) was purchased from Lapi Gelatine S.p.A (Empoli, Italy). All chemicals were of analytical grade.

6.3.2 Collection and preparation of fish sample

Fresh yellow stripe trevally (*S. leptolepis*) with an average weight of 90–100 g/fish were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were kept in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon the arrival, fish were immediately washed, filleted, and minced to uniformity using a mincer with a hole diameter of 0.5 cm.

6.3.3 Preparation of fish protein isolate

Prior to the isolation of fish protein, the prepared mince was subjected to washing as per the method of Toyohara *et al*. (1990) with slight modifications. Fish

mince was homogenized with 5 volumes of cold 0.05 M NaCl (2–4 °C) at a speed of 13,000 rpm for 2 min, using an IKA Labortechnik homogenizer (Selangor, Malaysia). The washed mince was filtered through two layers of cheesecloth. The washing process was repeated twice. Washed mince obtained was stored on ice until used.

Washed mince was added with cold distilled water at the ratio of 1:9 (w/v), followed by homogenization for 1 min at a speed of 13,000 rpm. The pH of the homogenates was then adjusted to 11 using 2 M NaOH. The resulting mixture was centrifuged at 10,000×g for 20 min at 4 °C using a refrigerated centrifuge (Avanti-JE Centrifuge, Beckman 163 Coulter Inc., Fullerton, CA, USA). The supernatant was collected and the pH was adjusted to 5.5 using 2 M HCl. The precipitate was then filtered through 4 layers of cheese-cloth. The retentate was dewatered by centrifugation at 12,000×g for 20 min at 4 °C. The final pH of the sample was adjusted to pH 7.0 using 2 M NaOH. The sample was referred to as “fish protein isolate; FPI”. FPI was used for film preparation.

6.3.4 Preparation of film-forming solution (FFS) and film casting

The film-forming solution (FFS) from FPI was prepared according to the method of Chinabhark *et al*. (2007). FPI was added with 3 volumes of distilled water and homogenized at 13,000 rpm for 1 min using a homogenizer. The protein concentration of the mixture determined by Kjeldahl method (AOAC, 2000) was adjusted to 3% (w/v). Glycerol at different levels, 30 and 50% (w/w) of protein, was used as a plasticizer. The mixtures were stirred gently for 30 min at room temperature. Subsequently, the pH of the mixture was adjusted to 3 or 11 using 1 N HCl and 1 N NaOH, respectively, to solubilize the protein. The solutions were filtered through 2 layers of cheese-cloth to remove undissolved debris. The filtrate was used for film casting.

FFS from FSG was prepared by adding gelatin powder in distilled water to obtain the protein concentration of 3% (w/v). The mixture was heated at 70 °C for 30 min. Glycerol at concentrations of 30 and 50% (w/w) of protein content

was used as a plasticizer. The mixtures were stirred gently for 30 min at room temperature prior to casting.

FFSs of the blend were prepared by mixing FPI–FFS and FSG–FFS with the same level of glycerol (30 or 50%) to obtain the different FPI: FSG ratios (10:0, 8:2, 6:4, 5:5 and 0:10). Then, the blend FFS was stirred gently at room temperature for 10 min. Prior to casting, FFS samples were degassed for 10 min using the sonicating bath (Elmasonic S 30 H, Singen, Germany).

To prepare the film, 4 g of FFS was cast onto a rimmed silicone resin plate (50 × 50 mm^2) and air-blown for 12 h at room temperature prior to further drying at 25 °C and 50±5% relative humidity (RH) for 24 h in an environmental chamber (WTB Binder, Tuttlingen, Germany). Finally, films were manually peeled off and used for analyses.

6.3.5 Determination of film properties

Prior to testing, films were conditioned for 48 h at 50 ± 5% relative humidity (RH) at 25 ± 0.5 °C in an environmental chamber (WTB Binder, Tuttlingen, Germany). For SEM, ATR-FTIR and TGA analyses, films were dried in a desiccator containing dried silica gel for 1 week and in a desiccator containing P_2O_5 for 2 weeks at room temperature (28–30 °C) to obtain the most dehydrated films and to minimize the plasticizing effect due to water.

6.3.5.1 Film thickness

The thickness of film was measured using a digital micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mituyoto Corp., Kawasaki-shi, Japan). Ten random locations around each film sample were used for thickness determination.

6.3.5.2 Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of films were determined as described by Iwata *et al*. (2000) using the Universal Testing Machine

(Lloyd Instruments, Hampshire, UK). Ten samples (2 cm × 5 cm) with initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile loading using a 100 N load cell with the cross-head speed of 30 mm/min until the samples were broken. The maximum load and the final extension at break were used for calculation of TS and EAB, respectively.

6.3.5.3 Water vapor permeability (WVP)

WVP was measured using a modified ASTM method (American Society for Testing and Materials, 1989) as described by Shiku *et al*. (2004). The film was sealed on an aluminum permeation cup containing dried silica gel (0% RH; 0 Pa water vapor pressure at 30 °C) with silicone vacuum grease and a rubber gasket to hold the film in place. The cups were placed in a desiccator containing the distilled water at 30 °C (99±1% RH; 4244.9 Pa vapor pressure at 30 °C). The cups were weighed at 1 h intervals over a 10 h period. WVP of the film was calculated as follows:

$$\text{WVP (g m}^{-1}\text{ s}^{-1}\text{ Pa}^{-1}) = w\, l\, A^{-1}\, t^{-1}\, (P_2 - P_1)^{-1}$$

where w is the weight gain of the cup (g); l is the film thickness (m); A is the exposed area of film (m^2); t is the time of gain (s); ($P_2 - P_1$) is the vapor pressure difference across the film (4244.9 Pa at 30 °C). Three films were used for WVP testing.

6.3.5.4 Film solubility

Film solubility was determined according to the method of Gennadios *et al*. (1998). The conditioned film samples (2 cm × 5 cm) were weighed and placed in a 50 mL centrifuge tube containing 10 mL of distilled water with 0.1% (w/v) sodium azide, used as antimicrobial agent. The mixture was shaken at a speed of 250 rpm using a shaker (Heidolth Inkubator 10000, Schwabach, Germany) at 30 °C for 24 h. Undissolved debris was removed by centrifugation at 3000×g for 20 min. The pellet was dried at 105 °C for 24 h to obtain the dry insolubilized matter. The weight of solubilized dry matter was calculated by subtracting the weight of insolubilized dry

matter from the initial weight of dry matter and expressed as a percentage of the total weight.

6.3.5.5 Color

Color of film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, VA, USA). Color of the film was expressed as *L** – (lightness), *a** – (redness/greenness) and *b**– (yellowness/blueness) values. Total difference in color (Δ*E**) was calculated according to the following equation (Gennadios *et al*., 1996):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where Δ*L**, Δ*a** and Δ*b** are the differences between the corresponding color parameter of the samples and that of white standard (*L** = 93.58, *a** = - 0.88, *b** = 0.46).

6.3.5.6 Light transmittance and transparency value

The light transmittance of films was measured at the ultraviolet and visible range (200–800 nm) using a UV–visible spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) according to the method of Shiku *et al*. (2004). The transparency value of film was calculated using the following equation (Han and Floros, 1997):

$$\text{Transparency value} = \frac{-\log T_{600}}{x}$$

where T_{600} is the fractional transmittance at 600 nm and *x* is the film thickness (mm). The greater transparency value represents the lower transparency of film.

6.3.6 Characterisation of the selected films

FPI/FSG (5:5) blend films (pH 3 and 11) containing 30% glycerol were subjected to characterisation, in comparison with FPI films and FSG films with corresponding pH.

6.3.6.1 Attenuated total reflectance-Fourier transforms infrared (ATR-FTIR) spectroscopy

FTIR spectra of films samples were determined using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at 25 °C as described by Nuthong *et al*. (2009). Samples were placed onto the crystal cells and the cells were clamped into the mount of FTIR spectrometer. The spectra in the range of 650–4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 °C.

6.3.6.2 Thermo-gravimetric analysis (TGA)

Dried films were scanned using a thermo-gravimetric analyser (TG A-7, Perkin Elmer, Norwalk, CT, USA) from 40 to 600 °C at a rate of 10 °C/min (Nuthong *et al*., 2009). Nitrogen was used as the purge gas at a flow rate of 20 mL/min.

6.3.6.3 Microstructure

Microstructure of upper surface and freeze-fractured cross-section of the film samples was determined using a scanning electron microscope (SEM) (Quanta400, FEI, Tokyo, Japan) at an accelerating voltage of 15 kV. Prior to visualisation, the film samples were mounted on brass stub and sputtered with gold in order to make the sample conductive, and photographs were taken at 7000× magnification for surface. For cross-section, freeze-fractured films were mounted around stubs using double sided adhesive tape, coated with gold and observed at the 4000× magnification.

6.3.7 Statistical analysis

Experiments were run in triplicate using different three lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparisons were

carried out by Duncan's multiple range test. For pair comparison, *T*-test was used (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

6.4 Results and discussion

6.4.1 Properties of blend film based on FPI and FSG at different ratios

The blend films with different FPI/FSG ratios and pHs containing glycerol at varying levels exhibited the different properties and molecular characteristics.

6.4.1.1 Thickness

Thickness of FPI film, FSG film and FPI/FSG blend films prepared at pH 3 and 11 in the presence of 30 and 50% glycerol is shown in Table 13. FPI films had similar thickness to FSG films ($P > 0.05$). At both acidic and alkaline pH, the extended or stretched chain of both FPI and FSG facilitated the molecular alignment to form the ordered network in the similar fashion. Thickness of blend films was similar to those of FPI or FSG films ($P > 0.05$). It was noted that blend films with FPI/FSG ratios of 6:4 and 5:5 had higher thickness than FSG films ($P < 0.05$). When FSG was incorporated, the arrangement between myofibrillar proteins in FPI with FSG might not occur in the ordered and uniform fashion. This plausibly led to the protruded film network. Hoque *et al*. (2011c) reported that all films based on cuttlefish skin gelatin had the increased thickness, when mungbean protein isolate was incorporated. However, the similar thickness in soy protein isolate (SPI) and gelatin based blend film, regardless of the proportion of SPI, was reported by Denavi *et al*. (2009). Nevertheless, different pH (3 or 11) and glycerol contents had no pronounced impact on thickness of the resulting films ($P > 0.05$). This suggested that glycerol might distribute uniformly in the film network. Due to its small molecule, glycerol did not cause the protrusion of film matrix like the large polymer or compounds incorporated into the film. Negligible differences in thickness of gelatin-based films with the different levels of glycerol were reported (Vanin *et al*., 2005).

6.4.1.2 Mechanical properties

Mechanical properties of FPI film, FSG film and FPI/FSG blend films with varying blend composition, pHs and glycerol contents are shown in Table 13. FSG films exhibited the higher TS and EAB, compared with FPI films ($P < 0.05$), regardless of glycerol content and pH of film forming solution. Amongst all film samples, FPI film had the lowest TS and EAB ($P < 0.05$), suggesting that proteins in FPI could not form the strong film network. This might be governed by the lower reactivity of myofibrillar protein molecules in cross-linking or interaction between protein molecules. It was noted that FSG and FPI films prepared at pH 11 showed no difference in TS and EAB, compared with those prepared at pH 3 ($P > 0.05$), when the same level of glycerol content was used. This result was in agreement with Hamaguchi *et al*. (2007) who reported that film forming solution with pH 3 or 11 yielded the films from blue marlin (*Makaira mazara*) muscle with similar TS and EAB.

For FPI/FSG blend films, TS and EAB increased with increasing FSG contents, irrespective of glycerol content and pH of film forming solution ($P < 0.05$). FPI/FSG (5:5) blend film prepared at pH 11 had higher TS but lower EAB than that prepared at pH 3 ($P < 0.05$). Alkaline pH might favor the solubilisation of protein molecules, which resulted in greater extension or stretching of proteins and subsequent alignment of partially denatured myofibrillar protein and the stretched fibrous gelatin molecules. As a result, interaction between protein molecules upon film network formation could be enhanced. The increased EAB of film prepared at acidic pH was more likely caused by the presence of weaker bonds stabilising film matrix. The higher TS and EAB of the FPI/FSG blend films, excepted at 8:2 FPI/FSG, than FPI films observed in the present study suggested the reinforcement of the film matrix, which was probably induced by a certain degree of cross-linking between chains of both FPI and FSG. FPI and FSG at an appropriate ratio might undergo interaction, in the way which higher inter-junctions with the longer strands took place. This resulted in higher chain entanglement and higher molecular slippage upon tensile deformation. Furthermore, FSG, which is known to undergo interaction mainly via hydrogen bonds, more likely cross-linked with FSG itself or FPI via hydrogen bonds.

This resulted in the enhanced mechanical properties of resulting blend films. TS and EAB of composite films made from type-B–bovine-bone gelatin and soy protein isolate improved progressively with increasing proportions of gelatin (Cao *et al*., 2007). Li *et al*. (2006) reported the increases in TS and EAB when gelatin was incorporated with konjac glucomannan. However, it was noted in this study that the blend films with FPI/FSG = 8:2 showed similar TS as compared to the FPI films. This result suggested that even though the FSG added imparted the molecular reinforcing effect, it might decrease the strong interaction between FPI molecules especially the disulphide covalent bond.

All films with 50% glycerol showed the lower TS but higher EAB than did those containing 30% glycerol ($P < 0.05$), irrespective of pH of film forming solution. The glycerol at higher level was able to lower the interaction between chains to a higher extent. When hydrophilic plasticizer was incorporated in the film structure, the interactions and the proximity between protein chains were reduced (Gontard *et al*., 1993; Jongjareonrak *et al*., 2006). Also the mobility of macromolecules increased, leading to the decrease in TS with the concomitant increase in EAB of films (Gontard *et al*., 1993). The result was in accordance with Jongjareonrak *et al*. (2006) who reported that the decreased TS and the increased EAB were obtained in gelatin film from bigeye snapper skin with increasing glycerol content. The decreases in TS of myofibrillar protein films from Atlantic sardine were also observed with increasing glycerol content (Cuq *et al*., 1997). Thus, the incorporation of FSG at a particular level in conjunction with the lower glycerol content could maneuver the strength and flexibility of resulting FPI/FSG blend films.

6.4.1.3 Water vapor permeability (WVP)

WVP of FPI film, FSG film and FPI/FSG blend films, prepared at pH 3 and 11, containing glycerol at 30 and 50% is shown in Table 13. FPI films had the highest WVP, whilst FSG films had the lowest WVP ($P < 0.05$), regardless of glycerol content and pH of film forming solution. The lowest WVP of FSG film was in agreement with the highest TS (Table 13). The lowered permeation of water vapor through FSG film was plausibly determined by the stronger interaction and higher

degree of organisation of protein molecules in film network. The film with high compactness could prevent the penetration of water vapor more effectively (Heng *et al*., 2003). FPI films probably had less compactness with less ordered network. According to Chen (1995), simple, linear polymeric chains, can be firmly packed, whereas molecules with voluminous chains such as muscle proteins, are more loosely packed, presenting greater permeability. Normally, fish muscle protein is hydrophilic, due to its polar amino acids and large number of hydroxyl group (–OH). As a consequence, muscle protein film has the lowered moisture barrier property (Paschoalick *et al*., 2003). However, no difference in WVP was observed between the same film prepared at pH 3 and 11, when the same glycerol content was used ($P > 0.05$). Bigeye snapper surimi film and blue marlin edible film prepared from FFS with acidic and alkaline pH had similar WVP (Chinnabhark *et al*., 2007; Hamaguchi *et al*., 2007).

For FPI/FSG blend films, WVP decreased with increasing FSG content ($P < 0.05$), irrespective of glycerol content and pH of film forming solution. All FPI/FSG blend films had the lower WVP than did FPI film ($P < 0.05$). For FPI/FSG blend film, similar WVP was obtained in films prepared at pH 11 and pH 3 ($P > 0.05$). According to McHugh and Krochta (1994), the structuring of a polymer inside the film matrix significantly affected the water vapor barrier property. Interaction between FPI and FSG plausibly led to the reduced interstitial space amongst protein chains in film matrix, with the consequent decrease in WVP (Di-Pierro *et al*., 2005). Furthermore, the introduction of bonding between FPI and FSG could lower the available or free charged or polar residues of proteins, particularly FPI. This might result in the decreased WVP of blend films. It was noted that the moisture content in different films was varying. The moisture content of FPI films and FSG films were approximately 20-22% and 11-12%, respectively. This was probably due to the difference in association of water in the film network, as determined by different proteins and amino acid compositions. The different moisture content in different films might affect the hydration and water vapor permeability of films to some degree.

When films with the same pH and blend ratio was tested, WVP increased with increasing glycerol content ($P < 0.05$). These results suggested that hydroxyl group (-OH) of hydrophilic glycerol was able to interact with water as evidenced by the increased WVP of film. Moreover, the insertion of plasticizer between chains of macromolecules increased the free volume of the system and favored the mobility of polymeric chains. Consequently, film network became less dense and more permeable (Gontard *et al*., 1993). Increased WVP was also found in bigeye snapper skin gelatin film (Jongjareonrak *et al*., 2006), pig skin gelatin film (Vanin *et al*., 2005) and fish muscle protein film (Paschoalick *et al*., 2003) when glycerol content increased. Thus, blend ratio and glycerol content had the profound impact on WVP of FPI/FSG blend films.

6.4.1.4 Film solubility

Film solubility of FPI film, FSG film and FPI/FSG blend films with different blend composition, pHs and glycerol contents is presented in Table 13. Amongst all films, FPI film had the lowest solubility and FSG has the highest solubility ($P < 0.05$), regardless of glycerol content and pH of FFS. Fish muscle protein films were mostly stabilized by various bonds, including disulphide covalent bonds (Chinabhark *et al*., 2007). This resulted in the decreased solubility of films. Films prepared at alkaline condition had the lowered film solubility, compared with those prepared at pH 3 ($P < 0.05$), irrespective of glycerol content. The strong protein–protein interactions at alkaline pH mainly via disulphide bonds led to the lowered solubility of film (Shiku *et al*., 2003). For FSG film, it was reported to contain high hydrophilic amino acids (Hoque *et al*., 2011a). High solubility of FSG film was associated with the weak bond, particularly hydrogen bond. This film could be hydrated in the presence of water, leading to the ease of solubilisation.

Film solubility of FPI/FSG blend films prepared at both pHs increased with increasing levels of FSG ($P < 0.05$), regardless of glycerol content (Table 13), simply due to the additive effect of FSG added. Moreover, the incorporated FSG more likely impeded the formation of disulphide covalent bond between FPI molecules,

Table 13. Thickness, mechanical properties, water vapor permeability and film solubility of FPI, FSG and FPI/FSG blend films as affected by blend composition, pH and glycerol content at 50 ± 5% relative humidity (RH) and 25 ± 0.5 °C

Glycerol (%)	pH level	FPI:FSG[a] (w/w)	Thickness (mm)	TS (MPa)	EAB (%)	WVP ($\times 10^{-11}$ gm^{-1} s^{-1} Pa^{-1})	Film solubility (%)
30	11	10:0	0.035 ± 0.002 abA	10.22 ± 0.90 eA	25.64 ± 5.13 hB	4.09 ± 0.14 aB	19.48 ± 1.92 hB
	11	8:2	0.035 ± 0.002 abA	10.77 ± 1.17 deA	37.46 ± 5.64 fgB	3.74 ± 0.18 bB	28.92 ± 1.32 gB
	11	6:4	0.036 ± 0.002 aA	12.57 ± 0.78 cA	53.43 ± 6.68 cdB	3.41 ± 0.26 cB	37.37 ± 2.27 fB
	11	5:5	0.036 ± 0.001 aA	13.98 ± 0.42 bA	64.27 ± 5.09 bB	3.29 ± 0.18 cB	42.49 ± 3.60 eB
	11	0:10	0.033 ± 0.001 bA	27.24 ± 0.65 aA	48.49 ± 6.94 deB	2.64 ± 0.06 dB	80.18 ± 3.28 aB
	3	10:0	0.034 ± 0.002 abA	9.54 ± 0.65 eA	31.95 ± 3.92 ghB	4.17 ± 0.09 aB	36.22 ± 2.24 fB
	3	8:2	0.035 ± 0.003 abA	10.17 ± 0.79 eA	41.69 ± 4.60 efB	3.78 ± 0.15 bB	47.24 ± 3.31 dB
	3	6:4	0.035 ± 0.002 abA	11.77 ± 0.44 cdA	57.36 ± 6.01 bcB	3.51 ± 0.13 bcB	57.79 ± 2.22 cB
	3	5:5	0.036 ± 0.001 aA	12.38 ± 1.00 cA	75.19 ± 6.45 aB	3.39 ± 0.16 cB	63.43 ± 3.04 bB
	3	0:10	0.032 ± 0.002 bA	26.88 ± 0.81 aA	51.38 ± 5.44 cdB	2.77 ± 0.16 dB	83.31 ± 2.95 aB
50	11	10:0	0.035 ± 0.002 abA	7.93 ± 1.18 eB	48.78 ± 6.33 fA	5.13 ± 0.12 aA	25.72 ± 2.0 fA
	11	8:2	0.036 ± 0.002 aA	8.38 ± 0.73 deB	60.51 ± 6.62 deA	4.84 ± 0.11 bA	37.79 ± 2.94 eA
	11	6:4	0.036 ± 0.002 aA	10.06 ± 0.88 bcB	81.34 ± 7.14 cA	4.49 ± 0.11 cdA	48.58 ± 3.37 dA
	11	5:5	0.036 ± 0.002 aA	11.16 ± 0.34 bB	89.07 ± 4.29 bcA	4.36 ± 0.09 dA	57.76 ± 2.99 cA
	11	0:10	0.032 ± 0.002 bA	18.98 ± 1.14 aB	84.21 ± 6.34 bcA	3.97 ± 0.16 eA	90.39 ± 2.71 aA
	3	10:0	0.033 ± 0.003 abA	7.53 ± 0.92 eB	54.33 ± 5.03 efA	5.34 ± 0.21 aA	48.97 ± 2.84 dA
	3	8:2	0.035 ± 0.002 abA	8.14 ± 0.85 eB	65.83 ± 5.75 dA	4.87 ± 0.16 bA	56.64 ± 2.86 cA
	3	6:4	0.036 ± 0.002 aA	9.53 ± 0.78 cdB	88.66 ± 5.36 bcA	4.67 ± 0.09 bcA	68.47 ± 2.65 bA
	3	5:5	0.036 ± 0.002 aA	9.71 ± 0.37 cdB	98.83 ± 5.05 aA	4.44 ± 0.12 cdA	73.17 ± 2.96 bA
	3	0:10	0.032 ± 0.001 bA	18.35 ± 1.05 aB	92.34 ± 5.06 abA	3.89 ± 0.15 eA	91.77 ± 3.15 aA

Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column under the same glycerol content indicate significant differences ($P < 0.05$).

Different uppercase letters in the same column under the same FPI/FSG ratio and pH indicate significant differences ($P < 0.05$).

[a] FPI: yellow stripe trevally fish protein isolate; FSG: fish skin gelatin.

resulting in the ease of disintegration and solubilisation of FPI/FSG blend film in water. However, the lower film solubility was found in blend films prepared at pH 11 than those prepared at pH 3. Hoque *et al*. (2011c) also found the higher solubility of cuttlefish skin gelatin (CG) and mungbean protein isolate (MPI) (CG/MPI) blend film with increasing CG content. The increase in gelatin content increased solubility of soy protein isolate and cod gelatin blend films (Denavi *et al*., 2009).

When film with the same pH and blend ratio was tested, solubility increased with increasing glycerol content ($P < 0.05$). Glycerol, a hydrophilic plasticizer, could enhance film solubility in water. The solubility of plasticized-protein film is in part due to leaching out of plasticizer (glycerol) in water (Denavi *et al*., 2009; Hoque *et al*., 2011c). The solubility of films from fish myofibrillar protein increased with increasing plasticizer content (Cuq *et al*., 1997). The result suggested that solubility of films was affected by pH of FFS, glycerol content and FPI/FSG ratio.

6.4.1.5 Color

Table 14 shows the color of FPI film, FSG film and FPI/FSG blend films prepared at pH 3 and 11 with 30 and 50% glycerol. FSG films prepared at both pHs had the highest L^*-value (lightness) ($P < 0.05$), regardless of glycerol content. The highest a^*-value (redness/greenness) but lowest b^*-value (yellowness) were obtained for FSG film with pH 3 ($P < 0.05$). Lower a^* and higher b^*-values were found in FSG films prepared at pH 11, compared with those prepared at pH 3, at both glycerol contents ($P < 0.05$). FPI films and most blend films prepared at pH 11 had the lower L^*-value and a^*-value but higher b^*-value and ΔE^* (total color difference), compared with those prepared at pH 3 at both glycerol contents ($P < 0.05$). This result suggested that an alkaline condition could induce the formation of yellowish pigment, especially via Maillard reaction. Alkaline pH favors the reductone formation over furfural production from the amadori products, leading to color development in films (Tongnuanchan *et al*., 2011b; Bates *et al*., 1998).

It was noticeable that the increases in L^*- and a^*-values with coincidental decreases in b^*- and ΔE^*-values were obtained as higher amount of FSG was incorporated, irrespective of pHs and glycerol contents. Such changes in color of resulting blend films were most likely attributed to the lower coloring components existing in FSG, as compared with FPI. The decrease in b^*-value of blend films with higher level of FSG incorporated was mainly associated with the lower content of FPI, whose some lipids were still retained. Lipids, especially membrane lipids, were reported to induce the formation of yellow discoloration, especially via Maillard reaction, in films (Tongnuanchan *et al*., 2011b).

Glycerol content did not show the marked effect on color. Gennadios *et al*. (1996) and Vanin *et al*. (2005) reported that neither plasticizer type nor concentration had effect on color difference of films from egg albumen and gelatin. Hoque *et al*. (2011a) also reported that glycerol content had no significant effect on color of the gelatin-based films.

Table 14. Color of FPI, FSG and FPI/FSG blend films as affected by blend composition, pH and glycerol content

Glycerol (%)	pH level	FPI:FSG[a] (w/w)	Color parameter			
			L*	***a****	***b****	**Δ*E****
30	11	10:0	89.46 ± 0.12 dA	-2.26 ± 0.07 iA	7.33 ± 0.36 aA	7.67 ± 0.38 aA
	11	8:2	89.93 ± 0.22 cA	-2.10 ± 0.08 hA	6.16 ± 0.24 bA	6.41 ± 0.14 bA
	11	6:4	90.15 ± 0.24 bA	-2.02 ± 0.02 gA	5.39 ± 0.24 cA	5.58 ± 0.31 cA
	11	5:5	90.28 ± 0.21 bA	-1.70 ± 0.03 fA	3.99 ± 0.32 dA	4.28 ± 0.39 dA
	11	0:10	90.67 ± 0.31 aA	-1.27 ± 0.03 cA	2.01 ± 0.21 hA	2.46 ± 0.26 hA
	3	10:0	89.87 ± 0.03 cA	-1.51 ± 0.04 eA	3.56 ± 0.13 eA	4.02 ± 0.13 eA
	3	8:2	90.27 ± 0.21 bA	-1.45 ± 0.04 dA	3.35 ± 0.16 fA	4.11 ± 0.10 deA
	3	6:4	90.29 ± 0.19 bA	-1.27 ± 0.03 cA	2.26 ± 0.14 gA	3.09 ± 0.14 fA
	3	5:5	90.37 ± 0.19 bA	-1.21 ± 0.05 bA	1.94 ± 0.04 hA	2.75 ± 0.20 gA
	3	0:10	90.81 ± 0.35 aA	-1.14 ± 0.03 aA	1.28 ± 0.07 iA	2.63 ± 0.21 ghA
50	11	10:0	89.41 ± 0.20 dA	-2.28 ± 0.07 iA	7.36 ± 0.33 aA	7.70 ± 0.35 aA
	11	8:2	89.86 ± 0.18 cA	-2.11 ± 0.04 hA	6.10 ± 0.15 bA	6.37 ± 0.12 bA
	11	6:4	90.14 ± 0.27 bA	-2.02 ± 0.02 gA	5.34 ± 0.34 cA	5.60 ± 0.43 cA
	11	5:5	90.25 ± 0.17 bA	-1.71 ± 0.05 fA	3.96 ± 0.33 dA	4.26 ± 0.30 dA
	11	0:10	90.62 ± 0.24 aA	-1.28 ± 0.02 cA	1.97 ± 0.19 hA	2.44 ± 0.12 hA
	3	10:0	89.84 ± 0.06 cA	-1.53 ± 0.03 eA	3.53 ± 0.14 eA	3.99 ± 0.12 eA
	3	8:2	90.24 ± 0.13 bA	-1.44 ± 0.04 dA	3.32 ± 0.17 fA	4.15 ± 0.11 deA
	3	6:4	90.25 ± 0.17 bA	-1.26 ± 0.03 cA	2.23 ± 0.12 gA	3.05 ± 0.11 fA
	3	5:5	90.36 ± 0.17 bA	-1.20 ± 0.04 bA	1.93 ± 0.03 hA	2.75 ± 0.21 gA
	3	0:10	90.79 ± 0.38 aA	-1.15 ± 0.02 aA	1.27 ± 0.05 iA	2.59 ± 0.21 ghA

Values are given as mean ± SD (n = 3).
Different lowercase letters in the same column under the same glycerol content indicate significant differences ($P < 0.05$).
Different uppercase letters in the same column under the same FPI/FSG ratio and pH indicate significant differences ($P < 0.05$).
[a] FPI: yellow stripe trevally fish protein isolate; FSG: fish skin gelatin.

6.4.1.6 Light transmittance and transparency value

Transmission of UV and visible light at wavelength range of 200–800 nm of FPI film, FSG film and FPI/FSG blend films prepared at various pH and glycerol contents is presented in Table 15. The transmission of UV light was very low at 200 and 280 nm for FPI films and all blend films with different FPI/FSG ratios, compared with FSG films, irrespective of pH level and glycerol content. Amongst all films, FPI films had the lowest transmission in both UV and visible range. Fish muscle protein films exhibited the good UV barrier properties (Tongnuanchan *et al*., 2011b; Shiku *et al*., 2003), owing to their high content of aromatic amino acids that absorb UV light (Hamaguchi *et al*., 2007). At both pHs, percent transmittance (%T) of films increased with increasing FSG content. The result suggested that FSG, especially at higher amount, with less light transmission barrier, more likely contributed to the increased light transmittance of blend films at both UV (280 nm) and visible ranges, as compared with FPI films. Light transmission of films was most likely governed by the arrangement or alignment of polymer in film network. %T in visible range (350–800 nm) of FPI and FPI/FSG blend films prepared at pH 3 was much higher than those of films prepared at pH 11.

FPI films showed the highest transparency values, whereas FSG films had the lowest values ($P < 0.05$). Noted that the greater transparency value represents the lower transparency of film. For FPI/FSG blend films, the decreases in transparency value were obtained as FSG amount increased ($P < 0.05$), indicating the increases in transparency of resulting films. The transparency value of FPI film and FPI/FSG blend films prepared at pH 3 was lower than that of films prepared at pH 11, regardless of glycerol content. The result indicated that the former was more transparent than the latter ($P < 0.05$). Due to the lower Maillard reaction in film prepared at acidic pH, this might result in higher transparency of films in comparison with films prepared at pH 11. At pH 11, Maillard pigments might play a role in lowering the transmission of light. These results were in agreement with Tongnuanchan *et al*. (2011b) who reported that films based on red tilapia muscle protein prepared at acidic condition (pH 3) were more transparent than those prepared at alkaline condition (pH 11).

No differences in transparency value were observed between films with 30% glycerol and those with 50% glycerol ($P > 0.05$) (Table 15). Both plasticizer type and concentration had no effect on transparency of film from pig skin gelatin (Vanin *et al*., 2005). Nemet *et al*. (2010) also reported that glycerol content did not affect the transparency of chicken breast protein edible films. Generally, the blend ratio and pHs of FFS had the impact on film transparency. Therefore, FPI/FSG blend films were more transparent and clear for packaging the products, in comparison with FPI film with less transparency.

Table 15. Light transmittance and transparency values of FPI, FSG and FPI/FSG blend films as affected by blend composition, pH and glycerol content

Glycerol (%)	pH level	FPI:FSG[a] (w/w)	Light transmittance (%)								Transparency values
			200	280	350	400	500	600	700	800	
30	11	10:0	0.00	0.03	48.82	56.40	67.83	71.04	73.52	76.17	4.24 ± 0.02 aA
	11	8:2	0.00	2.83	55.54	58.59	70.09	71.48	75.24	78.51	4.17 ± 0.02 bA
	11	6:4	0.01	3.86	49.97	57.19	69.02	73.02	74.06	80.87	3.98 ± 0.03 cA
	11	5:5	0.00	5.01	57.86	59.59	71.54	74.53	77.81	79.23	3.87 ± 0.02 dA
	11	0:10	0.00	26.83	74.17	77.59	79.24	81.79	84.26	84.87	2.82 ± 0.05 iA
	3	10:0	0.00	0.04	62.45	69.82	71.06	73.70	77.71	78.48	3.68 ± 0.04 eA
	3	8:2	0.00	2.91	66.22	71.56	73.65	75.38	78.34	79.14	3.61 ± 0.03 fA
	3	6:4	0.00	4.01	71.08	74.97	75.23	76.76	79.27	80.85	3.38 ± 0.02 gA
	3	5:5	0.00	5.30	72.44	74.19	75.17	77.87	80.53	81.39	3.29 ± 0.02 hA
	3	0:10	0.02	30.04	78.56	81.55	84.34	85.22	86.17	86.85	2.79 ± 0.03 iA
50	11	10:0	0.01	0.04	48.77	56.54	68.78	70.57	73.85	77.04	4.20 ± 0.03 aA
	11	8:2	0.00	2.57	49.51	57.43	68.89	71.42	74.46	77.13	4.18 ± 0.02 aA
	11	6:4	0.00	3.81	56.26	61.26	71.59	73.32	75.61	78.82	3.96 ± 0.03 bA
	11	5:5	0.00	5.22	58.21	59.26	72.26	74.13	77.98	78.50	3.82 ± 0.04 cA
	11	0:10	0.01	27.21	74.84	77.43	78.69	81.90	84.73	85.24	2.80 ± 0.04 hA
	3	10:0	0.00	0.05	63.57	70.24	72.34	73.89	77.82	78.27	3.65 ± 0.03 dA
	3	8:2	0.00	2.76	61.89	72.31	73.86	75.15	78.64	79.76	3.54 ± 0.04 eA
	3	6:4	0.00	4.26	72.29	74.27	76.11	77.34	79.17	80.59	3.38 ± 0.03 fA
	3	5:5	0.00	5.84	71.97	74.66	76.12	77.48	80.98	81.86	3.26 ± 0.02 gA
	3	0:10	0.02	29.78	77.79	80.87	84.00	85.11	85.97	86.51	2.78 ± 0.02 hA

Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column under the same glycerol content indicate significant differences ($P < 0.05$).

Different uppercase letters in the same column under the same FPI/FSG ratio and pH indicate significant differences ($P < 0.05$).

[a] FPI: yellow stripe trevally fish protein isolate; FSG: fish skin gelatin.

6.4.2 Characteristics of blend films prepared from the selected FPI/FSG ratio

Blend films based on FPI and FSG at a ratio of 5:5 prepared at both pHs 3 and 11, containing 30% glycerol, which showed the improved mechanical and water vapor barrier properties, were characterized, in comparison with FSG and FPI films prepared at corresponding pH.

6.4.2.1 FTIR spectroscopy

FTIR spectra of FPI films, FSG films and FPI/FSG (5:5) blend films containing 30% glycerol, prepared at pH 3 and 11, are illustrated in Figure 26. Films prepared at acidic and alkaline conditions showed the similar major peaks but the amplitudes of peaks varied slightly. Generally, all films had the similar spectra in the range of 1800–600 cm^{-1}, covering amide-I, II and III bands. All films exhibited the major bands at around 1633 cm^{-1} (amide-I, representing C= O stretching/hydrogen bonding coupled with COO), 1536 cm^{-1} (amide-II, arising from bending vibration of N-H groups and stretching vibrations of C–N groups) and 1238 cm^{-1} (amide-III, representing the vibrations in plane of C–N and N–H groups of bound amide or vibrations of CH_2 groups of glycine) (Aewsiri *et al*., 2009; Muyonga *et al*., 2004). Yakimets *et al*. (2005) reported the similar result for bovine skin gelatin film where amide-I, amide-II and amide-III peaks were found at the wavenumbers of 1633, 1536 and 1240 cm^{-1}, respectively. The band situated at the wavenumber of 1036–1039 cm^{-1} was found in all film samples, corresponding to the interactions arising between plasticizer (OH group of glycerol) and film structure (Bergo and Sobral, 2007; Hoque *et al*., 2011a). Generally, similar spectra were obtained between all fish protein isolate and gelatin films in the range of 1800–600 cm^{-1}, covering amide-I, amide-II and amide-III. An amide-A band was observed at the wavenumber of 3270–3280 cm^{-1} for all film samples. Amide-B band was also found at 2926–2928 cm^{-1} for all film samples. The amide-A band represents the NH-stretching coupled with hydrogen bonding and amide-B band represents the asymmetric stretching vibration of CH as well as NH_3^+ (Muyonga *et al*., 2004; Ahmad and Benjakul, 2011).

For amide-A peak, FPI/FSG blend film had the lower wavenumber (pH 3=3271 cm^{-1}; pH 11= 3270 cm^{-1}), compared with that of FPI (pH 3=3273 cm^{-1}; pH 11=3273 cm^{-1}) and FSG films (pH 3=3279 cm^{-1}; pH 11=3280 cm^{-1}). The amplitude at these corresponding wavenumbers was also decreased. The shift to lower wavenumber and lower amplitude of the amide-A peak suggested interaction between FSG and FPI taken place in the blend film matrix, mainly via hydrogen bonding. Typically, the decrease in vibrational wavenumber and broadening of the OH and NH vibration bands could be indicative of interaction between polymers in the film via hydrogen bonding (Xie *et al*., 2006; Hoque *et al*., 2011c). The shift to lower wavenumber of the amide-A peak in CG/MPI blend film indicated an increase in hydrogen bonding between two components (Hoque *et al*., 2011c). Cao *et al*. (2007) reported that interactions between the amino and carboxyl groups of SPI and gelatin determined mechanical properties of composite films and hydrogen bonding was mainly responsible for those interactions. FT-IR results reconfirmed the interaction between FPI and FSG in the film matrix.

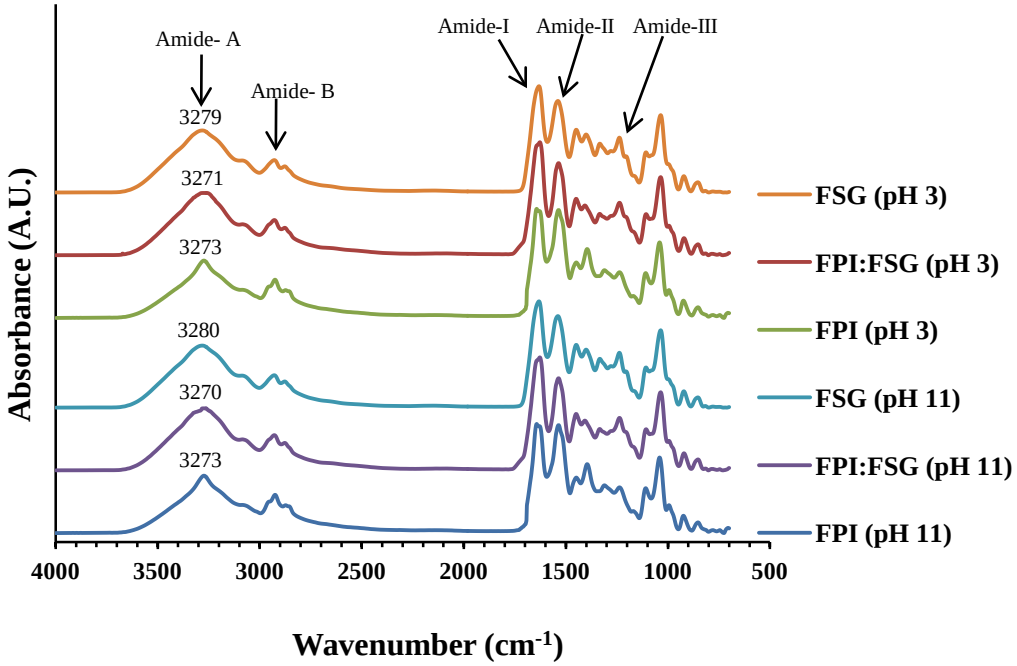


Figure 26. FT-IR spectra of FPI, FSG and FPI/FSG (5:5) blend films prepared at pH 3 and 11 containing 30% glycerol.

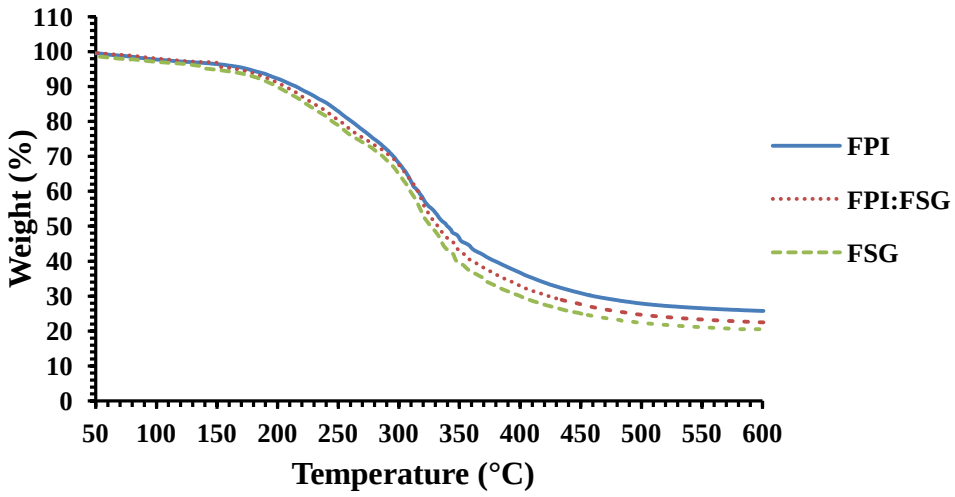
6.4.2.2 Thermo-gravimetric analysis (TGA)

TGA thermograms revealing thermal degradation behavior of all films are shown in Figure 27. Their corresponding degradation temperatures and weight loss are presented in Table 16. Three main stages of weight loss were observed for all films. The first stage weight loss (w_1 = 2.08–3.27%) was observed over the temperature (T_{d1}) ranging from 48.08 to 53.66 °C up to approximately 155 °C, possibly associated with the loss of free and bound water adsorbed in the film. The similar result was found in cuttlefish skin gelatin film (Hoque *et al*., 2011a) and porcine plasma protein film added with different cross-linking agents (Nuthong *et al*., 2009). The second stage weight loss (w_2 = 23.51–27.0%) appeared at the onset temperature of 195.07–213.04 °C (T_{d2}), depending on the film samples. This was most likely due to the degradation or decomposition of lower molecular weight protein fractions and glycerol compounds. For the third stage of weight loss (w_3 = 43.72–48.02%), T_{d3} of 294.01–311.52 °C were observed for all films but varied with film samples. This was possibly due to the decomposition of highly interacted proteins in film matrix.

The results suggested that FPI films and FPI/FSG blend films showed higher heat resistance than FSG films. However, films prepared at pH 11 showed higher heat stability than those prepared at pH 3. Amongst all films, FPI film prepared at pH 11 had the highest degradation/decomposition temperature (T_{d2} = 213.04 and T_{d3} = 311.52). Higher amount of disulphide and hydrophobic interaction between proteins in FPI at alkaline conditions might contribute to heat resistance of their films. FPI/FSG blend films had higher degradation temperature than FSG films. The interaction between FPI and FSG mostly yielded the stronger film network, leading to higher heat resistance of the resulting blend films than FSG films. Hoque *et al*. (2011c) also observed higher heat stability of CG/MPI film, where higher amount of bonding between MPI and gelatin molecules yielded stronger film network with heat resistance. Additionally, all films had residual mass (representing char content) at 600 °C in the range of 21.71–30.69%. The highest char content observed in FPI prepared at pH 11 was most likely ascribed to the highest covalent bonding formed in the protein network, resulted in the greatest thermal stability of the film. Difference in

char content was most likely owing to different amounts of covalent protein-protein cross-link as well as compositions of proteins obtained from different sources and pH during preparation processes.

A) **pH 3**



B) **pH 11**

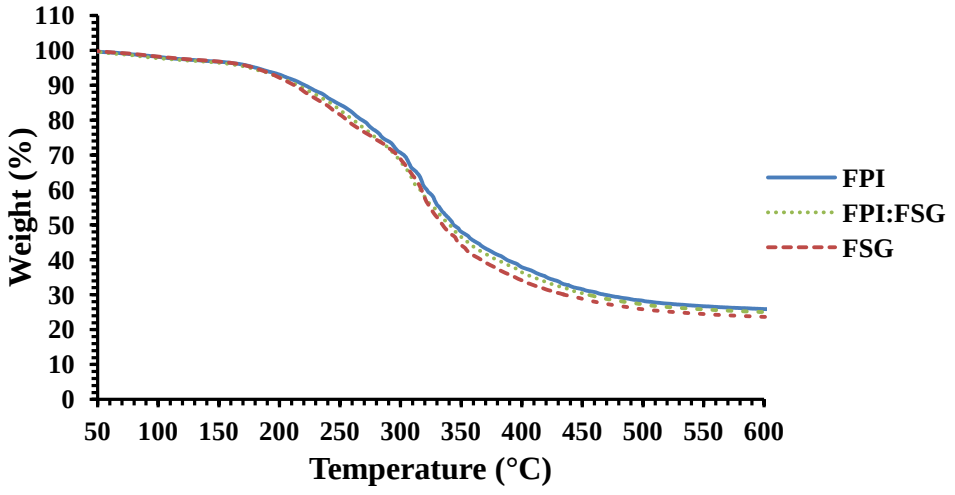


Figure 27. TGA curves of FPI, FSG and FPI/FSG (5:5) blend films prepared at pH 3 (A) and 11 (B) containing 30% glycerol.

Table 16. Thermal degradation temperature (T_d, °C) and weight loss (Δw, %) of FPI, FSG and FPI/FSG (5:5) blend films prepared at pH 3 and 11 containing 30% glycerol

pH level	FPI:FSG[a] (w/w)	Δ_1		Δ_2		Δ_3		Residue (%)
		T_{d1},onset	Δw_1	T_{d2},onset	Δw_2	T_{d3},onset	Δw_3	
11	10:0	48.08	2.08	213.04	23.51	311.52	43.72	30.69
11	5:5	50.16	2.36	204.47	24.6	305.5	44.61	28.43
11	0:10	53.66	2.89	199.11	26.7	300.88	46.32	24.09
3	10:0	48.6	2.53	203.33	24.63	306.51	44.04	28.8
3	5:5	49.38	3.04	198.1	26.75	301.01	47.01	23.2
3	0:10	50.52	3.27	195.07	27.0	294.01	48.02	21.71

Δ_1, Δ_2, and Δ_3 denote the first, second and third stage weight loss, respectively, of film during heating scan.

[a] FPI: yellow stripe trevally fish protein isolate; FSG: fish skin gelatin.

6.4.2.3 Microstructure

Surface and freeze-fractured cross-sectional images of FPI films, FSG films and FPI/FSG (5:5) blend film prepared at pH 3 and 11 containing 30% glycerol are depicted in Figure 28. FSG films prepared at pH 3 and 11 had the smoothest surface, compared with others and had crack-free cross-section. This indicated that those films had the homogeneous structure on both surface and cross-section. This was associated with the better mechanical and physical properties of films at both pH (pH 3 and 11), compared with other films. For FPI films prepared at pH 3 and 11, the smooth surface was observed. However, the rough surface was noticeable in both FPI/FSG blend films. Arrangements of molecules of two types of proteins during film formation might take place in different ways, compared with those occurring with the single protein. From SEM images, no distinct separation observed in the matrix of FPI/FSG blend film, indicating the compatibility between FPI and FSG. The compatibility of FPI and FSG more likely favored their molecular interaction in the film matrix, thereby resulting in the improved mechanical and physical properties of FPI/FSG blend film.

For cross-section, continuous and crack-free cross-sectional structure was observed in FSG films (at both pHs), compared with those observed in FPI/FSG blend film and FPI film. The discontinuous zone or micro-cracks were more profound in FPI film at both pH 3 and 11. This was more likely related with the less interaction of molecules of FPI. This could be associated with the poorer TS and WVP of FPI film, compared with other films. Tongnuanchan *et al*. (2011a) observed similar results for cross-sectional structure of red tilapia FPI film prepared at pH 3 and 11. However, FPI/FSG blend film had much lower cracks than FPI film. This was plausibly due to the greater interaction between FPI and FSG protein molecules in film matrix. Thus, the microstructures of films were governed by molecular organisation in the film network, which depended on types of proteins and the interaction of proteins in film matrix.

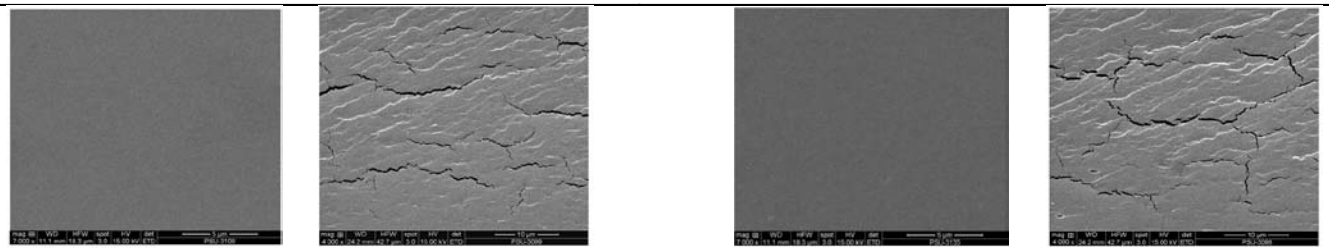
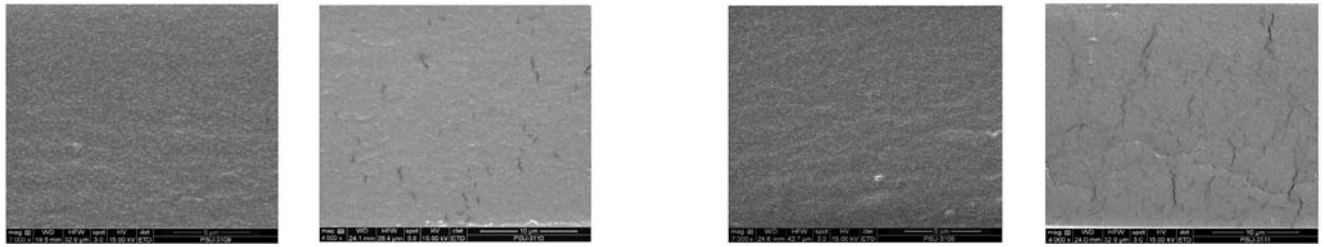
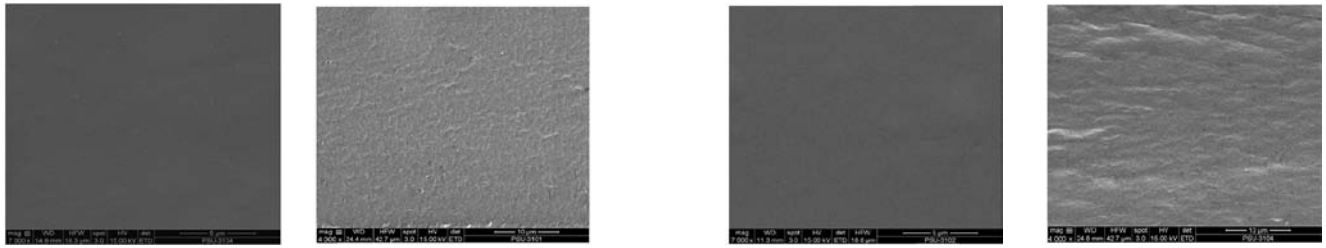
Film sample	pH 11		pH 3	
	Surface	Cross-section	Surface	Cross-section
FPI				
FPI:FSG (5:5)				
FSG				

Figure 28. SEM micrographs of surface and freeze-fractured cross-section of FPI, FSG and FPI/FSG (5:5) blend films prepared at pH 3 and 11 containing 30% glycerol. Magnification: 7000× and 4000× for surface and cross-section, respectively.

6.5 Conclusion

Properties of FPI films could be modified by blending with FSG at a ratio of 5:5 with lower glycerol content (30%) at both acidic and alkaline pH. The blend between FPI and FSG was compatible and the interaction between these two different proteins via hydrogen bonds and other interaction was enhanced. Blend film showed the improved mechanical and water vapor barrier properties, compared with FPI films. The improved properties of film were more likely attributed to the appropriate interaction and arrangement between FPI and FSG molecules in the blend film matrix. Thus, FPI/FSG blend films with improved elongation and water vapor barrier properties are expected to release the antimicrobial and antioxidant components, thus serving as the potential active food packaging to ensure food safety and to extend the shelf-life of packaged foods.

CHAPTER 7

Characteristics and antimicrobial properties of fish protein isolate/fish skin gelatin-zinc oxide (ZnO) nanocomposite films

7.1 Abstract

Antimicrobial nanocomposite films based on fish protein isolate (FPI)/fish skin gelatin (FSG) (1/1 w/w) prepared at pH 3 and 11 containing ZnO nanoparticles (ZnONP) at different levels (0-4% w/w of protein) were characterized. At both pH 3 and 11, tensile strength (TS) increased, whilst elongation at break (EAB) and water vapor permeability (WVP) decreased as ZnONP content increased ($P < 0.05$). FPI/FSG-ZnO nanocomposite films had lower b^*-values (yellowness) and ΔE^*-values (total color difference) than the control FPI/FSG film ($P < 0.05$). Transparency of nanocomposite films decreased slightly, but UV barrier property was improved. Those changes were more pronounced with increasing ZnONP content. At all ZnONP levels, films prepared at pH 11 were less transparent than those prepared at pH 3 ($P < 0.05$). FTIR spectroscopic analysis revealed that there was interaction between ZnONP and protein in the film matrix. Based on thermogravimetric analysis, ZnONP could improve thermal stability of the nanocomposites films. X-ray diffraction (XRD) analysis confirmed the crystalline structure of the ZnONP in the nanocomposite films. FPI/FSG-ZnO nanocomposite films, especially those prepared at pH 3, exhibited strong antibacterial activity against food-borne pathogenic and spoilage bacteria. Thus, FPI/FSG-ZnO nanocomposite films could be used as an active food packaging material to ensure safety and to extend the shelf-life of packaged foods.

7.2 Introduction

Biopolymers produced from various natural resources such as proteins, starch and cellulose have been considered as attractive alternatives for non-biodegradable petroleum based plastics since they are abundant, renewable, inexpensive, environmentally-friendly, and biodegradable. Biopolymers not only act

as barriers to moisture, water vapor, gases, and solutes, but also serve as carriers of wide variety of additives, such as antimicrobials, antioxidants, antifungal agents, colorants, and other nutrients (Han, 2000). Amongst biopolymer materials, proteins from different sources have been impressively used for the development of biodegradable films due to their abundance, diverse molecularity and good film-forming ability (Iwata *et al*., 2000; Prodpran *et al*., 2007).

Fish protein isolate (FPI) prepared by alkaline solubilization was reported as the promising starting material for preparation of films with negligible yellow discoloration (Tongnuanchan *et al*., 2011a). However, FPI based film is rigid and shows poor water vapor barrier property, owing to hydrophilicity of amino acids in protein molecules and to the significant amounts of hydrophilic plasticizers required for film flexibility (Prodpran *et al*., 2007; McHugh *et al*., 1994). Recently, properties of FPI films from yellow stripe trevally, an abundant trash dark muscle fish, could be modified by blending with FSG at a ratio of 1:1 with lower glycerol content (30%) at both acidic and alkaline pH. The blend between FPI and FSG was compatible and the interaction between these two different proteins via hydrogen bond and other interactions was enhanced. Blend films showed the improved mechanical and water vapor barrier properties, compared with FPI films (Arfat *et al*., 2014). However, FPI/FSG blend films still have poorer mechanical as well as water-vapor barrier property, in comparison with synthetic films. Nanotechnology has been implemented to biopolymer films for improving mechanical, thermal and water vapor barrier properties (Kovacevic *et al*., 2008). Furthermore, it can generate active packaging systems (Emamifar *et al*., 2011).

Bionanocomposites represent the new generation of nanocomposites, and comprise of natural polymer matrix and organic/inorganic filler with at least one dimension on the nanometer scale (Alebooyeh *et al*., 2012). Nanofillers have excellent interfacial interactions on polymer branches due to their large specific surface area and high surface energy, thus significantly enhancing polymer properties such as thermal, mechanical, and water barrier properties (Kovacevic *et al*., 2008). Recently, the incorporation of ZnO nanoparticles as functional filler into the biopolymer films such as starch based films have been reported to improve

mechanical and water vapor barrier properties (Alebooyeh *et al*., 2012; Yu *et al*., 2009). ZnO is currently listed as a generally recognized as safe (GRAS) material by the Food and Drug Administration (21CFR182.8991) and has previously shown strong antimicrobial activity against food borne pathogens and spoilage bacteria (Espitia *et al*., 2012, 2013; Zhang *et al*., 2008). However, there is no report on ZnO nanocomposite protein films, especially those from fish protein isolates and fish skin gelatin. This work aimed to characterize FPI/FSG-ZnO nanocomposite films and to determine antimicrobial activity against Gram-positive *Listeria monocytogenes* (food borne pathogen) and Gram-negative *Pseudomonas aeruginosa* (food spoilage bacteria).

7.3 Materials and methods

7.3.1 Chemicals

Zinc oxide nanoparticles (ZnONP) (particle size: 20 – 40 nm, specific surface area: 26.22 m^2/g) was purchased from Nano materials technology Co. Ltd. (Bangkok, Thailand). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Merck (Darmstadt, Germany). Commercial fish skin gelatin (FSG) from tilapia (~240 bloom) was purchased from Lapi Gelatine S.p.A (Empoli, Italy). All chemicals were of analytical grade. *Listeria monocytogenes* DMST 1327 was obtained from Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand and *Pseudomonas aeruginosa* TISTR 781 was obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand.

7.3.2 Collection and preparation of fish Sample

Fresh yellow stripe trevally (*S. leptolepis*) with an average weight of 90–100 g/fish were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were kept in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon the arrival, fish were immediately washed, filleted, and minced to uniformity using a mincer with a hole diameter of 0.5 cm.

7.3.3 Preparation of fish protein isolate

Prior to the isolation of fish protein, the prepared mince was subjected to washing as per the method of Toyohara *et al*. (1990) with slight modifications. Fish mince was homogenized with 5 volumes of cold 0.05 M NaCl (2–4 °C) at a speed of 13,000 rpm for 2 min, using an IKA Labortechnik homogenizer (Selangor, Malaysia). The washed mince was filtered through two layers of cheese-cloth. The washing process was repeated twice. Washed mince obtained was stored on ice until used.

Washed mince was added with cold distilled water at the ratio of 1:9 (w/v), followed by homogenization for 1 min at a speed of 13,000 rpm. The pH of the homogenates was then adjusted to 11 using 2 M NaOH. The resulting mixture was centrifuged at 10,000×g for 20 min at 4 °C using a refrigerated centrifuge (Avanti-JE Centrifuge, Beckman 163 Coulter Inc., Fullerton, CA, USA). The supernatant was collected and the pH was adjusted to 5.5 using 2 M HCl. The precipitate was then filtered through 4 layers of cheese-cloth. The retentate was dewatered by centrifugation at 12,000×g for 20 min at 4 °C. The final pH of the sample was adjusted to pH 7.0 using 2 M NaOH. The sample was referred to as “fish protein isolate; FPI”. FPI was used for film preparation.

7.3.4 Preparation of FPI/FSG-ZnONP film-forming solution (FFS) and film casting

The film-forming solution from FPI (FFS-FPI) was prepared according to the method of Chinabhark *et al*. (2007). FPI was added with 3 volumes of distilled water and homogenized at 13,000 rpm for 1 min using a homogenizer. Subsequently, the pH of the mixture was adjusted to 3 or 11 using 1 N HCl and 1 N NaOH, respectively, to solubilize the protein. The solutions were filtered through 2 layers of cheese-cloth to remove undissolved debris. The protein concentration of the filtrate determined by Kjeldahl method (AOAC, 2000) was adjusted to 3 % (w/v). Glycerol at 30% (w/w) of protein was used as a plasticizer. The mixture was stirred gently for 30 min at room temperature and was used for preparing blend film forming solution.

Film-forming solution from FSG (FFS-FSG) was prepared by adding gelatin powder in distilled water to obtain the protein concentration of 3% (w/v). The pH of the mixture was adjusted to 3 or 11 using 1 N HCl and 1 N NaOH, respectively. The mixture was heated at 70 °C for 30 min. Glycerol at concentrations of 30 % (w/w) of protein content was used as a plasticizer. For each pH, film-forming solution of the FPI/FSG blend was prepared by mixing FPI–FFS and FSG–FFS at a ratio of 1:1 (v/v).

ZnONP (0, 1, 2, 3 and 4% of protein, w/w) were mixed with distilled water and the suspension were stirred for 5 min and then homogenized for 1 min at the speed of 5,000 rpm (IKA Labortechnik homogenizer, Selangor, Malaysia). ZnONP suspension was gradually added into FPI/FSG blend film-forming solution in droplets. The mixture was stirred for 5 min. Final volume was made up to 100 ml using distilled water previously adjusted to corresponding pH. To improve the mixing and uniform distribution of protein and ZnONP, the suspensions were homogenized with three passes through a high pressure homogenizer (Microfluidizer M-110EH, Microfluidics Corp., Newton, MA, USA) with an operating pressure of 1,500 bars. Suspensions were gently stirred for 30 min at room temperature and were referred to as a film-forming suspension (FFS). Prior to casting, FFS samples were degassed for 10 min using the sonicating bath (Elmasonic S 30 H, Singen, Germany). To prepare the film, 4 g of FFS was cast onto a rimmed silicone resin plate (5×5 cm^2), air-blown for 12 h at 25 °C, followed by drying in an environmental chamber (Binder GmbH, Tuttlingen, Germany) at 25 ± 0.5 °C and 50 ± 5% relative humidity (RH) for 24 h. Dried film samples were manually peeled off and subjected to analyses.

7.3.5 Determination of film properties

Prior to testing, films were conditioned for 48 h at 50 ± 5% relative humidity (RH) at 25 ± 0.5 °C. For XRD, ATR-FTIR, TGA and SEM analyses, films were dried in a desiccator containing dried silica gel for 1 week and in a desiccator containing P_2O_5 for 2 weeks at room temperature (28–30 °C) to obtain the most dehydrated films and to minimize the plasticizing effect due to water.

7.3.5.1 Film thickness

The thickness of film was measured using a digital micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mituyoto Corp., Kawasaki-shi, Japan). Ten random locations around each film sample were used for thickness determination.

7.3.5.2 Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of films were determined as described by Iwata *et al*. (2000) using the Universal Testing Machine (Lloyd Instruments, Hampshire, UK). Ten samples (2 cm×5 cm) with initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile loading using a 100 N load cell with the cross-head speed of 30 mm/min until the samples were broken. The maximum load and the final extension at break were used for calculation of TS and EAB, respectively.

7.3.5.3 Water vapor permeability (WVP)

WVP was measured using a modified ASTM method (American Society for Testing and Materials, 1989) as described by Shiku *et al*. (2004). The film was sealed on an aluminum permeation cup containing dried silica gel (0% RH; 0 Pa water vapor pressure at 30 °C) with silicone vacuum grease and a rubber gasket to hold the film in place. The cups were placed in a desiccator containing the distilled water at 30 °C (99±1% RH; 4244.9 Pa vapor pressure at 30 °C). The cups were weighed at 1 h intervals over a 10 h period. WVP of the film was calculated as follows:

$$\text{WVP (g m}^{-1}\text{ s}^{-1}\text{ Pa}^{-1}) = w\, l\, A^{-1}\, t^{-1}\, (P_2 - P_1)^{-1}$$

where w is the weight gain of the cup (g); l is the film thickness (m); A is the exposed area of film (m^2); t is the time of gain (s); ($P_2 - P_1$) is the vapor pressure difference across the film (4244.9 Pa at 30 °C). Three films were used for WVP testing.

7.3.5.4 Color

Color of film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, VA, USA). Color of the film was expressed as L^* – (lightness), a^* – (redness/greenness) and b^*– (yellowness/blueness) values. Total difference in color (ΔE^*) was calculated according to the following equation (Gennadios *et al*., 1996):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differences between the corresponding color parameter of the samples and that of white standard (L^* = 93.58, a^* = - 0.88, b^* = 0.46).

7.3.5.5 Light transmittance and transparency value

The light transmittance of films was measured at the ultraviolet and visible range (200–800 nm) using a UV–visible spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) according to the method of Shiku et al. (2004). The transparency value of film was calculated using the following equation (Han and Floros, 1997):

$$\text{Transparency value} = \frac{-\log T_{600}}{x}$$

where T_{600} is the fractional transmittance at 600 nm and x is the film thickness (mm). The greater transparency value represents the lower transparency of film.

7.3.6 Characterisation of the selected films

FPI/FSG films (pH 3 and 11) incorporated with 3% ZnONP were subjected to characterization, in comparison with the control FPI/FSG films with corresponding pH.

7.3.6.1 Wide angle X-ray diffraction (WAXD) characterization

WAXD measurements were carried out by using a wide angle X-ray diffractometer (Philips X'Pert MPD, Almelo, Netherland) with the following conditions: Cu source; operating at room temperature; 40 kV and 30 mA current. The samples were cut into the circular shape of 30 mm diameter and placed in a sample holder. Then, the set was placed inside the chamber of the apparatus, in order to perform the measurements. The measurement angles (2θ) were varied from 5º to 75º.

7.3.6.2 Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

FTIR spectra of film samples were determined using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at 25 °C as described by Nuthong *et al*. (2009). Samples were placed onto the crystal cells and the cells were clamped into the mount of FTIR spectrometer. The spectra in the range of 650–4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 °C.

7.3.6.3 Thermo-gravimetric analysis (TGA)

Dried films were scanned using a thermo-gravimetric analyser (TGA-7, Perkin Elmer, Norwalk, CT, USA) from 40 to 600 °C at a rate of 10 °C/min (Nuthong *et al*., 2009). Nitrogen was used as the purge gas at a flow rate of 20 mL/min.

7.3.6.4 Scanning electron microscopy (SEM)

Morphology of surface and freeze-fractured cross-section of film samples were visualized using a scanning electron microscope (SEM) (Quanta400, FEI, Eindhoven, the Netherlands) at an accelerating voltage of 15 kV. Prior to visualisation, the film samples were mounted on brass stub and sputtered with gold in

order to make the sample conductive, and photographs were taken at 7000× magnification for surface. For cross-section, freeze-fractured films were mounted around stubs using double sided adhesive tape, coated with gold and observed at the 3500× magnification.

7.3.7 Antimicrobial properties

Antimicrobial activity of film samples was assessed using the agar diffusion method according to Ponce *et al*. (2008). The zone of inhibition on solid media was used for determining the antimicrobial activity of films against typical food pathogenic and spoilage bacteria including Gram-positive bacteria (*Listeria monocytogenes*) and Gram-negative bacteria (*Pseudomonas aeruginosa*). Film samples were cut into a disc shape of 10 mm diameter using a circular knife and then placed on Mueller Hinton (Merck, Darmstadt, Germany) agar plates, which had been previously smeared with 100 µl of inoculums containing approximately 10^6–10^7 CFU/ml of tested bacteria. The plates were then incubated at 37 °C for 24 h. The antimicrobial activity of nanocomposite films was determined by measuring the diameter of the bacterial growth inhibition zone around the film.

7.3.8 Statistical analysis

Experiments were run in triplicate using different three lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, U.S.A.).

7.4 Results and discussion

7.4.1 Properties of FPI/FSG-ZnO nanocomposite films

FPI/FSG films prepared at pH 3 and 11, containing ZnONP at varying levels, exhibited the different properties.

7.4.1.1 Thickness

Thickness of FPI/FSG films prepared at pH 3 and 11 with various levels of ZnONP is shown in Table 17. Different pHs (3 or 11) had no pronounced impact on thickness of the resulting films ($P > 0.05$). The extended or stretched chains of both FPI and FSG facilitated the molecular alignment to form the ordered network in the similar fashion, regardless of pH used. It was noted that the thickness of films increased as the levels of ZnONP incorporated increased ($P < 0.05$), irrespective of pH of FFS. The increases in the thickness with the addition of ZnONP could be due to the increase in solid content associated with ZnONP. Nanoparticles distributed throughout the film matrix resulted in the increased thickness of resulting films after drying. This behavior was also observed by Sothornvit *et al*. (2009) for whey protein isolate based nanocomposite films.

7.4.1.2 Mechanical properties

Mechanical properties of FPI/FSG films prepared at pH 3 and 11 without and with the addition of ZnONP at various levels are shown in Table 17. The control FPI/FSG film (without ZnONP) prepared at pH 11 had higher TS ($P < 0.05$) but lower EAB ($P > 0.05$) than those prepared at pH 3. Alkaline pH might favor the solubilization of protein molecules, which resulted in greater extension or stretching of proteins. The subsequent alignment of partially denatured myofibrillar protein and the stretched fibrous gelatin molecules more likely led to the formation of strong film network with high junction zones. Moreover, it was reported that FPI prepared at acidic pH had higher degradation (Tongnuanchan *et al*., 2011a). As a result, the lower strength of films containing FPI prepared at pH 3 was obtained. The slightly increased

EAB of film prepared at acidic pH was more likely caused by the presence of weaker bonds stabilising film matrix (Tongnuanchan *et al*., 2011a).

When ZnONP was incorporated into film, ZnONP exhibited an obvious reinforcing effect. As the ZnONP content increased up to 3%, TS increased, but the elongation at break of the composites decreased ($P < 0.05$), irrespective of pH of FFS. However the films prepared at pH 11 had higher TS ($P < 0.05$) and lower EAB ($P > 0.05$), as compared with those prepared at pH 3. The higher TS of ZnONP added films prepared at pH 11 was likely due to the enhanced salt bridges formation between Zn^{2+} and negatively charged residues, mainly COO^{-} of protein side chains or C-termini. Similar interactions in ZnO–whey protein nanocomposite were reported (Shi *et al*., 2008). A significant increase in TS with ZnONP incorporation was consistent with other studies on reinforced protein films by nanoparticles (Rouhi *et al*., 2013). The ZnONP incorporated into the protein matrix limited the moving scale of protein chain segments and generated an interactive force against the protein chains. This was evidenced by increased TS but decreased EAB. The nanoparticles have high surface energy and large specific surface area, giving rise to strong interfacial interactions between polymer and the nanoparticles and bring about a significant enhancement in the polymer properties (Kovacevic *et al*., 2008; Rouhi *et al*., 2013).

However, at the ZnONP concentration above 3%, the sharp decreases in both TS and EAB were noticeable ($P < 0.05$). The decrease of the mechanical strength of the nanocomposite film might be attributed to the non-uniform distribution of ZnONP and aggregation of excess nanoparticles in protein matrix. An increase in incompatibility of the protein/ZnO composites with excess ZnONP content resulted in discontinuous film matrix. ZnO is hydrophobic and tends to aggregate when certain concentration is reached to reduce energy dissipated in the system (Zhou *et al*., 2009). The aggregate could impede the arrangement of protein domains and amorphous network of FPI/FSG film and consequently reduce mechanical properties of films. Similar results for TiO_2 nanoparticles incorporated whey protein isolate films were reported (Zhou *et al*., 2009). The concentration of zinc was the major factor affecting

Table 17. Thickness, mechanical properties and water vapor permeability of FPI/FSG films as affected by pH and ZnONP content

pH level	ZnONP level (% w/w)	Thickness (mm)	TS (MPa)	EAB (%)	WVP ($\times 10^{-11}$ gm^{-1} s^{-1} Pa^{-1})
11	0	0.036 ± 0.001d	13.07 ± 0.60d	64.31 ± 5.64ab	3.37 ± 0.18bc
	1	0.038 ± 0.001cd	14.29 ± 0.63c	61.43 ± 4.55bc	3.11 ± 0.22cd
	2	0.039± 0.001bc	15.83± 0.86b	56.82± 4.48cd	2.67± 0.18e
	3	0.041± 0.001ab	17.76± 0.93a	49.20± 4.14ef	2.09± 0.14f
	4	0.042±0.002a	9.43±0.53f	39.43±4.63g	4.19±0.21a
3	0	0.036 ± 0.001d	11.66 ± 0.77e	70.33 ± 5.13a	3.64 ± 0.16b
	1	0.038 ± 0.001cd	12.21± 0.86de	66.19± 4.37ab	3.48± 0.14b
	2	0.039± 0.001bc	13.09± 0.76d	61.57± 4.57bc	3.03± 0.21d
	3	0.040± .002abc	14.18± 0.69c	53.33± 5.36de	2. 68± 0.19e
	4	0.042±0.002a	8.97± 0.57f	43.47± 3.59fg	4.47± 0.11a

Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

structure and spatial organization of protein aggregates in film matrix. Thus, the incorporation of ZnONP at a particular level could maneuver the strength of resulting FPI/FSG films.

7.4.1.3 Water vapor permeability (WVP)

WVP of FPI/FSG films prepared at pH 3 and 11 containing ZnONP at various levels are shown in Table 17. Fish muscle protein and gelatin are hydrophilic, due to its polar amino acids. Hydroxyl group (–OH) of glycerol used as a plasticizer also contribute to hydrophilicity of film. As a consequence, muscle protein and gelatin films had the lowered moisture barrier property (Prodpran *et al*., 2007).

WVP of FPI/FSG films decreased as the levels of ZnONP was increased up to 3% ($P < 0.05$), regardless of pH of FFS. However, FPI/FSG-ZnO nanocomposite films prepared at pH 11 with the same level of ZnONP had lower WVP than those prepared at pH 3 ($P < 0.05$). This result was in agreement with the higher mechanical properties of the formers, in which the stronger and more compact structure was formed. This effectively prevented the migration of water vapor through the films (Chen, 1995). At a level of 3%, ZnONP dispersed well in the matrix with few paths for water molecules to pass through. The significant decrease in WVP of film after the addition of ZnONP may be attributed to the greater water resistance of ZnONP. Thus, the incorporation of these nanoparticles to the protein matrix introduces a tortuous pathway for water vapor molecules to pass through (Alebooyeh *et al*., 2012; Yu *et al*., 2009). The improvement of water resistance in other nanocomposite systems containing ZnO fillers such as starch nanocomposites has also been reported (Alebooyeh *et al*., 2012).

When ZnONP at a level of 4% was incorporated, the superfluous filler congregated easily, decreasing the effective content of filler. Increased WVP of films containing 4% ZnONP was associated with the poor mechanical properties of films caused by pronounced coagulation of proteins at high Zn^{2+} concentration. Arfat and Benjakul (2012b) reported that disproportionate addition of Zn^{2+} causes protein coagulation in muscle proteins from yellow stripe trevally. Coagulated proteins along

with the agglomerated ZnONP contributed to the poor integrity of film matrix. As a consequence, increased WVP of films was obtained. Thus, both pH and ZnONP level had the profound impact on WVP of FPI/FSG films.

7.4.1.4 Color

Table 18 shows the color of FPI/FSG films prepared at pH 3 and 11 with varying levels of ZnONP. FPI/FSG films prepared at pH 11 had the lower L^*-value (lightness) and a^*-value (redness/greenness) but higher b^*-value (yellowness) and ΔE^* (total color difference), compared with those prepared at pH 3, regardless of ZnONP content ($P < 0.05$). This result suggested that an alkaline condition could induce the formation of yellowish pigment, especially via Maillard reaction. Alkaline pH favors the reductone formation over furfural production from the amadori products, leading to color development in protein-based films (Tongnuanchan *et al*., 2011a; Bates *et al*., 1998).

At both pH 3 and 11, the addition of ZnONP into FPI/FSG film did not have a marked impact on L^*- and a^*-values ($P > 0.05$). However, decreases in b^*- and ΔE^*-values were obtained as ZnONP with increasing amount was incorporated, regardless of pH of FFS ($P < 0.05$). These results were in agreement with Espitia *et al*. (2013) who reported that increasing amount of ZnO markedly decreased b^*- and ΔE^*-values, whilst had no significant effect on L^*- and a^*-values of methyl cellulose (MC)-ZnO nanocomposite films. Such changes in color of resulting films were most likely attributed to the whitening effect due to the presence of ZnO nanoparticles (Espitia *et al*., 2013). The results suggested that ZnONP decreased yellowish color in resulting nanocomposite films**.**

Table 18. Color of FPI/FSG films as affected by pH and ZnONP content

pH level	ZnONP level (% w/w)	Color parameters			
		L*	***a****	***b****	**Δ*E****
11	**0**	90.19 ± 0.19d	-1.89 ± 0.02b	3.84 ± 0.17a	4.31 ± 0.19a
	1	90.24± 0.23cd	-1.87± 0.03b	3.45± 0.14b	3.94± 0.11b
	2	90.29± 0.13bcd	-1.88± 0.02b	3.18± 0.1c	3.76± 0.13b
	3	90.36± 0.17abcd	-1.88± 0.04b	2.91± 0.09d	3.44± 0.04c
	4	90.40± 0.20abcd	-1.85± 0.04b	2.62± 0.05e	3.09± 0.09d
3	**0**	90.52 ± 0.17abc	-1.37 ± 0.04a	2.26 ± 0.06f	2.96 ± 0.08d
	1	90.57± 0.15ab	-1.35± 0.01a	2.03± 0.04g	2.70± 0.13e
	2	90.6± 0.10ab	-1.34± 0.03a	1.79± 0.06h	2.46± 0.06f
	3	90.62± 0.13a	-1.35± 0.02a	1.58± 0.07i	2.32± 0.09f
	4	90.64± 0.16a	-1.33± 0.04a	1.36± 0.06j	2.26± 0.13f

Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

7.4.1.5 Light transmittance and transparency value

Transmission of UV and visible light at wavelength range of 200–800 nm of FPI/FSG films prepared at different pH (3 or 11) and incorporated with ZnONP at different levels are shown in Table 19. Control films (without ZnONP) had a good barrier property in the UV-ranges (200–280 nm), irrespective of pH of FFS. Protein based films are generally known to have excellent UV barrier properties due to the presence of high content of aromatic amino acids which can absorb UV light (Prodpran *et al*., 2012; Hoque *et al*., 2011c).

The percent transmittance (%T) of films in both UV and visible range decreased as the levels of ZnONP increased, regardless of pH of FFS. However, the %T of FPI/FSG-ZnO nanocomposite films prepared at pH 3 was higher than those of films prepared at pH 11. The results indicated that ZnONP were able to impede the light transmission through the film. This was mainly due to the hindrance of light passage or light scattering by the nanoparticles dispersed in the film matrix (Kanmani

and Rhim, 2014). The incorporation of ZnONP into the FPI/FSG films greatly reduced the transmission of UV light at 280 nm. Rouhi *et al*. (2013) reported that the gelatin films added with ZnO nanorods had lower transmission in the UV range. The FPI/FSG reinforced with ZnONP could be used as UV-shielding packaging films to prevent UV light, which can induce lipid oxidation of various foods. With increasing levels of ZnONP, the films also showed the lower transmittance in the visible ranges. ZnONP in film could act as the barrier towards light.

Control FPI/FSG films prepared at pH 3 showed the lower transparency values, than those prepared at pH 11, indicating that the former was more transparent. These results were in agreement with Tongnuanchan *et al*. (2011a) who reported that films based on red tilapia muscle protein prepared at acidic condition (pH 3) were more transparent than those prepared at alkaline condition (pH 11). When FPI/FSG films were added with ZnONP, the higher transparency values were observed ($P < 0.05$). The greater transparency value represents the lower transparency of film. For FPI/FSG-ZnO nanocomposite films, the transparency value increased as the level of ZnONP content increased ($P < 0.05$), indicating the decrease in transparency of resulting films. This was more likely due to the opaqueness of ZnONP, which were distributed throughout the films. Similarly, Kanmani and Rhim (2014) reported that the blending of silver nanoparticles with gelatin protein films reduced transparency of the films. Thus both pH of FFS and the level of ZnONP had the impact on transparency and light barrier properties of FPI/FSG films.

Table 19. Light transmittance and transparency values of FPI/FSG films as affected by pH and ZnONP content

pH level	ZnONP level (% w/w)	Light transmittance (%)								Transparency values
		200	280	350	400	500	600	700	800	
11	**0**	0.00	6.72	54.73	60.17	71.22	72.68	79.49	82.15	3.95 ± 0.03e
	1	0.00	1.68	49.5	50.58	65.2	70.12	76.07	78.78	4.04± 0.03d
	2	0.00	0.79	45.72	46.58	62.88	68.79	70.19	74.12	4.14± 0.04c
	3	0.00	0.33	41.57	40.98	58.91	67.22	68.77	72.43	4.22± 0.05c
	4	0.00	0.14	38.18	37.02	54.95	61.34	63.24	68.16	5.04± 0.07a
3	**0**	0.00	7.30	68.44	72.19	75.17	77.27	86.42	87.11	3.18 ± 0.02i
	1	0.00	1.84	61.66	63.26	70.2	75.01	80.59	83.37	3.29± 0.05h
	2	0.00	0.86	50.14	54.04	66.18	73.5	77.32	80.94	3.42± 0.04g
	3	0.00	0.45	47.15	48.12	61.04	72.23	74.23	77.17	3.51± 0.08f
	4	0.00	0.21	43.3	44.56	57.88	65.15	70.04	73.83	4.44± 0.07b

Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

7.4.2 Characteristics of FPI/FSG-ZnO nanocomposite films

The FPI/FSG films prepared at pH 3 and 11, containing ZnONP at 3% level, which showed the improved mechanical and water vapor barrier properties, were characterized, in comparison with FPI/FSG films without ZnONP prepared at corresponding pH.

7.4.2.1 Wide angle X-ray diffraction (WAXD) pattern

FPI/FSG films prepared at pH 3 and 11 without and with 3% ZnONP were analysed using WAXD technique in comparision with ZnONP (Figure 29). Pure ZnO nanoparticles showed the main characteristic peaks at 2θ = 31.7, 34.4, 36.2, 47.5, 56.6, 62.7 and 67.9, matching with the standard (JCPDS76–0704), confirming the hexagonal wurtzite structure of ZnO nanoparticles (Bajpai *et al*., 2012). Films prepared at acidic and alkaline conditions showed almost similar pattern of WAXD. FPI/FSG film exhibited two broad diffraction peaks at 2θ around 9.72° and 21.87°, which represent its amorphous structure. Almost similar WAXD characteristic has been reported for films prepared from pig skin gelatin (Maria *et al*., 2008).

As shown in Figure 29B, the control film prepared from FFS at both pHs did not reveal any sharp diffraction peak, whilst the FPI/FSG-ZnO nanocomposite film revealed distinctive diffraction peaks at 2θ = 31.7, 34.4, 36.2, 47.5, 56.6, 62.7 and 67.9, corresponding to the (100), (002), (101), (102), (110), (103) and (200) planes of hexagonal wurtzite structure of ZnO (Bajpai *et al*., 2012). These results confirmed that the hexagonal wurtzite structure of ZnO nanoparticles was not affected after their incorporation in protein matrix. These typical WAXD peaks revealed the successful formation of FPI/FSG-ZnO nanocomposite.

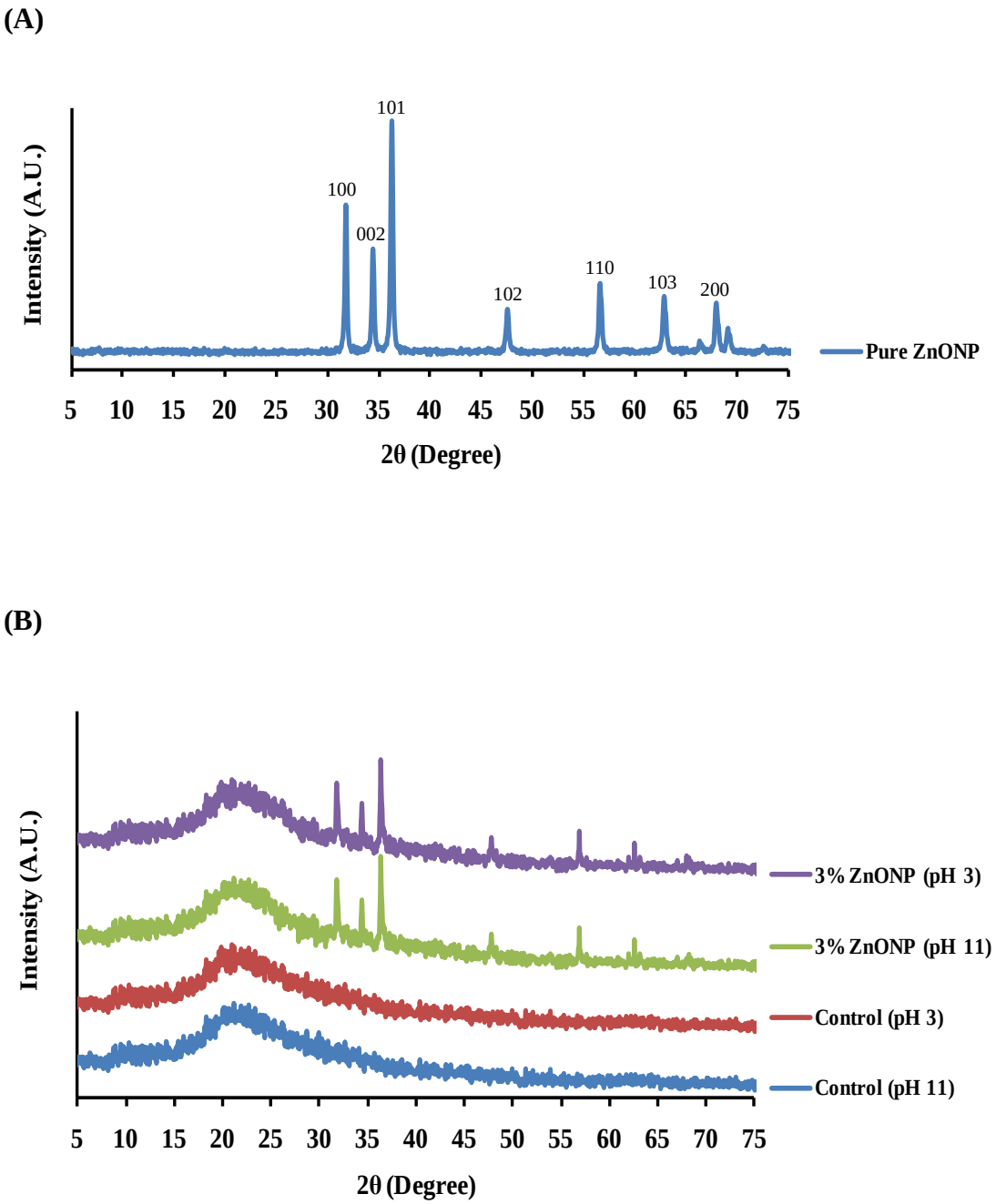


Figure 29. WAXD patterns of pure ZnONP (A) and FPI/FSG films prepared at pH 3 and 11 without and with 3% (w/w) ZnONP (B).

7.4.2.2 FTIR spectroscopy

FTIR spectra of FPI/FSG films prepared at pH 3 and 11 without and with 3% ZnONP are shown in Figure 30. Films prepared at acidic and alkaline conditions showed the similar major peaks but the amplitudes of peaks varied slightly. Generally, all films had the similar spectra in the range of 1800–600 cm^{-1}, covering amide-I, II and III bands. All films exhibited the major bands at around 1629-1632 cm^{-1} (amide-I, representing C= O stretching/hydrogen bonding coupled with COO), 1536 cm^{-1} (amide-II, arising from bending vibration of N-H groups and stretching vibrations of C–N groups) and 1238 cm^{-1} (amide-III, representing the vibrations in plane of C–N and N–H groups of bound amide or vibrations of CH_2 groups of glycine) (Muyonga *et al*., 2004). Yakimets *et al*. (2005) reported the similar result for bovine skin gelatin film, where amide -I, amide-II and amide-III peaks were found at the wavenumbers of 1633, 1536 and 1240 cm^{-1}, respectively. The band situated at the wavenumber of 1036–1039 cm^{-1} was found in all film samples, corresponding to the interactions arising between plasticizer (OH group of glycerol) and film structure (Hoque *et al*., 2011c). An amide-A band was observed at the wavenumber of 3275–3280 cm^{-1} for all film samples. Amide-B band was also found at 2926–2928 cm^{-1} for all film samples. The amide-A band represents the NH-stretching coupled with hydrogen bonding and amide-B band represents the asymmetric stretching vibration of CH as well as NH_3^+ (Muyonga *et al*., 2004; Hoque *et al*., 2011c).

The slight shift of FTIR spectra of films with 3% ZnONP in the amide-I region to the lower wavenumber were mainly associated with interaction of carbonyl of polypeptide chains with ZnONP. The shifts of the amide-I peak to a lower wavenumber were related to a decrease in the molecular order (Rouhi *et al*., 2013). Furthermore, for films prepared at pH 3, the amide-A band from the N-H stretching vibration of the hydrogen bonded N-H group became visible at wavenumbers 3,280 and 3,278 cm^{-1} for the control film and 3% ZnONP incorporated film, respectively, and at 3,278 and 3,275 cm^{-1} for the control film and 3% ZnONP incorporated film prepared at pH 11, respectively. This result clearly showed that amide-A band shifted to lower wavenumber when ZnONP was added. The shift to the lower wave number in the amide-A region suggested that N-H groups with protein chain interact with

ZnONP, mainly via hydrogen bonding (Nikoo *et al*., 2011). Rouhi *et al*. (2013) reported similar results from gelatin-based nanocomposite films incorporated with ZnO nanorods. Therefore, FTIR results reconfirm the interaction between protein molecules and ZnONP which was responsible for the improved mechanical and water vapor barrier properties of resulting FPI/FSG nanocomposite films (Table 17).

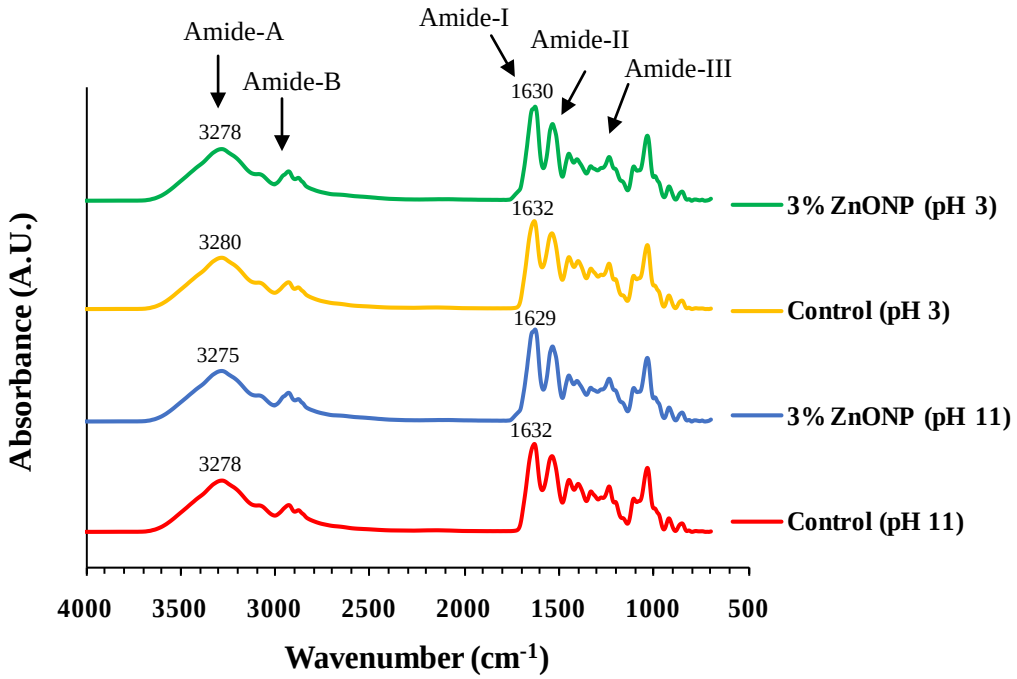


Figure 30. ATR-FTIR spectra of FPI/FSG films prepared at pH 3 and 11 without and with 3% (w/w) ZnONP.

7.4.2.3 Thermo-gravimetric analysis (TGA)

TGA thermograms revealing thermal degradation behavior of FPI/FSG films prepared at pH 3 and 11 without and with 3% ZnONP are shown in Figure 31. Their corresponding degradation temperatures and weight loss are presented in Table 20. Three main stages of weight loss were observed for all films. The first stage weight loss (Δw_1 = 2.28–3.14%) was observed over the temperature (T_{d1}) ranging from 47.22 to 51.27 °C up to approximately 150 °C, possibly associated with the loss of free and bound water adsorbed in the film. The similar result was found in cuttlefish skin gelatin film (Hoque *et al*., 2011c) and porcine plasma protein film added with different cross-linking agents (Nuthong *et al*., 2009). The second stage weight loss (Δw_2 = 24.66–27.86%) appeared at the onset temperature of 195.23–213.47 °C (T_{d2}), depending on the film samples. This was most likely due to the degradation or decomposition of lower molecular weight protein fractions and glycerol compounds. For the third stage of weight loss (Δw_3 = 42.64–45.61%), T_{d3} of 299.1–319.52 °C were observed for all films but varied with film samples. This was possibly due to the decomposition of highly interacted proteins in film matrix.

The results suggested that films with ZnONP showed the enhanced thermal stability in comparison with the films without ZnONP. ZnONP in the FPI/FSG matrix could insert within film matrix or acted as thermal insulator or mass transport barrier to the volatile products generated during decomposition. This resulted in delay of thermal degradation and improved thermal stability of the composites. The increase in the thermal stability was in agreement with the increase in mechanical resistance due to the strong film network, more energy thus being required to decompose the films. Carboxymethylcellulose/ZnO nanocomposite film with a high concentration of ZnONP showed enhanced thermal stability in comparison with control film (Espitia et al., 2013). Nevertheless, films prepared at pH 11 showed higher heat stability than those prepared at pH 3, irrespective of ZnONP addition. Amongst all films, films prepared at pH 11 and containing 3% ZnONP had the highest thermal degradation/decomposition temperature (T_{d2} = 213.47 and T_{d3} = 319.52). Higher amount of disulphide and hydrophobic interaction between proteins in FPI at alkaline conditions might contribute to heat resistance of their films

(Tongnuanchan *et al*., 2011a). Moreover, at alkaline pH, the salt bridge interactions between ZnO and proteins mostly yielded the stronger film network, leading to higher heat resistance of the resulting nanocomposite films than control films.

Additionally, all films had residual mass (representing char content) at 600 °C in the range of 23.39–30.42%. The highest char content observed in nanocomposite film prepared at pH 11 containing 3% ZnONP was most likely ascribed to the high thermal stability of the ZnONP and the highest bonding formed in the protein network. Thus, both pH and incorporated ZnONP, affected thermal property of FPI/FSG films.

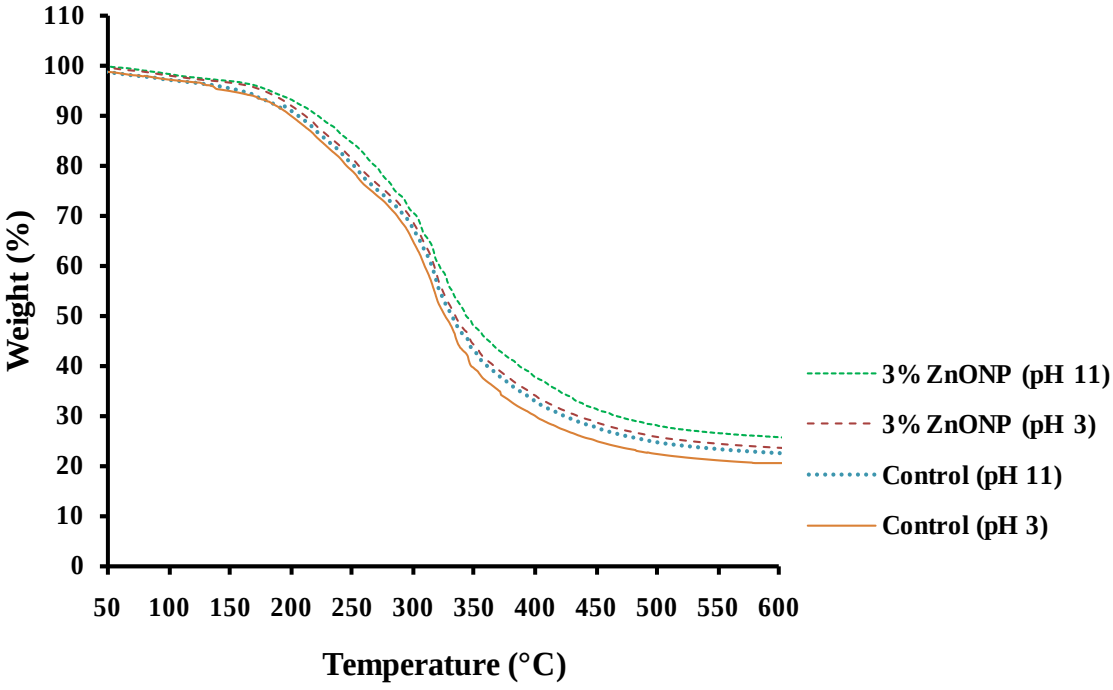


Figure 31. TGA curves of FPI/FSG films prepared at pH 3 and 11 without and with 3% (w/w) ZnONP.

Table 20. Thermal degradation temperature (T_d, °C) and weight loss (Δw, %) of FPI/FSG films prepared at pH 3 and 11 containing 0 and 3% ZnONP

pH level	ZnONP level (% w/w)	Δ_1		Δ_2		Δ_3		Residue (%)
		T_{d1},onset	Δw_1	T_{d2},onset	Δw_2	T_{d3},onset	Δw_3	
11	0	49.17	2.55	201.04	26.08	303.22	44.86	26.51
11	3	51.27	2.28	213.47	24.66	319.52	42.64	30.42
3	0	47.22	3.14	195.23	27.86	299.1	45.61	23.39
3	3	49.82	2.87	200.37	25.79	306.01	43.43	27.91

Δ_1, Δ_2, and Δ_3 denote the first, second and third stage weight loss, respectively, of film during heating scan.

7.4.2.4 Scanning electron microscopy

SEM micrographs of the surface and freeze-fractured cross-section of FPI/FSG films prepared at pH 3 and 11 without and with 3% ZnONP are illustrated in Figure 32. At both pHs 3 and 11, the slightly rough surface was noticeable in the control FPI/FSG film (without ZnONP). Arrangements or alignment of FPI and FSG might not occur in ordered fashion. From SEM images, no distinct separation observed in the matrix of FPI/FSG film. Roughness of surface structure was more pronounced in films incorporated with ZnONP than that found in the control film, regardless of pH of FFS. The roughness of film was attributed to ZnONP distributed throughout film matrix. This might be also associated with the coexisting protruded film structure as indicated by the increased thickness of resulting films (Table 17). Furthermore, nanocomposite films showed distinctive ZnONP images on their films surfaces, in which ZnONP were uniformly dispersed in the films.

The discontinuous zone or micro-cracks were found in FPI/FSG film prepared at pHs 3 and 11. This was more likely related with the less interaction between molecules of FPI and FSG. This could be associated with the poorer TS and WVP of FPI/FSG films. However, crack-free cross-sectional structure with distinctive ZnONP images was observed in FPI/FSG films incorporated with 3% ZnONP (at both pHs), compared with those observed in FPI/FSG blend film. This was plausibly due to the filler property of ZnONP and its interaction with FPI/FSG protein molecules in film matrix. The ZnONP could impede the water molecules to transfer or penetrate through the film (Alebooyeh *et al*., 2012). As a result, WVP of ZnONP incorporated films was lowered, compared with the control film, as shown in Table 17. Thus, the microstructures of films were markedly governed by ZnONP incorporation.

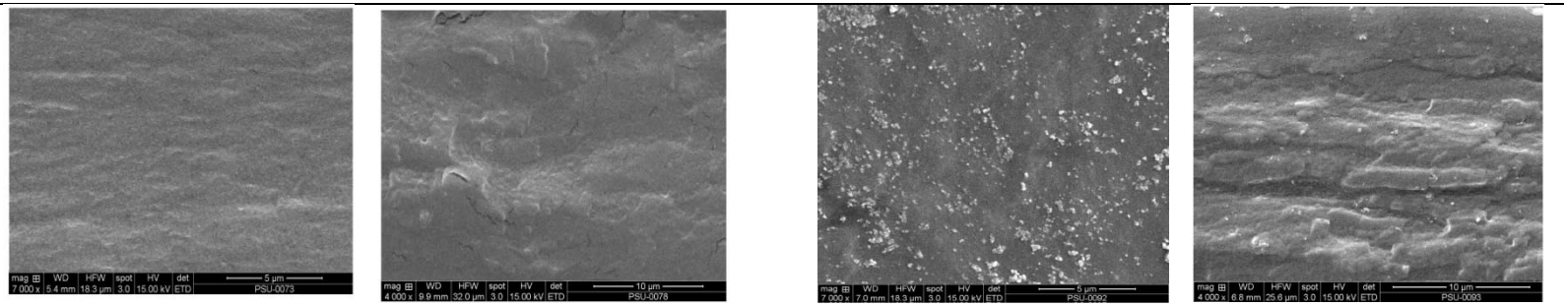
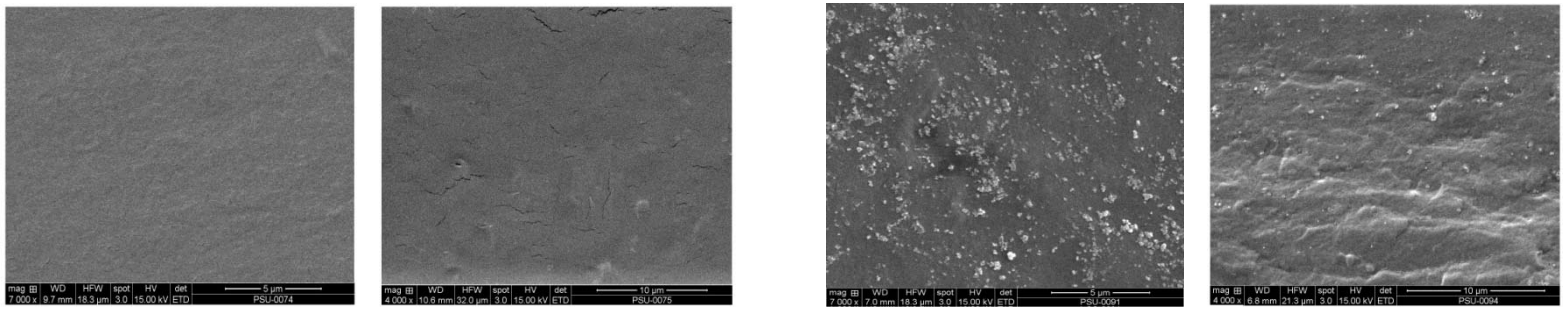
pH level	0% ZnONP (% w/w)		3% ZnONP (% w/w)	
	Surface	Cross-section	Surface	Cross-section
11				
3				

Figure 32. SEM micrographs of surface and freeze-fractured cross-section of FPI/FSG films prepared at pH 3 and 11 without and with 3% (w/w) ZnONP. Magnification: 7000× and 4000× for surface and cross-section, respectively.

7.4.3 Antimicrobial activity of FPI/FSG-ZnO nanocomposite films

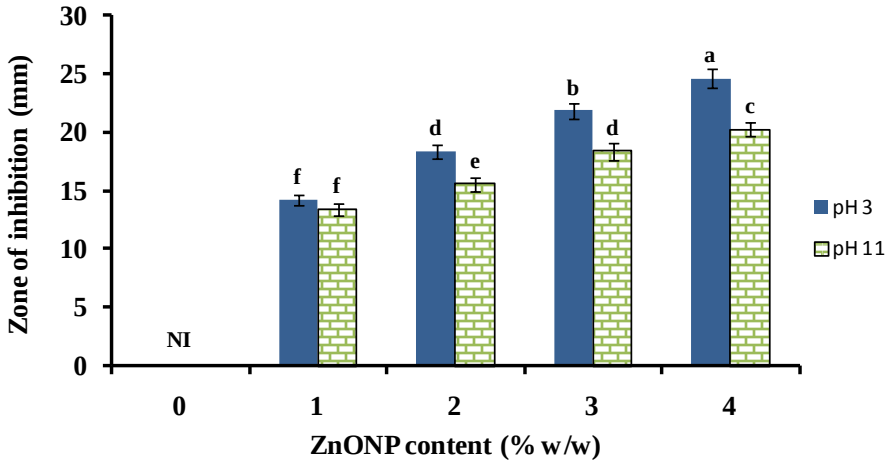
Antibacterial activity of the FPI/FSG-ZnO nanocomposite films prepared at pH 3 and 11 with different ZnONP contents was tested using Gram-positive food pathogenic bacteria (*L. monocytogenes*) and Gram-negative food spoilage bacteria (*P. aeruginosa*) in comparison with the control FPI/FSG films, as shown in Figure 33. At both pHs 3 and 11, the inhibition zone of ZnO-incorporated films increased with increasing ZnONP contents ($P < 0.05$). At the same ZnONP content, antibacterial activity of FPI/FSG-ZnO nanocomposite films prepared at pH 3 was higher than those prepared at pH 11 ($P < 0.05$). This was more likely governed by higher solubility of the films prepared at pH 3. Higher solubility of films prepared at pH 3 was obtained than those prepared at pH 11 (data not shown). ZnONP could be co-released with solubilized film matrix to a higher extent. The antimicrobial component releasing rate of gelatin films increased with increasing solubility (Yehuala and Emire, 2013).

Excellent antimicrobial activity of ZnO nanoparticles and the mechanism of the action against the microorganisms have been demonstrated (Espitia *et al*., 2013; Zhang *et al*., 2010). The release of Zn^{2+} ions from the powder could penetrate through the cell wall of microorganism and react with interior components that finally affect the viability of the cells. ZnO has also been known to mediate the generation of hydrogenperoxide (H_2O_2), a powerful oxidizing agent causing damage to the cell membrane of bacteria (Tayel *et al*., 2011).

At the same ZnONP level, the antibacterial effect of the FPI/FSG-ZnO nanocomposite films on *L. monocytogenes* was stronger than on *P. aeruginosa*, regardless of pH of FFS. Tam *et al*. (2008) demonstrated that ZnO was more effective for killing Gram-positive than Gram-negative bacteria. Gram-positive bacteria typically have one cytoplasmic membrane and cell wall composed of peptidoglycan (Ma and Zhang, 2009). More complex cell wall structure of gram-negative bacteria, with a layer of peptidoglycan between outer membrane and cytoplasmic membrane might prevent ZnO penetration into the cells and interacting with their internal components (Ma and Zhang, 2009). Antibacterial action of ZnO is suggested to occur

through its interaction with specific cell compounds like surface proteins (e.g., adhesions) and teichoic acids plus lipoids (forming lipoteichoic acids), which may be found in Gram-positive rather than in Gram-negative bacteria (Tayel *et al*., 2011). However, further studies are required to clarify the exact antibacterial mechanism of ZnONP. Thus, FPI/FSG nanocomposite films, especially those prepared at pH 3, had the potential to control both *L. monocytogenes* and *P. aeruginosa* in foods.

(A)



(B)

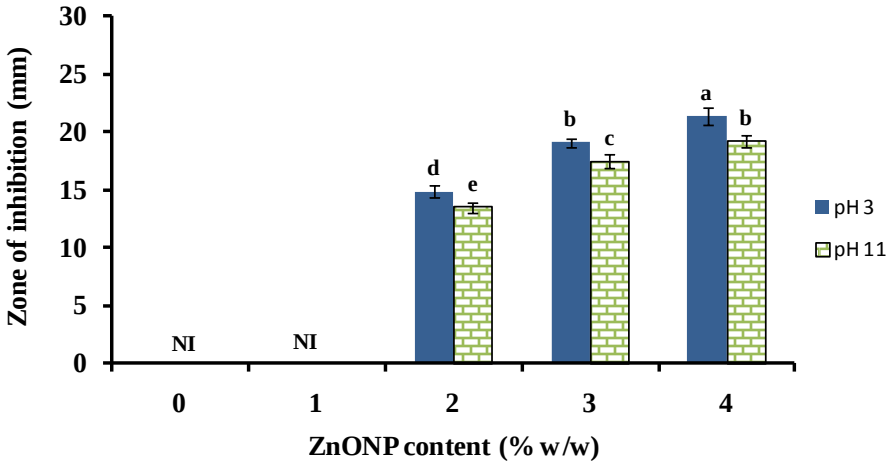


Figure 33. Effects of ZnONP content on antimicrobial activity of FPI/FSG films prepared at pH 3 and 11 against *L. monocytogenes* (A) and *P. aeruginosa* (B). Bars represent the standard deviation (n=3). Different lowercase letters on the bars indicate significant differences ($P < 0.05$). NI indicates the absence of inhibitory zone.

7.5 Conclusion

The antimicrobial nanocomposites films based on FPI/FSG were developed by addition of ZnONP. Properties of FPI/FSG films could be enhanced by incorporating 3% ZnONP at both acidic and alkaline pH. Nanocomposite film showed the improved mechanical, water vapor barrier and thermal properties, compared with control films (without ZnONP). Incorporation of ZnONP yielded film with lower yellow discoloration. Moreover, FPI/FSG-ZnO nanocomposites films greatly inhibited the growth of Gram-positive and Gram-negative food borne pathogenic and spoilage bacteria. Thus, FPI/FSG-ZnO nanocomposite films could be used as an active food packaging to prevent growth of pathogen and spoilage bacteria in foods.

CHAPTER 8

Properties and antimicrobial activity of fish protein isolate/fish skin gelatin film containing basil leaf essential oil and zinc oxide nanoparticles

8.1 Abstract

Composite films based on fish protein isolate (FPI) and fish skin gelatin (FSG) blend incorporated with 50 and 100% (w/w, protein) basil leaf essential oil (BEO) in the absence and presence of 3% (w/w, protein) ZnO nanoparticles (ZnONP) were prepared and characterized. Tensile strength (TS) decreased, whilst elongation at break (EAB) increased as BEO level increased ($P < 0.05$). However, ZnONP addition resulted in higher TS but lower EAB ($P < 0.05$). The lowest water vapor permeability (WVP) was observed for the film incorporated with 100% BEO and 3% ZnONP ($P < 0.05$). BEO and ZnONP incorporation decreased transparency of FPI/FSG films ($P < 0.05$). FTIR spectra indicated that films added with BEO exhibited higher hydrophobicity. Both BEO and ZnONP had a marked impact on thermal stability of the films. Microstructural study revealed that presence of ZnONP prevented bilayer formation of film containing 100% BEO. FPI/FSG films incorporated with 100% BEO, especially in combination with ZnONP, exhibited strong antibacterial activity against food pathogenic and spoilage bacteria and thus could be used as active food packaging material.

8.2 Introduction

Biopolymers have been widely paid attention over the last decades due to their advantages and potential applications in food industries (Rhim and Ng, 2007; Kanmani and Rhim, 2014). Biopolymer films are the excellent vehicles for incorporating a wide variety of additives, such as antimicrobials, antioxidants, antifungal agents, colorants, and other nutrients, thus improving food quality and extending shelf-life of foods and products (Rhim and Ng, 2007). Amongst biopolymers, proteins from different sources have been impressively used for the development of biodegradable films due to their relative abundance and good film-forming ability (Gennadios *et al*., 1996; Prodpran *et al*., 2007).

Fish protein isolate (FPI) prepared by alkaline solubilization was reported as the promising starting material for preparation of films with negligible yellow discoloration (Tongnuanchan *et al*., 2011a). However, FPI based film is rigid and thus requiring the addition of high amount of hydrophilic plasticizer (Tongnuanchan *et al*., 2011a). Plasticized-FPI films have the poor water vapor barrier property, owing to hydrophilicity of amino acids in protein molecules and to the significant amounts of hydrophilic plasticizers required for film flexibility (Prodpran *et al*., 2007). Recently, properties of FPI films from yellow stripe trevally, an abundant trash dark muscle fish, could be modified by blending with fish skin gelatin (FSG) at a ratio of 5:5 with lower glycerol content (30%) (Arfat *et al*., 2014). FPI/FSG blend films showed the improved mechanical and water vapor barrier properties, compared with FPI films. However, FPI/FSG blend films still have poorer mechanical as well as water vapor barrier properties, in comparison with synthetic films.

Nanotechnology has been implemented by incorporating various nano-fillers to biopolymer films for improving mechanical, thermal and water vapor barrier properties (Kovacevic *et al*., 2008). Among nano-fillers, ZnO nanoparticles (ZnONP) have the excellent ability for nano-scale dispersion and interfacial interactions in protein matrix due to their large specific surface area and high surface energy (Rouhi *et al*., 2013). Recently, the incorporation of ZnONP as functional filler into the biopolymer films such as starch based films have been reported to improve mechanical and water vapor barrier properties (Alebooyeh *et al*., 2012; Yu *et al*., 2009). ZnO is currently listed as a generally recognized as safe (GRAS) material by the Food and Drug Administration (21CFR182.8991) and has previously shown strong *in vitro* antimicrobial activity against food borne pathogens and spoilage bacteria (Espitia *et al*., 2013; Zhang *et al*., 2008).

Antimicrobial biodegradable films have received increasing attention because of their potential to delay microbial spoilage of foods (Emiroglu *et al*., 2010; Rhim and Ng, 2007). Films containing essential oils have been known as active packaging, which shows the effectiveness in retarding the microbial growth (Ahmad *et al*., 2012a). Additionally, essential oils are hydrophobic in nature and the

incorporation of essential oils could improve the water vapor barrier property and impart flexibility of protein films (Tongnuanchan *et al*., 2013a). The use of essential oil exhibited the increased antimicrobial activity, when combined together with various nanoparticles (Allahverdiyev *et al*., 2011). To the best of our knowledge, no information regarding the combined effect of essential oils and ZnONP on properties of protein based films have been reported. The present study aimed to investigate the combined effects of ZnONP and basil leaf essential oil (BEO) on physico-mechanical, thermal and antimicrobial activity of FPI/FSG blend films against *L. monocytogenes* (food borne pathogen) and *P. aureginosa* (food spoilage bacteria).

8.3 Materials and methods

8.3.1 Chemicals

Zinc oxide nanoparticle (ZnONP) (particle size: 20-40 nm, specific surface area: 26.22 m^2/g) was purchased from Nano materials technology Co. Ltd. (Bangkok, Thailand). Sodium hydroxide and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Commercial fish skin gelatin (FSG) from tilapia (~240 bloom) was purchased from Lapi Gelatine S.p.A (Empoli, Italy). Essential oil from the leaves of basil (*Ocimum basilicum*) was obtained from Botanicessence (Bangkok, Thailand). All chemicals were of analytical grade. *Listeria monocytogenes* DMST 1327 was obtained from Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand and *Pseudomonas aeruginosa* TISTR 781 was obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand.

8.3.2 Collection and preparation of fish sample

Fresh yellow stripe trevally (*Selaroides leptolepis*) with an average weight of 90–100 g/fish were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were kept in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon the arrival, fish were immediately washed, filleted, and minced to uniformity using a Model HC 5000 mincer (Microfluidics, Massachusetts, USA) with a hole diameter of 0.5 cm.

8.3.3 Preparation of fish protein isolate

Prior to the isolation of fish protein, the prepared mince was subjected to washing as per the method of Toyohara *et al*. (1990) with slight modifications. Fish mince was homogenized with 5 volumes of cold 0.05 M NaCl (2–4 °C) at a speed of 13,000 rpm for 2 min, using an IKA Labortechnik homogenizer (Selangor, Malaysia). The washed mince was filtered through two layers of cheese-cloth. The washing process was repeated twice. Washed mince obtained was stored on ice until used.

Washed mince was added with cold distilled water at the ratio of 1:9 (w/v), followed by homogenization for 1 min at a speed of 13,000 rpm. The pH of homogenate was then adjusted to 11 using 2 M NaOH. The resulting mixture was centrifuged at 10,000×g for 20 min at 4 °C using a refrigerated centrifuge (Avanti-JE Centrifuge, Beckman 163 Coulter Inc., Fullerton, CA, USA). The supernatant was collected and the pH was adjusted to 5.5 using 2 M HCl. The precipitate was then filtered through 4 layers of cheese-cloth. The retentate was dewatered by centrifugation at 12,000×g for 20 min at 4 °C. The final pH of the sample was adjusted to pH 7.0 using 2 M NaOH. The sample was referred to as “fish protein isolate; FPI”. FPI was used for film preparation.

8.3.4 Preparation of fish protein isolate/fish skin gelatin film added with BEO and ZnONP

Firstly, film-forming solution was prepared according to the method of Chinabhark *et al*. (2007). FPI was added with 3 volumes of distilled water and homogenized at 13,000 rpm for 1 min using a homogenizer. Subsequently, the pH of the mixture was adjusted to 3 using 1 N HCl, to solubilize the protein. The obtained solution was filtered through 2 layers of cheese-cloth to remove undissolved debris. The protein concentration of the filtrate determined by the Kjeldahl method (AOAC, 2000) was adjusted to 3 % (w/v). Glycerol at 30% (w/w) of protein was used as a plasticizer. The mixtures were stirred gently for 30 min at room temperature and was used for preparing blend FFS.

Prior to blending, FSG powder was dissolved in distilled water to obtain the protein concentration of 3% (w/v). The pH of the mixture was adjusted to 3 using 1 N HCl. The solution was heated at 70 °C for 30 min. Glycerol at concentrations of 30 % (w/w) of protein content was used as a plasticizer. Thereafter, both FPI and FSG solutions were mixed at a ratio of 1:1 (v/v). The obtained solution was added without and with 3% ZnONP (w/w, protein content) in droplets. Before addition of ZnONP, ZnONP was suspended in distilled water and homogenized for 1 min at 5,000 rpm (IKA Labortechnik homogenizer, Selangor, Malaysia). The obtained FPI/FSG/ZnONP suspension was stirred for 5 min and then homogenized for 30 sec at the speed of 5,000 rpm.

BEO previously mixed with Tween 20 at 25% (w/w, based on essential oil) was added to the FPI/FSG/ZnONP suspension at levels of 50% and 100% (w/w, protein content). Final volume was made up to 100 ml using distilled water previously adjusted to pH 3. To obtain the uniform distribution of BEO and ZnONP, the suspensions were homogenized with three passes through a high pressure homogenizer (Microfluidizer M-110EH, Microfluidics Corp., Newton, MA, USA) with an operating pressure of 1,500 bars. Suspensions were gently stirred for 30 min at room temperature and were referred to as a film-forming suspension (FFS). Prior to casting, FFS samples were degassed for 10 min using the sonicating bath (Elmasonic S 30 H, Singen, Germany). To prepare the film, 4 g of FFS was cast onto a rimmed silicone resin plate (5×5 cm^2), air-blown for 12 h at 25 °C, followed by drying in an environmental chamber (Binder GmbH, Tuttlingen, Germany) at 25 ± 0.5 °C and 50 ± 5% relative humidity (RH) for 24 h. Dried film samples were manually peeled off and subjected to analyses.

8.3.5 Determination of properties and antimicrobial activity of films

Prior to testing, films were conditioned for 48 h at 50 ± 5% relative humidity (RH) at 25 ± 0.5 °C. For ATR-FTIR, TGA and SEM, films were dried in a desiccator containing dried silica gel for 1 week and in a desiccator containing P_2O_5 for 2 weeks at room temperature (28–30 °C) to obtain the most dehydrated films and to minimize the plasticizing effect due to water.

8.3.5.1 Film thickness

The thickness of film was measured using a digital micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mituyoto Corp., Kawasaki-shi, Japan). Ten random locations around each film sample were used for thickness determination.

8.3.5.2 Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of films were determined as described by Iwata *et al*. (2000) using the Universal Testing Machine (Lloyd Instruments, Hampshire, UK). Ten samples (2 cm×5 cm) with initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile loading using a 100 N load cell with the cross-head speed of 30 mm/min until the samples were broken. The maximum load and the final extension at break were used for calculation of TS and EAB, respectively.

8.3.5.3 Water vapor permeability (WVP)

WVP was measured using a modified ASTM method (American Society for Testing and Materials, 1989) as described by Shiku *et al*. (2004). The film was sealed on an aluminum permeation cup containing dried silica gel (0% RH) with silicone vacuum grease and a rubber gasket to hold the film in place. The cups were placed in a desiccator containing the distilled water at 30 °C. The cups were weighed at 1 h intervals over a 10 h period. WVP of the film was calculated as follows:

$$\text{WVP (g m}^{-1}\text{ s}^{-1}\text{ Pa}^{-1}) = w\, l\, A^{-1}\, t^{-1}\, (P_2 - P_1)^{-1}$$

where w is the weight gain of the cup (g); l is the film thickness (m); A is the exposed area of film (m^2); t is the time of gain (s); ($P_2 - P_1$) is the vapor pressure difference across the film (4244.9 Pa at 30 °C).

8.3.5.4 Color

Color of film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, VA, USA). Color of the film was expressed as L^*

– (lightness), *a** – (redness/greenness) and *b**– (yellowness/blueness) values. Total difference in color (Δ*E**) was calculated according to the following equation (Gennadios *et al*., 1996):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where Δ*L**, Δ*a** and Δ*b** are the differences between the corresponding color parameter of the samples and that of white standard (*L** = 92.65, *a** = - 0.92, *b** = 0.49).

8.3.5.5 Light transmittance and transparency value

The light transmittance of films was measured at the ultraviolet and visible range (200–800 nm) using a UV–visible spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) according to the method of Shiku *et al*. (2004). The transparency value of film was calculated using the following equation (Han and Floros, 1997):

$$\text{Transparency value} = \frac{-\log T_{600}}{x}$$

where T_{600} is the fractional transmittance at 600 nm and *x* is the film thickness (mm). The greater transparency value represents the lower transparency of film.

8.3.5.6 Antimicrobial properties

Antimicrobial activity of film samples was assessed using the agar diffusion method according to Ponce *et al*. (2008). The zone of inhibition on solid media was used for determining antimicrobial effects of films against typical food pathogenic and spoilage bacteria including Gram-positive bacteria (*Listeria monocytogenes*) and Gram-negative bacteria (*Pseudomonas aeruginosa*). Film samples were cut into a disc shape of 10 mm diameter using a circular knife and then placed on Mueller Hinton (Merck, Darmstadt, Germany) agar plates, which had been previously smeared with 100 µl of inoculum containing approximately 10^6–

10^7 CFU/ml of tested bacteria. The plates were then incubated at 37 °C for 24 h. The antimicrobial activity of nanocomposite films was determined by measuring the diameter of the bacterial growth inhibition zone around the film.

8.3.6 Characterisation of the selected films

The FPI/FSG films incorporated without and with BEO at 100% (w/w protein), in the absence and presence of 3% ZnONP were characterized, in comparison with the control FPI/FSG films (without BEO and ZnONP).

8.3.6.1 Attenuated total reflectance-Fourier transforms infrared (ATR-FTIR) spectroscopy

FTIR spectra of film samples were determined using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at 25 °C as described by Nuthong *et al*. (2009). Samples were placed onto the crystal cells and the cells were clamped into the mount of FTIR spectrometer. The spectra in the range of 650–4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 °C.

8.3.6.2 Thermo-gravimetric analysis (TGA)

Films were scanned using a thermo-gravimetric analyser (TGA-7, Perkin Elmer, Norwalk, CT, USA) from 40 to 600 °C at a rate of 10 °C/min (Nuthong *et al*., 2009). Nitrogen was used as the purge gas at a flow rate of 20 mL/min.

8.3.6.3 Scanning electron microscopy (SEM)

Morphology of surface and freeze-fractured cross-section of film samples were visualized using a scanning electron microscope (SEM) (Quanta400, FEI, Eindhoven, the Netherlands) at an accelerating voltage of 15 kV. Prior to visualisation, the film samples were mounted on brass stub and sputtered with gold in

order to make the sample conductive, and photographs were taken at 7000× magnification for surface visualisation. For cross-section, freeze-fractured films were mounted around stubs using double sided adhesive tape, coated with gold and observed at the 3500× magnification.

8.3.7 Statistical analysis

Experiments were run in triplicate using different three lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, U.S.A.).

8.4 Results and discussion

8.4.1 Properties of FPI/FSG films incorporated with BEO and ZnONP

FPI/FSG films incorporated with BEO at 50 and 100% (w/w) in the absence and presence of 3% ZnONP, exhibited the different properties and antimicrobial activity.

8.4.1.1 Thickness

Thickness of FPI/FSG films incorporated with BEO at different levels in the absence and presence of 3% ZnONP is shown in Table 21. Thickness of films increased with the increasing levels of BEO ($P < 0.05$), regardless of ZnONP. It was plausibly due to the protruded structures mediated by interaction between chemical components present in BEO and protein matrix. The results suggested that protein chains could not form the compact and ordered film network in the presence of BEO as indicated by increased thickness. Moreover, the increases in thickness with the addition of BEO could be due to the increase in solid content in films. Ahmad *et al*. (2012a) reported the increase in thickness of gelatin based films incorporated with bergamot essential oil.

Films containing 3% ZnONP had higher thickness, compared with those without ZnONP ($P < 0.05$), irrespective of BEO level. At 100% BEO level, films incorporated with ZnONP had the highest thickness ($P < 0.05$). This was plausibly due to the combined effect of 3% ZnONP and 100% BEO, inserting between protein network as well as the increased solid content of resulting films. This behavior was also observed by Sothornvit *et al*. (2009) for whey protein isolate nanocomposite films.

8.4.1.2 Mechanical properties

Mechanical properties of FPI/FSG films containing BEO at various levels without and with 3% ZnONP are shown in Table 21. Films incorporated with BEO showed the lower TS, but higher EAB, compared with the control (without essential oil), regardless of ZnONP addition ($P < 0.05$). Amongst all films added with BEO, the lowest TS but the highest EAB were found in films added with 100% BEO ($P < 0.05$). The incorporation of essential oil into FPI/FSG blend film could enhance the development of heterogeneous film matrix, leading to discontinuity of film network. As a result, the lower rigidity and strength of film matrix was obtained. Hydrogen bonding and hydrophobic interaction were the major bonds stabilising FPI and FSG films network (Arfat *et al*., 2014). Addition of lipid or oil in protein-based films may hinder protein–protein interaction and provide flexible domains within the film (Limpisophon *et al*., 2010). The results suggested that essential oil functioned as plasticizer in resulting film. Plasticizers are molecules, which penetrate into biopolymer matrix, thereby increasing the volume of empty spaces between the chains of molecules and causing a decrease in the strength of intermolecular forces along the matrix (Hoque *et al*., 2011c). The addition of lipids, oils or fatty acids with increasing level decreased TS of protein-based films e.g., films from commercial fish skin gelatin (Tongnuanchan *et al*., 2013a) and fish muscle protein (Tanaka *et al*., 2001).

At all BEO levels, FPI/FSG films incorporated with 3% ZnONP showed the higher TS and the lower EAB ($P < 0.05$), as compared with those without ZnONP. ZnONP more likely acted as the reinforcing filler in the protein matrix, thereby enhancing the strength of resulting films. Reinforcing effect of nanoparticles

in gelatin based film was reported by Rouhi *et al*. (2013). The ZnONP incorporated generated an interactive force with protein molecules and limited the molecular mobility of protein chain (Rouhi *et al*., 2013). The nanoparticles have high surface energy and large specific surface area, giving rise to strong interfacial interactions between polymers and the nanoparticles. This brings about a significant enhancement in mechanical properties of polymers (Kovacevic *et al*., 2008; Rouhi *et al*., 2013). Thus, both BEO and ZnONP incorporated in FPI/FSG film directly determined mechanical properties of resulting composite films.

8.4.1.3 Water vapor permeability (WVP)

WVP of FPI/FSG films varied, depending on the levels of BEO and ZnONP added as shown in Table 21. FPI/FSG film is known to have poor water barrier properties due to the presence of polar amino acids and hydroxyl group (–OH), mainly from glycerol (Prodpran *et al*., 2007; Arfat *et al*., 2014). WVP of FPI/FSG films decreased with increasing amount of BEO ($P < 0.05$). Incorporation of BEO, nonpolar or hydrophobic materials, increased the hydrophobicity of films, thereby lowering the adsorptivity as well as diffusivity of water vapor through the film as indicated by lower WVP. Monoterpene hydrocarbon was generally found in essential oils (Turina *et al*., 2006) and contributed to hydrophobicity of film. The presence of a hydrophobic disperse phase, even at small ratio, limits water vapor transfer since it interferes hydrophilic phase and increases the tortuosity factor of mass transfer (Atares *et al*., 2010). Furthermore, water vapor transfer normally occurs through the hydrophilic portion of film network and depends on hydrophilic/hydrophobic ratio of film constituent (Tongnuanchan *et al*., 2012). This result was in agreement with Pires *et al*. (2011) who reported that thyme essential oil incorporated into hake protein based film decreased WVP of resulting films, especially with increasing amount of essential oil.

WVP of films decreased when 3% ZnONP was incorporated ($P < 0.05$), regardless of BEO addition. The significant decrease in WVP after the addition of ZnONP was mainly attributed to the water resistance of incorporated ZnONP. Moreover, the incorporation of these nanoparticles to the protein matrix introduces a

tortuous pathway for water vapor molecules to pass through (Alebooyeh *et al*., 2012; Yu *et al*., 2009). The improvements of water resistance in nanocomposite biopolymer films, e.g. nanocomposite starch (Alebooyeh *et al*., 2012) and nanocomposite gelatin (Rouhi *et al*., 2013) have been reported. Amongst all films, those containing 100% BEO and 3% ZnONP had the lowest WVP ($P < 0.05$). Films incorporated with 100% BEO without and with 3% ZnONP had the decreases in WVP by 26.2, and 54.9%, respectively, in comparison with the control film (without essential oils and ZnONP) ($P < 0.05$). Thus, both BEO level and ZnONP had the profound impact on WVP of FPI/FSG films.

Table 21. Thickness, mechanical properties and water vapor permeability of FPI/FSG films as affected by BEO and ZnONP incorporation

ZnONP level	Essential oil level	Thickness (mm)	TS (MPa)	EAB (%)	WVP ($\times 10^{-11}$ gm^{-1} s^{-1} Pa^{-1})
0	0	0.036 ± 0.001d	11.94 ± 0.82c	68.38 ± 4.89c	3.62 ± 0.17a
	50	0.040 ± 0.002c	9.52 ± 0.72d	89.26 ± 5.41b	3.06 ± 0.14b
	100	0.045 ± 0.002b	7.23 ± 0.52e	110.05 ± 7.09a	2.67 ± 0.13c
3	0	0.041 ± 0.002c	14.70 ± 0.36a	56.39 ± 5.67d	2. 86 ± 0.15bc
	50	0.045 ± 0.001b	13.05 ± 0.58b	70.18 ± 5.29c	2.36 ± 0.16d
	100	0.050 ± 0.002a	11.17 ± 0.80c	85.51 ± 5.50b	1.63 ± 0.10e

Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

8.4.1.4 Color

The color expressed as *L**, *a**, *b** and Δ*E** values of FPI/FSG films added with BEO at various levels in the absence and presence of 3% ZnONP is shown in Table 22. As the levels of BEO increased, higher *b** and Δ*E** values were observed in resulting films with the concomitant lower *L** and *a** values, regardless of ZnONP incorporation ($P < 0.05$) (Table 22). The changes in color of resulting films were most likely attributed to the coloring components in essential oil. This result was in agreement with Tongnuanchan *et al*. (2013a) who reported that increasing amount of root essential oil markedly increased *b** andΔE values with the concomitant decreases in *L** and *a** values of fish skin gelatin films. Nevertheless, the addition of thyme oil (0–0.25 ml oil/g protein) into hake protein film did not have a significant impact on color attributes (Pires *et al*., 2011).

At all levels of BEO incorporation, the addition of ZnONP did not have a marked impact on *L**- and *a**-values of resulting films ($P > 0.05$). However, the decreases in *b**- and Δ*E**-values were obtained as ZnONP was incorporated ($P < 0.05$). These results were in accordance with Espitia *et al*. (2013) who reported that increasing amount of ZnONP markedly decreased *b**- and Δ*E**-values, whilst it had no significant effect on *L**- and *a**-values of methyl cellulose (MC)-ZnO nanocomposites. Such changes in color of resulting films were most likely caused by the whitening effect of ZnONP (Espitia *et al*., 2013). The results suggested that ZnONP decreased yellowish color in BEO incorporated FPI/FSG films.

Table 22. Color of FPI/FSG films as affected by BEO and ZnONP incorporation

ZnONP level	Essential oil level	Color			
		L*	***a****	***b****	**Δ*E****
0	**0**	90.54 ± 0.13a	-1.37 ± 0.04a	2.13 ± 0.14e	2.71 ± 0.08e
	50	90.11 ± 0.07b	-1.68 ± 0.03b	3.46 ± 0.04c	3.98 ± 0.10c
	100	89.87 ± 0.06c	-1.93 ± 0.13c	4.17 ± 0.10a	4.72 ± 0.13a
3	**0**	90.65 ± 0.11a	-1.41 ± 0.06a	1.81 ± 0.08f	2.45 ± 0.12f
	50	90.24 ± 0.06b	-1.66 ± 0.04b	3.06 ± 0.03d	3.60 ± 0.08d
	100	89.93 ± 0.09c	-1.90 ± 0.09c	3.69 ± 0.11b	4.31 ± 0.09b

Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

8.4.1.5 Light transmittance and transparency value

Transmission of UV and visible light at wavelength range of 200-800 nm of FPI/FSG films incorporated with 50 and 100% BEO without and with 3% ZnONP are shown in Table 23. Control FPI/FSG films (without BEO and ZnONP) had a good barrier property in the UV range (200-280 nm). Protein based films are generally known to have the excellent UV barrier properties due to the presence of high content of aromatic amino acids which can absorb UV light (Hoque *et al*., 2011c; Arfat *et al*., 2014).

Light transmittance of films at wavelength of 280 nm decreased markedly from 7.81% (control film) to 0.08% and 0% upon the incorporation of BEO at levels of 50 and 100%, respectively. Thus, the incorporation of BEO to film could improve the UV-barrier property, especially for films added with higher level of BEO. The transmission of visible light in the range of 350-800 nm of films incorporated with BEO decreased with increasing levels of BEO. The decrease in light transmittance was possibly caused by light scattering of lipid droplets distributed throughout the protein network (Tongnuanchan *et al*., 2013a). The barrier property towards UV and visible light became more pronounced when ZnONP was

incorporated. This was plausibly due to the hindrance of light passage or light scattering by the nanoparticles dispersed in the film matrix (Kanmani and Rhim, 2014). Rouhi *et al*. (2013) reported that the gelatin films added with ZnO nanorods had lower transmission for UV light. The FPI/FSG films incorporated with BEO and reinforced with ZnONP could be used as potential packaging films to prevent UV light, which can induce lipid oxidation of various foods.

The transparency values of all film samples are presented in Table 23. The control film had the lowest transparency value, compared with those films added with BEO ($P < 0.05$). The lower transparency value represents the greater transparency of film. The transparency value of films increased as the level of BEO incorporated increased ($P < 0.05$). The result indicated that films containing BEO became less transparent. The decreases in transparency of films were related with the decrease in light transmittance when BEO was incorporated. Moreover, the coloring components in BEO as well as the heterogeneous film network were contributable to the decreased transparency of films. This result was in agreement with Tongnuanchan *et al*. (2012) who reported that the addition of citrus essential oils lowered the transparency of fish skin gelatin film. Addition of thyme essential oil also decreased the transparency of hake protein film (Pires *et al*., 2011). The films added with ZnONP had higher transparency value ($P < 0.05$), regardless of essential oil incorporation. This indicated the decrease in transparency of resulting films. Similarly, Sothornvit *et al*. (2010) reported that the addition of nanoparticles to whey protein films resulted in the reduced transparency of the films. Moreover, transparency value of film also varied with the thickness of film. Thus, the incorporation of BEO and ZnONP had the profound impact on light transmittance and transparency of films.

Table 23. Light transmittance and transparency values of FPI/FSG films as affected by BEO and ZnONP incorporation

ZnONP level	Essential oil level	Light transmittance (%)								Transparency values
		200	280	350	400	500	600	700	800	
0	0	0.00	7.81	68.32	71.76	75.04	77.34	85.32	87.69	3.10 ± 0.05e
	50	0.00	0.08	35.78	62.01	66.27	71.68	83.66	86.08	3.62 ± 0.04c
	100	0.00	0.00	17.89	50.88	59.87	66.13	81.98	85.75	3.99 ± 0.06b
3	0	0.00	0.45	47.15	56.12	68.04	71.87	77.23	80.17	3.51 ± 0.04d
	50	0.00	0.00	29.71	43.13	59.71	66.32	76.87	78.21	3.96 ± 0.04b
	100	0.00	0.00	13.68	36.00	53.84	61.44	74.58	77.68	4.23 ± 0.07a

Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

8.4.1.6 Antimicrobial activity of FPI/FSG films incorporated with BEO and ZnONP

Antibacterial activity of the FPI/FSG films incorporated with BEO at various levels in the absence and presence of 3% ZnONP toward Gram-positive food pathogenic bacteria (*L. monocytogenes*) and Gram-negative food spoilage bacteria (*P. aeruginosa*) in comparison with the control films is shown in Figure 34. The inhibition zone of BEO incorporated films increased with increasing BEO contents ($P < 0.05$), regardless of ZnONP. At the same BEO level, the films showed the higher inhibition toward Gram-positive bacteria (*L. monocytogenes*), compared with Gram-negative bacteria (*P. aeruginosa*). Essential oils have been employed as food additive for the preservation of a wide range of foods, including vegetables, rice, fruit, dairy products, fish, meat and poultry (Burt, 2004). The antimicrobial activity of the BEO could be explained by the significant amount of linalool, an oxygenated monoterpenoid. This compound was shown to have antimicrobial activity (Hussain *et al*., 2008). Linalool has the potential to act as either a protein denaturing agent or as a solvent dehydrating agent, contributing to its antimicrobial activity (Suppakul *et al*., 2003). The mechanism of antimicrobial activity of essential oils is related to its ability to penetrate the cell wall of a bacterium and attack on the phospholipid bilayer present in cell membranes. This causes increased permeability and leakage of cytoplasm, or in their interaction with enzymes located on the cell wall (Emiroglu *et al*., 2010). Gram-negative bacteria have an additional external membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Sharma *et al*., 2010b). Suppakul *et al*. (2003) reported that basil essential oils exhibited powerful antimicrobial activity against a wide range of microorganisms. Bozin *et al*. (2006) showed that the Gram-positive strains of bacteria showed higher sensitivity to *O. basilicum* essential oils.

At the same BEO level incorporated, antibacterial activity of FPI/FSG films containing 3% ZnONP was higher than that without 3% ZnONP ($P < 0.05$). This was governed by the strong antimicrobial activity of ZnONP against both microorganisms. Amongst all films, that incorporated with 100% BEO and 3% ZnONP had the highest antimicrobial activity ($P < 0.05$). This was more likely due to

combined antimicrobial effect of BEO and ZnONP distributed throughout the films matrix. Antimicrobial activity of ZnO nanoparticles and the mechanism of inhibition against the microorganisms was demonstrated (Espitia *et al*., 2013; Zhang *et al*., 2010). The release of Zn^{2+} ions from the powder could penetrate through the cell wall of microorganism and react with interior components that finally affect the viability of the cells. ZnONP has been known to mediate the generation of hydrogen peroxide (H_2O_2), a powerful oxidising agent causing damage to the cell membrane of bacteria (Tayel *et al*., 2011). At the same BEO level, film containing 3% ZnONP showed higher antibacterial effect on *L. monocytogenes* than *P. aeruginosa* ($P < 0.05$). Tam *et al*. (2008) demonstrated that ZnO was more effective for killing Gram-positive than Gram-negative bacteria. Gram-positive bacteria typically have one cytoplasmic membrane and cell wall composed of peptidoglycan (Ma and Zhang, 2009). More complex cell wall structure of gram-negative bacteria, with a layer of peptidoglycan between outer membrane and cytoplasmic membrane might prevent ZnO penetration into the cells (Ma and Zhang, 2009). Antibacterial action of ZnO is suggested to occur through its interaction with specific cell compounds like surface proteins (e.g., adhesions) and teichoic acids plus lipoids (forming lipoteichoic acids), which may be found in Gram-positive rather than in Gram-negative bacteria (Tayel *et al*., 2011). However, further studies are required to clarify the exact antibacterial mechanism of ZnONP. Thus, FPI/FSG films incorporated with 100% BEO and 3% ZnONP had the potential to control both *L. monocytogenes* and *P. aeruginosa* in foods.

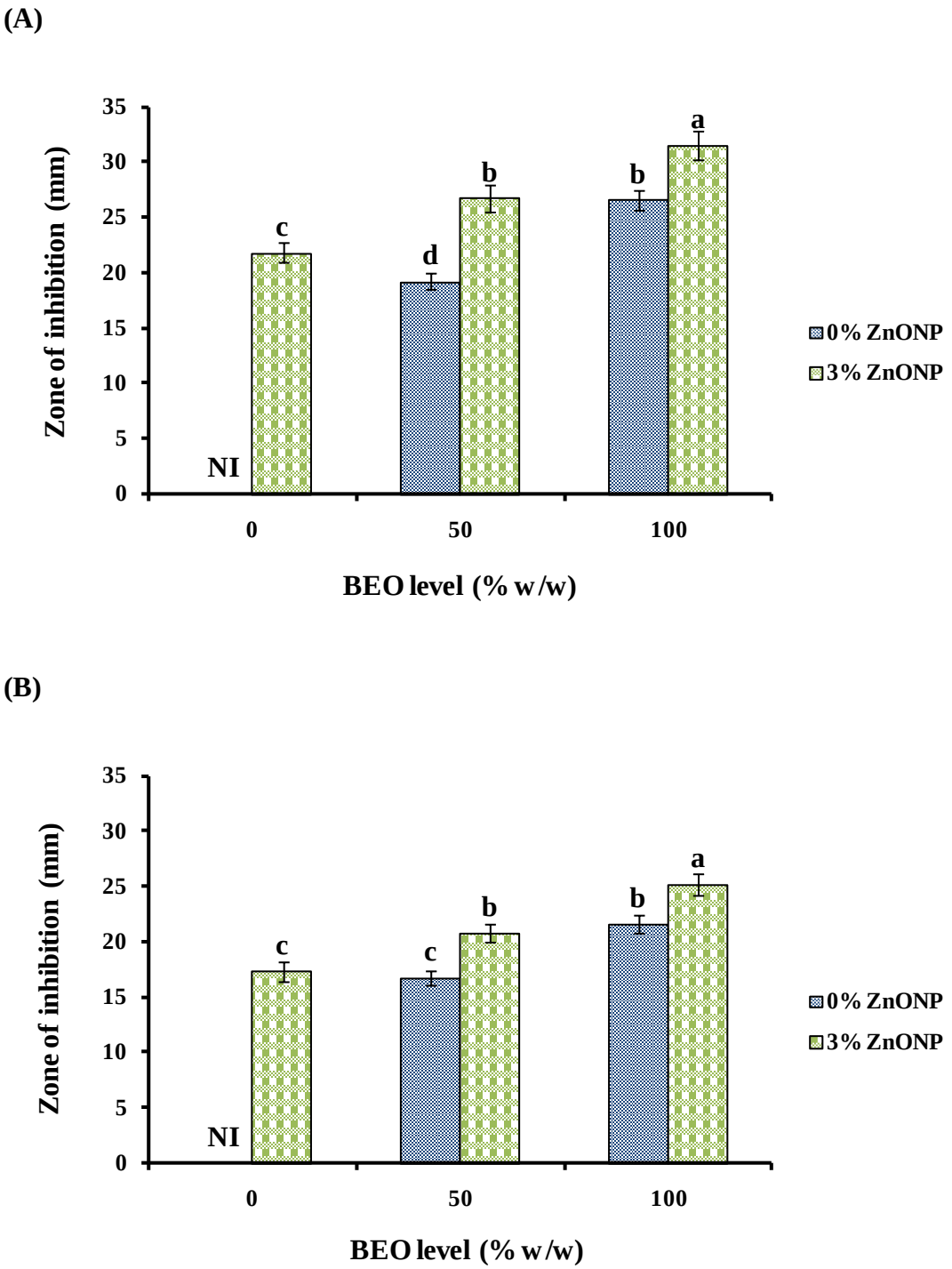


Figure 34. Antimicrobial activity of FPI/FSG films against *L. monocytogenes* (A) and *P. aeruginosa* (B) as influenced by BEO and ZnONP. Bars represent the standard deviation (n=3). Different lowercase letters on the bars indicate significant differences ($P < 0.05$). NI indicates the absence of inhibitory zone.

8.4.2 Characteristics of FPI/FSG films incorporated with BEO and ZnONP

The FPI/FSG films containing 100% BEO in the absence and presence of 3% ZnONP were characterized, in comparison with the control films (without BEO and ZnONP).

8.4.2.1 FTIR spectroscopy

FTIR spectra of FPI/FSG films as influenced by BEO and ZnONP incorporation are illustrated in Figure 35. All films exhibited the major bands at wavenumber of 1631 cm^{-1} (amide-I, representing C= O stretching/hydrogen bonding coupled with COO), 1537 cm^{-1} (amide-II, arising from bending vibration of N-H groups and stretching vibrations of C–N groups) and 1238 cm^{-1} (amide-III, illustrating the vibrations in plane of C–N and N–H groups of bound amide or vibrations of CH_2 groups of glycine) (Muyonga *et al*., 2004). Yakimets *et al*. (2005) reported the similar result for bovine skin gelatin film where amide -I, amide-II and amide-III peaks were found at the wavenumbers of 1633, 1536 and 1240 cm^{-1}, respectively. The band situated at the wavenumber of 1036-1039 cm^{-1} was found in all film samples, corresponding to the interactions arising between plasticizer (OH group of glycerol) and film structure (Hoque *et al*., 2011c). An amide-A band was observed at the wavenumber of 3275–3280 cm^{-1} and amide-B band was found at 2922–2924 cm^{-1} for all film samples. The amide-A band represents the NH-stretching coupled with hydrogen bonding and amide-B band represents the asymmetric stretching vibration of CH as well as NH_3^+ (Muyonga *et al*., 2004).

Amplitude of peaks at wavenumbers 2862 cm^{-1} and 2922 cm^{-1} increased when the films were incorporated with BEO. Those peaks represent the methylene asymmetrical and symmetrical stretching vibration of the aliphatic C–H in CH_2 and CH_3 groups, respectively (Guillen and Cabo, 2004; Muik *et al*., 2007). Both the methylene asymmetrical stretching bands at approximately 2853 cm^{-1} and methylene symmetrical stretching band near 2924 cm^{-1} were obviously present in most lipids (Guillen and Cabo, 2004). The higher amplitude of these peaks was obtained in film incorporated with BEO, indicating the presence of BEO containing

hydrocarbon in film matrix. Similar results for lemon essential oil incorporated gelatin films were reported (Tongnuanchan *et al*., 2012). Furthermore, the peak was observed at wavenumber of 1745 cm^{-1} for film incorporated with BEO, regardless of ZnONP addition. It more likely represented the C=O stretching vibration of aldehyde or ester carbonyl groups (Guillen and Cabo, 2004; Muik *et al*., 2007). Aldehyde, ketone and ester are the main chemical groups in essential oils (Mohamed *et al*., 2010). It was noted that no peak at 1745 cm^{-1} was found in the control film. FPI/FSG films incorporated with BEO had the highest amplitude of peaks at wavenumbers of 2862 cm^{-1} and 2922 cm^{-1}.

The slight shift of FTIR spectra of all films incorporated with 3% ZnONP in the amide-I region to the lower wavenumber was mainly associated with interaction of carbonyl of polypeptide chains with ZnONP. The shifts of the amide-I peak to a lower wavenumber were related to a decrease in the molecular order (Rouhi *et al*., 2013). Furthermore, the amide-A band from the N-H stretching vibration of the hydrogen bonded N-H group became visible at wavenumbers 3,280 and 3,275 cm^{-1} for the control film and 3% ZnONP incorporated film, respectively. Amide-A band of the film incorporated with 3% ZnONP and 100% BEO was found at 3,275 cm^{-1}. This result clearly showed that amide-A band shifted to lower wavenumber when ZnONP was added. The shift to the lower wavenumber in the amide-A region suggested that N-H groups of protein chain interacted with ZnONP, mainly via hydrogen bonding (Nikoo *et al*., 2011). Rouhi *et al*. (2013) reported similar results from gelatin-based nanocomposite films incorporated with ZnO nanorods. Therefore, incorporation of BEO and ZnONP altered the molecular organisation and intermolecular interaction in the film matrix.

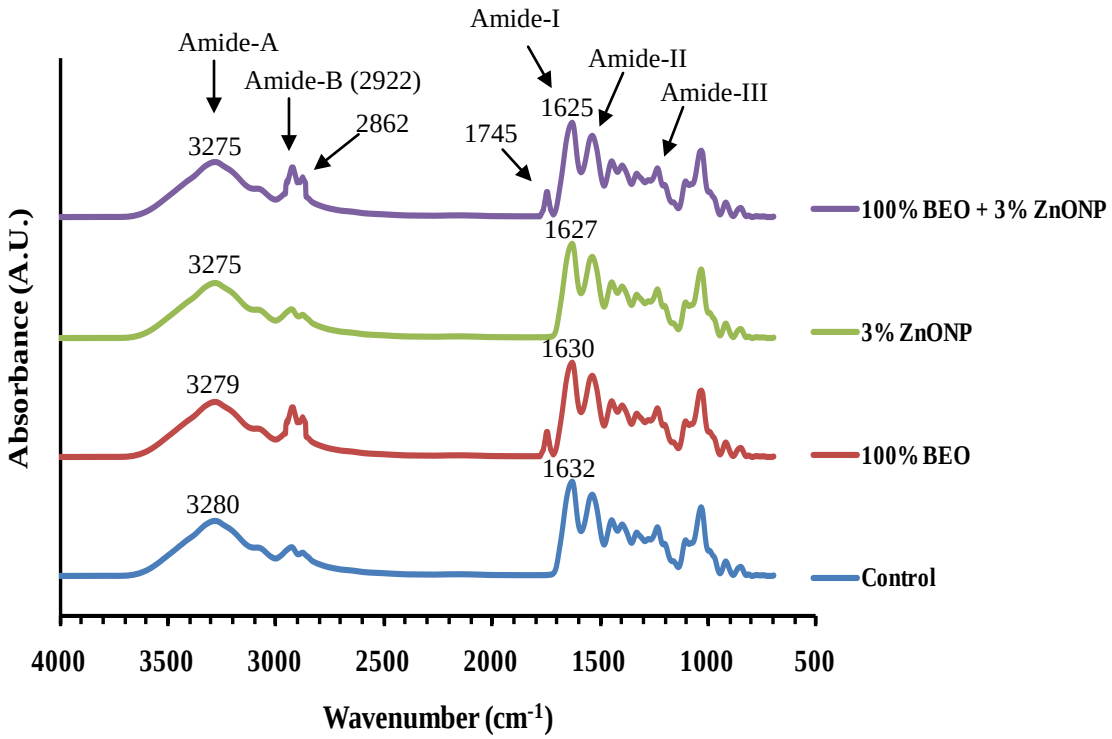


Figure 35. ATR- FTIR spectra of FPI/FSG films as influenced by BEO and ZnONP incorporation.

8.4.2.2 Thermo-gravimetric analysis (TGA)

TGA thermograms revealing thermal degradation behavior of FPI/FSG films as affected by BEO and ZnONP incorporation are shown in Figure 36. Their corresponding degradation temperatures (T_d), weight loss (Δw) and residue are presented in Table 24. Three main stages of weight loss were observed for all films. The first stage weight loss (Δw_1 = 2.53–3.22%) was observed over the temperature (T_{d1}) ranging from 47.88 to 50.86 °C, possibly associated with the loss of free water adsorbed in the film, as well as other volatile compounds absorbed in the film. The similar result was found in cuttlefish skin gelatin film (Hoque *et al.*, 2011c) and porcine plasma protein film added with different cross-linking agents (Nuthong *et al.*, 2009). The second stage weight loss (Δw_2 = 24.39–28.61%) appeared at the onset temperature of 192.47–202.45 °C (T_{d2}), depending on the film samples. This transition revealed the degradation of film matrix. This was most likely due to the

degradation or decomposition of lower molecular weight protein fractions and glycerol compounds (Tongnuanchan *et al*., 2012). For the third stage of weight loss (Δw_3 = 43.76–46.35%), T_{d3} of 293.5–307.34 °C were observed for all films but varied with film samples. This was possibly caused by the loss or decomposition of larger-size or highly interacted proteins and high temperature stable components in film matrix.

It was noted that slightly lower first stage weight loss was found in film incorporated with BEO. Lower T_{d2} and T_{d3} with coincidental higher weight loss were found in films incorporated with BEO, compared with the control films, regardless of ZnONP addition. This result was in agreement with the lowered TS and higher EAB of films incorporated with BEO (Table 21). This might be owing to the plasticizing effect of BEO, which might impede interaction between protein molecules in the film network. A reduction in thermal stability can be promoted by changes in the protein structure and provoked by the rupture of low energy intermolecular bonds which maintain the protein conformation (Kaminska and Sionkowska, 1999). Increase in weight loss on the second and third stages of film incorporated with all essential oils, especially at 100% level, was plausibly associated with the lower protein interaction (Tongnuanchan et al., 2013a). However, the films with 3% ZnONP showed enhanced thermal stability in comparison with the films without ZnONP, irrespective of BEO incorporation, as evidenced by the higher T_{d2} and T_{d3} and lower weight loss for Δw_2 and Δw_3. ZnONP in the protein matrix could insert within film matrix or acted as thermal insulator or mass transport barrier to the volatile products generated during thermal decomposition. This resulted in the improved thermal stability of the composites. Carboxymethylcellulose/ZnO nanocomposite film with a high concentration of ZnONP showed enhanced thermal stability in comparison with control film (Espitia *et al*., 2013).

Additionally, all films had residual mass (representing char content) at 600 °C in the range of 22.01–29.12%. The highest char content observed in nanocomposite film without BEO was most likely ascribed to the high thermal stability of the ZnO nanoparticles and highest bondings formed in the protein network. In general, the lower residue (or char content) from thermal degradation was

found in films incorporated with BEO, compared with control film, regardless of ZnONP addition. Thus, both BEO and ZnONP had the marked impact on thermal stability of FPI/FSG films.

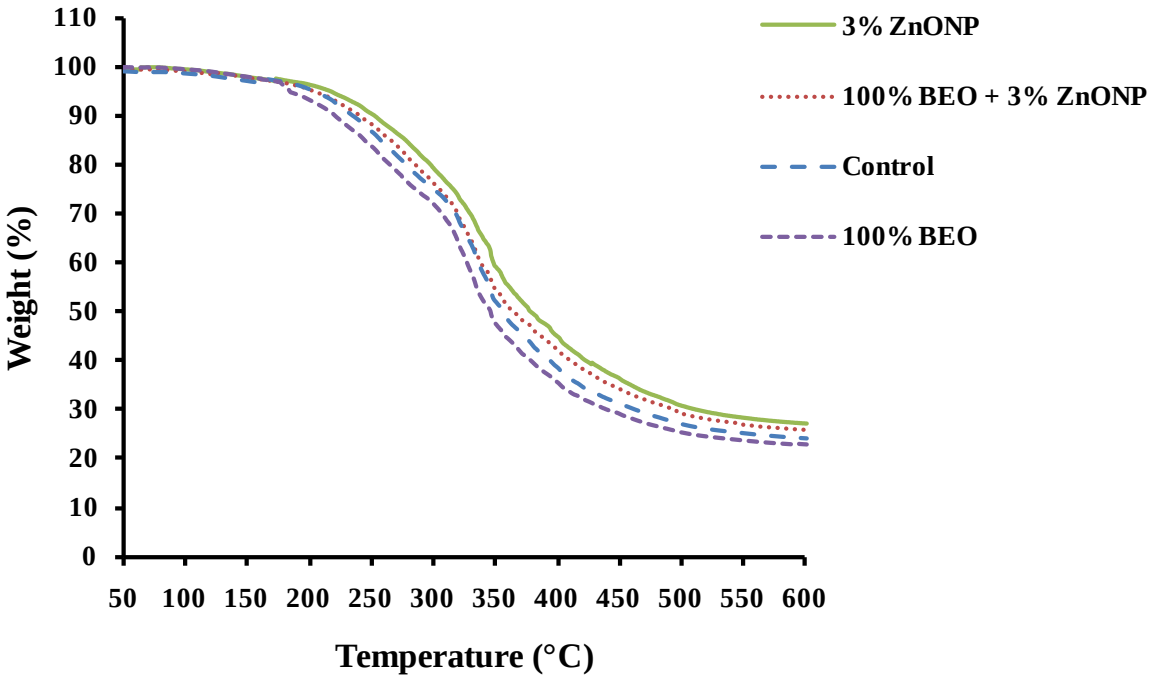


Figure 36. TGA curves of FPI/FSG films as influenced by BEO and ZnONP incorporation.

Table 24. Thermal degradation temperature (T_d, °C) and weight lossΔ(w, %) of FPI/FSG films as affected by 100% BEO and 3% ZnONP incorporation

ZnONP level (w/w)	**Essential oil level**	Δ_1		Δ_2		Δ_3		**Residue (%)**
		T_{d1}**,onset**	Δw_1	T_{d2}**,onset**	Δw_2	T_{d3}**,onset**	Δw_3	
0	0	47.88	3.22	197.04	26.23	301.65	45.72	24.83
	100	48.39	3.03	192.47	28.61	293.50	46.35	22.01
3	0	50.06	2.73	202.45	24.39	307.34	43.76	29.12
	100	50.86	2.53	198.50	26.07	303.93	44.24	27.16

Δ_1, Δ_2, and Δ_3 denote the first, second and third stage weight loss, respectively, of film during heating scan.

8.4.2.3 Film morphology

SEM micrographs of the surface and freeze-fractured cross-section of FPI/FSG films without and with 100% BEO in the absence and presence of 3% ZnONP are illustrated in Figure 37. The slightly rough surface was noticeable in the control FPI/FSG blend film (without BEO and ZnONP). No obvious separation was observed in the matrix of FPI/FSG blend film, indicating the compatibility between FPI and FSG. Roughness of surface structure was more pronounced in films incorporated with BEO and ZnONP than that found in the control film. The increases in the roughness could be due to the distribution of ZnONP as well as BEO droplet throughout the film matrix. This might be also associated with the coexisting of protruded film structure as indicated by the increased thickness of resulting films (Table 21). Furthermore, nanocomposite films showed distinctive ZnONP images on their films surfaces. Those ZnONP were uniformly dispersed in the nanocomposite films, leading to effective force transfer from protein matrix to ZnONP reinforcing phase. This could be associated with the higher TS of FPI/FSG nanocomposite film with 3% ZnONP, compared with other films (Table 21).

FPI/FSG films incorporated with 100% BEO showed the phase separation between oil phase (upper) and protein phase (lower). The higher content of essential oil droplets could enhance creaming and phase separation. BEO with low density at high content might be separated and localized at the upper surface of film, thereby forming the bilayer microstructure (Tongnuanchan *et al*., 2013a). The hydrophobic oil droplet could hinder the water molecules to transfer through the film (Karbowiak *et al*., 2007), thus contributing to lower WVP of BEO incorporated films, compared with the control film, as shown in Table 21. However, no phase separation was observed in FPI/FSG films incorporated with BEO in the presence of ZnONP. Nanoparticles are shown to be highly effective emulsifying agents due to their strong adsorption at oil-water and air-water interface (Larson-Smith and Pozzo, 2012).

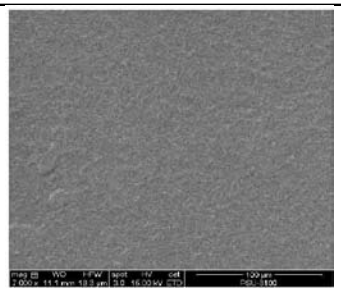
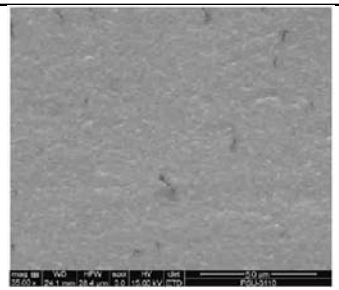
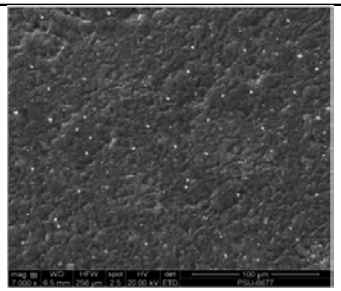
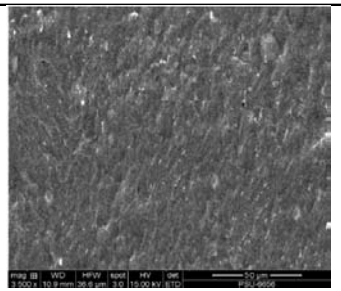
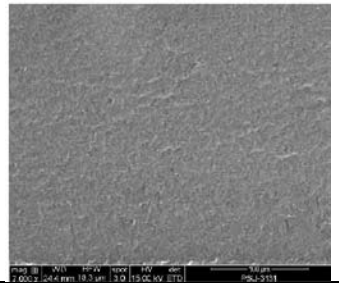
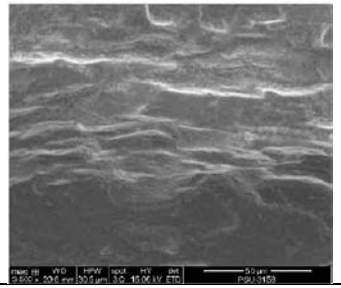
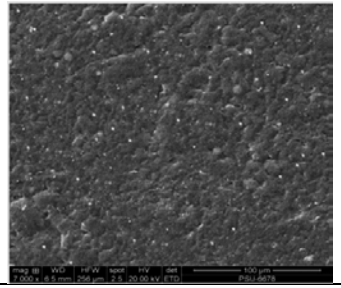
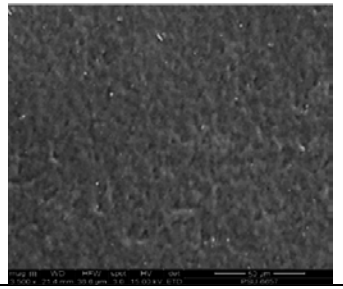
BEO level (% w/w)	0% ZnONP		3% ZnONP	
	Surface	Cross-section	Surface	Cross-section
0				
100				

Figure 37. SEM micrographs of surface and cross-section of FPI/FSG films as influenced by BEO and ZnONP incorporation.

Magnification: 7000× and 3500× for surface and cross-section, respectively.

8.5 Conclusion

The active nanocomposite films based on FPI/FSG blend incorporated with BEO and ZnONP were developed. The developed films showed the improved mechanical and water vapor barrier properties as well as thermal stability, compared with control films (without BEO and ZnONP). Moreover, FPI/FSG films incorporated with BEO greatly inhibited the growth of Gram-positive and Gram-negative food borne pathogenic and spoilage bacteria. Antimicrobial activities of BEO incorporated FPI/FSG films were more pronounced in the presence of ZnONP. Thus, FPI/FSG films incorporated with BEO and ZnONP could be used as an active food packaging to prevent growth of pathogen and spoilage bacteria in foods.

CHAPTER 9

Shelf-life extension of refrigerated sea bass slices wrapped with fish protein isolate/fish skin gelatin-ZnO nanocomposite film incorporated with basil leaf essential oil

9.1 Abstract

Microbiological, chemical and sensory changes of sea bass slices wrapped with composite films based on fish protein isolate (FPI) and fish skin gelatin (FSG) incorporated with 3% ZnO nanoparticles (ZnONP) and 100% basil leaf essential oil (BEO) (w/w, protein content) during storage of 12 days at 4 °C were investigated. Sea bass slices wrapped with FPI/FSG-ZnONP-BEO film had the lowest growth of psychrophilic bacteria, lactic acid bacteria (LAB) and spoilage microorganisms including pseudomonads, H_2S-producing bacteria and Enterobacteriaceae throughout storage of 12 days in comparison with those wrapped with FPI/FSG-BEO, FPI/FSG-ZnONP, FPI/FSG film, polypropylene film (PP film) and the control (without wrapping), respectively ($P < 0.05$). Lowered increases in pH, total volatile base (TVB), peroxide value (PV) and TBARS value were also found in FPI/FSG-ZnO-BEO film wrapped samples, compared with others ($P < 0.05$). Sensory evaluation revealed that the shelf-life of sea bass slices was longest for samples wrapped with FPI/FSG-ZnONP-BEO film (12 days), followed by FPI/FSG-BEO film (10 days) and FPI/FSG-ZnONP film (8 days), as compared to the control (6 days) ($P < 0.05$). Therefore, the incorporation of ZnONP and BEO into FPI/FSG film could enhance the antimicrobial and antioxidative properties of the film, thereby maintaining the quality and extending the shelf-life of the wrapped sea bass slices stored at refrigerated temperature.

9.2 Introduction

Generally fish muscle slices are known for their high nutritional value, due to high contents of protein, phospholipids, polyunsaturated fatty acids (PUFA) and essential minerals (Simopoulos, 2002). Ready-to-cook fish have become increasingly popular. Due to the rapid microbial growth and lipid oxidation, such perishable products have the limited shelf-life (Masniyom *et al*., 2002). With increasing demand for high quality ready-to-cook fish with the extended shelf-life, several innovative techniques for maintaining quality and safety of products have been developed (Maftoonazad and Badii, 2009). To satisfy these requirements, the uses of conventional chemical additives in food formulation have been decreased. The alternative use of novel natural additives has gained the increasing attention (Sánchez-González *et al*., 2011).

Biopolymer films are excellent vehicles for incorporating a wide variety of additives, such as antimicrobials, antioxidants, antifungal agents, colors, and other nutrients (Rhim and Ng, 2007). Active biopolymer packaging systems, based on the incorporation of antimicrobial or antioxidative substances in biodegradable food packaging materials could delay microbial spoilage of food, control undesirable changes and extend shelf-life of food products (Emiroglu *et al*., 2010). The uses of essential oils (EOs) as sources of antimicrobials and antioxidants have long been acknowledged and the biopolymer packaging industry has recently paid more attention to their applications as natural antimicrobials and antioxidants for smart packaging (Holley and Patel, 2005; Sánchez-González *et al*., 2011). Chitosan coating incorporated with cinnamon essential oil was reported to extend the shelf-life of rainbow trout during storage at 4 °C for 16 days, mainly by retarding the microbial growth and lowering the lipid oxidation (Ojagh *et al*., 2010). *In-vitro* studies have revealed significant antimicrobial and antioxidant effects of essential oil from basil leaf, which are abundant and have low market value with great potential for use in food preservation (Hussain *et al*., 2008; Suppakul *et al*., 2003). Additionally, essential oils are hydrophobic in nature and the incorporation of essential oils could improve the water vapor barrier property and impart flexibility of protein films (Tongnuanchan *et al*., 2013b). The use of essential oil exhibited the enhanced antimicrobial activity,

when combined together with various nanoparticles (Allahverdiyev *et al*., 2011). ZnONP is currently listed as a generally recognized as safe (GRAS) material by the Food and Drug Administration (21CFR182.8991) and has previously shown strong antimicrobial activity against food borne pathogens and spoilage bacteria (Espitia *et al*., 2013; Zhang *et al*., 2010).

Sea bass (*Lates calcalifer*) is very popular in tropical areas of South-East Asia (Masniyom *et al*., 2002). It is commonly sold as whole fish or as fillets. However, microbial spoilage and lipid oxidation shorten the shelf-life of sea bass slices (Masniyom *et al*., 2002). Recently, FPI/FSG films have been prepared and showed the improved mechanical and water vapor barrier properties, compared with films prepared from single material (Arfat *et al*., 2014). To widen the application as the active packaging, the incorporation with ZnONP and BEO having antimicrobial and antioxidative activities could be a promising means. Nevertheless, there is no information on the use of FPI/FSG film containing ZnONP and essential oil for the shelf-life extension of fish slices. Therefore, the objective of this investigation was to study the impact of FPI/FSG-ZnO nanocomposite film incorporated with BEO on the shelf-life extension of sea bass slices stored at 4 °C.

9.3 Materials and methods

9.3.1 Chemicals

Zinc oxide nanoparticles (ZnONP) (particle size: 20-40 nm, specific surface area: 26.22 m^2/g) were purchased from Nano materials technology Co. Ltd. (Bangkok, Thailand). Commercial fish skin gelatin (FSG) from tilapia (~240 bloom) was obtained from Lapi Gelatine S.p.A (Empoli, Italy). Basil (*Ocimum basilicum*) leaf essential oil (BEO) was purchased from Botanicessence essential oils (Suanlung, BKK, Thailand). Ammonium thiocyanate, ferrous chloride and 2-thiobarbituric acid (TBA) were obtained from Fluka Chemical Co. (Buchs, Switzerland). Chloroform and methanol were procured from Lab-Scan (Bangkok, Thailand). Trichloroacetic acid (TCA), hydrochloric acid, sodium chloride, MRS culture media, standard plate count agar, triple sugar iron agar (IA), and eosin methylene blue agar (EMB) were

purchased from Merck (Darmstadt, Germany). Pseudomonas isolation agar was purchased from Difco (Detroit, Michigan, USA). All chemicals were of analytical grade.

9.3.2 Collection and preparation of fish sample

Fresh yellow stripe trevally (*Selaroides leptolepis*) with an average weight of 90-100 g/fish were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were kept in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon the arrival, fish were immediately washed, filleted, and minced to uniformity using a Model HC 5000 mincer (Microfluidics, Massachusetts, USA) with a hole diameter of 0.5 cm. Fresh sea bass (*Lates calcarifer*) were purchased from a local market in Hat Yai, Songkhla, Thailand and transported in ice with fish/ice ratio of 1:2 (w/w) to the laboratory within 30 min. Sea bass were filleted and sliced to a thickness of 1.5 cm.

9.3.3 Preparation of fish protein isolate

Prior to the isolation of fish protein, the prepared yellow stripe trevally mince was subjected to washing as per the method of Toyohara *et al*. (1990) with slight modifications. Fish mince was homogenized with 5 volumes of cold 0.05 M NaCl (2-4 °C) at a speed of 13,000 rpm for 2 min, using an IKA Labortechnik homogenizer (Selangor, Malaysia). The washed mince was filtered through two layers of cheese-cloth. The washing process was repeated twice. Washed mince obtained was stored on ice until used. Washed mince was added with cold distilled water at the ratio of 1:9 (w/v), followed by homogenization for 1 min at a speed of 13,000 rpm. The pH of homogenate was then adjusted to 11 using 2 M NaOH. The resulting mixture was centrifuged at 10,000×g for 20 min at 4 °C using a refrigerated centrifuge (Avanti-JE Centrifuge, Beckman 163 Coulter Inc., Fullerton, CA, USA). The supernatant was collected and the pH was adjusted to 5.5 using 2 M HCl. The precipitate was then filtered through 4 layers of cheese-cloth. The retentate was dewatered by centrifugation at 12,000×g for 20 min at 4 °C. The final pH of the

sample was adjusted to pH 7.0 using 2 M NaOH. The sample was referred to as “fish protein isolate; FPI”. FPI was used for film preparation.

9.3.4 Preparation of fish protein isolate/fish skin gelatin film containing ZnONP and BEO

Firstly, film-forming solution was prepared according to the method of Chinabhark *et al*. (2007). FPI was added with 3 volumes of distilled water and homogenized at 13,000 rpm for 1 min using a homogenizer. Subsequently, the pH of the mixture was adjusted to 3 using 1 N HCl to solubilize the protein. The obtained solution was filtered through 2 layers of cheese-cloth to remove undissolved debris. The protein concentration of the filtrate determined by the Kjeldahl method (AOAC, 2000) was adjusted to 3 % (w/v). Glycerol at 30% (w/w) of protein was used as a plasticizer. The mixtures were stirred gently for 30 min at room temperature and was used for preparing blend FFS.

Prior to blending, FSG powder was dissolved in distilled water to obtain the protein concentration of 3% (w/v). The pH of the mixture was adjusted to 3 using 1 N HCl. The solution was heated at 70 °C for 30 min and cooled at room temperature (28-30 °C) for 20 min. Glycerol at concentrations of 30 % (w/w) of protein content was used as a plasticizer. Thereafter, both FPI and FSG solutions were mixed at a ratio of 1:1 (v/v). The obtained solution was added without and with 3% ZnONP (w/w, protein content) in droplets. Before addition of ZnONP, ZnONP was suspended in distilled water and homogenized for 1 min at 5,000 rpm. The obtained FPI/FSG/ZnONP suspension was stirred for 5 min and then homogenized for 30 sec at the speed of 5,000 rpm.

BEO previously mixed with Tween 20 at 25% (w/w, based on essential oil) was added to FPI/FSG solution or FPI/FSG/ZnONP suspension at 100% (w/w, protein content). Final volume was made up to 100 ml using distilled water previously adjusted to pH 3. To obtain the uniform distribution of BEO in suspensions, the mixtures were homogenized with three passes using a high pressure homogenizer (Microfluidizer M-110EH, Microfluidics Corp., Newton, MA, USA) with an

operating pressure of 1,500 bars. Suspensions were gently stirred for 30 min at room temperature and were referred to as a film-forming suspension (FFS).

Prior to casting, all FFS samples were degassed for 10 min using the sonicating bath (Elmasonic S 30 H, Singen, Germany). To prepare the film, 4 g of FFS was cast onto a rimmed silicone resin plate (5×5 cm^2), air-blown for 12 h at 25 °C, followed by drying in an environmental chamber (Binder GmbH, Tuttlingen, Germany) at 25 ± 0.5 °C and 50 ± 5% relative humidity (RH) for 24 h. Dried film samples were manually peeled off and subjected to analyses.

9.3.5 Effect of FPI/FSG-ZnO nanocomposite film incorporated with BEO on quality changes of sea bass slices

Fish slices (4×4 cm^2) were wrapped with films (5×5 cm^2), in which all sides were completely covered. Films used included PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film. Subsequently, the samples were placed in polystyrene trays (9×7 cm^2), wrapped with an extensible polypropylene film (Thickness # 11 μm, MMP Corporation Ltd., Bangkok, Thailand) prior to storage at 4 °C for 12 days. Control samples were prepared in the same manner except that the slices were not covered with any film. During storage, the samples were randomly taken every 2 days for analyses.

9.3.6 Microbiological analyses

Microbiological analyses were performed by the spread plate method (Sallam, 2007). Samples were collected aseptically and used as the composite sample. The sample (25 g) was placed in a Stomacher bag containing 225 ml of 0.85% saline solution. After mixing for 1 min in a Stomacher blender (Stomacher M400, Seward Ltd., Worthington, England), further serial dilutions were made from the homogenate using 0.85% saline solution as the diluent. Appropriate dilutions were used for microbiological analyses.

9.3.6.1 Total viable count and psychrophilic bacterial count

Total viable count and psychrophilic bacterial count were determined by spreading 0.1 ml of an appropriately diluted homogenate on plate count agar (HiMedia, Mumbai, India) containing 1% NaCl, followed by incubation at 35 °C for 3 days and 4 °C for 10 days, respectively (Sallam, 2007).

9.3.6.2 Pseudomonas count

Pseudomonas count was determined by spreading 0.1 ml of an appropriately diluted homogenate on Pseudomonas isolation agar (Difco, Detroit, MI, USA). Then the plates were incubated at 25 °C for 48 h (Escudero-Gilete *et al*., 2014).

9.3.6.3 Hydrogen sulphide (H_2S) producing bacterial count

H_2S-producing bacteria were grown on triple sugar iron agar (HiMedia, Mumbai, India) by the spread plate method using 0.1 ml of an appropriately diluted homogenate. Plates were incubated at 25 °C for 3 days. Black colonies, due to the precipitation of ferrous sulphide on this medium, were counted (Sallam, 2007).

9.3.6.4 Enterobacteriaceae count

For the determination of enterobacterial count, 0.1 ml of an appropriately diluted homogenate was spread on Eosin methylene blue agar (EMB) (HiMedia, Mumbai, India) and incubated at 37 °C for 24 h (Ahmad *et al*., 2012b).

9.3.6.5 Lactic acid bacteria count

Lactic acid bacterial count was determined by spreading 0.1 ml of an appropriately diluted homogenate on double-layered plates of deMann-Rogosa Sharpe medium (MRS) Agar (HiMedia, Mumbai, India). Then the plates were incubated at 37 °C for 72 h (Asahara *et al*., 2001).

9.3.7 Chemical analyses

9.3.7.1 Determination of total volatile base (TVB) content

TVB content was determined following the method of Conway and Byrne (1936). Sample (5 g) was homogenized with 4% of trichloroacetic acid at a ratio of 1: 2 (w/v). The homogenate was filtered through Whatman No. 1 paper (Schleicher & Schuell, Maidstone, England). The filtrate (1 ml) was placed in the outer ring. The inner ring solution (1% boric acid containing the Conway indicator) was pipetted into the inner ring. To initiate the reaction, K_2CO_3 (1 ml) was mixed with the filtrate. The Conway unit was closed and incubated at 37 °C for 60 min. The inner ring solution was then titrated with 0.02 N HCl until the green color turned to pink.

9.3.7.2 pH measurement

pH measurement was performed by the method described by Lopez-Caballero *et al*. (2007) with a slight modification. Sample (2 g) was homogenized with 10 volumes of deionized water for 1 min. The homogenate was kept at room temperature for 5 min. The pH was determined using a pH-meter (Sartorious North America, Edgewood, NY, USA).

9.3.7.3 Determination of peroxide value (PV)

PV was determined according to the method of Richards and Hultin (2002) with a slight modification. Ground sample (1 g) was homogenized at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, v/v). The homogenate was then filtered using a Whatman No.1 filter paper. Two milliliters of 0.5% NaCl was then added to 7 ml of the filtrate. The mixture was vortexed at a moderate speed for 30 s and then centrifuged at 3,000×*g* for 3 min at 4 °C using a refrigerated centrifuge to separate the sample into two phases. The lower phase (3 ml) was carefully pipetted out and 2 ml of cold chloroform: methanol (2:1) mixture was added. Then 25 µl of 30% (w/v) ammonium thiocyanate and 25 µl of 20 mM iron (II) chloride were added to the mixture. The reaction mixture was allowed to stand for

20 min at room temperature prior to reading the absorbance at 500 nm. The blank was prepared in the same manner, except deionized water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0.5 to 2 mg/l. PV was expressed as mg cumene hydroperoxide/kg sample.

9.3.7.4 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). The ground sample (0.5 g) was homogenized with 2.5 ml of solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was heated in boiling water bath (95-100 °C) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600×g at 25 °C for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane at the concentrations ranging from 0 to 6 ppm. TBARS were calculated and expressed as mg malonaldehyde/kg sample.

9.3.8 Sensory evaluation

Sensory evaluation of sea bass slices was undertaken by a panel consisting of 8 trained panellists, who were graduate students in the Food Science and Technology programme, aged 25-30 years and familiar with fish consumption. Prior to the evaluation, the panellists were trained for three sessions with 1 h each to enable panellists to discuss and assess each attribute. Slices placed on white tray were presented to panellists. The sensory evaluation was based on a five-point-scale to determine: texture (5, firm; 1, very soft); color (5, no discoloration; 1, extreme discoloration); odor (5, extremely desirable; 1, extremely unacceptable/off-odors); and overall quality (5, extremely desirable; 1, extremely unacceptable) of the samples. For shelf-life prediction, the rejection was claimed when the sensory score was below 4.0 (Ojagh *et al*., 2010).

9.3.9 Statistical analyses

Experiments were performed in triplicate (n=3) and a completely randomized design (CRD) was used. Data were presented as means ± standard deviation and $P < 0.05$ was considered significant. Analysis of variance (ANOVA) was performed and the mean comparisons were done by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows, SPSS Inc., Chicago, IL, USA).

9.4 Results and discussion

9.4.1 Changes in microbial loads

Total viable count (TVC) of sea bass slices wrapped without and with films (PP, FPI/FSG, FPI/FSG-ZnONP, FPI/FSG-BEO and FPI/FSG-ZnONP-BEO) during storage at 4 °C for 12 days is shown in Figure 38A. TVC of all samples at day 0 was around 3.7 log CFU/g, indicating the presence of some microorganisms, probably contaminated during slice preparation, packaging, etc. TVC of the control sample (without wrapping) increased during the first 4 days of storage, with an approximate value of 6.8 log CFU/g at day 4 and this load became higher and reached 8.9 log CFU/g at day 12. The control had the higher increase in TVC, compared with others, throughout the storage of 12 days ($P < 0.05$). For PP and FPI/FSG film wrapped samples, slightly lower TVC was observed, compared with that of control samples during refrigerated storage ($P < 0.05$). For FPI/FSG-ZnONP, FPI/FSG-BEO and FPI/FSG-ZnONP-BEO-film wrapped samples, TVC increased gradually and reached the value of 7.3, 6.6 and 5.9 log CFU/g at day 12, respectively. Thus, FPI/FSG-ZnONP, FPI/FSG-BEO and FPI/FSG-ZnONP-BEO films were able to retard the microbial growth of sea bass slices. Amongst all films, FPI/FSG-ZnONP-BEO had the highest inhibitory effect on microbial growth. The delay in microbial spoilage of sea bass slices was plausibly due to the combined antimicrobial effect of BEO and ZnONP distributed throughout the films matrix. After 12 days of storage, FPI/FSG-ZnONP-BEO film wrapped samples had the lower TVC by approximately 3 log CFU/g, as compared to the control samples ($P < 0.05$). Generally, the total

number of microorganisms varies enormously and ranges from 10^2 to 10^7 CFU/cm^2 in fish (Liston, 1980). Antimicrobial activity of ZnONP and the mechanism of inhibition against the microorganisms have been demonstrated (Espitia *et al*., 2013; Zhang *et al*., 2010). The release of Zn^{2+} ions from the powder could penetrate through the cell wall of microorganism and react with interior components that finally affect the viability of cells. ZnONP has been known to mediate the generation of hydrogen peroxide (H_2O_2), a powerful oxidising agent causing damage to the cell membrane of bacteria (Tayel *et al*., 2011). Emamifar *et al*. (2010) confirmed that active packaging based on low-density polyethylene (LDPE) containing ZnONP prolonged the shelf-life of fresh orange juice up to 28 days by reducing the rate of microbial growth. Microbial inhibition by film containing BEO could be attributed to its hydrophobic nature. BEO and its main components, especially linalool, an oxygenated monoterpenoid, could penetrate through the cell wall of a bacterium and attack on the phospholipid bilayer present in cell membranes (Hussain *et al*., 2008). This causes the increased permeability and leakage of cytoplasm, or in their interaction with enzymes located on the cell wall (Emiroglu *et al*., 2010). Linalool has the potential to act as either a protein denaturing agent or as a solvent dehydrating agent, contributing to its antimicrobial activity (Emiroglu *et al*., 2010). Films incorporated with essential oils could retard the microbial growth in rainbow trout (Mexis *et al*., 2009) and bologna slices (Zivanovic *et al*., 2005).

The increases in psychrophilic bacterial count in all samples were noticeable with increasing storage time up to day 12 ($P < 0.05$) (Figure 38B). During the storage, the lowest psychrophilic bacterial count was obtained in the samples wrapped with FPI/FSG-ZnO-BEO film, followed by those wrapped with FPI/FSG-BEO film, FPI/FSG-ZnONP film, FPI/FSG film and PP film, respectively. The control sample showed the highest psychrophilic bacterial count during the storage ($P < 0.05$). At the end of storage (day 12), psychrophilic bacterial count of the control, those wrapped with PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film, and FPI/FSG-ZnONP-BEO film was 7.7, 7.2, 6.8, 5.5, 5.2 and 4.7 log CFU/g, respectively. The result indicated the combined antimicrobial activity of ZnONP and

BEO toward psychrophilic bacteria in sea bass slices during storage at 4 °C for 12 days.

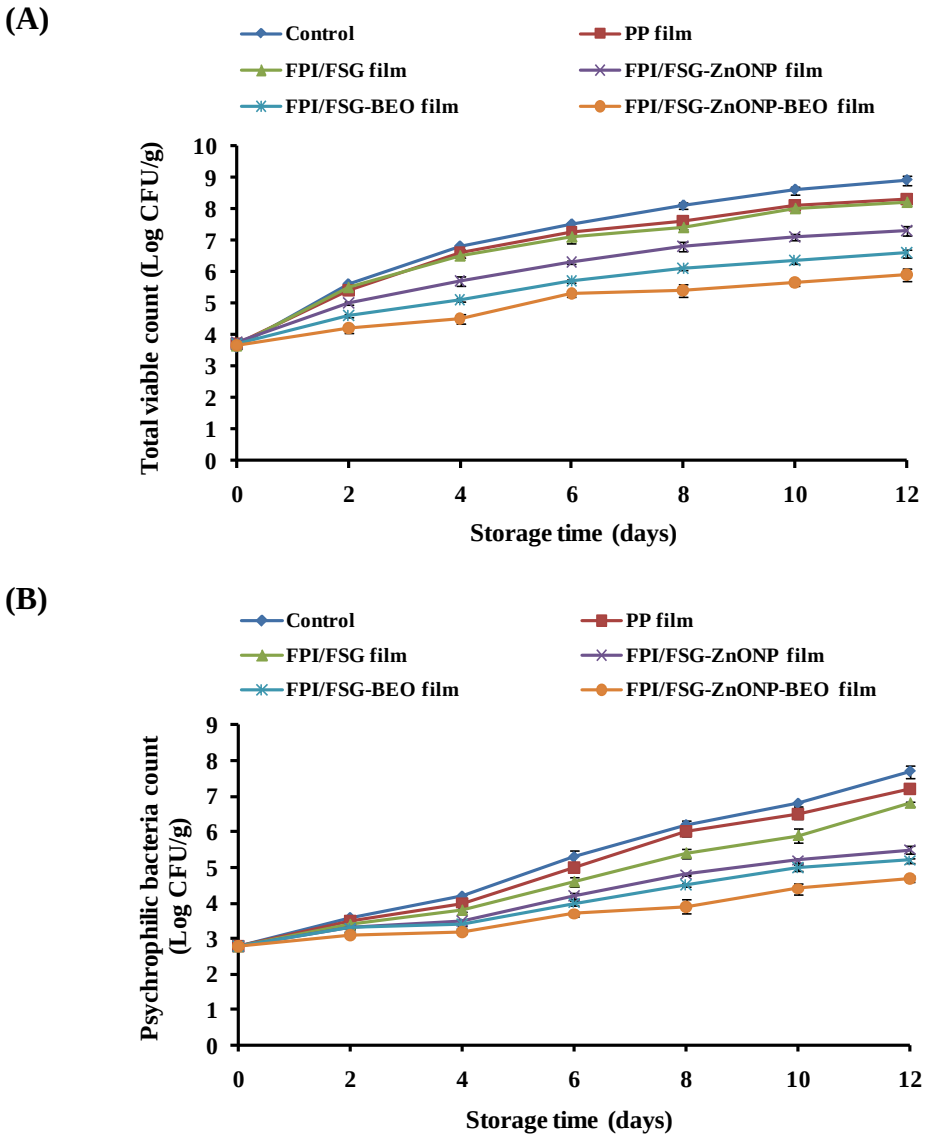


Figure 38. Total viable bacterial count (A) and psychrophilic bacterial count (B) of sea bass slices wrapped without and with films (PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film) during storage at 4 °C for 12 days. Control: unwrapped samples, PP film: samples wrapped with Polypropylene film, FPI/FSG film: samples wrapped with fish protein isolate/fish skin gelatin film, FPI/FSG-ZnONP film: samples wrapped with fish protein isolate/fish skin gelatin film incorporated with zinc oxide nanoparticles, FPI/FSG-BEO film: samples wrapped with fish protein isolate/fish skin gelatin film incorporated with basil leaf essential oil, FPI/FSG-ZnONP-BEO film: samples wrapped with fish protein isolate/fish skin gelatin film incorporated with zinc oxide nanoparticles and basil leaf essential oil. Bars represent the standard deviation (n=3).

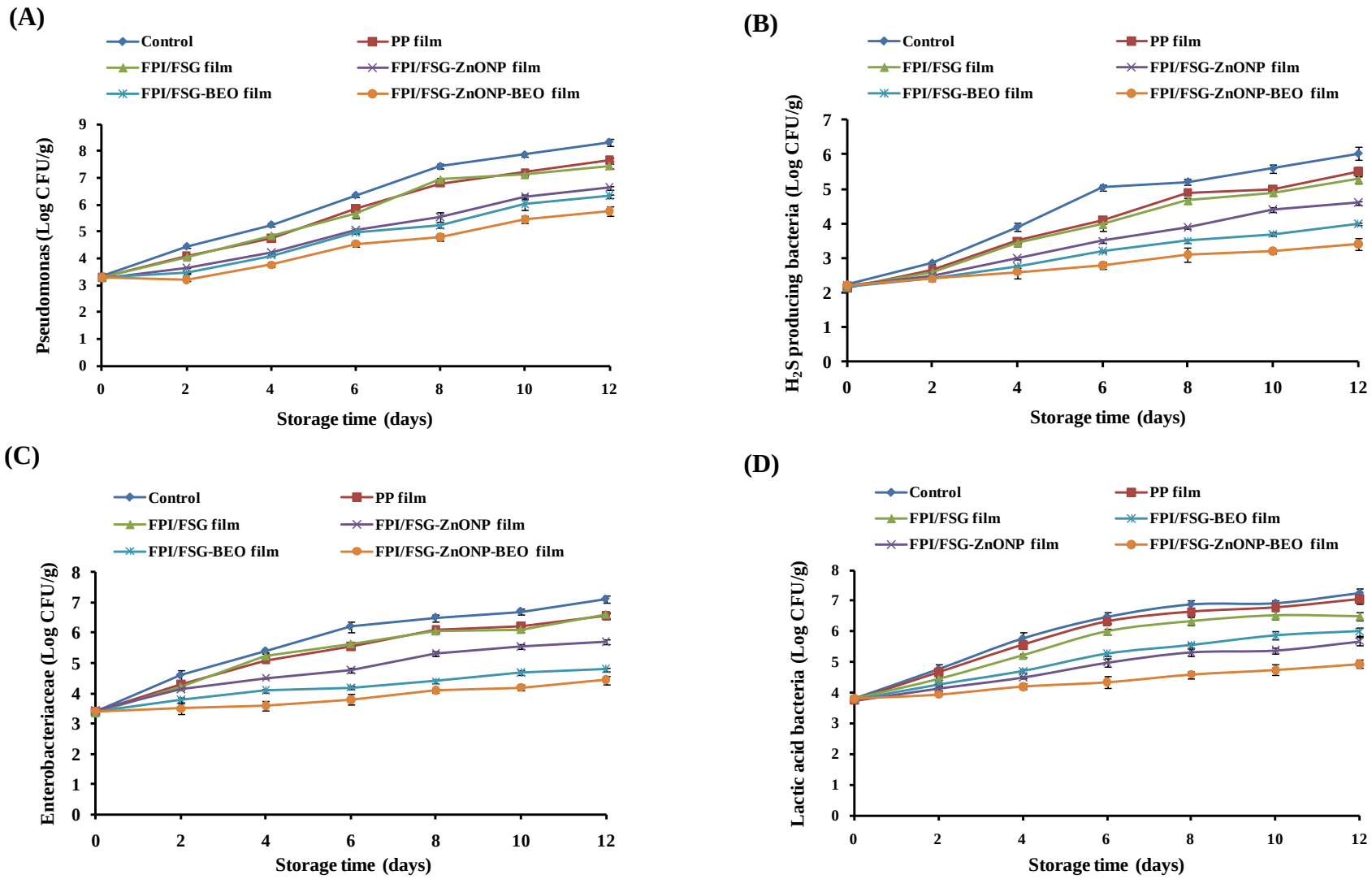


Figure 39. Pseudomonas (A), H_2S-producing bacteria (B), Enterobacteriaceae (C) and Lactic acid bacteria (D) counts of sea bass slices wrapped without and with films (PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film) during storage at 4 °C for 12 days. Key: see the caption for Figure 38. Bars represent the standard deviation (n=3).

Pseudomonads (Figure 39A) in sea bass slices increased over the entire storage period. Pseudomonads are generally recognized to dominate in the meat system and contribute to spoilage. This can be attributed to their ability to degrade glucose and amino acids even under refrigeration conditions (Ercolini *et al*., 2006). Pseudomonas count of all samples at day 0 was around 3.3 log CFU/g. Pseudomonas of the control sample increased during the storage, with an approximate value of 8.3 log CFU/g at day 12 ($P < 0.05$). FPI/FSG-ZnONP, FPI/FSG-BEO and FPI/FSG-ZnONP-BEO films had a profound effect on the inhibition of Pseudomonas growth. For FPI/FSG-ZnONP, FPI/FSG-BEO and FPI/FSG-ZnONP-BEO film wrapped samples, pseudomonas increased gradually ($P < 0.05$) and reached the value of 6.6, 6.3 and 5.8 log CFU/g at day 12, respectively. Amongst all samples, those wrapped with FPI/FSG-ZnONP-BEO film had the lowest pseudomonas count. The sample showed the lower count by 2.6 log CFU/g, as compared to the control samples, after 12 days of storage. This was due to the combined antimicrobial effect of ZnONP and BEO distributed throughout the films matrix. Both ZnONP and BEO showed the strong antimicrobial activity against Pseudomonads (Suppakul *et al*., 2003). ZnONP could potentially be used as an effective antibacterial agent to protect food related bacteria including Pseudomonads (Tayel *et al*., 2011). As shown in Figure 39A, PP and FPI/FSG films also inhibited the growth of Pseudomonads to some extent. Pseudomonads are strictly aerobic microorganisms and are unable to survive in the limited oxygen or absence of oxygen.

Changes in H_2S-producing bacterial count of sea bass slices during 12 days of refrigerated storage are illustrated in Figure 39B. Counts of H_2S-producing bacteria, including *Shewanella putrefaciens* have been used as spoilage indicators of seafood products (Stamatis and Arkoudelos, 2007). *S. putrefaciens* produces very intense and unpleasant off-odors associated with H_2S formation and reduces TMAO to TMA (Sivertsvik *et al*., 2002). The lowest H_2S-producing bacterial count was found in samples wrapped with FPI/FSG-ZnONP-BEO film as compared to others during 12 days of storage ($P < 0.05$). Therefore, ZnONP and BEO incorporated into FPI/FSG film were shown to strongly retard the growth of spoilage bacteria, which were able to produce H_2S. At the end of storage, the control, those wrapped with PP

film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film had H_2S-producing bacterial counts of 6.02, 5.5, 5.2, 4.6, 4.0 and 3.4 log CFU/g, respectively. Specific spoilage organisms such as H_2S-producing bacteria are mostly predominant during the spoilage of fish and fish products, producing very intense and unpleasant off-odors and rejection (Sivertsvik *et al*., 2002). Tayel *et al*. (2011) reported that nanosized ZnO suspensions are active in inhibiting the growth of H_2S-producing bacteria. Oregano essential oil inhibited growth of H_2S producing bacteria in rainbow trout stored at 4 °C (Mexis *et al*., 2009). For the sea bass slices, gelatin film and gelatin film incorporated with lemon grass essential oil inhibited the growth of H_2S-producing bacteria during 12 days of refrigerated storage and had bacterial counts of 3.6 and 2.5 log CFU/g, respectively, as compared to control (4.2 log CFU/g). (Ahmad *et al*., 2012b). Inhibition of H_2S producing bacteria growth by PP film and FPI/FSG film was also observed. It was suggested that H_2S-producing bacteria might proliferate at lower level when the surface of the sample had the lower oxygen level (Ahmad *et al*., 2012b).

With respect to Enterobacteriaceae (Figure 39C), considered as a hygiene indicator (Zeitoun *et al*., 1994), the initial count at day 0 was 3.4 log CFU/g, but reached 7.1 log CFU/g in the control samples at day 12. PP film and FPI/FSG film showed a significant impact on the inhibition of Enterobacteriaceae and the counts of 6.5 and 6.6 log CFU/g were obtained at the end of storage (day 12), for samples wrapped with PP and FPI/FSG films, respectively. Aerobic microorganisms might have the retarded growth when the surface of slice samples was covered by PP and FPI/FSG film. PP and protein based film were reported to have the excellent oxygen barrier property (Tihminlioglu *et al*., 2010; Chiou *et al*., 2008). Nevertheless, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film wrapped samples had the lower counts by 1.4, 2.3 and 2.6 log CFU/g, as compared to the control, at day 12 of storage. The lowest count observed in FPI/FSG-ZnONP-BEO film wrapped samples was more likely associated with inhibitory action of ZnONP and BEO against the spoilage bacteria ($P < 0.05$). The combined use of ZnONP and BEO was therefore able to inhibit the growth of Enterobacteriaceae effectively during the storage as their population remained below 5 log CFU/g. Tassou *et al*. (1995)

reported that treatment of fresh sea bream fillets with a mixture of olive oil, lemon and essential oil (oregano) reduced the final Enterobacteriaceae counts by approximately 2.5 log CFU/g, compared to the control.

Lactic acid bacteria (LAB) (Figure 39D) are facultative anaerobic bacteria that can grow under both anaerobic and aerobic conditions and constitute a substantial part of the natural microflora of stored food products under anaerobic conditions (Mastromatteo *et al*., 2009). The initial LAB count was 3.8 log CFU/g in sea bass slices at day 0. LAB count gradually increased when storage time increased ($P < 0.05$). LAB increased to the levels of 7.2, 7.0, 6.5, 5.7, 6.0 and 4.9 log CFU/g at the end of storage (day 12) for the control, PP, FPI/FSG, FPI/FSG-ZnONP, FPI/FSG-BEO and FPI/FSG-ZnONP-BEO film wrapped samples, respectively (Figure 39D). The use of the BEO was slightly less effective than ZnONP. The limited action of BEO as compared to ZnONP was possibly attributed to the high tolerance of LAB against the action of essential oils (Holley and Patel, 2005). Although LAB is the most resistant among the Gram-positive bacteria towards the antimicrobial action of essential oils, thyme oil along with modified atmosphere packaging (MAP) was able to retard their growth (Kostaki *et al*., 2009). The combined use of ZnONP and BEO was the most effective for retardation of LAB growth during the storage as their population remained below 5 log CFU/g at the end of refrigerated storage. LDPE nanocomposite packaging containing Ag and ZnO caused the reduction of *Lactobacillus plantarum* growth in orange juice (Emamifar *et al*., 2011). Ahmad *et al*. (2012b) reported a reduction of 1.4 log CFU/g in LAB populations in the sea bass slices wrapped with gelatin film incorporated with lemon grass essential oil. Chouliara *et al*. (2007) also found a reduction of 1.1 log CFU/g in LAB for chopped chicken meat after 6 days of storage with the addition of 0.1% oregano essential oil. It was noted that FPI/FSG film also showed the slight antimicrobial activity. This was possibly owing to the antimicrobial peptides present in fish protein isolate and fish skin gelatin. Gómez-Guillén *et al*. (2010) reported that gelatin peptides showed antimicrobial activity toward several microorganisms.

9.4.2 Changes in chemical compositions

9.4.2.1 Total volatile base content

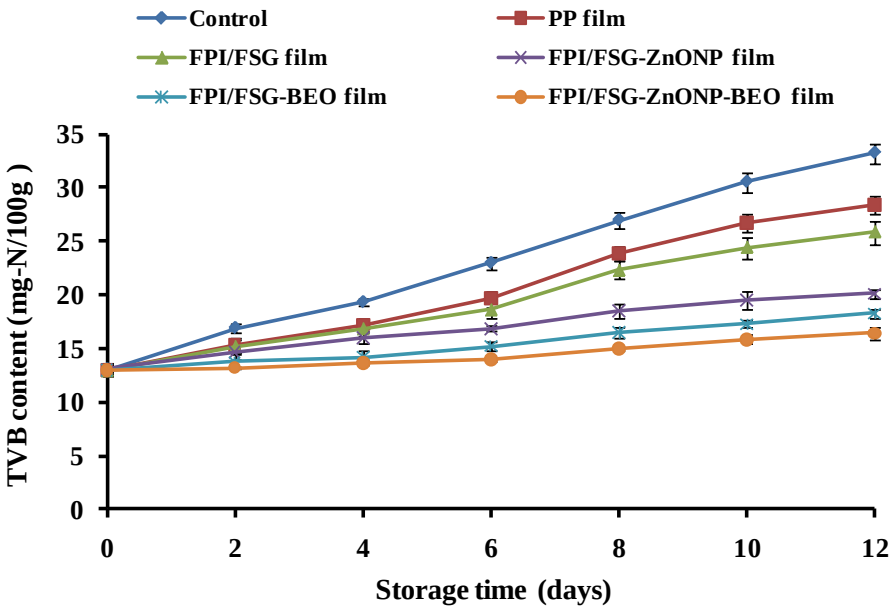
TVB content of sea bass slices wrapped without and with films (PP, FPI/FSG, FPI/FSG-ZnONP, FPI/FSG-BEO and FPI/FSG-ZnONP-BEO) during storage at 4 °C for 12 days is shown in Figure 40A. TVB content of all samples at day 0 was approximately 13 mg N/100 g, indicating the good quality of the fresh samples. As the storage time increased, a continuous increase in TVB content was observed in all samples ($P < 0.05$), but the increasing rate varied amongst samples wrapped with different films. A rapid increase in TVB content was noticed in the control, which reached a value of 33.24 mg N/100 g muscle at day 12. However, the gradual increase in TVB content was observed throughout the storage of 12 days for PP, FPI/FSG, FPI/FSG-ZnONP, FPI/FSG-BEO and FPI/FSG-ZnONP-BEO wrapped samples, reaching a value of 28.45, 25.87, 20.12, 18.27 and 16.44 mg N/100 g at day 12, respectively. Since TVB is produced mainly by bacterial decomposition of fish flesh, the higher values of total viable counts in the control indicated the higher spoilage. The lower TVB values of wrapped samples, especially with FPI/FSG-ZnONP-BEO film, could be attributed to either more rapid reduction at bacterial population or decreased capacity of bacteria for oxidative de-amination of non-protein nitrogen compounds or both (Fan *et al*., 2008). Silver carp fillets coated with chitosan biopolymer incorporated with nanoclay had the lower TVB-N content as compared to the control (chitosan only) during 12 days of storage (Abdollahi *et al*., 2013). Harpaz *et al*. (2003) found that addition of oregano or thyme oil (0.05%, v/v) in sea bass maintained the TVB values below the limit (30 mg N/100 g) up to 35 days of storage at 0 to 2 °C. At the end of the storage period (12 days), TVB contents of sea bass slices wrapped with gelatin film and gelatin film incorporated with lemongrass essential oil were 18.34 and 15.84 mg N/100 g, respectively (Ahmad *et al*., 2012b). TVB value in freshly caught fish is typically between 5 and 20 mg N/100 g, and TVB value of 30-35 mg N/100 g has been established as an upper acceptability limit for fresh fish by the European Commision (Commission Decision 95/149/EC, 1995). TVB represents the sum of ammonia, dimethylamine (DMA), trimethylamine (TMA) and other basic volatile nitrogenous compounds. DMA and TMA were the

degradation products of trimethylamine oxide (TMAO), a typical compound, which has an important role in osmoregulation. DMA is mostly produced by endogenous enzymes and TMA is generated by bacterial enzymes (Zeisel *et al*., 1985). TVB content was in accordance with microbial load (Figure 38A) in the corresponding samples. Thus, the combined inhibitory effect of ZnONP and BEO in FPI/FSG-ZnONP-BEO films against microbial growth could retard or lower the production of microbial degradation products.

9.4.2.2 pH

pH of sea bass slices wrapped without and with films during storage at 4 °C for 12 days is shown in Figure 40B. pH of the control increased from 5.95 to 7.86 after storage for 12 days. Generally, pH of all samples slightly increased, when the storage time increased ($P < 0.05$). Such a trend was associated with the accumulation of basic compounds such as ammonia and TMA, etc. generated from microbial enzymatic actions (Lopez-Caballero *et al*., 2007). The pH of refrigerated sea bass muscle was increased to 7.7 after 15 days of storage, most likely due to the production of basic amines (Masniyom *et al*., 2002). The lower increase in the pH of sea bass slices wrapped with FPI/FSG-ZnONP-BEO film was observed, when compared with other samples ($P < 0.05$). The result was in agreement with the lower microbial count and TVB content in FPI/FSG-ZnONP-BEO film wrapped samples. Similar results for sea bass slices wrapped with LEO incorporated gelatin films were reported (Ahmad *et al*., 2012b).

(A)



(B)

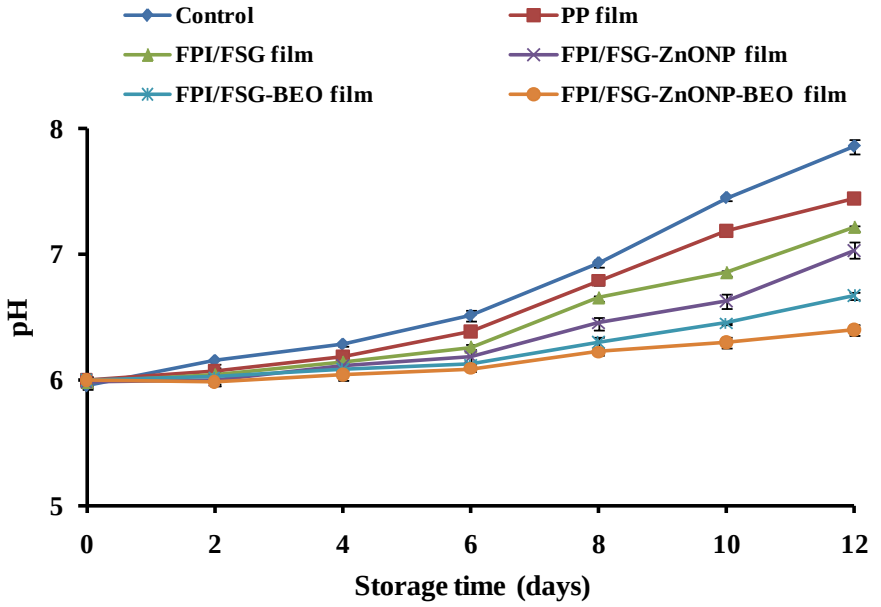


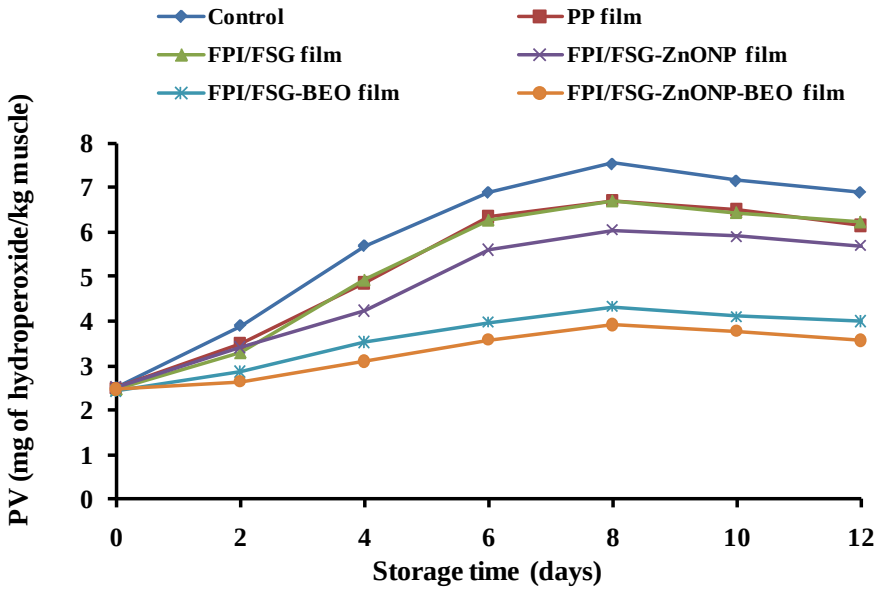
Figure 40. TVB content (A) and pH (B) of sea bass slices wrapped without and with films (PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film) during storage at 4 °C for 12 days. Key: see the caption for Figure 38. Bars represent the standard deviation (n=3).

9.4.2.3 Peroxide value (PV)

Changes in PV of refrigerated sea bass slices wrapped without and with various films are presented in Fig. 41A. PV of all samples increased during the early stage of storage and reached a maximum by day 8. Subsequently, it decreased until the end of the storage period. PV shows the amount of oxidized substances, which are usually hydroperoxides and are the primary products of autoxidation (Yanishlieva and Marinova, 2001). The PV increase in the early stages shows lipid oxidation and formation of hydroperoxides with a rate higher than that of their decomposition. Its decrease after reaching the maximum value is related to hydroperoxide degradation, producing the secondary lipid peroxidation products (Boselli *et al*., 2005). During the storage, a higher increase in PV was observed in the control samples as compared to others ($P < 0.05$). PP and protein based films have low oxygen permeability, which more likely produced an oxygen-resistant layer on the surface of fish slice and decreased lipid oxidation (Tihminlioglu *et al*., 2010; Chiou *et al*., 2008). Samples wrapped with FPI/FSG-ZnONP film showed the lower PV in comparison with the control, PP and FPI/FSG wrapped samples. It is well known that nanoparticles improve the barrier properties of biopolymers due to its functional filler property and the formation of a tortuous path for the molecule diffusion (Arora and Padua, 2010). This more likely decreased the oxygen permeability of FPI/FSG-ZnONP film, compared with FPI/FSG film. Thus, the decrease in PV in the samples wrapped with FPI/FSG-ZnONP was more likely related with their higher oxygen barrier properties. However, the lowest PV during the storage period was observed in the samples wrapped with FPI/FSG-ZnONP-BEO film ($P < 0.05$). This could be caused by the combined effect of ZnONP (oxygen barrier) and antioxidant activity of BEO, which was mediated by polyphenols (Suppakul *et al*., 2003). Phenolic antioxidants do not function as oxygen absorbers; they prevent the formation of fatty acid free radicals, which react with or absorb oxygen in the autoxidation process. This delays the onset of the autoxidative process in fat or oil (Turhan *et al*., 2009). The results were in agreement with Abdollahi *et al*. (2013) who reported that silver carp fillets coated with chitosan nanocomposites activated with

rosemary essential oil had the lower PV, compared with the samples coated with chitosan and chitosan nanocomposite film.

(A)



(B)

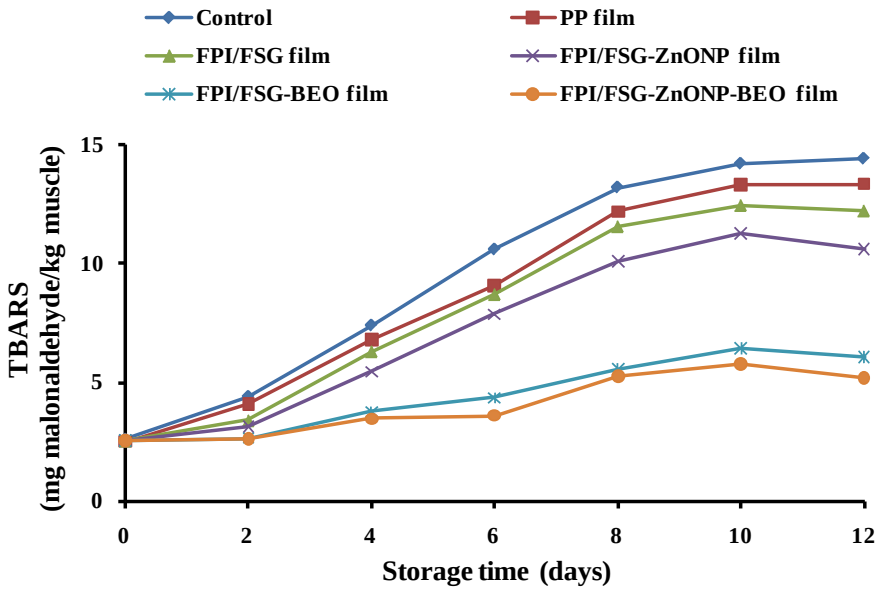


Figure 41. Peroxide value (A) and TBARS value (B) of sea bass slices wrapped without and with films (PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film) during storage at 4 °C for 12 days. Key: see the caption for Figure 38. Bars represent the standard deviation (n=3).

9.4.2.4 TBARS value

During the storage, a higher increase in TBARS was observed in the control samples, followed by those wrapped with PP, FPI/FSG, FPI/FSG-ZnONP, FPI/FSG-BEO and FPI/FSG-ZnONP-BEO film, respectively ($P < 0.05$) (Fig. 41B). TBARS value is an index of lipid oxidation, in which malondialdehyde (MDA) content is measured (Benjakul *et al*., 2005b). Attacks of oxygen against the double bond in fatty acids can cause initiation of free radical chain reactions in lipid oxidation (Abdollahi *et al*., 2013). Samples wrapped with PP and FPI/FSG films also showed the lower TBARS than the control ($P < 0.05$). These films might function as a barrier to oxygen permeability and only a small amount of oxygen could therefore contact with samples. As a result, the lower oxidation rate was obtained. TBARS value in the sample wrapped with FPI/FSG-ZnONP film was lower than those wrapped with PP or FPI/FSG, which might be related to well-known and documented excellent oxygen barrier properties of bionanocomposite films (Arora and Padua, 2010). However, sea bass slices wrapped with FPI/FSG-ZnONP-BEO film showed the lowest TBARS value ($P < 0.05$). The result suggested that lipid oxidation in sea bass slices could be retarded when film incorporated with BEO was applied, probably due to the antioxidant activity of BEO as well as low oxygen permeability characteristics of film incorporated with nanoparticles and essential oil (Abdollahi *et al*., 2013). Thus, the incorporation of BEO enhanced the antioxidant property of the resulting film, as evidenced by lower TBARS of samples wrapped with FPI/FSG-BEO and FPI/FSG-ZnONP-BEO films, compared to other films. Tongnuanchan *et al*. (2013b) reported DPPH radical– and ABTS radical–scavenging activities of gelatin films incorporated with BEO. The antioxidant activities of the essential oils have been attributed to the redox properties, ability to scavenge a variety of reactive species such as superoxide, hydroxyl and peroxyl radicals and hypochlorous acid, singlet oxygen quenching, metal ion chelation and decomposition of peroxides (Perumalla and Hettiarachchy, 2011). During the refrigerated storage, psychrotrophic bacteria, mainly Pseudomonas species, produce lipase and phospholipase, causing an increase in free fatty acids (Koka and Weimer, 2001). These free fatty acids are highly vulnerable to oxidation and form unstable lipid hydroperoxide. This hydroperoxide is readily

decomposed to shorter chain products such as aldehydes, which can be detected as TBARS (Benjakul *et al*., 2005b). Lowered lipid oxidation was in agreement with the lower microbial growth of sea bass slices wrapped with different films (Figure 38).

9.4.3 Sensory evaluation

Sensory properties of sea bass slices wrapped without (control) and with different films during refrigerated storage are given in Table 25. The fish samples were considered to be acceptable for human consumption until the sensory score reached 4 (Ojagh *et al*., 2010). The score for texture, odor, color and overall quality decreased ($P < 0.05$) over the storage of 12 days. Texture, odor, color and overall quality of control samples were given 'unacceptable' scores by the 8th day. Due to high microbial growth and lipid oxidation, the control samples (unwrapped) of sea bass slices showed spoilage as evidenced by the formation of slime and off-odor with discoloration after 6 days storage. However, the samples wrapped with the films especially FPI/FSG-ZnO-BEO possessed the higher scores than others. The score below critical score of 4 (overall quality) was obtained at day 10 and 12 for sea bass slices wrapped with FPI/FSG-ZnONP and FPI/FSG-BEO, respectively. However, the score higher than 4 was found for the sample wrapped with FPI/FSG-ZnONP-BEO after 12 days of storage. Based on sensory analysis, the samples wrapped with FPI/FSG-ZnO-BEO film had the longer shelf-life by 6 days, as compared to the control. The antioxidant, antimicrobial and gas barrier effects of films, especially FPI/FSG-ZnO-BEO, could minimize the oxidative effects, thereby prolonging the shelf-life, whilst maintaining quality. Adding ZnONP and BEO to FPI/FSG film provided the beneficial effects on color, odor and overall quality of the refrigerated sea bass slices over a period of 12 days. The results were in good agreement with those reported by Mahmoud *et al*. (2004) for carp treated with carvacrol–thymol solution (1%, v/v) stored at 5 °C. An extension of 8 days based on odor and taste evaluation was reported for the carp treated with the essential oil mixture. Mejlhom and Dalgaard (2002) also reported that the use of oregano oil (0.05%, v/w) extended the shelf-life of cod from 11-12 days to 21-26 days. It is noteworthy that the presence of BEO in FPI/FSG-BEO and FPI/FSG-ZnO-BEO did not showed detrimental effect

Table 25. Sensory properties of sea bass slices wrapped without and with films (PP film, FPI/FSG film, FPI/FSG-ZnONP film, FPI/FSG-BEO film and FPI/FSG-ZnONP-BEO film) during storage at 4 °C for 12 days

Sensory attributes	Treatments	Storage (days)						
		0	2	4	6	8	10	12
Texture	Control	5±0.00aA	5±0.00aA	4.6±0.14aB	4.1±0.09cC	3.66±0.13bD	3.0±0.13dE	2.06±0.14eF
	PP film	5±0.00aA	4.75±0.22aB	4.7±0.12aB	4.45±0.11bC	4.13±0.12aD	3.53±0.16cE	2.88±0.09dF
	FPI/FSG film	5±0.00aA	4.93±0.12aA	4.76±0.08aAB	4.56±0.09abB	4.11±0.26aC	3.85±0.23bC	3.25±0.18cD
	FPI/FSG-ZnONP film	5±0.00aA	4.90±0.10aA	4.8±0.13aA	4.55±0.11bB	4.17±0.21aC	4.02±0.16abC	3.62±0.08bD
	FPI/FSG-BEO film	5±0.00aA	4.85±0.12aAB	4.7±0.16aB	4.65±0.11abB	4.24±0.14aC	3.98±0.06abD	3.79±0.13bD
	FPI/FSG-ZnONP-BEO film	5±0.00aA	4.82±0.16aA	4.78±0.18aA	4.76±0.14aA	4.41±0.18aB	4.18±0.11aBC	4.05±0.10aC
Odor	Control	5±0.00aA	4.75±0.17aB	4.31±0.12cC	4.09±0.21cD	2.06±0.04dE	1.0±0.00dF	1.0±0.00eF
	PP film	5±0.00aA	4.8±0.12aA	4.43±0.12bcB	4.22±0.17bcB	3.63±0.31cC	2.97±0.21cD	2.48±0.18dE
	FPI/FSG film	4.8±0.16aA	4.61±0.10aAB	4.50±0.08abB	4.16±0.09bcC	3.78±0.18bcD	3.05±0.22cE	2.67±0.15dF
	FPI/FSG-ZnONP film	4.93±0.12aA	4.65±0.09aB	4.58±0.1abB	4.37±0.14abC	4.08±0.16abD	3.82±0.12bE	3.41±0.08cF
	FPI/FSG-BEO film	4.8±0.21aA	4.70±0.1aA	4.58±0.03abAB	4.38±0.11abB	4.14±0.15aC	3.96±0.14abCD	3.74±0.13bD
	FPI/FSG-ZnONP-BEO film	4.8±0.16aA	4.75±0.09aA	4.65±0.11aAB	4.50±0.08aBC	4.37±0.11aC	4.19±0.07aD	4.05±0.04aD
Color	Control	5±0.00aA	4.65±0.13bB	4.40±0.14cC	4.1±0.04cD	3.66±0.04eE	2.40±0.18dF	1.76±0.17fG
	PP film	4.9±0.10aA	4.8±0.08abA	4.5±0.12abcB	4.35±0.12bB	3.90±0.12dC	3.33±0.12cD	2.78±0.13eE
	FPI/FSG film	5.0±0.00aA	4.8±0.05abB	4.45±0.08bcC	4.30±0.08bD	4.1±0.08cdE	3.45±0.08cF	3.05±0.09dG
	FPI/FSG-ZnONP film	4.9±0.10aA	4.85±0.09abA	4.6±0.14abcB	4.55±0.14aB	4.27±0.14bcC	3.92±0.14bD	3.42±0.16cE
	FPI/FSG-BEO film	4.9±0.10aA	4.8±0.16abAB	4.65±0.12abB	4.6±0.06aB	4.34±0.13bC	4.13±0.16abC	3.81±0.15bD
	FPI/FSG-ZnONP-BEO film	5.0±0.00aA	4.9±0.07aA	4.7±0.07aB	4.6±0.13aB	4.55±0.11aB	4.30±0.12aC	4.10±0.11aD
Overall	Control	5±0.00aA	4.6±0.12bB	4.30±0.10cC	4.05±0.04cD	3.16±0.05dE	1.85±0.15dF	1.0±0.00dG
	PP film	5±0.00aA	4.70±0.09abB	4.40±0.06bcC	4.15±0.12cD	3.53±0.12cE	2.75±0.10cF	2.18±0.18cG
	FPI/FSG film	4.9±0.15aA	4.65±0.08abB	4.40±0.08bcC	4.06±0.08cD	3.48±0.06cE	2.68±0.12cF	2.17±0.15cG
	FPI/FSG-ZnONP film	4.9±0.10aA	4.8±0.10aA	4.50±0.12bB	4.37±0.12bB	4.09±0.14bC	3.72±0.16bD	3.41±0.10bE
	FPI/FSG-BEO film	5.0±0.00aA	4.8±0.08aB	4.50±0.10bC	4.40±0.08bC	4.21±0.10abD	4.08±0.06aD	3.86±0.10aE
	FPI/FSG-ZnONP-BEO film	4.9±0.10aA	4.8±0.11aAB	4.70±0.08aBC	4.60±0.10aC	4.37±0.09aD	4.19±0.08aE	4.03±0.05aF

Different lowercase letters in the same column within the same sensory attribute indicate significant differences ($P < 0.05$).

Different uppercase letters in the same row indicate significant differences ($P < 0.05$).

on odor of sea bass slices.

9.5 Conclusion

The active nanocomposite films based on FPI/FSG incorporated with ZnONP and BEO could retard microbial growth and lipid oxidation in refrigerated sea bass slices more effectively than PP, FPI/FSG, FPI/FSG-ZnONP and FPI/FSG-BEO films. FPI/FSG-ZnONP-BEO film could extend shelf-life of sea bass slice up to 12 days at refrigerated temperature, which was 6 days longer than the control. Thus, FPI/FSG-ZnONP-BEO film could be used as an active packaging to maintain quality and extend shelf-life of sea bass slices at 4 °C.

CHAPTER 10
SUMMARY AND FUTURE WORKS

10.1 Summary

1. Yellow stripe trevally muscle could be used as the new raw material for surimi which yielded the gel with high deformation. Setting temperature showed significant effect on textural properties and cross-linking of myofibrillar proteins. Setting at 40 °C for an appropriate time could improve gelling properties of surimi produced from yellow stripe trevally.

2. Addition of $ZnSO_4$ could induce higher aggregation of NAM, compared with $ZnCl_2$, mainly by induction of higher hydrophobic interaction, disulphide bond and ionic interaction. Kamaboko gel incorporated with 60 μmol/kg $ZnSO_4$ yielded the gel with increased breaking force, deformation and whiteness. Therefore, $ZnSO_4$ at an appropriate level could serve as promising additive to improve the strength and water holding capacity of gel from surimi manufactured from dark fleshed fish.

3. Gel with improved properties could be obtained from protein isolate from yellow stripe trevally phosphorylated with STPP in conjunction with addition of $ZnSO_4$ at an appropriate level.

4. Properties of FPI films could be modified by blending with FSG at a ratio of 5:5 with lower glycerol content (30%) at both acidic and alkaline pH. pH and FPI/FSG ratio affected the yellow discoloration of film mediated by Maillard reaction. Blend film showed the improved mechanical and water vapor barrier properties, compared with FPI films.

5. Mechanical, water vapor barrier and thermal properties of FPI/FSG films could be improved by incorporating 3% ZnONP at both acidic and alkaline pH. Incorporation of ZnONP yielded film with lower yellow discoloration. Moreover, FPI/FSG-ZnONP nanocomposite films, especially those prepared at pH 3, exhibited

strong antibacterial activity against Gram-positive and Gram-negative food borne pathogenic and spoilage bacteria.

6. The active antimicrobial nanocomposite films with the lowest WVP were prepared by addition of 100% BEO and 3% ZnONP. FPI/FSG films incorporated with 100% BEO and 3% ZnONP effectively retarded the microbial growth and lipid oxidation of refrigerated sea bass slices during storage at 4 °C, thereby extending the shelf-life of sea bass slices.

10.2 Future works

1. Influence of zinc salts on proteolytic and transglutaminase mediated cross-linking activities of fish muscle should be studied.

2. FPI/FSG film incorporated with nano-encapsulated essential oil should be prepared and characterized.

3. The sealability of active film should be intensively investigated for application as active biodegradable food packaging material.

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