Antioxidative, Antimicrobial and Fish Skin Gelatin Cross-Linking Activities of Seaweed Extracts

Saowapa Rattaya

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Science and Technology

Prince of Songkla University

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ชื่อวิทยานิพนธ์	กิจกรรมต้านออกซิเดชัน กิจกรรมยับยั้งจุลินทรีย์และกิจกรรมการเชื่อมประสานเจลาตินจากหนังปลาของสารสกัดจากสาหร่ายทะเล
ผู้เขียน	นางสาวเสาวภา รัตญา
สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีอาหาร
ปีการศึกษา	2551

บทคัดย่อ

จากการศึกษากิจกรรมการต้านออกซิเดชันและปริมาณสารประกอบฟีนอลิกของสารสกัดจากสาหร่ายทะเลสีน้ำตาล 2 สายพันธุ์ (*Turbinaria ornata* และ *Sargassum polycystum*) ด้วยตัวทำละลาย 2 ชนิด คือ เมธานอลและเอธานอล พบว่าสาหร่ายทะเลสายพันธุ์ *T. ornata* ที่สกัดด้วยเมธานอลมีปริมาณของสารประกอบฟีนอลิกสูงสุด (2.07 มก. คาเทชิน/กรัม นน. แห้ง) (p 0.05) และมีความสามารถในการต้านออกซิเดชันสูงสุดโดยตรวจสอบจากความสามารถในการกำจัดอนุมูลอิสระ DPPH ABTS และความสามารถในการรีดิวซ์ RAP ที่สูงสุด (p 0.05) กิจกรรมการต้านออกซิเดชันเพิ่มขึ้นเมื่อระดับความเข้มข้นของสารสกัดที่ใช้เพิ่มขึ้น (100-500 มก/ลิตร) โดยมีความสัมพันธ์ระหว่างกิจกรรมการต้านอนุมูลอิสระ ABTS และ DPPH, DPPH และ RAP, ABTS และ RAP ดังนั้นสารสกัดจากสาหร่ายทะเลจึงมีความสามารถในการต้านอนุมูลอิสระและความสามารถในการให้อิเลคตรอน จากการทดสอบประสิทธิภาพในการต้านออกซิเดชันของสารสกัดจากสาหร่าย *T. ornata* ที่ระดับความเข้มข้น 100-500 มก./ลิตรในระบบเลซิติน-ลิโปโซมและลิโนเลอิก พบว่าสารสกัดจากสาหร่ายทะเลทั้งที่กำจัดและไม่กำจัดคลอโรฟิลล์มีประสิทธิภาพในการต้านออกซิเดชันแต่ประสิทธิภาพต่ำกว่าสารต้านออกซิเดชันสังเคราะห์ BHT ที่ระดับ 50 และ 200 มก./ลิตร สำหรับการศึกษาประสิทธิภาพการยับยั้งจุลินทรีย์พบว่าสารสกัดจากสาหร่ายทะเลทั้ง 2 สายพันธุ์ไม่สามารถยับยั้ง *Bacillus subtilis, Salmonella enteritidis* และ *Aspergillus niger* แต่สามารถยับยั้ง *Staphylococcus aureus* ได้ที่ระดับความเข้มข้น 500 มก./ลิตร

จากการศึกษาการเปลี่ยนแปลงของสารละลายฟิล์มเจลาตินที่เติมสารสกัดจากสาหร่ายทะเล (*T. ornata*) ที่ระดับพีเอชต่างๆ (8, 9 และ 10) และความเข้มข้นแตกต่างกัน (ร้อยละ 3 และ 6) พบว่าสารละลายฟิล์มที่เติมสารสกัดสาหร่ายที่ระดับพีเอช 10 ความเข้มข้นร้อยละ 6 มีปริมาณหมู่อะมิโนอิสระต่ำสุด (p 0.05) สารละลายฟิล์มที่ปรับพีเอชให้เท่ากับพีเอชของสารสกัดสาหร่ายทะเลที่เติมลงไปมีค่าการต้านอะนุมูลอิสระ DPPH และ ABTS สูงกว่าสารละลายฟิล์มที่ไม่มีการปรับพีเอช เมื่อศึกษารูปแบบโมเลกุลของสารละลายฟิล์มเจลาตินพบว่าสารละลายฟิล์มที่เติมและไม่เติมสารสกัดจากสาหร่ายทะเลมีย่อยสลายของสายโปรตีนชนิดบีตาและแอลฟา

เมื่อทำการศึกษาฟิล์มเจลาตินที่เตรียมจากสารละลายฟิล์มที่ไม่เติมและเติมสารสกัดสาหร่ายทะเลที่ระดับความเข้มข้นร้อยละ 6 พีเอช 9 และ 10 พบว่าฟิล์มเจลาตินที่เติมสารสกัดสาหร่ายทะเลมีค่าระยะยืดเมื่อขาดสูงกว่าฟิล์มที่ไม่เติมสารสกัด (p 0.05) แต่ไม่มีความแตกต่างของค่าการทนต่อแรงดึงและความโปร่งใส (p>0.05) ฟิล์มเจลาตินที่เติมสารสกัดสาหร่ายทะเลมีค่าการซึมผ่านไอน้ำ การละลายของฟิล์ม การละลายของโปรตีน การย่อยสลายโดยเอนไซม์ และการละลายของโปรตีนในตัวทำละลายชนิดต่างๆ ลดลงเมื่อเปรียบเทียบกับฟิล์มที่ไม่เติมสารสกัด ผิวหน้าของฟิล์มเจลาตินในชุดควบคุมมีลักษณะเรียบและการเติมสารสกัดจากสาหร่ายทะเลมีผลให้ฟิล์มที่ได้มีพื้นที่ผิวที่ขรุขระ และอุณหภูมิในการเปลี่ยนสถานะของฟิล์มเจลาตินที่เติมสารสกัดสาหร่ายทะเลที่ระดับพีเอช 10 มีค่า 178.96 องศาเซลเซียส

จากการศึกษาการเก็บรักษาฟิล์มเจลาตินที่ความชื้นสัมพัทธ์ร้อยละ 54 และอุณหภูมิห้อง (28-30 °ซ) พบว่าฟิล์มเจลาตินที่ไม่มีเติมสารสกัดสาหร่ายทะเลมีค่าการทนต่อแรงดึงเพิ่มขึ้นอย่างต่อเนื่องและค่าระยะยืดเมื่อขาดลดลงอย่างชัดเจนในช่วง 3 สัปดาห์แรก (p 0.05) ส่วนฟิล์มเจลาตินที่เติมสารสกัดสาหร่ายทะเลมีการเปลี่ยนแปลงสมบัติทางกลเพียงเล็กน้อย ฟิล์มเจลาตินทุกตัวอย่างมีการเปลี่ยนแปลงสมบัติการป้องกันการซึมผ่านของไอน้ำเพียงเล็กน้อยตลอดระยะเวลาการเก็บรักษา 4 สัปดาห์ (p 0.05) ความชื้นของฟิล์มทุกตัวอย่างมีค่าเพิ่มขึ้นอย่างช้าๆมื่อมีการเก็บรักษาที่ Aw ต่ำ (0.18-0.64) และค่าความชื้นเพิ่มขึ้นอย่างรวดเร็วเมื่อเก็บรักษาที่ Aw สูงขึ้น (0.64-0.90) ในระหว่างการเก็บรักษาการละลายของฟิล์มและการละลายของโปรตีนมีค่าลดลงอย่างต่อเนื่องเมื่อระยะเวลาการเก็บรักษาเพิ่มขึ้น ฟิล์มมีลักษณะคล้ำขึ้นและมีสีเหลืองเพิ่มขึ้นโดยเฉพาะอย่างยิ่งตัวอย่างฟิล์มในชุดควบคุมที่ไม่มีการเติมสารสกัดสาหร่ายทะเลซึ่งมีสีเหลืองเกิดขึ้นอย่างเด่นชัด

Thesis Title	Antioxidative, antimicrobial and fish skin gelatin cross-linking activities of seaweed extracts
Author	Miss Saowapa Rattaya
Major program	Food Science and Technology
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ABSTRACT

Antioxidative activity and total phenolic content of methanolic and ethanolic extracts from brown seaweeds, *Turbinaria ornata* and *Sargassum polycystum*, were determined. Among all extracts, methanolic extract of *T. ornata* contained the highest total phenolic content (2.07 mg catechin/g dry seaweed) ($p<0.05$) and exhibited the highest antioxidative activity as indicated by the greatest ABTS and DPPH radical scavenging activity as well as reducing activity power (RAP), compared with other extracts ($p<0.05$). When different concentrations of seaweed extracts (100-500 mg/L) were used, antioxidative activities were dose-dependent. Correlations between ABTS and DPPH radical scavenging activity; DPPH radical scavenging activity and RAP; ABTS radical scavenging activity and RAP were observed. Therefore, antioxidants in seaweed extracts possessed the capability of scavenging the radicals together with reducing power. The efficacy in prevention of lipid oxidation of methanolic extract of *T. ornata* in lecithin-liposome and linoleic oxidation systems was studied. The extract at levels of 100-500 mg/L could retard the oxidation, regardless of chlorophyll removal but its efficacy was lower than that of BHT at levels of 50 and 200 mg/L. For antimicrobial activity, all extracts could not inhibit the growth of *B. subtilis*, *S. enteritidis* and *A. niger*, while *S. aureus* was inhibited with the extracts at 500 mg/L.

Effects of the extract from *T. ornata* with different pHs (8, 9 and 10) and concentrations (3 and 6%) on the changes of gelatin film forming solutions (FFS) were studied. FFS incorporated with 6% extract having pHs 9 or 10 showed the lowest free amino group content ($p<0.05$). FFS incorporated with seaweed extract and subjected to pH adjustment had the higher DPPH and ABTS scavenging activities than those without pH adjustment ($p<0.05$). The degradation of β and α - component of occurred in all FFS, regardless of seaweed extract addition.

When gelatin based film from FFS incorporated with the extract at pHs 9 or 10 were prepared and investigated in comparison with the control film, those incorporated with the extract exhibited the higher elongation at break (EAB) than those without the extract ($p<0.05$). However, no differences in tensile strength (TS) and transparency between films without and with the extract were observed ($p>0.05$). The lower water vapor permeability (WVP), film solubility, protein solubility, degree of hydrolysis and protein solubility in various solvents were found in gelatin film containing the extract, compared with those without the extract ($p<0.05$). Gelatin film without seaweed extract had smooth surface while the rougher surface was noticeable in film incorporated with seaweed extract. The transition temperature of gelatin film incorporated with 6% seaweed extract at pH 10 shifted to the higher temperature (178.96°C).

During the storage under 54% relative humidity at room temperature (28-30°C), TS of gelatin film without seaweed extract increased continuously, while EAB decreased markedly when storage time increase up to 3 weeks ($p<0.05$). Gelatin film incorporated with seaweed extract had a lesser changes in TS and EAB, compared with the control film. Slight decreases in water barrier property of all films were obtained throughout the storage time up to 4 weeks ($p<0.05$). Moisture content of all gelatin films increased slowly at Aw of 0.18-0.64 and increased rapidly at Aw of 0.64-0.90. Film solubility and protein solubility decreased continuously during storage. All films became darker and more yellowish as storage time increased, especially in the control film.

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CONTENTS

Page

Content……………………………………………………………………………viii

List of tables…………………………………………………………………….....xiii

List of Figures……………………………………………………………….….xv

Chapter

1. Introduction and Literature Review…………….……………..…….….………….... 1

Introduction…………………………………..……….……………..…………. 1

Literature review……………...……………………………………………….….. 2

1. Seaweed……………………………………………………………...…....2

1.1 Division: Cyanophyta (blue-green algae)………………………….........2

1.2 Division: Chlorophyta (green algae)……………………………..…….. 3

1.3 Division: Rhodophyta (red algae)………………………………..….. 3

1.4 Division: Pheophyta (brown algae)……………………………......3

2. Lipid oxidation………………………………………………………........ 5

2.1 Initiation………………………………………………….…………….. 6

2.2 Propagation……………………………………………….……………..6

2.3 Termination…………………………………………………………...… 7

3 Antioxidants...…………………………………………………….……….... 7

3.1 Antioxidants and their mode of action………………….………….... 7

3.2 Antioxidants from plant extracts……………………….…………..... 9

3.3 Antioxidative activity of seaweed extracts……………….……..........11

3.4 Chlorophyll………………………………………………….……......12

4 Antimicrobials..…………………………………………….…..….…..... 14

4.1 Antimicrobials from plant extracts…………………….………......14

4.2 Antimicrobial activity of seaweed extracts……………..……..... 16

5 Gelatin……………………………………………………….….…….... 17

5.1 Functional properties of gelatin……………………………….....20

6 Protein film properties………………………..…………...…...21

6.1 Barrier properties……………………………..…………….. 21

6.2 Mechanical properties……………………..………..…..... 22

6.3 Solubility……………………………..…………….…... 23

.

CONTENTS (Continued)

Chapter **Page**

6.4 Use of protein cross-linkers for the improvement of film properties 23
Objectives of study.. 27
2. Materials and Methods.. 28
1. Materials.. 28
2. Chemicals and enzyme.. 28
3. Instruments.. 29
4. Methods.. 30
4.1 Extraction and characterization of seaweed extracts........................ 30
4.1.1 Preparation of seaweed extracts........................ 30
4.1.2 Chlorophyll content........................ 30
4.1.3 Total phenolic content........................ 30
4.2 Study on antioxidative activity of seaweed extracts........................ 31
4.2.1 DPPH radical scavenging activity........................ 31
4.2.2 ABTS radical scavenging activity........................ 31
4.2.3 Reducing power........................ 32
4.2.4 Determination of antioxidative activity of seaweed extract in different system 32
4.2.4.1 Lecithin liposome system........................ 32
4.2.4.2 Linoleic oxidation system........................ 32
4.3 Study on antimicrobial activity of seaweed extracts........................ 33
4.3.1 Microorganism........................ 33
4.3.2 Determination of antimicrobial activity of seaweed extracts........................ 33
4.4 Study on gelatin cross-linking activity of seaweed extracts and the effect on properties of gelatin film........................ 34
4.4.1 Fish skin preparation........................ 34
4.4.2 Extraction of fish gelatin 34

CONTENTS (Continued)

Chapter **Page**

4.4.3 Effect of pH and the concentration of *T. ornata* extracts on properties of gelatin film solutions.................. 34

4.4.3.1 Preparation of film-forming solutions.................. 34

4.4.3.2 Determination of gelatin FFS.................. 35

4.4.3.3 Film preparation.................. 35

4.4.3.4 Determination of properties of resulting films.................. 36

4.4.4 Characterization of gelatin film incorporated with seaweed extract.................. 36

4.4.4.1 Film thickness.................. 36

4.4.4.2 Mechanical properties.................. 36

4.4.4.3 Water vapor permeability 36

4.4.4.4 Color and film transparency.................. 37

4.4.4.5 Film solubility.................. 37

4.4.4.6 Microstructure.................. 37

4.4.4.7 Hydrolysis by protease.................. 37

4.4.4.8 Protein solubility in various solvents.................. 38

4.4.4.9 Thermal properties.................. 38

4.5 Moisture sorption isotherm.................. 39

4.6 Changes of gelatin films during storage.................. 39

5. Statistical analysis.................. 39

3. Results and Discussion.................. 40

1. Extraction and study on properties of seaweed extracts.................. 40

1.1 Total phenolic content and total chlorophyll content of brown seaweed extracts.................. 40

1.2 Antioxidative activities of brown seaweed extracts.................. 42

1.2.1 DPPH radical scavenging activity.................. 42

CONTENTS (Continued)

Chapter **Page**

1.2.2 ABTS radical scavenging activity……………….……….... 44

1.2.3 Reducing activity power……………………….…….….... 46

1.3 The correlation between antioxidative activities tested by different assays………………………………………..……... 46

1.4 Antioxidative activity of seaweed extract in different systems…..…. 49

1.4.1 Lecithin liposome system…………………………….…... 49

1.4.2 Linoleic oxidation system…………………………….......49

1.5 Antimicrobial activity of brown seaweed extracts…………………... 51

2. Effect of the incorporation of seaweed extract on cross-linking and film properties of bigeye snapper skin gelatin ……….......................54

2.1 Compositions and protein pattern of bigeye snapper skin gelatin…..... 54

2.2 Effect of pH and concentration of seaweed extract on characteristics of gelatin film forming solutions and properties of resulting films…………..56

2.2.1 Characteristic of film forming solution…………………..……56

2.2.1.1 Free amino group content and radical scavenging activities……………………..…..….56

2.2.1.2 Protein pattern………………………………… 59

2.2.2 Properties of gelatin based film…………………………..…..…59

2.2.2.1 Thickness and mechanical properties of gelatin films…………………………………...59

2.2.2.2 Light transition and transparency of gelatin films…………………………….….. 61

2.2.2.3 Color and gelatin films…………………………..63

2.3 Characterization of bigeye snapper skin gelatin film incorporated with seaweed extract………………………………….... 63

2.3.1 Mechanical and physical properties……………………….....66

2.3.2 Moisture content and color…………………………………. 67

CONTENTS (Continued)

Chapter **Page**

2.3.3 Light transmission and film transparency………………….... 67

2.3.4 Film solubility and protein solubility……………………….. 68

2.3.5 Surface characteristic……………………………………......69

2.3.6 Hydrolysis by protease…………………………………….... 69

2.3.7 Protein solubility in various solvents……………………...... 71

2.3.8 Thermal properties………………………………….. ... 72

2.4 Moisture sorption isotherm………………………………………. ...73

2.5 Changes of gelatin films during storage…………………….....…...75

2.5.1 Changes in mechanical properties……………….……… 75

2.5.2 Changes in color and transparency……………….….…....75

2.5.3 Changes in film solubility and protein solubility……….….. 79

2.5.4 Changes in water vapor permeability…………………......79

4. Conclusion and future works…………………………………….....…......82

Conclusion……………………………………………………….....……82

Suggestion……………………………………………………….…..… 82

References………………………………………………………….......... 83

Appendix…………………………………………………………..…........ 108

Vitae………………………………………………………………….........117

LIST OF TABLES

Table **Page**

1. Amino acid composition of gelatin from different fish species……………...…19
2. Barrier properties, mechanical properties and soluble matter of gelatin films……………………………………………………………..…22
3. Total phenolic content (TPC) of methanolic and ethanolic extract of *T. ornata* and *S. polycystum* without and with chlorophyll removal..41
4. Total chlorophyll content of methanolic and ethanolic extract of *T. ornata* and *S. polycystum* without and with chlorophyll removal……………………………………………………...….........41
5. Inhibition zone of different microorganisms in the presence of methanolic and ethanolic extracts of *T. ornata* and *S. polycystum* with and without chlorophyll removal at 500 mg/L. ………….…………..…...53
6. Proximate composition and hydroxyproline content of bigeye snapper skin gelatin ………………………………………………………….............55
7. TNBS and radical scavenging activities of gelatin FFS incorporated with seaweed extract adjusted to different pHs at different concentrations without and with pH adjustment to various FFS pHs.…………………………………………………...……..…….....58
8. Thickness and mechanical properties of gelatin film prepared from FFS incorporated with seaweed extract adjusted to different pHs at different concentrations without and with pH adjustment to various FFS pHs. ………..……....62
9. Light transmission and transparency of gelatin film prepared from FFS incorporated with seaweed extract adjusted to different pHs at different concentrations without and with pH adjustment to various FFS pHs. ……………………………………………………….……….....65
10. Color of gelatin film prepared from FFS incorporated with seaweed extract adjusted to different pHs at different concentrations without and with pH adjustment to various FFS pHs. ..………...………...…..……....65

LIST OF TABLES (Continued)

Table **Page**

11. Physical and mechanical properties of gelatin films incorporated with seaweed extract at different pHs. ……………………………………..….66
12. Moisture content and color of gelatin films incorporated with seaweed extract at different pHs.……………………………………………67
13. Light transmission and transparency of gelatin films incorporated with seaweed extract at different pHs. ……………………….……………..….68
14. Film solubility and protein solubility of gelatin films incorporated with seaweed extract at different pHs……………………………………….69
15. Degree of hydrolysis (%) of gelatin films incorporated with seaweed extract at different pHs by α- chymotrypsin…………...…….71
16. Protein solubility (%)* of gelatin films incorporated with seaweed extract at different pHs...72
17. Transition temperatures and enthalpy of gelatin films incorporated with seaweed extract at different pHs……………………………………….73

LIST OF FIGURES

Figure **Page**

1. *Sargassum polycystum*……………………………………………………......…......4
2. *Turbinaria ornata*……………………………………………….......................5
3. Structures of lanosol and lanosol derivertive in red seaweed (*Osmundaria serrata*) ………………………………………………………......11
4. Basic structures of chlorophyll……………………………….…….........13
5. Reactions of a phenolic acid with amino side chains of polypeptides……....25
6. DPPH radical scavenging activity (µmol TE/ml) of methanolic (ME) and ethanolic (EE) extracts of *T. ornata* and *S. polycystum* without (a) and with (b) chlorophyll removal at various concentrations. Bars represent the standard deviation from triplicate determinations. …….......43
7. ABTS radical scavenging activity (µmol TE/ml) of methanolic (ME) and ethanolic (EE) extracts of *T. ornata* and *S. polycystum* without (a) and with (b) chlorophyll removal at various concentrations. Bars represent the standard deviation from triplicate determinations.……....45
8. Reducing activity power (µmol TE/ml) of methanolic (ME) and ethanolic (EE) extracts of *T. ornata* and *S. polycystum* without (a) and with (b) chlorophyll removal at various concentrations. Bars represent the standard deviation from triplicate determinations……………………..…..47
9. Correlation between ABTS and DPPH radical scavenging activity (a), RAPand ABTS radical scavenging activity (b) and RAP and DPPH radical scavenging activity (c) of the extract of *T. ornata* and *S. polycystum* without and with chlorophyll removal. ……………….............48
10. Changes in TBARS (mg MDA/ml liposome) of lecithin liposome system containing methanolic extract of *T. ornata* without (a) and with (b) chlorophyll removal at different levels. Bars represent the standard deviation from triplicate determinations. ……………………...50
11. Changes in A_{500} of linoleic acid system containing methanolic extract of *T. ornata* without (a) and with (b) chlorophyll removal at different levels. Bars represent the standard deviation from triplicate determinations. ……………………………………………………52

LIST OF FIGURES (Continued)

Figure **Page**

12. Protein pattern of bigeye snapper skin gelatin under reducing (SR) and non-reducing (SN) conditions. CTI: collagen type I from calf skin. ……………………………………………………….………....55
13. Protein patterns of gelatin forming solution (FFS); incorporated with seaweed extract at pH 8 (A); incorporated with seaweed extract at pH 8 and the mixture was adjusted to pH 8 (B); incorporated with seaweed extract at pH 9 (C); incorporated with seaweed extract at pH 9 and the mixture was adjusted to pH 9 (D); incorporated with seaweed extract at pH 10 (E); incorporated with seaweed extract at pH 10 and the mixture was adjusted to pH 10 (F). Numbers designate the concentration of seaweed extract (%). CTI: collagen type I from calf skin; con: control…………………………...60
14. SEM micrographs of gelatin films. Upper surface (A), lower surface (B) and cross section (C). Control film (without seaweed extract) (1), film incorporated with 6% seaweed extract; pH 9 (2) and film incorporated with 6% seaweed extract, pH 10 (3). …………......…70
15. Moisture sorption isotherms (at 28-30°C) of gelatin films incorporated without and with seaweed extract at different pHs. SE: Seaweed extract. Bars represent standard deviation from triplicate determinations. ……………………………………….…….…......74
16. Changes in tensile strength and elongation at break of gelatin films incorporated without and with seaweed extract at different pHs during storage at room temperature (28-30°C). Bars represent the standard deviation from nine determinations. ……………………..……76
17. Changes in transparency value of gelatin-based films incorporated without and with seaweed extract at different pHs during storage at room temperature (28-30°C). Bars represent the standard deviation from five determinations. ……………………………………..…..77

LIST OF FIGURES (Continued)

Figure **Page**

18. Changes in L*, a* and b*-values of gelatin films incorporated without and with seaweed extract at different pHs during storage at room temperature (28-30°C). Bars represent the standard deviation from five determinations…………………………….................78
19. Changes in film solubility and protein solubility of gelatin films incorporated without and with seaweed extract at different pHs during storage at room temperature (28-30°C). Bars represent the standard deviation from five determinations. …………….……….........…..80
20. Changes in water vapor permeability of gelatin films incorporated without and with seaweed extract at different pHs during storage at room temperature (28-30°C). Bars represent the standard deviation from five determinations. ………………………….………..........81

CHAPTER 1

INTRODUCTION

Lipid oxidation and microbial spoilage are the major deteriorative processes in foods, leading to unacceptability for the consumers and a loss in nutritional value. Additionally, oxidation leads to health disorders such as altherosclerosis and cancerogenesis. Hence, the antioxidants and antimicrobial agents have been widely used to maintain the quality, acceptability and safety of foods. (Koleva *et al.,* 2003). Nevertheless, synthetic antioxidant and antimicrobial agents in food products are under strict regulation due to the potential health hazards (Kranl *et al.,* 2005). Therefore, the search for natural antioxidants and antimicrobial as alternatives to synthetic counterpart is of great interest.

Seaweeds or marine macroalgae are potential renewable resources in the marine environment. About 6000 species of seaweeds have been identified and are grouped into different classes including green (Chlorophytes), brown (Pheophytes) and red (Rhodophytes) algae (Abbott, 1995) Total global seaweed production of the world in the year 2004 was greater than 15 million metric tones (FAO, 2006). Seaweeds have been widely used for the production of hydrocolloids such as agar, etc. Additionally, marine algae extracts were demonstrated to have strong antioxidant properties (Kuda *et al.,* 2005), protective effects against liver injury caused by carbon tetrachloride, antimicrobial activity and antiviral properties (Newman *et al.,* 2003). Active compounds from brown seaweeds were identified as phylopheophytin in *Eisenia bicyclis* and fucoxantine in *Hijikia fusiformis* (Yan *et al.*, 1999). However, algae grown in Thailand are still underutilized. Only small portion has been used as food, animal fodder, fertilizers, and for the production of hydrocolloids (Aungtonya and Liao, 2002). To maximize the utilization of seaweeds, particularly brown seaweed, the uses of their extracts as natural antioxidant and antimicrobial should be focused. Furthermore, natural phenolic compounds in seaweeds can be served as cross-linkers of proteins or peptides. Mechanical properties of gelatin were improved by phenolic compounds. (Strass and Gibson, 2004).

So far, edible film or packaging has been paid increasing interest owing to its environmental friendly aspect. Gelatin based film is another biomaterial which can be used as alternative packaging**.** Due to the restriction of uses of gelatin from land animals, marine gelatin has been intensively extracted and utilized. Gelatin has been attracted the attention for the development of edible films due to its abundance and biodegradability (Bigi *et al.*, 2002). Generally, gelatin from marine sources showed lower functional properties than that of mammalian (Norland, 1990; Leuenberger, 1991). Therefore, the use of gelatin for film formation in a promising means to maximize the utilization of marine gelatin. The incorporation of phenolic compounds from natural sources, especially seaweed extracts, may improve functional properties of gelatin film. The information gained will be of benefit for the maximized use of seaweeds as natural antioxidant and antimicrobial agents. Additionally, edible gelatin based film with the improved properties can be produced for further uses.

Literature Reviews

1. Seaweeds

In Thailand, there are a variety of seaweeds. The total number of 132 genera and 333 species consists of 28 genera and 63 species of the Divition Cyanophyta (blue-green algae), 29 genera and 91 species of the Division Clorophyta (green algae), 20 genera and 48 species of the Division Phaeophyta (brown algae) and 55 genera and 131 species of the Division Rhodophyta (red algae) (Lewmanomont, 1995; Lewmanomont and Ogawa, 1995).

1.1 Division: Cyanophyta (blue-green algae)

The blue-green algae are the only prokaryotic algae, having no distinct nucleus, and pigments are not localized in chromotophores. The photosynthetic pigments consist of chlorophyll *a,* carotenes, xanthophylls and phycobilins. The blue-green algae are more closely related to the prokaryotic bacteria than to the eukaryotic algae. The term blue-green bacteria (cyanobacteria) is recognized instead of blue-green algae. This group of algae ranges from unicels, colonies to unbranched and branched filaments. Some blue-green algae can fix elemental nitrogen. The blue-green algae are of less significance in the marine flora than the other groups of algae

(Lewmanomont, 1978).

1.2 Division: Chlorophyta (green algae)

The green algae are grass-green in color because of the photosynthetic and associated pigments, chlorophyll *a* and *b,* carotenes and xanthophylls, similar to higher plants. The pigments are in various shapes of plastids or chloroplasts. The cell wall consists of cellulose and pectin. The green algae have a wide range of form, varying from unicellular, colonial, filamentous, membranous and tubular types. The members of this division in marine habitats are macroscopic filamentous, thalloid and siphonous forms (Lewmanomont, 1978).

1.3 Division: Rhodophyta (red algae)

The division Rhodophyta, comprising the red algae, is readily distinguishable from other groups of eukaryotic algae by a combination of characteristics. The red algae contain pigments, chlorophyll *a* and *d,* carotenes, xanthophylls and phycobilins especially phycoerythrin, which is typically the predominant accessory pigment. The color varies from red to brown or green due to the amount of the pigments. The cell walls of the red algae consist of mucilaginous substances such as agar and carrageenan, which are used for various industrial purposes. The great majority of Rhodophyta are filamentous, foliose or more massive forms. Most of the members of the red algae are marine in habitat, only a few are freshwater and terrestrial. Some are partial or complete parasites on other marine algae (Lewmanomont, 1978).

1.4 Division: Pheophyta (brown algae)

The brown algae are brownish in color due to the large amounts of the caroteniod fucoxanthin masking the remaining pigments, chlorophyll *a* and *c,* β-carotene and other xanthophylls. Cell walls are composed of alginic acid which can be extracted as algin or alginate, used for various industrial purposes. The brown algae range in size from small filaments to the largest marine algae. Most members of this division are almost exclusively of marine occurrence. Most of the brown algae grow in the intertidal belt and the upper littoral region (Lewmanomont, 1978).

1.4.1 *Sargassum polycystum*

Sargassum polycystum has thallus 35 cm tall, with yellowish brown color, attached with discoid holfast; main axis cylindrical and rough due to the presence of numerous outgrowth, supporting alternately arranged branches bearing leaves and vesicles. In young thalli, leaves are longer and broader measuring 13 - 42 mm long including the stalk and 2.5 - 11.5 mm wide; leaves are generally oblong slightly tapered, retuse (slightly rounded) or emarginate at the tip finally serrated throughout the margin; mature thalli fewer leaves smaller, 7 - 15 mm long including stalk and 17 - 4 mm wide, oblanceolate, oblong with tapered bases. The apices are rounded and obtuse to acute outer margin is coarsely serrate; prominant midrib at a short distance from triplex of the leaves. Cryptostomates are scattered on the surface of the blade. Pedunculate vesicles are ovate or spherical with a diameter of 1.5 - 3 mm. These are tipped with spinose or thin leaf-like extensions and with few cryptostomates. Vesicles may be solitary or may form clusters attached to the primary or secondary branches and are more numerous but smaller in mature thalli (Ang, 1986) (Figure 1.).

Figure 1. *Sargassum polycystum*
Source: Lewmanomont, (1995)

1.4.2 ***Turbinaria ornata***

Turbinaria ornate, a common tropical brown algae, is widely distributed from the Indo-Pacific through the Caribbean (Ang, 1986). The erect thalli are attached to rocks by discoid holdfasts. The blades are coarse subpyramidal to turbinate with their centers concave and usually with vesicles (pneumatocysts) encircled by rows of coarse teeth. *T. ornata* shows morphological plasticity depending on the degree of wave exposure. Pneumatocyst production increased in their blades when plants were transplanted to a sheltered area (Stewart, 2004). *T. ornata* has invaded large areas on the intertidal and shallow subtidal shores in French Polynesia and Hawaii (Stiger and Payri, 1999; Smith *et al.,* 2002). It is also common and abundant on the shore at Samui Island (Mayakul and Prathep, 2005) and Koh Pling, Sirinart Marine National Park, Phuket Island, Southern Thailand (Prathep, 2005), where it occurs over a wide range from sheltered to very exposed shores and from the intertidal to shallow subtidal zones (Figure 2.).

Figure 2. *Turbinaria ornata*
Source: Lewmanomont, (1995)

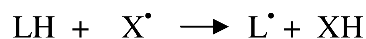
2. Lipid oxidation

Lipid oxidation is one of the major causes of food spoilage. It is of great economic concern to the food industry because it leads to the development of off flavors and off odors in edible oils and fat-containing foods. This renders these foods less acceptable. In addition, oxidative reactions can decrease the nutritional quality of food and certain oxidation products are potentially toxic (Fennema, 1996).

The lipid oxidation proceeds in three distinct steps (Gordon, 2001).

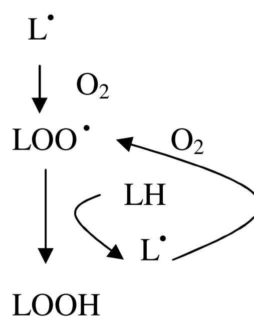
2.1 Initiation

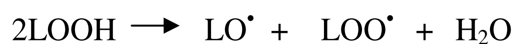
Lipid radicals are formed from lipid molecules. Abstraction of a hydrogen atom by a reactive species such as a hydroxyl radical may lead to initiation of lipid oxidation.

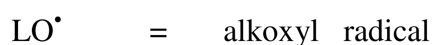


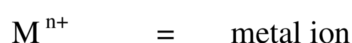
2.2 Propagation

Propagation reaction involves the process in which one lipid radical is converted into a different lipid radical. These reactions commonly involve abstraction of a hydrogen atom from a lipid molecule or addition of oxygen to an alkyl radical. The enthalpy of reaction is relatively low, compared with that of the initiation reactions. Therefore propagation reactions occur more rapidly, compared with initiation reactions.



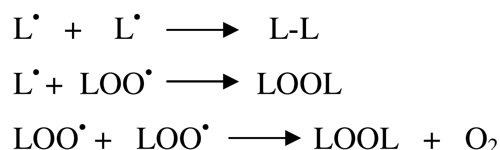






2.3 Termination

Free radicals combine to form molecules with a full complement of electrons with low energy reactions. The reactions are limited by the low concentration of radicals and by the requirement for radicals with the correct orientation for reaction to collide.



$$L^{\bullet} + L^{\bullet} \longrightarrow L\text{-}L$$
$$L^{\bullet} + LOO^{\bullet} \longrightarrow LOOL$$
$$LOO^{\bullet} + LOO^{\bullet} \longrightarrow LOOL + O_2$$

3. Antioxidants

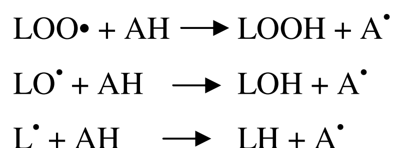
3.1 Antioxidants and their mode of action

Antioxidative can function in food systems by different modes of action, in which the initiation and propagation steps are retarded or terminated. Functions of antioxidants are shown as follows:

3.1.1 Radical scavenger

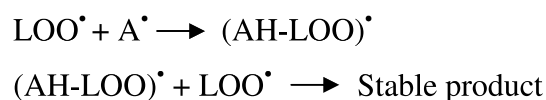
Antioxidants can retard or inhibit lipid oxidation by inactivating or scavenging free radicals, thus inhibiting initiation and propagation reactions. Free radical scavengers or chain-breaking antioxidants are capable of accepting a radical from oxidizing lipids species such as peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals to form stable end products (Decker, 1998; Akoh and Min, 1998). Antioxidants can scavenge free radical either as hydrogen donors or as electron donors, which form charge-transfer complexes (Namiki, 1990; Osawa, 1994).

Hydrogen donor



$$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet}$$
$$LO^{\bullet} + AH \longrightarrow LOH + A^{\bullet}$$
$$L^{\bullet} + AH \longrightarrow LH + A^{\bullet}$$

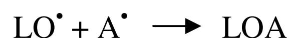
Electron donor



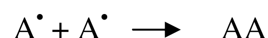
$$LOO^{\bullet} + A^{\bullet} \longrightarrow (AH\text{-}LOO)^{\bullet}$$
$$(AH\text{-}LOO)^{\bullet} + LOO^{\bullet} \longrightarrow \text{Stable product}$$

The free antioxidant radicals ($A^{\bullet}$) may undergo additional reactions that remove radical from the system. Termination reactions of antioxidant with other free antioxidant radicals or lipid radicals can form nonradical species.

$$LOO^{\bullet} + A^{\bullet} \longrightarrow LOOA$$



$$LO^{\bullet} + A^{\bullet} \longrightarrow LOA$$



$$A^{\bullet} + A^{\bullet} \longrightarrow AA$$

3.1.2 Peroxide decomposer

Some phenols, amine, dithiopropionic acid and thiopropionic acid function by decomposing the lipid peroxide into stable end products such as alcohol, ketone and aldehyde (Dziezak, 1986; Namiki, 1990).

3.1.2.1 Singlet oxygen quenchers

Singlet oxygen is generated from the triplet state oxygen. The mechanism of converting triplet oxygen to singlet oxygen is initiated by the transfer of the photosensitizer to its electronically excited state due to the absorption of light in the visible or near-UV region. Subsequently, the photosensitizer is able to transfer its excess energy to an oxygen molecule, giving rise to singlet oxygen (Shahidi and Wanasundara, 1992). Thus, the singlet oxygen can react with a lipid molecule to yield a hydroperoxide. Singlet oxygen reacts about 1,000-10,000 times as fast as normal oxygen with methyl linoleates (Jadhav *et al.*, 1996). Lipid oxidation initiated by xanthine oxidase can be inhibited by β-carotene because of its ability to quence singlet oxygen (Rajalakshmi and Narasimhan, 1996; Namiki, 1990). The Maillard reaction derived from xylose-lysine, tryptophan-glucose and glucose-glycine model systems had a high scavenging effect on active oxygen (Tanaka *et al.*, 1992; Yen and Hsieh, 1995; Yoshimura *et al.*, 1997).

3.1.2.2 Lipoxygenase inhibitor

Lipoxygenase is a non-heme iron-containing enzyme that catalyzes the oxygenation of the 1,4-pentadiene sequence of polyunsaturated fatty acid to produce their corresponding hydroperoxide (Salas *et al.*, 1999). Free-radical intermediates occur during lipoxygenase catalysis, and these can lead to cooxidation of easily oxidized compounds, e.g. carotenoids and polyphenols (Rajalakshmi and Narasimhan, 1996).

3.1.3 Synergists

3.1.3.1 Chelating agents

Chelating agents are not antioxidants; however, they play a valuable role in stabilizing foods. Chelating agents that improve the shelf-life of lipid containing food are EDTA, citric acid and phosphoric acid derivatives (Jadhav *et al*., 1996). Chelating agents form stable complexes with prooxidant metals such as iron and copper. Chelating agents bind metal ions and forms sigma bonds with a metal. An unshared pair electron in their molecule structure promotes the chelating action (Dziezak, 1986; Rajalakshmi and Narasimhan, 1996; Jadhav *et al*., 1996).

3.1.3.2 Reducing agents or oxygen scavengers

Reducing agents or oxygen scavengers function by various mechanisms. They may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant or react with free oxygen and remove it in a closed system. (Giese, 1996; Rajalakshmi and Narasimhan, 1996). Ascorbic acid is a strong reducing agent, readily losing H^+ to become dehydroascorbic acid, which also has vitamin C activity. However, vitamin C activity is lost, when the lactone ring of dehydroascorbic acid is hydrolyzed to yield diketogluconic acid (Gordon, 2001).

3.2 Antioxidants from plant extracts

In recent years, research has focused on natural extract to replace synthetic additives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) that might be carcinogenic (Whysner *et al.,* 1994) and even toxic (Moure *et al.,* 2001). Plants including herbs and spices have many phytochemicals, which are potential sources of natural antioxidants, e.g. phenolic diterpenes, flavonoids, tannins and phenolic acids (Dawidowicz *et al.,* 2006). These compounds have antioxidant, anti-inflammatory, anticancer and antimicrobial activities (Lee *et al.,* 2004).

Phenolic substances including monophenols with a single benzene ring, such as 3-ethylphenol and 3, 4-dimethylphenol are found in fruits and seeds. Hydroxycinnamic acid group involves cafeic and ferulic acid. Flavonoids and their glycosides which include catechins, proanthocyanins, anthocyanidins and flavonols are common in plants (Johnson, 2001). The overall effectiveness of the natural antioxidant is dependent on the involvement of the phenolic hydrogen in the radical reactions, the stability of the natural antioxidant radical formed during radical

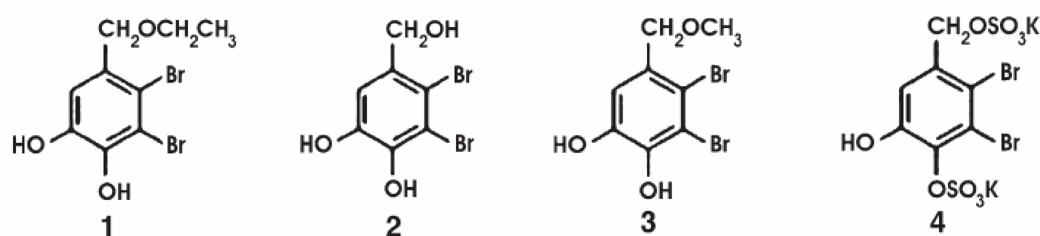
reactions, and chemical substitutions present on the structure (Huang *et al.,* 2004). The electron-donating capacity of methyl, ethyl and tertiary butyl substitutions at positions *ortho* and *para* to the hydroxyl groups greatly enhances the antioxidant oxidation activity of phenolic compounds (Pokorny *et al*. 2001).

The major phenolics present in tea (*Camellia sinensis*) leaves are flavan-3-ols, which constitute up to 30% of their dry weight. Depending on the steriochemical configuration of the 30, 40-dihydroxyphenyl and hydroxyl groups at the 2- and 3-positions of the C-ring, catechins can exist as two isomers: trans-catechins and cis-epicatechins. Catechin can be modified by esterification with gallic acid to form (-)-catechin-3-gallate, (-)-epicatechin-3-gallate, (-)-epigallocatechin-3-gallate and (-)-gallocatechin-3-gallate (Friedman *et al.,* 2005). Other related compounds found in tea are gallic, coumaric and caffeic acids, as well as the purine alkaloids, theobromine and caffeine (Surak and Singh, 2006). Mayachiew and Devahastin (2008) reported that ethanolic Indian gooseberry and galangal extracts consisted of 1,8-cineole, β-caryophyllene, β-bisabolene and β-selinene, whereas α-selinene, farnesene, 1,2-benzenedicarboxylic acid, germacrene B and pentadecane were the minor components. Those phenolic compounds possessed antibacterial (*Staphylococcus aureus*) and antioxidant (β-carotene bleaching method) activities. Ethanolic and water extracts of *Smilax excelsa* L. leaves that used for consumption in the daily diet in Black Sea region of Turkey contained high levels of total phenolic compounds and showed scavenging activities against DPPH, superoxide radicals and hydrogen peroxide (Ozsoy *et al.*, 2008). Liang *et al.* (2005) reported that polyphenolic compounds from guava leaves were gallic acid, quercetin, procatechuic acid, chlorogenic acid, caffeic acid, kaempferol and ferulic acid. These compounds were supposed to play an important role in the antioxidant activity of guava leaves. Phenolic contents and free radical scavenging activity in guava leaves had the low correlation, indicating that not only the phenolic compounds were involved in the antioxidant activity (Tachakittirungrod *et al.,* 2007). Moreover, Maisuthisakul *et al.* (2008) reported that flavonoid and phenolic compounds were the major components of 28 Thai plants, which exhibited high DPPH radical scavenging activity. Phenolic compounds of the leaves of *Mallotus japonicus* included mallotinic acid, mallotusinic acid, corilagin, geraniin, rutin and ellagic acid. These active compounds were subjected to DPPH, superoxide and hydroxyl radical scavenging activities.

Furthermore, *M. japonicus* exhibited the higher DPPH-radical scavenging activity than did green tea, rooibos tea and red wine. (Tabata *et al.,* 2008).

3.3 Antioxidative activity of seaweed extracts

Seaweeds are considered to be a rich source of antioxidants (Cahyana *et al.,* 1992). The potential antioxidant compounds were identified as some pigments (fucoxanthin, astaxanthin, carotenoid e.g.) and polyphenols (phenolic acid, flavonoid, tannins e.g.) (Yoshie and Suzuki, 2000). Those compounds are widely distributed in plants or seaweeds and are known to exhibit antioxidative activities via reactive oxygen species scavenging activity and the inhibition of lipid peroxidation (Yan *et al.,* 1999; Athukorala *et al.*, 2003; Heo *et al.,* 2005; Siriwardhana *et al.,* 2003). Additionally, active antioxidant compounds were identified as fucoxanthin in *Hijikia fusiformis* (Yan *et al.,* 1999) and phlorotannins in *Sargassum kjellamanianum* (Yan *et al.,* 1996). Flodin and Whitfield (1999) found phenol, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenyllactic acid, 4-hydroxybenzaldehyde, 3,5-dibromo-4-hydroxybenzoic acid and 2,4,6-tribromophenol in *Ulva lactuca*. Barreto and Meyer (2006) identified lanoaol and lanosol derivertives in red seaweed (*Osmundaria serrata*) and their structure was shown in Figure 3. Thunbergols A , thunbergols B and tetraprenyltoluquinol have been isolated from the brown alga *Sargassum thunbergii*. These compounds exhibited DPPH radical scavenging activity (Seo *et al*., 2006).



lanosol **lanosol-1-methyl ether** **lanosol-1-ethyl ether** **lanosol 1,4-disulfate ester**

Figure 3. Structures of lanosol and lanosol derivertive in red seaweed (*Osmundaria serrata*)

Source: Barreto and Meyer (2006)

When seven species of brown seaweeds (*Ecklonia cava, Ishige okamura, Sargassum fullvelum, S. horneri, S. coreanum, S. thumbergii* and *Scytosipon lomentaria*) were enzymatically hydrolyzed by five carbohydrate degrading enzymes (AMG, Celluclast, Termamyl, Ultraflo and Viscozyme) and five proteases (Alcalase, Flavourzyme, Kojizyme, Neutrase, Protamex), *S. horneri* hydrolyzed by Ultraflo and Alcalase at 2 mg/ml exhibited the highest radical scavenging activity, superoxide anion scavenging activity, hydroxyl radical scavenging activity and hydrogen peroxide scavenging activity and the activities were higher than BHA, BHT and α-tocopherol at the same concentration (Heo *et al.,* 2005). Moreover, Nagai and Yukimoto (2003) prepared beverages from four seaweeds (*Undaria pinnatifida, Ecklonia cava, Hizikia fusifome* and *Ulva pertusa*). The beverages possessed high antioxidative activities by autoxidation test, super anion radical, DPPH radical and hydroxyl radical scavenging tests. Li *et al.* (2007) prepared the extracts of 23 microalgae using hexane, ethyl acetate and water by a three-step sequential procedure. *Synechococcus sp*., *Chlamydomonas nivalis* and *Nostoc ellipsosporum* with hexane extraction had the highest total phenolic content and thus possessed the greatest antioxidant activities. Generally, the correlation coefficients of phenolic content and antioxidant activities of three microalgae were very small. Thus, phenolic compounds were not a major contributor to the antioxidant capacities of these microalgae. Chen (1996) and Chen *et al.* (2005) found that apart from phenolic compounds from brown seaweed, carotenoids and polysaccharides could exhibit the antioxidative activity.

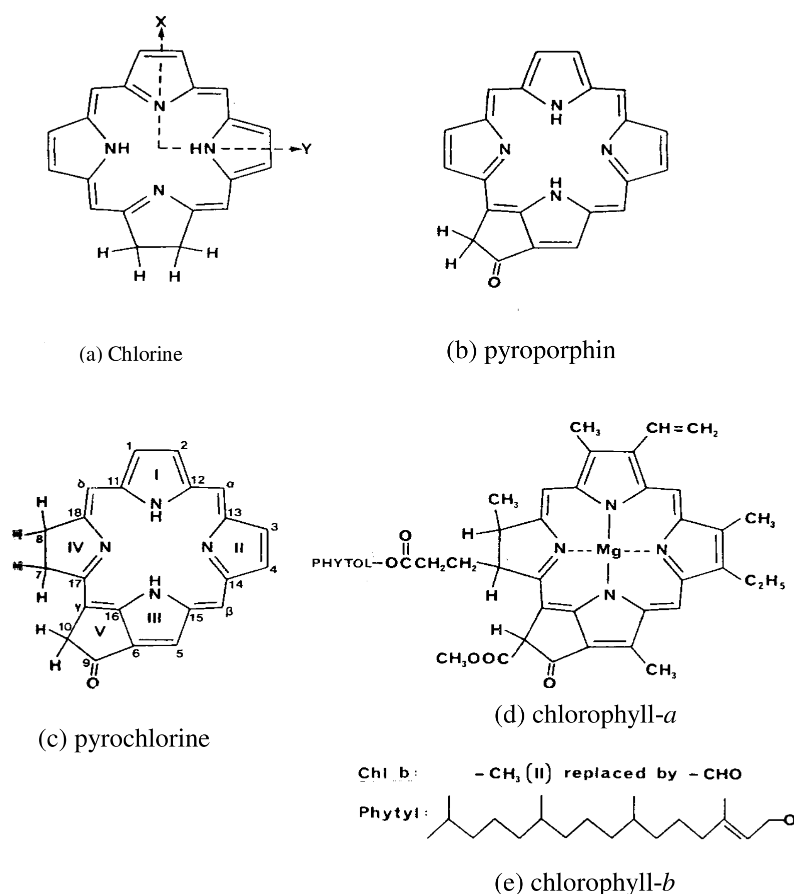
3.4 Chlorophyll

The chlorophylls are porphyrins complexed to magnesium. A few features of the chlorophylls distinguish them as a unique class of compounds from the nonphotosynthetic porphyrins. The characteristics of chlorophylls are summarized as follows (Wong, 1989):

3.4.1 The major chlorophylls (Chl *a* and Chl *b*) contain a reduced pyrrole ring. The porphyrin form with reduction on the pheriphery of one of the pyrrole rings is dihydroporphin (or chlorine).

3.4.2 Chlorophylls contain an alicyclic ring with a kero carbonyl and a carbomethyoxy group at C-9 and C-10, respectively. This structural feature is the basis for classification of compounds as chlorophyll. The porphin form with a cyclopentanone ring is designated as pyroporphin (Figure 4), and the corresponding chlorin form is pyrrochlorine.

3.4.3 Both Chl *a* and Chl *b* have a long-chain alcohol (phytol) esterified to the propionic acid side chains at C-7.



(a) Chlorine

(b) pyroporphin

(c) pyrochlorine

(d) chlorophyll-*a*

(e) chlorophyll-*b*

Figure 4. Basic structures of chlorophyll
Source: Wong (1989)

Chlorophylls and their derivatives are instable. Pro-oxidant activity of chlorophylls under light could be understood as a transfer of the energy of singlet-excited chlorophyll to oxygen that would form reactive oxygen species. Wanasundara and Shahidi (1998) indicated that aqueous ethanol extracts of green tea have a pro-

oxidant effect in edible marine oils (seal blubber oil and menhaden oil), perhaps due to the catalytic effect of their chlorophyll.

However, the some authors also reported that chlorophylls and pheophytins provide protection by preventing autoxidation of vegetable edible oils stored in the dark and suggested a hydrogen donating mechanism breaking the radical chain reactions. In addition, they stated that the intact chemical structure of porphyrin seems to be essential for antioxidant activity (Endo *et al.,* 1985; Usuki *et al.,* 1984). Hoshina *et al.* (1998) found that chlorophylls were better antioxidants than their metal free derivatives, which confirmed the importance of the porphyrin ring on the inhibition of lipid autoxidation. Sakata *et al.* (1992) reported that a related compound to pheophorbide *a*, isolated from clam, appeared to be the most important antioxidant in the organism. Ferruzzi *et al.* (2002) reported in *vitro* antioxidant and antimutagenic activities of water and lipid soluble chlorophyll derivatives.

4. Antimicrobials

4.1 Antimicrobials from plant extracts

Plant extracts have been reported to possess antimicrobial activity, mainly from phenolic compounds or essential oil. Antimicrobial activity of phenolic compounds can be classified into one or more following groups (Davidson, 1993):

4.1.1 Reaction with the cell membrane: Polyphenol attacked the cytoplasmic membrane of microorganisms, causing the release of cytoplasmic constituents. These compounds caused physical damage to the membrane or permeability barrier (Reed, 1995; Cowan, 1999)

4.1.2 Inactivation of essential enzymes: Polyphenol at high concentrations precipitated all proteins but selectively inhibited essential enzymes at lower concentrations (Prindle, 1983). Chipley (1974) found that 0.1 mM 2,4-dinitrophenol (DNP) noncompetitively inhibited enzymes associated with the cell envelopes of both *E. coli* and *S. enteritidis*. A conformational change in the membranes of the vesicle resulted in inhibition of the enzymes and that there was no direct effect of DNP.

4.1.3 Destruction or function inactivation of genetic material: Little work has been done on the effects of polyphenols on the genetic function of microbial cells. Surak and Singh (2006) reported that BHA and TBHQ inhibited the uptake and therefore synthesis of DNA, RNA, and protein in *Tetrahymena pyriformis*.

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). Prindle (1983) reported that antimicrobial activity of simple phenols; n-alkyl chains increased, depending on their chain length. Gram-negative bacteria are less susceptible to alkyl chains of more than five carbons. The position of the alkyl chain may or may not influence activity; n-alkyl chains are more effective than branched chains; polyhydroxy derivatives are generally less effective than monohydroxy compounds and separation of the alkyl group from the phenol nucleus by an oxygen (e.g., methoxy) decreases activity. Chan *et al.* (2007) reported that methanolic extract from fresh leaves of five *Etlingera* species inhibited Gram-positive bacteria (*Staphylococcus aureus*) as determined by the disc-diffusion method, but showed no antimicrobial activity on Gram-negative bacteria including *Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella cholerasuis.* Gram-negative bacteria have an outer membrane consisting of lipoprotein and lipopolysaccharide, which is selectively permeable and thus regulates access to the underlying structures (Chopra and Greenwood, 2001). This renders the Gram-negative bacteria generally less susceptible to plant extracts than the Gram-positive bacteria. Additionally, Williams and Harborne. (1977) reported that flaonoids were the major components in the leave of *Etlingera*, which were identified to be kaempferol 3-glucuronide, quercetin 3-glucuronide, quercetin 3-glucoside, and quercetin 3-rhamnoside. Moreover, Chiang *et al.* (2004) identified the bioactive components from *Bidens pilosa,* which widely used in Taiwan as a traditional medicine, as phenylpropanoid glucosides, polyacetylenes, diterpenes, flavonoids, and flavone glycosides. These compounds exhibited antimicrobial and antifungal activities through the disruption of bacterial and fungal membrane integrity (Rabe and Standen, 1997; Knobloch *et al.* 1989). The essential oils from the leaves and root of medicine plants extracts commonly used in Brazil (*Aloysia triphylla*, *Anthemis nobilis*, *Cymbopogon martini*, *Cymbopogon winterianus*, *Cyperus articulatus*, *Cyperus rotundus*, *Lippia alba*, *Mentha arvensis*, *Mikania glomerata*, *Mentha*

piperita, *Mentha* sp., *Stachys byzantina*, and *Solidago chilensis*) exhibited anti-*Candida* activity. Chemical analyses showed the presence of 1,8-cineole, geranial, germacrene-d, limonene, linalool, and menthol (Duarte *et al.,* 2005). Ndi *et al.* (2007) reported a search for antimicrobial compounds of diethyl ether extract from the leaves of *Eremophila serrulata* led to the isolation of two compounds, an o-naphthoquinone and a serrulatane diterpenoid. These compounds showed antimicrobial activity against Gram-positive bacteria (*Staphylococcus aureus, Streptococcus pyogenes* and *Streptococcus pneumoniae*), whereas no activity was observed for against in Gram-nagative bacteria tested.

4.2 Antimicrobial activity of seaweed extracts

The extracts from seaweed have been reported to exhibit the antimicrobial activity and can be used as natural antimicrobial agent. Bennamara *et al* (1999) reported that methoxybifurcarenone from brown algae (*Cystoseira tamariscifolias*) possessed antifungal activity (*Botrylis cinerea, Fusarium oxysporum* and *Verticallium alboatrum*) and antibacterial activity (*Agrobacterium tumefaciens* and *Escherichia coli*). Barreto and Meyer (2006) found that ethyl ether from red seaweed (*Osmundaria serrata*) exhibited bactericidal and fungicidal activity toward *Halomonas halophila, H. marina, Halomonas* sp., *Marinococcus* sp., *Pseudomonas* sp., *Vibrio alginolyticus, V. harveyi, Bacillus cereus, B. pumilus, B. subtilis, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Propionibacterium acnes. Salmonella typhimurium, Serratia marcescen* and *Staphylococcus aureus*. Additionally, methanolic extract of brown seaweed (*Stoechospermum marginatum*) showed bacteriacidal (Escherichia *coli, Psudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi, Shigella flexineri, Klebsiella* sp and *Vibrio cholerae*), whereas the extract of green seaweed (*Cladophora prolifera*) exhibited both bacteriacidal and fungicidal (*Aspergillus funigatus, Fusarium* sp., *Cryptococcus neoformans, Aspergillusniger, Rhodotorula* sp., *Norcardia* sp. and *Candida albicans*) activities (Ely *et al.,* 2004). Yang *et al.* (2006) isolated lasiodiplodin and the derivatives from the mycelium extracts of brown algae. These compounds exhibited the inhibitory activities toward *S.aureus, B. subtilis* and *Fusarium oxysporun* when minimal inhibitory concentrations at levels of 25, 50 and 100 µg/ml were used, respectively.

5. Gelatin

Gelatin is a protein derived from collagen, in which the source and type will influence the properties of the resulting gelatin. The most abundant current source of gelatin is from mammals, especially bovine and pork skin and bone. Because of the outbreak of bovine spongiform encephalopathy (BSE) and the food-and-mouth disease (FMD) crisis have resulted in anxiety among users from land-based animal gelatins (Helcke, 2000). In addition, gelatin produced from porcine skin or bone cannot be used for some foods due to esthetic and religious objections (Sadowska *et al.,* 2003). Furthermore, the increases in consumer need for kosher and halal foods have gained a demand for fish gelatin. Therefore, alternative sources, especially fish processing byproducts including skin, bone or scale, have been paid increasing attention for gelatin extraction. About 30% of such byproducts consists of skin and bone with high collagen content (Patil *et al*., 2000). Collagen is the fibrous protein that contributes to the unique physiological functions of connective tissues in skin, tendons, bones, cartilage, etc. The structural unit of collagen is tropocollagen. It is a rod-shaped protein (15 Å diameter, 3000 Å length) consisting of three polypeptide units (called α chains) intertwine to form a triple-helical structure. Each α chain coils in a left-handed helix with three residues per turn, and the three chains are twisted right-handed to form the triple helix (Wong, 1989). Collagen with different compositions from varying species is associated with the temperature of animals living habitat (Jongjareonrak *et al.,* 2006). In general, fish collagens have lower amino acid contents (from about 16% to 18%) than mammalian collagens (near 24%) and this may be the reason for the lower temperature of denaturation (Grossman and Bergman, 1992).

Gelatin is derived from denatured collagen and its properties and gelling abilities are dependent on various parameters (Johnston-Bank, 1990; Ledward, 1986). Thus, the use of fish skin for gelatin production has to take into accounts at least two different aspects. First, the wide diversity among the fish species, that present intrinsic differences in the collagen molecules present in their skin. Second, the higher susceptibility of the collagenous material from fish skin to degradation due to the lower content in intra- and interchain non-reducible crosslinks (Norland, 1990; Montero *et al.,* 1990). Furthermore, the thermal stability of collagen is related to the

content of imino acids (proline and hydroxyproline). The higher imino acid content, the more stable the helices. Collagen denatures at temperatures above 40°C to a mixture of random-coil single, double, and triples strands. Upon controlled cooling below the melting temperature, T_m, reformation of the helical form occurs (Wong, 1989). The yield and properties of gelatin depend on the kind of raw material, pre-treatment and process used (Gómez-Guillén *et al.*, 2005; Gómez-Guillén *et al.*, 2002; Gudmundsson and Hafsteinsson, 1997; Kolodziejska *et al.*, 2004a). Kolodziejska *et al.* (2004) reported that heating at 45°C for 15 min was the optimal conditions of fish skin (salmon and herring) gelatin extraction. However, increasing extraction temperature (70°C) and time (45-60 min) did not affect the yield of gelatin. The optimum operating condition for extracting the gelatin from channel catfish skin were 43.2°C for 5.73 h (Liu *et al.,* 2008). When pre-cooked tuna caudal fin was extracted for gelatin, the yield of only 1.99% on the basis of dry weight was obtained. This was because some collagen underwent denaturation and soluble gelatin could be leached out during pre-cooked (Aewsiri *et al.,* 2008). Yang *et al.* (2008) extracted gelatin from channel catfish (*Ictalurus punctatus*) skins pretreated with acetic acid (0.1 M for 180 min) and sodium hydroxide (0.1 M for 90 min). Acid pretreatment groups showed the higher gel strength and yield. Acid or alkali facilitated the fragmentation of the collagen chain, but any collagen that dissolved in the pretreatment solution resulted in a corresponding loss in gelatin yield (Yoshimura *et al*., 2000). Fernández-Díaz *et al.* (2003) investigated protein pattern of gelatin of founder skins, stored under different conditions (-12 and -20°C), compared with fresh skins. Gelatin from fresh skins showed some high molecular weight aggregates and bands corresponding to α, β and γ-components, whereas, gelatin from frozen skins showed less α and β-chains but more bands corresponding to higher molecular weight fragments; γ-components, especially in the case of skins frozen at -12°C. The molecular weight of a gelatin is largely responsible for its gelling behaviour (Johnston-Banks, 1990). Therefore, gelatin from fresh skin had the higher gel strength, while frozen skin had the lower gel strength (Gilsenan and Ross-Murphy, 2000). Gómez-Guillén *et al.* (2002) reported that slight differences in amino acid composition of gelatin extract from different species in Table 1.

In general, all of gelatins resembled the composition of 30% glycine (Gly) and 17% imino acids (Pro+Hyp). Similarly, the amino acid profile of bigeye snapper (*Priacanthus hamrur*) skin gelatin revealed high proportion of glycine and imino acids and the protein content of the dried gelatin was 94.6% (Binsi *et al.,* 2009).

Table 1. Amino acid composition of gelatin from different fish species.

	No. residues/1000 residues				
	Sole	Megrim	Cod	Hake	Squid
Hyp	61	60	50	59	80
Asx	48	48	52	49	65
Thr	20	20	25	22	24
Ser	44	41	64	49	37
Glx	72	72	78	74	90
Pro	113	115	106	114	95
Gly	352	350	344	331	327
Ala	122	123	96	119	89
Val	17	18	18	19	21
Met	10	13	17	15	13
Ile	8	8	11	9	18
Leu	21	21	22	23	32
Tyr	3	3	3	4	6
Phe	14	14	16	15	10
His	8	8	8	10	8
Hyl	5	5	6	5	15
Lys	27	27	29	28	13
Arg	55	54	56	54	57
Imino acids (Pro+Hyp)	174	175	156	173	175
% Pro hydroxylation	35.05	34.29	32.05	34.10	45.65
% Lys hydroxylation	15.62	15.62	17.14	15.15	54.86

Source: Gómez-Guillén *et al.*, (2002)

5.1 Functional properties of gelatin

In general, there are two methods to obtain gelatin from skins and bones, an acid process (gelatin A with isoelectric point at pH 6-9) and an alkaline process (gelatin B with isoelectric points at pH 5) (Stansby, 1987). Chemicals used for pretreatment as well as extraction condition such as temperature and time can influence the length of polypeptide chains and the functional properties of the gelatin (Kolodziejska *et al.,* 2004). Nevertheless, ionic strengths and pH values have a decisive influence on the biophysical properties (swelling, solubility, gelation, viscosity and water-binding capacity) of a protein (Hermansson, 2007). Normally, the functional properties of gelatins depend on the raw material as well as their processing conditions (Bell, 1989). Gelatin from salmon and cod skin extracted with same condition had the different physical properties. The salmon gelatin had the slightly higher gel strength and melting point than did cod gelatin (Arnesen and Gildberg, 2007). Gelatin from salmon had slightly higher imino acid contents. This may promote a slow regeneration of helical structures, resulting in the considerable gel strengthening (Arnesen and Gildberg, 2007; Gómez-Guillén *et al.*, 2002). Generally, fish gelatin has lower gel strength than mammalian gelatin (Norland, 1987). Bloom strength of the sin croaker and shortfin scad gelatin was 124.94 and 176.92 g, which were lower than that of bovine gelatin (239.98 g) (Cheow *et al.,* 2007). Higher hydroxyproline content in bovine gelatin was associated with the formation of hydrogen bonds between water molecules and free hydroxyl groups of amino acids in gelatin, which were essential for gel strength (Arnesen and Gildberg, 2002). However, Cho *et al.* (2005) found the higher gel strength of gelatin from the dosal skin of yellowfin, which is a tropical fish (15-31°C), compared with bovine and pocine gelatin. The gelatin prepared from the skins of the Atlantic halibut (*Hippoglossus hippoglossus*) was used for preparation of edible films (Carvalho *et al.,* 2008). Two types of dry gelatin were obtained depending on whether evaporation step at 60ºC to a target concentration before spray drying. The other one was directly spray dried without previous concentration. The intermediate evaporation step at 60°C induced thermal protein degradation, causing the resulting films to be less resistant and more extensible. Different mechanical behaviors of the films were the result of slight differences in the molecular weight distribution of fractions of both types of

gelatin (Carvalho *et al.,* 2008).

6. Protein film properties

Generally, edible film is defined as a thin, continuous layer of edible material (Torres, 1997). The materials used are mainly the renewable sources such as proteins, carbohydrates and lipids. Among these materials, protein-based films are generally superior to polysaccharide-based films in their mechanical and barrier properties (Cuq *et al*. 1998). Development of biopolymer films and coating from proteins has received increasing interest (Gennadious *et al.,* 1994; Choi and Han, 2002; Bigi *et al.,* 2001; Shiku *et al.,* 2004). Two major promising applications of such films are the replacement of plastic in food packaging and uses as edible films (Cao and Chang, 2001). Among all proteins, gelatin has been attracted the attention for the development of edible films due to its abundance and biodegradability (Bigi *et al.* 2002).

6.1 Barrier properties

Protein based films, as a function of their hydrophilic characteristics, present an effective barrier to gases (CO_2 and O_2) at low to intermediate relative humidity. However, high permeability to water vapor limits their application as a packaging material (McHugh and Krochta, 1994). Normally, the hydroxyl group of the polar macromolecules (proteins and polysaccharides) binds to water via hydrogen bonds, resulting in plasticizing of the polymeric matrix and altering its mechanical and barrier properties. The chemical nature of the macromolecule, structural/morphological characteristics of the polymeric matrix, chemical nature of the additives and degree of crosslinking, affect the barrier characteristics (Kester and Fennema, 1986; McHugh and Krochta, 1994; Stuchell and Krochta, 1994). The significant differences in WVP of bovine gelatin film and modified film indicate structural changes in the polymeric matrix as a function of the introduction of cross-linking that might have influenced the moisture diffusion coefficient within the gelatin network. The incorporation of plasticizer results in an increase in the free volume of the system, favoring solvent mobility and provoking an increase in water diffusion in the polymeric matrix (Sobral *et al.,* 2001), thus decreasing the barrier properties. The decrease in permeability could also be related to the decrease in free volume due to reticulation of the polymeric matrix. Film modified using

transglutaminase (TGase) presented a significant decrease in permeability as compared to both the native film and those modified chemically using glyoxal or formaldehyde (Table 2) (de Carvalho and Grosso, 2004). In general, films or coatings containing hydroxyl, ester or other polar groups tend to present low oxygen permeability (Torres, 1994). Many edible films are highly sensible to moisture, but due to the high degree of hydrophilic properties, present an excellent barrier to oxygen and to some aromatic components (Miller and Krochta, 1997). Normally, an increase in crystallinity, orientation, molecular mass or degree of cross-linking result in a decrease in permeability (Miller and Krochta, 1997).

Table 2. Barrier properties, mechanical properties and soluble matter of gelatin films.

Film	Water vapor permeability ($g.mm/m^2.h.kPa$)	TS (MPa)	EAB (%)	soluble matter (%)
NF	0.198 ± 0.003	15.12 ± 0.75	39.24 ± 0.80	31.08 ± 0.35
EMF	0.120 ± 0.001	14.63 ± 0.34	33.21 ± 2.53	25.51 ± 0.29
FMF	0.155 ± 0.008	23.10 ± 1.31	37.70 ± 1.94	25.76 ± 0.11
GMF	0.174 ± 0.007	14.97 ± 0.52	38.13 ± 0.44	24.27 ± 0.06

NF: native film, EMF: enzyme modified film, FMF: formaldehyde modified film, GMF: glyoxal modified film

Source: Adapted from de Carvalho and Grosso (2004)

6.2 Mechanical properties

Mechanical properties of protein film are generally poorer than synthetic films (Cuq, 2002; Gennadios *et al.,* 1994). Several factors, including surface charges, hydrophobicity, polymer chain length, etc., may significantly affect the mechanical properties of protein films (Kester and Fennema, 1986). Hydrogen bonds are considered important in contributing to the tensile strength (TS) of protein films (Meier, 1990). Type and level of plasticizer have a dramatic effect on film properties (Shellhammer and Krochta, 1997; Cuq, 2002). Moreover, enhanced cross-linking increases TS and elongation at break (EAB) of gelatin film. de Carvalho and Grosso (2004) reported that formaldehyde modified film had the highest TS compose with

other films (Table 2). The introduction of cross-linkages apparently reduced the mobility of the matrix, impeding the renaturation of the gelatin into triple helix structures during film preparation, leading to a loss of orientation in the form of fibers of the polymeric matrix (Babin and Dickinson, 2001). Chambi and Grosso (2006) used TGase to produce cross-linked casein, gelatin and casein-gelatin blend (100:0, 75:25, 50:50, 25:75 and 0:100) edible films. Enzymatic cross-linking also induced a substantial increase in the high molecular weight protein components in the film forming solutions. The casein-gelatin film showed significant greater elongation values with TGase treatment.

6.3 Solubility

Film solubility is an important property that relates to intended use. High molecular weight proteins are insoluble or slightly soluble in water and thus have potential for forming water-resistant films (Cuq, 2002). Low molecular weight protein chains such as monomers and small peptides, formed during the film-forming solution and immobilized in the film network, could thus constitute the water-soluble proteinic component of the films (Cuq *et al.,* 1995). de Carvalho and Grosso (2004) reported that the cross-linking of gelatin film as a result of enzyme and chemical treatments could have led to a reduction in the low molecular weight fractions, thus decreasing the solubility of the films

6.4 Use of protein cross-linkers for the improvement of film properties

6.4.1 Chemical cross-linking

Gelatin-based films can be potentially modified via chemical, physical or enzymatic cross-linking (Gennadious and Weller, 1990; Guilbert *et al.,* 1996). Cross-linking agents are natural or synthetic molecules containing at least two reactive groups that are able to form covalent inter- and/or intra-molecular links between protein chains. These agents, when used to prepare protein-based films, can strengthen the material through formation of new covalent bonds, while reducing film elasticity and solubility in water (Gennadious and Weller, 1992). Genipin isolated from the fruits was an alternate cross-linker to cross-link gelatin (Liang *et al.,* 2003). The cross-linking of fish gelatin with genipin and glutaraldehyde determined their dynamic rheological properties during cross-linking process. Bigi *et al.* (2002) reported that stable pig skin gelatin films were obtained by cross-linking with genipin

solutions (0-2.0 wt%). The films treated with genipin at relative high concentration had 15% of free ε- amino groups retained (Bigi *et al.,* 2001). Both Alaska pollock and Alaska pink salmon skin gelatins containing genipin showed faster cross-linking rates for samples with higher pH values (Chiou *et al.,* 2006). Cao *et al.* (2007) studied gelatin film cross-linked by ferulic and tannin acid obtained from plant at various concentrations (0-40 mg cross-linking agents/g gelatin) and various pHs (7-9). When the pH value of film-forming solution was 7, film cross-linked with ferulic acid had the maximum mechanical strength. For tannin acid-treated film, the maximum value was obtained at pH value of 9.

Strass and Gibson (2004) reported that protein cross-linking induced by phenolic compounds, resulting in the formation of rigid molecular structures, was via the reactions of ortho-quinones with proteins as shown in Figure 5. The diphenol moiety of a phenolic acid or other polyphenol (1) is readily oxidized to an orthoquinone, either enzymatically as in plant tissues, or by molecular oxygen. The quinone forms a dimer (2) in a side reaction, or reacts with amino or sulfhydryl side chains of polypeptides to form covalent C–N or C–S bonds with the phenolic ring, with regeneration of hydroquinone. The latter can be reoxidized and bind a second polypeptide, resulting in a cross-link (3). Alternatively, two quinones, each carrying one chain, can dimerize, also producing a cross-link (4). Moreover, the combination of polyphenol-protein was the double functionals of hydrophobic bonds and hydrogen bonds. Firstly, polyphenol, which contained hydrophobic groups such as galloyl group entered into hydrophobic district of protein by hydrophobic reaction. Then phenolic hydroxyl group of polyphenol combined with polar group of protein by hydrogen bond (Shi and Di, 2000).

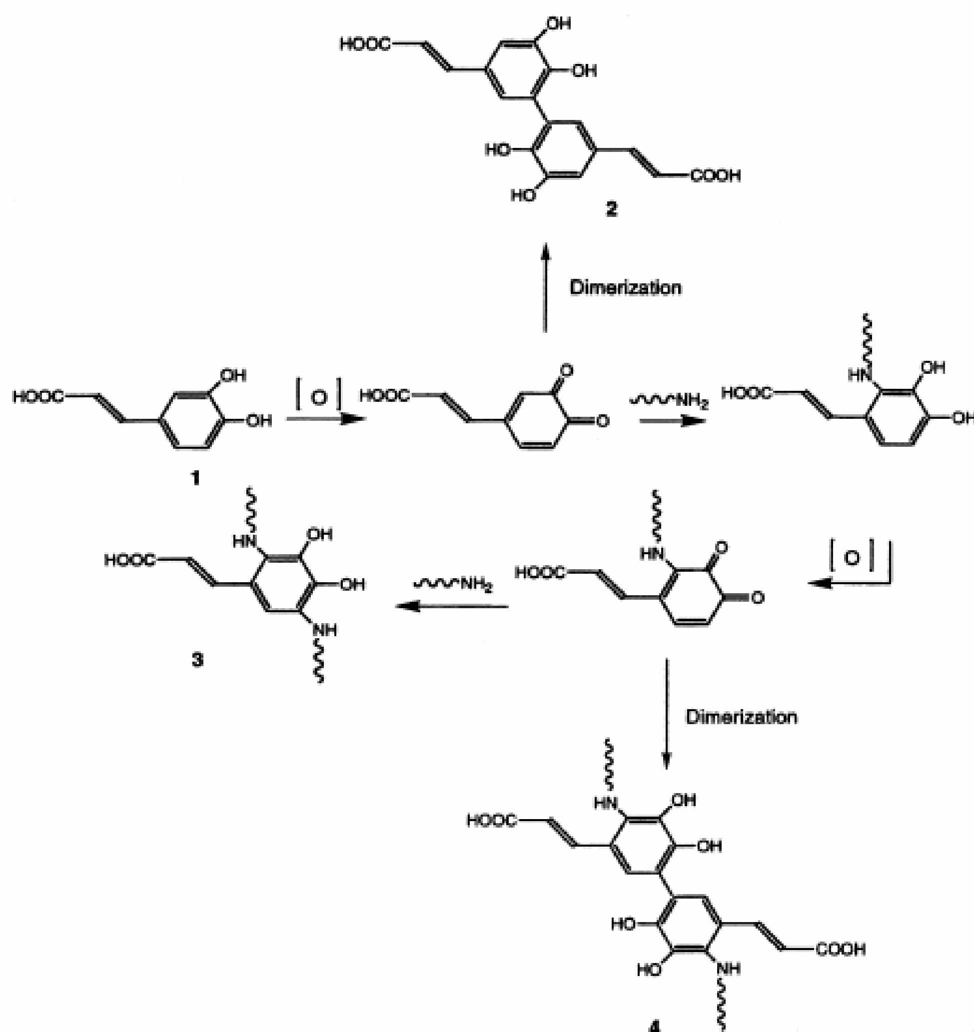


Figure 5. Reactions of a phenolic acid with amino side chains of polypeptides
Source: Strass and Gibson (2004)

Of chemical cross-linking agents, glutaraldehyde (GTA) has been used extensively because it has the advantage of being a fast-acting hardener for collageneous materials (Donohue *et al.,* 1983) and is especially used in gelatin reticulation in photographic films and in microcapsules produced by coaccervation, where the aldehyde chemically fixes the gelatin gel, improving its functional properties (Thies, 1995). Crossilnking of collagenous samples with GTA involves the reaction of free amino groups of lysine or hydroxylysine amino acid residues of the polypeptide chains with the aldehyde groups of glutaraldehyde (GTA) (Olde-Damink *et al.,* 1995). Bigi *et al.* (2001) investigated different degrees of GTA (0.05 to 2.5 wt %) for cross-linking pig skin gelatin film. In the range from 0.1 to 1

wt %, the extent of crosslinking increased from 60% to about 100%, determined by trinitrobenzensulfonic acid assay. Thermal property of uncrosslinked gelatin film exhibited an endothermic peak at 43ºC, while crosslinked gelatin film increased the thermalstability, as shown by the shift of the denaturation temperature to higher values.

6.4.2 Enzymatic cross-linking

Transglutaminase (TGase) catalyzed acyl-transfer reactions between β-carboxyamide groups of glutamine residues (acyl donor) and γ-amino groups of lysine residues (acyl acceptor), resulting in the formation of ε(γ-glutaminyl) lysine intra and intermolecular cross-linked proteins (Dejong and Koppelamn, 2002). TGase has been used to cross-link several protein molecules, such as caseinate (Bruno *et al*., 2005), whey protein (Di Pierro *et al*., 2006; Mahmoud and Savello, 1993; Oh *et al.,* 2004), soy protein (Mariniello *et al*., 2003), egg white and gelatin (Lim *et al.,* 1998, 1999). The increase in gel strength of gelatin subjected to the action of transglutaminase depended on the order and intensity by which the enzyme produced cross-links, and the extent to which these new covalent linkages could impede the physical cross-linkages occurring during renaturation and formation of the triple helix during gel formation (Babin and Dickinson, 2001). Piotrowska *et al.* (2008) reported that TGase cross-linking of Baltic cod skins effectively decreased film solubility in aqueous medium of different pHs at 25°C for 24 h, compared with unmodified film

Jongjareonrak *et al.* (2006) reported that the bloom strength of gelatin gel from bigeye snapper and brownstripe red snapper skins were increased with the addition of microbial transglutaminase (MTGase) at 0.005% and 0.01%, respectively. However, bloom strength decreased with further increase in MTGase concentration. Excessive formation of MTGase-catalyzed covalent bonds was detrimental to gel strength due to the formation of intramolecular covalent bonds. As a consequence, intermolecular aggregation might be impeded, leading to reduced gel network formation (Gómez-Guillén *et al*., 2001).

OBJECTIVES

1. To study antioxidative, antimicrobial and cross-linking activities toward fish skin gelatin of seaweed extracts.
2. To prepare and characterize gelatin-based films incorporated with seaweed extract.
3. To monitor the changes in properties of gelatin-based films incorporated with seaweed extract during storage.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

Collection and preparation of seaweed

Brown seaweeds, *Turbinaria ornata* and *Sargassum polycystum*, were collected freshly from Samui Island, Suradthanee Province, Thailand. Samples collected were washed thoroughly with tapwater, packed in polyethylene bag and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 5 h. Upon arrival, seaweeds were dried at 35°C for 24 h using an air-force oven. The dried seaweeds were powdered using a blender (National, MX-T2GN, Taipei, Taiwan). Then powder was sieved using a screen with a diameter of 0.5 mm. The seaweed powder was placed in the polyethylene bag and stored at 4°C until use.

Preparation of fish skin

Bigeye snapper (*Priacanthus tayenus*), off-load approximately 48 h after capture, were obtained from the dock in Songkhla, Thailand. Fish were stored in ice with a fish/ice ratio of 1:2 (w/w) and transported within 1 h to the Department of Food Technology, Prince of Songkla University, Hat Yai. Upon arrival, fish were washed with tap water. The skins were then removed, cut into small pieces (0.5x0.5 cm) and stored at -20°C until use.

2. Chemicals and enzyme

Sodium chloride (NaCl), urea and sodium dodecylsulfate (SDS) were purchased from Univar (Worksafe, Australia). Glycerol, α-chymotrypsin and Coomassie Brilliant Blue G250 were purchased from Wako Pure Chemical Industry, Ltd. (Tokyo, Japan). Methanol was obtained from Merk (Darmstadt, Germany). 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-l-picryl hydrazyl (DPPH), linoleic acid, *β*-mercaptoethanol (*β*-ME) and L-α-phosphatidylcholine (L-α-lecithin) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acrylamide, *N,N,N'N'*- tetramethylethylenediamine (TEMED), 2-thiobarbituric acid,

bis-acrylamide, Ferric chloride hexahydrate, 2-thiobarbituric acid, butylated hydroxyl toluene (BHT) and potassium persulfate were procured from Fluka Chemical Co. (Buchs, Swizerland)

3. Instruments

Instruments	Model	Company/Country
Refrigerated centrifuge	RC-5B plus	Sorvall, California, USA
pH meter	CG 842	Schott, Mainz, Germany
Homogenizer	model T25 basic	ULTRA TURREX®, Selangor Malaysia
Magnetic stirrer	Ro 15 power	IKA labortechnik, Stanfen, Germany
Electrophoresis apparatus	Mini-Protean II	Bio-Rad Laboratory int., California, USA
Double-beam spectrophotometer	UV-16001	Shimadzu, Kyoto, Japan
CIE colorimeter	Color Flex	HunterLab Reston, Virginia, USA
Differential scanning calorimeter	DSC 7	Perkin Elmer, Michigan, USA
Scanning Electron Microscope	JSM-5800 LV	JEOL, Tokyo, Japan
Vortex mixer	G-560E	Scientific Indrustries Inc., NY, USA
Oil bath	B-490	BUCHI, Flawil, Sweitzerland
Water bath	W350	Memmert, Schwabach, Germany
Microcentrifuge	MIKRO20	ZENTRIFUGEN,Hettich, Germany
Rotary evaporator	Rotavapor-R	Buchi, Flawil, Switzerland
Stirrer	RW 20.n	IKA LABORTECHNIK, Stanfen, Germany
Freeze dryer	Dura-Top™µp	FTS symtem, Inc., NY, USA

4. Methods

4.1 Extraction and characterization of seaweed extracts

4.1.1 Preparation of seaweed extracts

Seaweed powder (5g) was mixed with 150 ml of solvents, either methanol or ethanol following the method of Terada *et al.* (1987). The mixtures were homogenized at 10,000xg for 2 min using IKA LABOTECHNIK homogenizer (model T25 basic, ULTRA TURREX®, Selangor Malaysia). The homogenate was then stirred continuously at room temperature for 30 min. The mixtures were centrifuged at 5,000xg for 10 min at room temperature using a Sorvall Model RC-5B Plus refrigerated centrifuge (Newtown, CT, USA) to remove undissolved debris. A portion of extract was subjected to chlorophyll removal as per the method of Lanfer-Marquez *et al.* (2005). The extract was mixed with petroleum ether at a ratio of 5:2 (v/v) at room temperature. The extraction was repeated for 3 times. The layer of petroleum ether was drawn off. The solvent in the extracts without and with chlorophyll removal was removed by a rotary evaporator (Model Rotavapor-R, Brinkmann, Switzerland) at 40°C. The volume of evaporated extract was adjusted to 10 ml using the same solvent.

4.1.2 Chlorophyll content

Total chlorophyll content was determined spectrophotometrically according to the method of the AOAC (1990). Methanolic and ethanolic extracts of seaweed with and without chlorophyll removal prepared as previously described were dehydrated with anhydrous sodium sulfate. Immediately, the pigments were quantified spectrophotometrically at 660 and 642.50 nm. For the blank, methanol or ethanol were used instead of extracts. Total chlorophyll content (TCC) was calculated using the following equation:

$$TCC\ (\mu g/g\ \text{dry extract}) = 7.12\ (A_{660}) + 16.8(A_{642})$$

4.1.3 Total phenolic content

Total phenolic content was determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1997). One ml of seaweed extract was added with 200 µl of reagent (the mixture of Folin-Ciocalteu reagent and deionized water, 1:1 (v/v)) and mixed thoroughly. After 3 min, 3 ml of 2% Na_2CO_3

were added. The mixture was allowed to stand at room temperature for 30 min. The absorbance was measured at 760 nm using a UV-16001 spectrophotometer (Shimadzu, Kyoto, Japan). The concentration of total phenolic compounds in seaweed extract was calculated from the standard curve of catechin with the range of 0.01-0.1 mg/ml and expressed as mg catechin/g dry seaweed.

4.2 Study on antioxidative activity of seaweed extracts

Ethanol and methanol extract from both seaweeds without and with chlorophyll removal were subjected to determination of antioxidative activity.

4.2.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Wu *et al.* (2003) with a slight modification. Sample (1.5 ml) was added with 1.5 ml of 0.15 mM DPPH in 95% methanol. The mixture was then mixed vigorously and allowed to stand at room temperature in dark for 30 min. The absorbance of resulting solution was measured at 517 nm using an UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The control was prepared in the same manner except that the distilled water was used instead of sample. The sample blank was prepared by using ethanol or methanol instead of DPPH solution. The standard curve was prepared using Trolox in the range of 10-60 µM The activity was expressed as µmol Trolox equivalents (TE)/ml.

4.2.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined as per the method of Arnao *et al.* (2001) with a slight modification. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml of ABTS solution with 30 ml of methanol to obtain an absorbance of 1.1 ± 0.02 at 734 nm using an UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). ABTS solution was freshly prepared for each assay. Sample (150 µl) was mixed with 2.85 ml of ABTS solution and the mixture was left at room temperature for 2 h in dark. The sample blank was prepared by using methanol instead of ABTS solution. The absorbance was then measured at 734 nm using the spectrophotometer. The standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalent (TE)/ml.

4.2.3 Reducing power

Reducing power was determined according to the method of Wu *et al.* (2003) with a slight modification. Diluted sample (1 ml) was mixed with 1 ml of 0.2 M phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide. The mixtures were incubated at 50ºC for 20 min, followed by addition of 1 ml of 10% trichloroacetic acid. An aliquot (1 ml) of reaction mixture was added with 1 ml of distilled water and 200 µl of 0.1% $FeCl_3$. The absorbance of resulting solution was measured at 700 nm. Increased absorbance of the reaction mixture indicates the increased reducing power. The sample blank was prepared by using methanol instead of $FeCl_3$ solution.

4.2.4 Determination of antioxidative activity of seaweed extract in different systems

Methanolic extracts of *T. ornata* with and without chlorophyll removal at different concentrations were tested in different systems. BHT at levels of 50 and 200 mg/L was used for comparison purpose. For the control, methanol was added instead of the extract or BHT.

4.2.4.1 Lecithin liposome system

Antioxidative activity of seaweed extracts in lecithin liposome system was determined according to the method of Frankel *et al.* (1997). Lecithin was suspended in deionized water at a concentration of 8 mg/ml. The mixture was stirred with glass rod, followed by sonicating for 30 min using a sonicating bath (Transsonic 460/H, Elma, Germany). Seaweed extracts was mixed with lecithin liposome to obtain the final concentrations of 100, 200 and 500 mg/L. The liposome suspension was then sonicated for 2 min. To initiate the assay, 20 µl of 0.15 M cupric acetate were added. The mixture was shaken at 120 rpm using a shaker (UNIMAX 1010, Heidolph, Germany) at 37°C in dark for 0, 6, 12, 18, 24, 30, 42 and 48 h. Liposome oxidation was monitored by determining thiobarbituric acid-reactive substances (TBARS). TBARS values were calculated from the standard curve of (0 – 3 mg/L malonaldehyde (MDA)) and expressed as mg MDA/ml liposome.

4.2.4.2 Linoleic oxidation system

Antioxidative activity of seaweed extracts in linoleic oxidation system was tested as described by Sakanaka *et al.* (2004). Seaweed extracts were mixed with 10 ml of 50 mM linoleic acid in 99.5% ethanol to obtain the final concentrations of 100, 200 and 500 mg/l and the mixture was kept at 40ºC in dark.

During incubation, aliquots of the reaction mixtures were taken for measurement of the oxidation using the ferric thiocyanate method every day for totally 10 days. To 50 µl of the reaction mixture, 2.35 ml of 75% ethanol, 50 µl of 30% ammonium thiocyanate and 50 µl of 20 mmol/L ferrous chloride solution in 3.5% HCl were added and mixed thoroughly. After 3 min, the absorbance of the colored solution was measured at 500 nm. For the control, methanol was added instead of antioxidant in the assay system. BHT at level of 50 and 200 mg/L was also used.

4.3 Study on antimicrobial activity of seaweed extracts

4.3.1 Microorganisms

Bacillus subtilis, *Staphylococcus aureus, Salmonella enteriditis* and *Aspergillus niger* were obtained from the Department of Microbiology, Prince of Songkla University, Hat Yai, Thailand. The microorganisms were sub-cultured in slant plate count agar for *Bacillus subtilis*, *Staphylococcus aureus* and *Salmonella enteriditis* and potato dextrose agar (PDA) for *Aspergillus niger* and kept at 4°C until use.

4.3.2 Determination of antimicrobial activity of seaweed extracts

Antimicrobial activity was tested according to the method of Baydar *et al.* (2004). The microorganism sub-cultured in slant (1 loop) were incubated in tubes of nutrient broth (10 ml) at 37°C for 18-24 h to obtain approximately 10^7 -10^8 colony forming units/ml (CFU/ml). The solutions (0.1 ml) were spread on plate count agar (PCA) for *Bacillus subtilis*, *Staphylococcus aureus* and *Salmonella enteriditis* and potato dextrose agar (PDA) for *Aspergillus niger*. Sterile discs (5 mm) prepared from Whatman No. 4 filter paper were absorbed with 50 µl of either methanolic or ethanolic seaweed extract at different concentrations (0, 100, 200, 300, 400 and 500 mg/L). The discs were dried at 40°C for 30 min and placed onto surface of each microorganism plate and incubated at 37°C for 24 h. The inhibition zones were measured as the diameter of disc.

4.4 Study on gelatin cross-linking activity of seaweed extracts and the effect on properties of gelatin film

4.4.1 Fish skin preparation (as described in section 1)

4.4.2 Extraction of fish skin gelatin

Gelatin was extracted from fish skin according to the method of Gomez-Guillen *et al.* (2002). Skins were soaked in 0.025 N NaOH with a skin/solution ratio 1:10 (w/v) with a gentle stirring. The solution was changed every 1 h for 2 times to remove non-collagenous protein and pigments. Alkaline-treated skins were then washed with tap water until neutral or faintly basic pH of wash water were obtained. The skins were then soaked in 0.05 N acetic acid with skin/solution ratio of 1:10 (w/v). The solution was changed every 40 min for 3 times with a gentle stirring to swell the collagenous material in fish skin matrix. Acid-treated skins were washed as previously described. The swollen fish skins were soaked in distilled water with a skin/water ratio of 1:10 (w/v) at 45°C for 12 h with a continuous stirring to extract the gelatin from skin matter. The mixture was then filtered using two layers of cheesecloth. The resultant filtrate was freeze-dried (Dura-Top^{TM} µp, FTS symtem, New York, USA) with drying pressure of 50 mb and the vaporized temperature of 25°C. The dry matter was referred to as gelatin powder.

4.4.3 Effect of pH and the concentration of *T. ornata* extract on properties of gelatin film solutions

4.4.3.1 Preparation of film-forming solutions

Freeze dried gelatins were dissolved with de-ionized water to obtain the final protein concentration of 2% (w/v) determined by Biuret method (Robinson and Hodgen, 1940). The solution was added with glycerol at 50% (w/w) of protein content. Film forming solution (FFS) was stirred gently until the homogeneity was obtained and incubated at 60°C for 30 min. Methanolic extract of *T. ornata* was dissolved with de-ionized water adjusted to various pHs (8, 9 and 10) to obtain the concentrations of 10 mg/ml. The solutions were oxygenated for 30 min. Oxygenated extract was then added into FFS to obtain the different concentrations (0%, 3% and 6% w/w based on protein content. The mixtures were stirred at room temperature for 1 h prior to analyses or film formation.

4.4.3.2 Determination of gelatin FFS

(a) Free amino group content

Free amino group content of gelatin FFS were determined as described by Benjakul and Morrissey (1997). FFS (125 µL) was mixed with 2 ml of 0.2125 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% TNBS solution. The mixtures were then placed in water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. After being cooled down at room temperature for 15 min, the absorbance was measured at 420 nm using an UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The standard curve was prepared using L-leucine in the range of 0-3 mM. The activity was expressed as mM L-luecine.

(b) Protein pattern

Protein pattern of FFS using sodium dosecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) using 4% stacking gel and 7.5% separating gel. Sample were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol) in the presence of 10% β-ME. Sample (15µg) were loaded onto the gel. After electrophoresis using 15 mA/gel (model Mini Protein II, Biorad, USA), gel was stained with 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Type I collagen was used as a standard.

(c) Antioxidant activity

DPPH radical scavenging activity of FFS was determined according to the method of Wu *et al.* (2003) (section 4.2.1) and ABTS radical scavenging activity of film forming solution was determined according to the method of Arnao *et al.* (2001) (section 4.2.2)

4.4.3.3 Film preparation

FFS (from 4.4.3.1) obtained (4 g) was cast onto a rimmed silicone resin plate (50 x 50 mm) and air blown for 12 h at room temperature prior to further drying at 25°C and 50% relative humidity (RH) for 24 h in an environmental chamber (WTB Binder, Tuttlingen, Germany). The resulting films were manually peeled off and used for analysis.

4.4.3.4 Determination of properties of resulting films

(a) Film thickness

The thickness of film was measured using a micrometer (Gotech, Model GT-313-A, Gotech testing machines Inc, Taiwan). Nine random position of each film of nine films were used for thickness determination.

(b) Mechanical properties

The films were conditioned for 48 h at 25°C and 50% RH prior to testing. Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata *et al.* (2000) with a slight modification using the Universal Testing Machine (Lloyd Instruments, Hamsphire, UK). Nine samples (2x5 cm) with initial grip length of 3 cm were used for testing Cross-head speed was 0.5 mm/s. Load cell used was 100 N.

(c) Color and film transparency

Color of the film was determined as L*, a* and b* using CIE colorimeter (Hunter associates laboratory, Inc., VA, USA) (Paschoalick *et al.,* 2003). The films were subjected to the transmission measurement at 600 nm using the UV-16001 spectrophotometer (Shimadzu, Kyoto, Japan) as described by Han and Floros (1997). The transparency of the films was calculated by the following equation:

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

where T_{600} is the fractional transmittance at 600 nm and x is the film thickness (mm)

4.4.4 Characterization of gelatin based film incorporated with seaweed extract

The films obtained from section 4.4.3 with the highest mechanical properties were selected and subjected to the following analyses in comparison with the control film.

4.4.4.1 Film thickness as described in section 4.4.3.4

4.4.4.2 Mechanical properties as described in section 4.4.3.4

4.4.4.3 Water vapor permeability (WVP)

WVP of film was determined using a modified ASTM method (American Society for Testing & Materials, 1989) as described by Shiku *et al.* (2003). The film was sealed on a glass permeation cup containing silica gel (0% RH) with

silicone vacuum grease and a rubber band was used to hold the film in place. The cups were weighed at 1 h intervals over a 7 h period. WVP of film was calculated as follows

$$\text{WVP (gm}^{-1}\text{s}^{-1}\text{Pa}^{-1}) = wxA^{-1}t^{-1}(P_2\text{-}P_1)^{-1}$$

where w is the weight gain of the cup (g), x is the film thickness (m), A is the area of exposed film (m^2), t is the time of gain (s) and $(P_2\text{-}P_1)$ is the vapor pressure difference across the film (Pa). Five films were used for WVP testing and the measurement was run in duplicate.

4.4.4.4 Color and film transparency as described in section 4.4.3.4

4.4.4.5 Film solubility

Film solubility was determined according to the method of Gennadios *et al*. (1998) with a slight modification. The conditioned film samples (3x2 cm) were weighed and placed in 50 ml centrifuge tubes containing 10 ml of distilled water with 0.1% (w/v) sodium azide. The mixture was stirred continuously at 30 °C for 24 h using a magnetic stirrer (Model BIG SQUID, IKALABORTECKNIC, Stanfen, Germany). Undissolved dry matter was determined after centrifugation at 3000xg for 20 min and drying them at 105 °C for 24 h. The weight of solubilized dry matter was calculated by subtracting the weight of unsolubilized dry matter from the initial weight of dry matter and expressed as the percentage of total weight.

4.4.4.6 Microstructure

Microstructure of the film samples was determined using scanning electron microscopy (SEM). The film was coated with gold using Sputter coater (SPI-Module, PA, USA). The upper and lower surfaces as well as cross section were visualized at an acceleration voltage of 10 kV.

4.4.4.7 Hydrolysis by protease

Hydrolysis by protease was determined according to the modified method of Yildirim and Hettiarachychy (1998). Ground film samples (25 mg) was suspended in 50 ml of α-chymotrpsin (40 µg/ml) in 40 mM Tris-HCl buffer, pH 7.6. The mixture was then incubated at 37°C for 2 h. At 0, 30, 60, 90 and 120 min, the samples (2.5 ml) were taken and heated at 100°C for 3 min to inactivate the enzyme. After standing for 30 min at room temperature, the precipitate was removed by centrifugation (1800xg for 15 min). The peptide and amino acid content of supernatant was determined by Lowry method (Lowry, 1951) and degree of

hydrolysis was calculated as follows:

$$\text{Degree of hydrolysis (\%)} = \frac{A}{B} \times 100$$

where A = amount of peptide and amino acids in supernatant (μmol)

B = Amount of total protein in sample film was solubilized in 6 N HCl at 110°C for 24 h

4.4.4.8 Protein solubility in various solvents

Protein solubility of gelatin films in various solvents were determined as described by Chawla *et al.* (1996). The solvents used included

S1 : 0.6 M KCl

S2 : 20 mM Tris-HCl (pH 8.0)

S3 : 20 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS

S4 : 20 mM Tris-HCl (pH 8.0) containing 1 % (w/v) SDS and 8 mM Urea

S5 : 20 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS, 8 mM Urea and 2 % (v/v) *β*-ME

The film samples (0.5 g) were homogenized in various solvents for 1 min using a homogenizer (IKA Labortechnik, Malasia). The homogenate with S5 was heated in boiled water (100 °C) for 2 min. All homogenates were stirred at room temperature for 4 h. The resulting homogenate was centrifuged at 7,500xg for 30 min using a microcentrifuge (MIKRO 20, Hettich Zentrifugan, Germany). Protein in supernatant (10 ml) was precipitated by adding 50% (w/v) cold TCA to a final concentration of 10%. The mixture was kept at 4 °C for 18 h and centrifuged at 7,500xg for 30 min. The precipitate was washed with 10% TCA and solubilized in 0.5 M NaOH. The protein content was measured using the Biuret test (Robison and Hodgen, 1940). To obtain the total amount of protein, films were solubilized in 0.5 M NaOH. The solubility was reported as percentage of total protein.

4.4.4.9 Thermal properties

Measurment of the glass transition temperature (*Tg*) of films was performed by differential scaning calorimetry (DSC) as desclibed by Hernandez-Munoz *et al.* (2004). The instrument was calibrated with Indium as a standard. Film were conditioned over siligar gel at 25°C for 3 weeks before testing. Dry samples (10

mg) were placed in an hermetically sealed aluminium pan and heated at 5°C/min between 25 and 250°C.

4.5 Moisture sorption isotherms

Moisture sorption isotherms of the film samples were determined as described by Kim and Ustunal (2001). Prior to analysis, all films were conditioned at 25 °C over desiccant for 3 weeks. Moisture sorption isotherms of the films were determined at room temperature (28-30°C) under eight different humidity conditions (18±0.5, 23±0.5, 34±0.5, 46±0.5, 54±0.5, 64±0.5, 73±0.5, 90±0.5) prepared using different saturated salt solutions of $LiCl.H_2O$, $KC_2H_3O_2$, $MgCl_2$, $K_2CO_3.2H_2O$, $Mg(NO_3)_2.6H_2O$, $NaNO_2$, NaCl and KCl, respectively. The water activity (a_w) of each of salt solutions was calculated as % RH/100.

4.6 Changes of gelatin films during storage

Films obtained from section 4.4.3.4 with the highest mechanical properties were stored in a dessicator containing a saturated salt solution of $Mg(NO_3)_2.6H_2O$ (54% RH) at 25°C. Film samples were taken at week 0, 1, 2, 3 and 4 of storage for the following analyses:

4.6.1 Mechanical properties (as described in section 4.4.3.4)

4.6.2 Color, light transmission and film transparency (as described in section 4.4.6.4)

4.6.3 Film solubility and protein solubility (as described in section 4.4.4.5)

4.6.4 Water vapor permeability (as described in section 4.4.4.3)

5. Statistical analysis

All data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's Multiple Range Test (Steel and Torrie, 1980). SPSS statistic program (SPSS 11.0 for window, SPSS Inc., Chicago, IL, USA.) was used for data analysis.

CHAPTER 3

RESULTS AND DISCUSSION

1. Extraction and properties of seaweed extracts

1.1 Total phenolic content and total chlorophyll content of brown seaweed extracts

Total phenolic contents (TPC) of methanolic and ethanolic extracts of *T. ornata* and *S. polycystum* are presented in Table 3. Without chlorophyll removal, both methanolic and ethanolic of *T. ornata* contained higher TPC than those of *S. polycystum.* For the same seaweeds, methanolic extract had the greater TPC than did ethanolic counterpart. Various phenolic compounds including quercetin, myricetin (flavonoles); genistin, daidzein (iso-flavones); hesperidin (flavanones) and lutein (flavones) were found in methanolic extract of red, brown and green seaweeds collected from various areas in Japan (Yoshie *et al.,* 2000).

After chlorophyll removal, TPC of the same extract decreased, except for methanolic extract of *S. polycystum,* in which chlorophyll removal had no impact on TPC. During extraction of chlorophylls using petroleum ether, some phenolics, particulary non-polar, could be removed together with chlorophyll, leading to the lower TPC remained in the extract. Yan *et al.* (1999) reported some active compounds from brown seaweed, which were identified as phylopheophytin in *Eisenia bicyclis* and fucoxantine in *Hijikia fusiformis.* Methanol has been intensively used to extract plant phenols and its extraction efficiency was generally higher than ethanol (Kumaran and karunakaran, 2007). Lim *et al.* (2002) reported that phenolic compound of methanolic extract from *Sargassum siliquastrum* had higher than did ethyl acetated, chloromethane and buthanol.

Total chlorophyll content of different seaweed extracts is shown in Table 4. After chlorophyll removal, methanolic and ethanolic extracts had lower total chlorophyll content ($p<0.05$). Efficacy in chlorophyll removal varied with seaweed and solvents used. Methanolic extract of *S. polycystum* had the most decrease in chlorophyll content by 41.32 % after chlorophyll removal.

Table 3. Total phenolic content (TPC) of methanolic and ethanolic extract of *T. ornata* and *S. polycystum* without and with chlorophyll removal.

Extracts		Total phenolic content* (mg catechin/g dry seaweed)	
		T. ornata	*S. polycystum*
Methanolic	With chlorophyll removal	2.07 ± 0.06[bA**]	0.54 ± 0.09[aB]
	Without chlorophyll removal	2.18 ± 0.01[aA]	0.59 ± 0.01[aB]
Ethanolic	With chlorophyll removal	1.03 ± 0.04[dA]	0.22 ± 0.01[cB]
	Without chlorophyll removal	1.25 ± 0.01[cA]	0.32 ± 0.00[bB]

[*] Mean ± SD from triplicate determinations.

[**] Different letters within the same column indicate significant differences ($p<0.05$) and different capital letters within the same row indicate significant differences ($p<0.05$).

Table 4. Total chlorophyll content of methanolic and ethanolic extract of *T. ornata* and *S. polycystum* without and with chlorophyll removal.

Extracts		Total chlorophyll content* (µg chlorophyll/g dry seaweed)	
		T. ornata	*S. polycystum*
Methanolic	With chlorophyll removal	20.79 ± 0.29[dA**]	13.26 ± 0.10[dB]
	Without chlorophyll removal	35.23 ± 0.04[bA]	32.09 ± 0.15[bA]
Ethanolic	With chlorophyll removal	25.14 ± 0.27[cA]	21.57 ± 0.05[cA]
	Without chlorophyll removal	37.77 ± 0.44[aA]	37.18 ± 0.09[aA]

[*] Mean ± SD from triplicate determinations.

[**] Different letters within the same column indicate significant differences ($p<0.05$) and different capital letters within the same row indicate significant differences ($p<0.05$).

1.2 Antioxidative activities of brown seaweed extracts

1.2.1 DPPH radical scavenging activity

DPPH radical scavenging activity of methanolic and ethanolic extracts from *T. ornata* and *S. polycystum* without and with chlorophyll removal at different concentrations is shown in Figure 6. For extract without chlorophyll removal, methanolic extract of *T. ornata* and ethanolic extract of *S. polycystum* exhibited the highest DPPH radical scavenging activity. At the same concentration tested, methanolic extract of *S. polycystum* had the lowest activity. Due to the different TPC between methanolic extract of *T. ornata* and ethanolic extract of *S. polycystum* (Table 3), it was suggested that TPC was not correlated well with DPPH radical scavenging activity. It was also presumed that different types of phenolic compounds with different antioxidation activity were presented in both extracts. Polyphenolic constituent in seaweed were capable of functioning as free radical scavengers (Chew *et al.*, 2008). Polyphenols such as phlorotannins, which are bi-polar in nature, were found in brown seaweeds (Burtin, 2003). For each extract used, DPPH radical scavenging activity increased with increasing concentration ($p<0.05$). DPPH is one of the compounds that have a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Yamaguchi *et al.*, 1996). It is well accepted that the DPPH radical scavenging by antioxidants is attributable to their hydrogen donating ability (Chen and Ho, 1995). The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidant (Gülcin *et al.*, 2004). Therefore, DPPH radical scavenging activity varied with seaweed species. Methanolic extract of three Indian brown seaweeds (*Turbinaria conoides, Padina tetrastomatica* and *Sargassum marginatum*) had different DPPH radical scavenging activities. *T. conoides* extracts showed higher activity than those of *P. tetrastomatica* and *S. marginatum* (Chandini *et al.,* 2008).

After chlorophyll removal, all extracts exhibited the lower DPPH radical activity. Nevertheless, the activity increased as the concentration used increased ($p<0.05$). With chlorophyll removal, methanolic extract of *T. ornata* had the highest DPPH radical scavenging activity, whereas methanolic extract of *S. polycystum* exhibited the lowest activity at all concentrations tested ($p<0.05$). The result suggested that chlorophylls contributed to antioxidative activity of seaweed extracts. Endo *et al.* (1985) reported antioxidative activity of chlorophyll-a, followed by chlorophyll-b and pheophytin.

Additionally, chlorophyll removal using petroleum ether might remove some antioxidative compounds from the extracts. As a result, antioxidative compounds in the extracts could be affected by chlorophyll removal process.

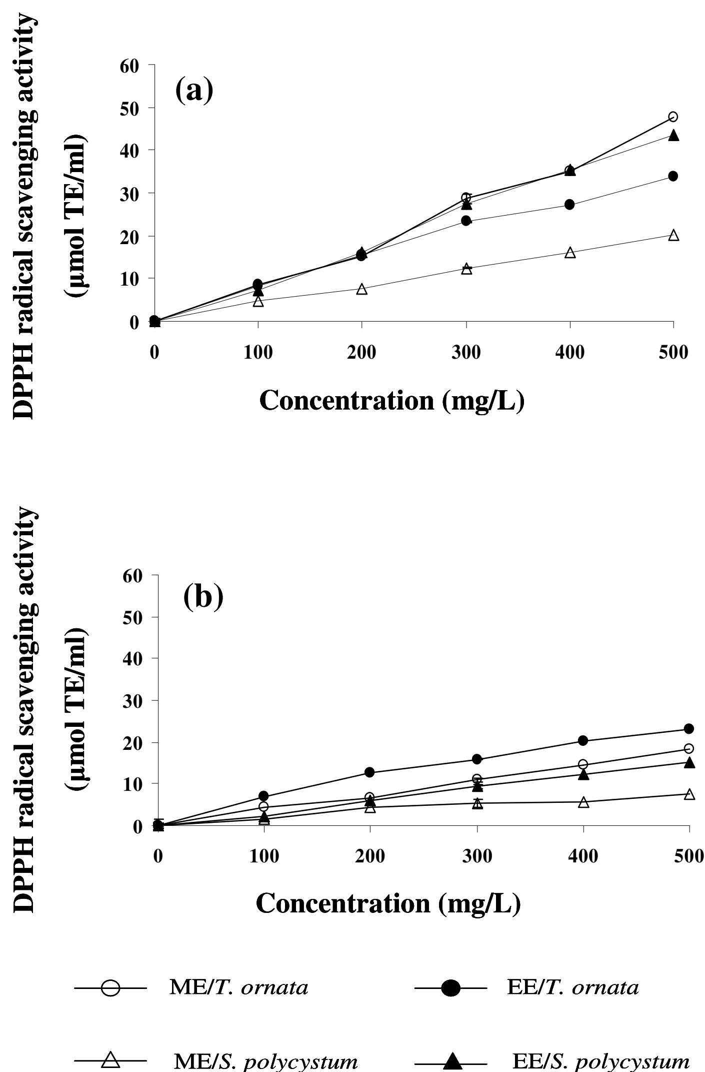


Figure 6. DPPH radical scavenging activity (µmol TE/ml) of methanolic (ME) and ethanolic (EE) extracts of *T. ornata* and *S. polycystum* without (a) and with (b) chlorophyll removal at various concentrations. Bars represent the standard deviation from triplicate determinations.

1.2.2 ABTS radical scavenging activity

Methanolic and ethanolic extracts of *T. ornata* and *S. polycystum* without and with chlorophyll removal at different concentrations showed different ABTS radical scavenging activity (Figure 7). ABTS radical scavenging activity of all extracts increased with increasing concentrations ($p<0.05$). For the extracts without chlorophyll removal, methanolic extract of *T. ornata* exhibited the highest activity ($p<0.05$), whereas methanolic extract of *S. polycystum* showed the lowest activity ($p<0.05$). For the extract of *T. ornata,* methanolic extract had the higher ABTS radical scavenging activity than did ethanolic counterpart. Nevertheless, ethanolic extract of *S. polycystum* showed the higher activity than did methanolic counterpart ($p<0.05$). Different results between DPPH and ABTS radical scavenging activities indicated the differences in mode of action of antioxidants, particularly in term of specificity in radical scavenging. Both of seaweed extracts without chlorophyll removal had higher ABTS radical scavenging activity than did those with chlorophyll removal, indicating that some antioxidative compounds could be removed by petroleum ether used in chlorophyll removing process. Furthermore, removal of chlorophylls, which had antioxidative activity, resulted in the lower activity. Hagerman *et al.* (1998) reported that molecular weight, the number of aromatic rings and nature of hydroxyls groups substitution, rather than the specific functional groups, determine antioxidative activity of phenolic compounds. $ABTS^{\bullet}$ assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants (scavengers of aqueous phase radicals) and of chain breaking antioxidants (scavenger of lipid peroxyl radical) (Leong and Shui, 2002). For the extracts with chlorophyll removal, ethanolic extract of *T. ornata* had the greater ABTS radical scavenging activity than did methanolic counterpart. No differences were noticeable between methanolic and ethanolic extracts of *S. polycystum.* This reconfirmed that chlorophyll removal affected the types of antioxidative compounds and composition of extracts.

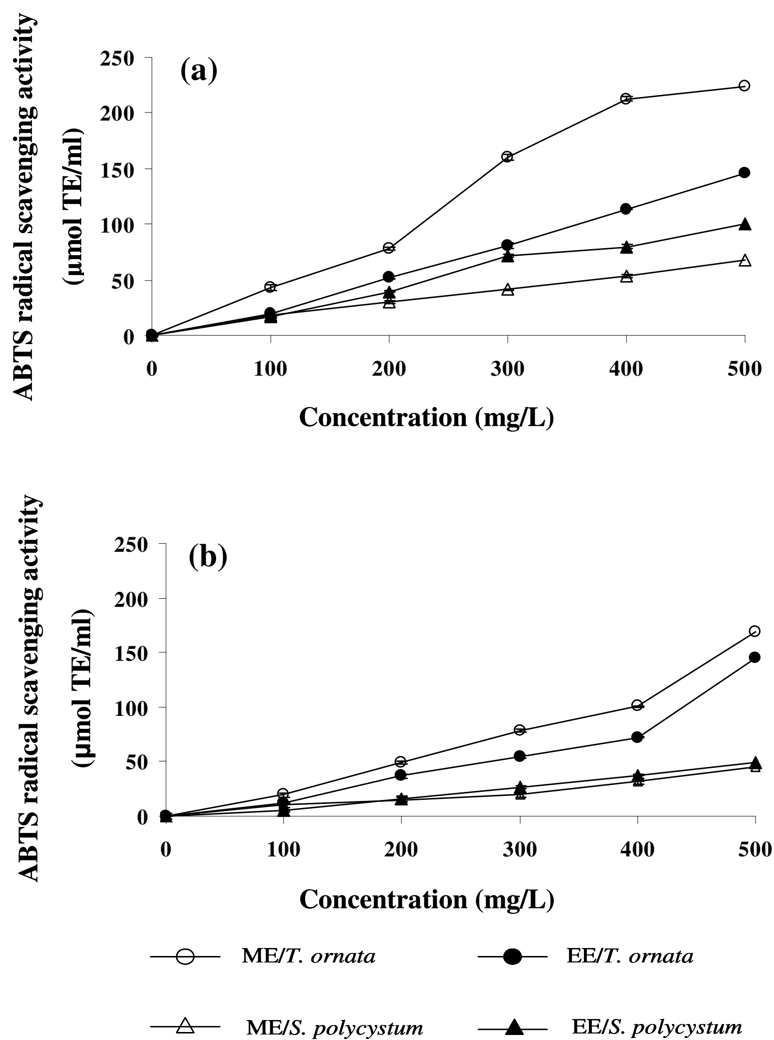


Figure 7. ABTS radical scavenging activity (µmol TE/ml) of methanolic (ME) and ethanolic (EE) extracts of *T. ornata* and *S. polycystum* without (a) and with (b) chlorophyll removal at various concentrations. Bars represent the standard deviation from triplicate determinations.

1.2.3 Reducing activity power

Among all extracts without chlorophyll removal, methanolic extract of *T. ornata* had the highest reducing activity power (RAP) and ethanolic extract of *S. polycystum* showed the lowest RAP ($p<0.05$) (Figure 8). After chlorophyll removal, slight decrease in RAP was generally found. In general, extracts from *T. ornta* possessed the greater RAP than did those of *S. polycystum.* For the same extract, RAP was not absolutely in accordance with DPPH and ABTS radical scavenging activities. The result suggested that different extracts contained antioxidative compounds with different functions in inhibiting lipid oxidation. Kuda *et al.* (2005) and Chandini *et al.* (2008) reported that methanolic extracts of *Turbinaria conoides* and *Padina tetrastomatica* had higher reducing power than did *Sargassum marginatum.* RAP of all extracts increased as the concentrations increased up to 500 mg/L ($p<0.05$). RAP indicated that all extracts were capable of donating the electrons to the radicals, in which propagation could be terminated or retarded.

1.3 The correlation between antioxidative activities tested by different assays.

The correlations between antioxidative activities determined by different assays and reported as Trolox equivalent antioxidant capacity (TEAC) were observed (Figure 9). Their linear correlation could be desclibed as: $TEAC_{ABTS} = 5.1562TEAC_{DPPH}$ ($R^2 = 0.8732$), $TEAC_{RAP} = 0.7186TEAC_{ABTS}$ ($R^2 = 0.7178$) and $TEAC_{RAP} = 3.8178TEAC_{DPPH}$ ($R^2 = 0.7150$). Zhao et al. (2008) also reported the correlation between antioxidative activity of barley extract tested by DPPH and ABTS radical scavenging activities and reducing power. Nevertheless, S´anchez *et al.* (2007) reported the best correlation between total polyphenol contents and antioxidant capacity determined by ABTS and DPPH radical scavenging activity method ($R^2 = 0.8927$ and 0.8052, respectively) in virgin olive oil. The result revealed that brown seaweed extracts possessed the ability in donating electron as well as the capability of scavenging various radicals. It could be inferred that the extracts could be used as antioxidants. Since methanolic extract of *T. ornata* had the highest antioxidative activity determined by all assays, it was used to prevent lipid oxidation in different systems.

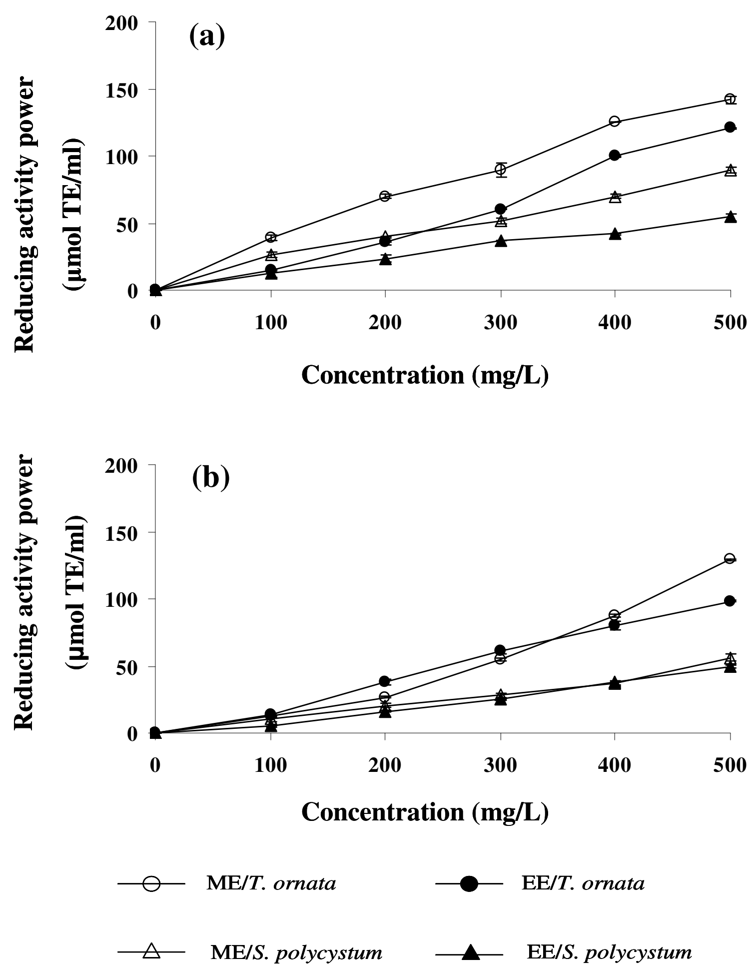


Figure 8. Reducing activity power (µmol TE/ml) of methanolic (ME) and ethanolic (EE) extracts of *T. ornata* and *S. polycystum* without (a) and with (b) chlorophyll removal at various concentrations. Bars represent the standard deviation from triplicate determinations.

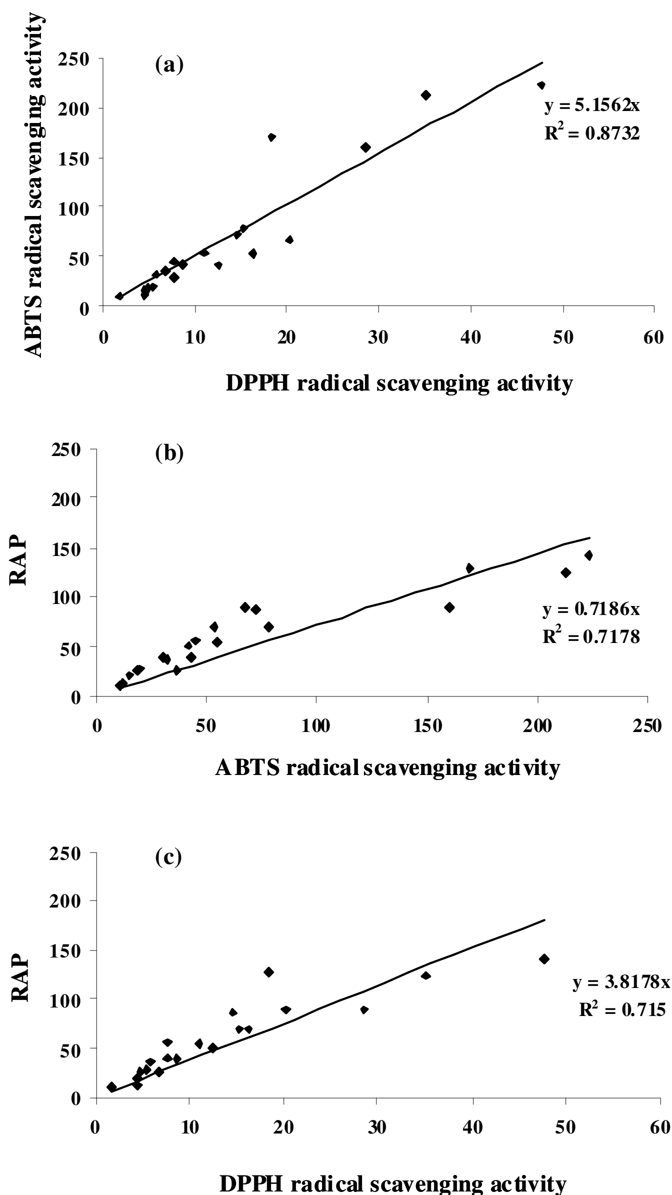


Figure 9. Correlation between ABTS and DPPH radical scavenging activity (a), RAP and ABTS radical scavenging activity (b) and RAP and DPPH radical scavenging activity (c) of the extract of *T. ornata* and *S. polycystum* without and with chlorophyll removal.

1.4 Antioxidative activity of seaweed extract in different systems

1.4.1 Lecithin liposome system

Antioxidative activities of methanolic extract of *T. ornata* at different concentrations in lecithin liposome system as monitored by the formation of TBARS during incubation at 37°C are shown in Figure 10. Generally, the increase in liposome oxidation was observed when incubation time increased ($p<0.05$). During the first 6-30 h of incubation, the sharp increases in TBARS were found in the control. Systems containing seaweed methanolic extract had lower TBARS than did the control throughout the incubation of 48 h. TBARS values in liposome system added with 50 and 200 mg/L BHT was very low throughout the incubation period of 48 h ($p<0.05$). In general, lower TBARS tended to be obtained in the sample containing the higher amount of extracts added. TBARS values represent the content of secondary lipid oxidation products, mainly aldehydes (or carbonyls), that contribute to off-flavours in oxidized oils (Wanasundara and Shahidi, 1998). The decrease in TBARS was found after 30 h of incubation. This might be due to the losses in secondary products with low molecular weight, leading to lower amount of such products (Stanhne, 1995). The extract with chlorophyll removal exhibited the higher antioxidant activity than did that without chlorophyll removal in this system ($p<0.05$). This was probably due to the pro-oxidative effect of chlorophylls and their derivatives in this system. Wanasundara and Shahidi (1998) reported that ethanolic green tea extract (GTE) with chlorophyll showed pro-oxidative activity in seal blubber oil (SBO) and menhaden oil (MHO). However, after dechlorophyllization, the extract exhibited antioxidant activity in both SBO and MHO oil. However, He and Shahidi (1997) found that GTE, despite the presence of chlorophyll, had an antioxidant effect when applied to white muscles of mackerel. Therefore, antioxidant/pro-oxidant activity of GTE was system-dependent (He and Shahidi, 1997). The results indicated that methanolic extract of *T. ornata* could inhibit lipid oxidation, irrespective of chlorophyll removal.

1.4.2 Linoleic oxidation system

The oxidation of linoleic acid as monitored by the increase in absorbance at 500 nm (A_{500}) in systems containing methanolic extract of *T. ornata* at different levels during the incubation at 40°C for 10 days are shown in Figure 11. All samples had a continuous increase in A_{500} throughout 10 days of incubation and the control showed the

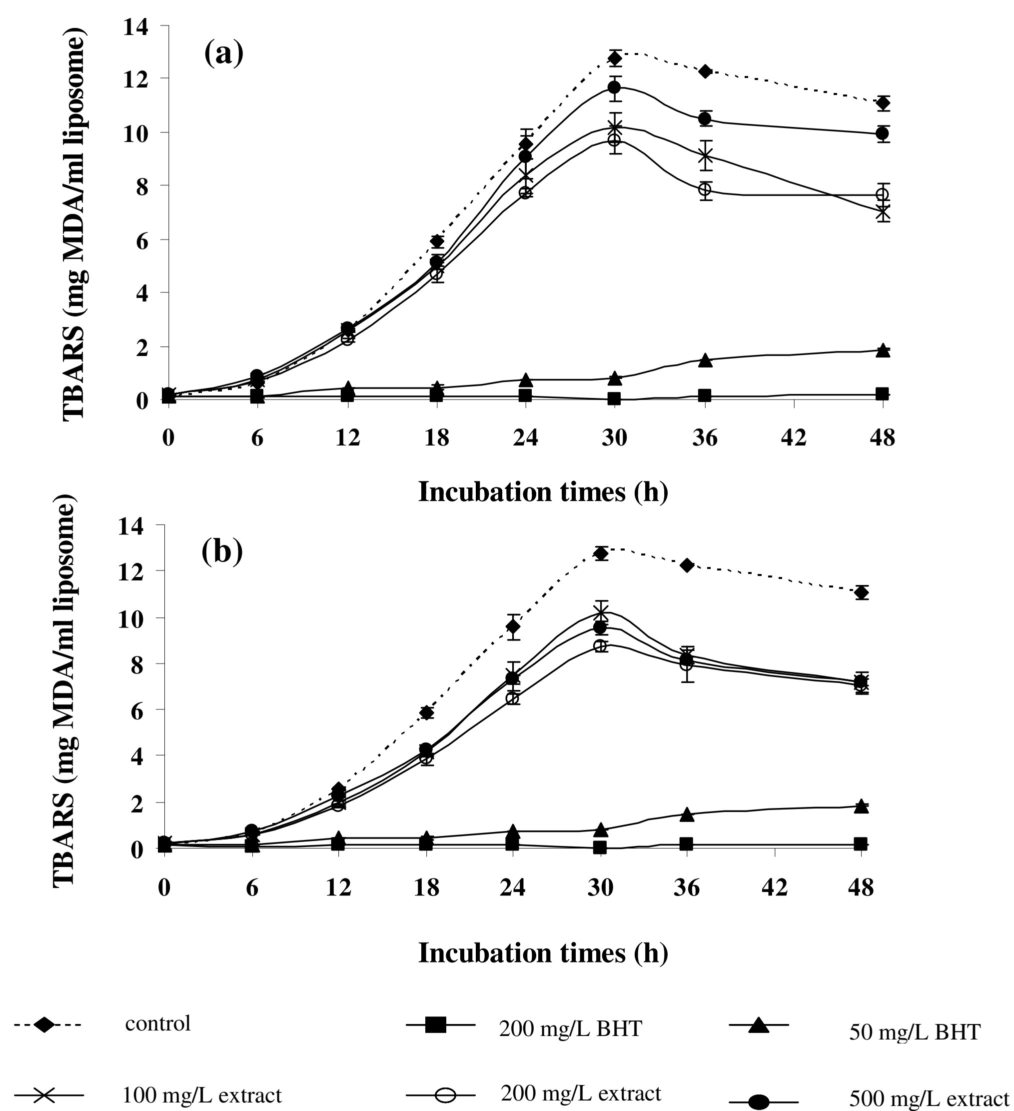


Figure 10. Changes in TBARS (mg MDA/ml liposome) of lecithin liposome system containing methanolic extract of *T. ornata* without (a) and with (b) chlorophyll removal at different levels. Bars represent the standard deviation from triplicate determinations.

highest rate of increase. During incubation, the system containing the extract with chlorophyll removal at all concentrations had the lower oxidation as evidenced by lower A_{500}, compared with the control ($p<0.05$). For systems added with the extract without chlorophyll removal, no significant difference in A_{500} was found, compared with the control, except after 7 days of incubation, when the lower A_{500} was found in sample added with the extract. Reaction medium which contains unsaturated fatty acids promotes the decomposition of chlorophyll to release the metal-free, behaving as prooxidant at higher concentrations (Usuki *et al.,* 1984). Santoso *et al.* (2004) reported that methanolic extract from seaweed contained not only polyphenolic compounds but also other compounds such as polyunsaturated fatty acid (e.g., eicosapentaenoic acid), minerals (e.g., copper, iron) and pigment (e.g., chlorophyll). These components might interact with polyphenolic compounds or affect directly to the emulsion system; therefore, they might act like a pro-oxidant. Linoleic acid is a C18: 2 fatty acid with two double bonds at C9 and C12 and is one of the major unsaturated fatty acid in vegetable oils. The isomeric structures of hydroperoxides and decomposition volatile compounds from linoleic acid or methyl linoleate by auto-oxidation and photosensitized oxidation have been studied extensively (Frankel, 1985). Lipid peroxidation is thought to proceed via radical-mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids (Rajapakse *et al.*, 2005). In food-related systems, anti-oxidant activity is mainly due to the chain-breaking inhibition of lipid peroxidation (Morales and Jimennez-Perez, 2001). Therefore, brown seaweed extract could retard lipid oxidation in the emulsion system. However, the efficiency was lower than BHT at both levels (50 and 200 mg/L).

1.5 Antimicrobial activity of brown seaweed extracts

Methanolic and ethanolic extracts of *T. ornata* and *S. polycystum* without and with chlorophyll removal at 500 mg/L was able to inhibit only gram-positive bacteria (*S. aureus*) but could not inhibit gram-negative bacteria (*S. enteritidis*), gram-positive bacteria, spore-forming rods (*B. subtilis*) and fungi (*A. niger*) (Table 5). Similar clear zone diameter was observed among all extracts tested, but methanolic extract of *T. ornata* tended to exhibit the highest inhibition against *S. aureus.* Ely *et al.* (2004) reported that the extract of green seaweed, *Cladophora prolifera,* (1.5 mg/ml) exhibited *S. aureus* and *Vibrio cholerae.* Antimicrobial activity of extract could be because phenols reacted

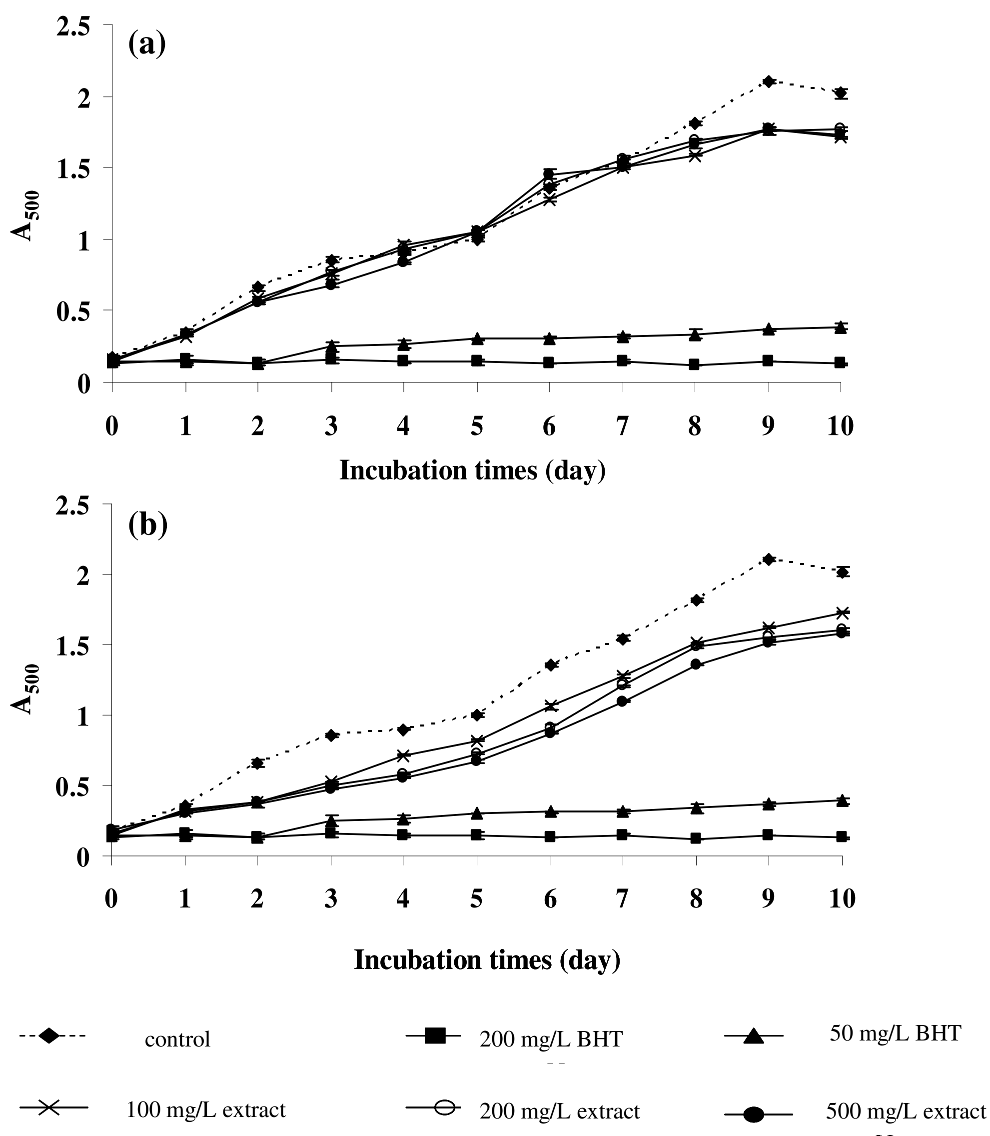


Figure 11. Changes in A_{500} of linoleic acid system containing methanolic extract of *T. ornata* without (a) and with (b) chlorophyll removal at different levels. Bars represent the standard deviation from triplicate determinations.

primarily with the phospholipids component of cell membrane (Ely *et al.,* 2004). Different active compounds in different brown seaweeds extracts such as spatane diterpenoids, 19-accetoxy-5, 15,18-trihydrxyapata-13,16-diene, from *Stoechospermum marginatum* (De Silva *et al.,* 1982), methoxybifurcarenone, from *Cystoseira tamariscifolia* (Bennamara *et al.,* 1999) and phlorotannins from *Ecklonia kurome* (Nagayama *et al.,* 2002) were reported.

Due to the higher antioxidative activity and high extraction yield of methanolic extract from *T. ornata,* the extract was used to modify gelatin film in further study.

Table 5. Inhibition zone of different microorganisms in the presence of methanolic and ethanolic extracts of *T. ornata* and *S. polycystum* with and without chlorophyll removal at 500 mg/L.

Seaweeds	Extracts	*B. subtilis*	*S.aureus*	*S.enteritidis*	*A. niger*
T. ornata	ME/W	-	6.0	-	-
	ME/W/O	-	6.0	-	-
	EE/W	-	6.0	-	-
	EE/W/O	-	6.0	-	-
S. polycystum	ME/W	-	7.0	-	-
	ME/W/O	-	6.0	-	-
	EE/W	-	6.0	-	-
	EE/W/O	-	6.5	-	-

The value is the distance (mm) across the zone of inhibition and the disc (diameter 5 mm).

Values are the mean values of triplicate determinations.

-: no inhibition

B. subtilis: *Bacillus subtilis*; *S. aureus*: *Staphylococcus aureus*;

S. enteritidis: *Salmonella enteriditis*; *A. niger*: *Aspergillus niger*

ME/W: methanolic extract with chlorophyll removal

ME/W/O: methanolic extract without chlorophyll removal

EE/W: ethanolic extract with chlorophyll removal

EE/W/O: ethanolic extract without chlorophyll removal

2. Effect of seaweed extract incorporation on cross-linking and film properties of bigeye snapper skin gelatin

2.1 Compositions and protein pattern of bigeye snapper skin gelatin

Proximate composition and hydroxyproline content of bigeye snapper skin gelatin are shown in Table 6. Bigeye snapper skin gelatin constituted protein as the major component (85.94 %), followed by moisture (9.31%), lipid (2.47%) and ash (3.18%), respectively. Cheow *et al.* (2007) reported that gelatin from sin croaker and shortfin scad skin contained 69.2% and 68.7% protein content and there were no the differences in moisture, fat and ash contents. The ash content of bigeye snapper skin gelatin was higher than the recommended maximum of 2.6% (Jones, 1997). This was possibly due to the incomplete demineralization during pretreatment. The yield of gelatin was 10.02%. Grossman and Bergman (1992) reported a yield of 15% for gelatin from tilapia skin. Similarly, Gugmundsson and Hafsteinsson (1997) also reported a gelatin yield of about 14% for cod skin. However, Cheow *el at.* (2007) found that the yields of gelatin for sin croaker and shortfin scad skins were 14.3% and 7.25%, respectively. During extraction, hot water (45°C) could break down H-bond stabilizing the collagen molecule. The lower yield of gelatin might be caused by the insufficient denaturation or unraveling of collagen during extraction (Jamilah and Harvinder, 2002).

Hydroxyproline content of bigeye snapper skin gelatin was 105.24±0.22 mg/g dry sample. Hydroxyproline is the unique imino acid in collagen and gelatin (Wong, 1989). Hydroxyproline plays an essential role in the stabilization of the triple-helix strands of collagen via its hydrogen bonding ability through its -OH group (Burjandze, 1979; Ledward, 1986). Gelatin with higher content of hydroxyproline is believed to have higher viscoelastic properties and its ability to develop triple helix structures, which are important for stabilizing the gelatin gel structure (Gómez-Guillèn *et al*., 2002).

Protein patterns of bigeye snapper skin gelatin are shown in Figure 12. Gelatin consisted of α-chains, β- and γ-chains as the major constituents. This was in accordance with the results obtained previously in gelatin from skin of another species of bigeye snapper (*Priacanthus macracanthus*) (Jongjareonrak *et al.,* 2005). Most fish collagens have been found to contain two α-chain variants, which are normally designated as α_1 and α_2, called type I collagen (Gòmez-Guillèn *et al.,* 2002; Nagai *et al.,* 2001).

Table 6. Proximate composition and hydroxyproline content of bigeye snapper skin gelatin

Compositions	Content (%)
Moisture	9.91±0.01*
Protein	85.94±0.18
Lipid	2.47±0.17
Ash	3.18±0.44
Hydroxyproline (mg/g dry basis)	105.24±0.22

*Mean ± SD from triplicate determinations.

Gòmez-Guillèn *et al.* (2002) reported that collagens from invertebrate present a really high cross-linking degree compared to vertebrate collagens, mainly due to the high increase in lysine hydroxylation that participate in different types of cross-links via Schiff-base formation. Moreover, hydroxylysine is normally glycosylated in invertebrate collagens. The electrophoresis patterns of gelatins from bigeye snapper skin under non-reducing and reducing conditions were similar, indicating the absence of the disulfide bond in the gelatin. Type I collagen had low amounts of cysteine (~0.2%) and methionine (~1.24-1.33%), which play an essential role in disulfide bond formation (Owusu-Apenten, 2002).

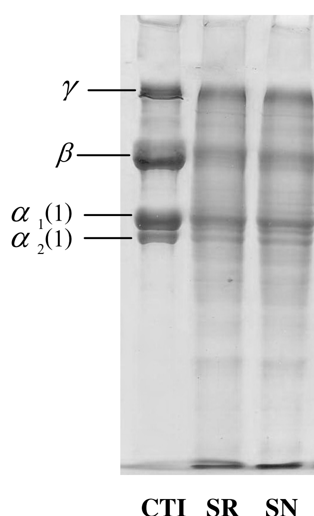


Figure 12. Protein pattern of bigeye snapper skin gelatin under reducing (SR) and non-reducing (SN) conditions. CTI: collagen type I from calf skin.

2.2 Effect of pH and concentration of seaweed extract on characteristics of gelatin film forming solutions and properties of resulting films

2.2.1 Characteristic of film forming solution

2.2.1.1 Free amino group content and radical scavenging activities

Free amino group content as determined by TNBS assay and radical scavenging activities of gelatin film forming solutions (FFS) containing seaweed (*T. ornata*) extract at different concentrations are shown in Table 7. FFS added with seaweed extract having different pHs showed the different free amino group content. Addition of seaweed extract with pHs of 9 and 10 resulted in the decrease in total free amino group content ($p<0.05$). No differences in total amino group content were found between FFS containing extract at levels of 3 and 6% ($p>0.05$). However, FFS added with the extract having a pH of 8 had the similar free amino group content to the control (FFS without extract) ($p>0.05$). The decrease in free amino group content indicated that cross-linking via free amino groups induced by the extract took place. Nevertheless, the cross-linking was more pronounced when extract was adjusted to very alkaline pH. Phenolic compounds in seaweed extract adjusted to alkaline pH might be responsible for protein cross-linking. The result was in agreement with Cao *et al.* (2007) who found that the maximum values of tensile strength and elastic modulus of bovine gelatin film was obtained when ferulic acid at pH 9 was incorporated in gelatin. Additionally, Strauss and Gibson (2004) reported that the addition of caffeic acid, coffee and grape juice adjusted to pH 8 resulted in the lower free amino groups in gelatin and increased in gel rigidity.

FFS incorporated with seaweed extract with different final pHs of FFS had no changes in free amino group content at all pHs used, except FFS with pH of 10 containing 6% seaweed extract, which had the lowest free amino group content ($p<0.05$). At alkaline pH, fewer amine groups become protonated, resulting in more free amine (Chiou *et al.,* 2006). Phenolic compounds at higher pH could be oxidized to quinone and then the quinone further reacts with amines on proteins (Cao *et al.,* 2007). From the result, pH 10 might be the appropriate pH for oxidizing the phenolic compounds to reactive quinone, which could induce the cross-linking of gelatin molecules, particularly at the higher concentration used. It was suggested that proteins underwent complete unfolding, in which the reactive groups, especially amino group, were exposed for cross-linking reaction. Aguilera-Morales *et al.* (2005) found tannins in green algae extract (*Enteromorpha* spp). Tannins interact with specific site of protein,

forming strong noncovalent bonds with such hydrophobic moieties of proteins such as proline and histidine residues (Shutava *et al.,* 2005).

DPPH and ABTS radical scavenging activities of FFS without seaweed extract were 26.67 and 27.69 µmole TE/mg protein, respectively. FFS containing 3% seaweed extract (without final pH adjustment) showed the decrease in antioxidative activity ($p<0.05$). Polyphenol-protein interactions could hinder the reactive group possessing antioxidant activities. At 6% seaweed extract, the increase in antioxidative activity was obtained, but was similar to that found in the control FFS ($p>0.05$). The control FFS displayed some antioxidant activity, as measured by both indices. This indicated the antioxidant activity of gelatin. Peptides or proteins have been known to exhibit the antioxidative activity (Kim *et al.,* 2001; Kim *et al.,* 2001a). Mendis *et al.* (2005) reported that the peptide, His-Gly-Pro-Leu-Gly-Pro-Leu present in hydrolysates of hoki (*Johnis belengerii*) skin gelatin, was a powerful scavenger of free radicals. Differences in the level of antioxidative activity of the different components have been reported to depend on the chemical form of the compounds present. Combining two or more phenolic acids, as in the case of rosmarinic acid (a dimmer of caffeic acid and hydrocaffeic acid), greatly increased the antioxidative activity, whereas esterification lowers the antioxidative activity, as in the case of chlorogenic acid (caffeic acid esterified with quinic acid) (Chen and Ho, 1997; Cuvelier *et al.,* 1992). For FFS containing no seaweed extract with final pH adjustment to different pHs, no differences in DPPH radical scavenging activity were noticeable when pH of FFS was adjusted to alkaline pH ($p>0.05$). pH adjustment of FFS to 8 or 10 rendered the increase in ABTS radical scavenging activity ($p<0.05$). At pH 10, no differences in ABTS radical scavenging activity were found when seaweed extracts were incorporated ($p>0.05$). However, at pHs 8 and 9, ABTS radical scavenging activity increased with increasing extract content ($p<0.05$). Addition of seaweed extract in FFS (pH 10) had no impact in antioxidative activity, both DPPH and ABTS radical scavenging activities ($p>0.05$). The result suggested that the excessive amount of seaweed extract was required for both cross-linking and antioxidative purposes. Practically, phenolic compounds to some level were involved in gelatin cross-linking. When bound with proteins, the losses in their antioxidative activity were obtained.

Table 7. TNBS and radical scavenging activities of gelatin FFS incorporated with seaweed extract adjusted to different pHs at different concentrations without and with pH adjustment to various FFS pHs.

pH of extract	Seaweed extract†† (%)	TNBS* (mM L-leucine)	DPPH radical scavenging activity* (µmol TE/mg protein)	ABTS radical scavenging activity* (µmol TE/mg protein)
control	0	2.24 ± 0.046^{a**}	26.67 ± 4.532^{bcd}	27.69 ± 1.54^{fgh}
pH8	3	2.16 ± 0.142^{abc}	26.08 ± 2.539^{bcd}	13.46 ± 2.69^{i}
	6	2.22 ± 0.141^{ab}	29.90 ± 4.353^{ab}	32.31 ± 1.54^{efgh}
	0 (pH 8)†	2.24 ± 0.087^{ab}	30.79 ± 1.437^{ab}	44.79 ± 6.52^{cde}
	3 (pH 8)†	2.27 ± 0.038^{a}	34.30 ± 0.460^{cde}	34.19 ± 6.07^{efgh}
	6 (pH 8)†	2.21 ± 0.055^{ab}	34.64 ± 2.874^{a}	65.14 ± 10.79^{a}
pH9	3	2.02 ± 0.027^{d}	29.88 ± 4.491^{ab}	12.69 ±5.00^{i}
	6	2.11 ± 0.060^{bcd}	27.69 ± 2.988^{bc}	36.92 ± 8.46^{defg}
	0 (pH 9)†	2.22 ± 0.013^{ab}	29.93 ± 0.115^{ab}	34.07 ± 6.79^{efgh}
	3 (pH 9)†	2.24 ± 0.008^{ab}	27.35 ± 1.437^{bc}	24.67 ± 7.33^{ghi}
	6 (pH 9)†	2.20 ± 0.067^{ab}	31.54 ± 0.920^{ab}	58.71 ± 8.24^{ab}
pH10	3	2.06 ± 0.033^{cd}	29.27 ± 3.367^{abc}	21.92 ± 4.23^{hi}
	6	2.06 ± 0.056^{cd}	21.53 ± 5.849^{dc}	38.85 ± 2.69^{cdef}
	0 (pH 10)†	2.26 ± 0.046^{a}	24.41 ± 0.460^{cde}	49.26 ± 11.96^{bc}
	3 (pH 10)†	2.15 ± 0.040^{abc}	19.36 ± 0.115^{e}	40.26 ± 11.96^{cdef}
	6 (pH 10)†	2.00 ± 0.096^{d}	20.39 ± 0.690^{e}	47.05 ± 8.61^{bcd}

*Mean ± SD from triplicate determinations.

**Different superscripts in the same column indicate the significant differences ($p<0.05$).

†† = based on protein content.

† = gelatin FFS was finally adjusted to the designated pHs.

2.2.1.2 Protein pattern

Protein patterns of gelatin FFS are shown in Figure 13. All FFS contained proteins with molecular weight lower than α-component and had the similar protein patterns. The disintegration or degradation of β and α-components more likely occurred. From the result, the degree of degradation might be higher than that of polymerization. Even though the decrease in free amino group content was noticeable in the presence of seaweed extract in some treatments, the cross-linking was negligible in FFS. Degraded gelatins in the solution might not undergo cross-linking effectively, owing to the low inter-conjunction. FFS were heated at 60°C to ensure the completely-solubilized gelatin, causing disintegration of β and α-components, due to autolytic activity by endogenous protenase. When FFS from bigeye snapper was incubated at 60°C for 30 min without proteinase inhibitors, no high MW cross-link with the decreased band intensity of α-chains was found. Degradation of cross-links and α-chains was mostly inhibited by 0.01 and 0.1 mM of soybean trypsin (Jongjareonrak *et al.*, 2006). Additionally, Intarasirisawat *et al.* (2007) reported that the major proteinase in bigeye snapper (*Priacanthus macracanthus*) skin was a serine proteinase. Similar results were observed by Nalinanon *et al.* (2008) who found that a serine protease in *P. tayenus* skin had the optimum activity at 45°C for 12 h. This protease showed the greastest degradation of β and α-components.

2.2.2 Properties of gelatin based films

2.2.2.1 Thickness and mechanical properties of gelatin films

Thickness and mechanical properties of bigeye snapper skin gelatin films prepared from FFS incorporated with seaweed extract with different pHs (8, 9 and 10) and films prepared from FFS containing seaweed extract with different final pHs are shown in Table 8. Thicknesses of films ranged from 28 to 35 μm. Similar thickness was found among all films ($p>0.05$), except the film prepared from FFS containing seaweed extract with pH of 8 at levels of 3 and 6%. The increase in thickness of those films might be due to the partial unfolding of gelatin at pH 8, in which some folded chains were present and could not form the compact network. When seaweed extract with pH 8 was used, the decrease in TS was found, regardless of seaweed extract concentration or final pH of FFS. When the extract with pHs, 9 or 10 was incorporated, FFS containing 3% seaweed extract and FFS containing 6% seaweed extract with final FFS pHs of 9 or 10

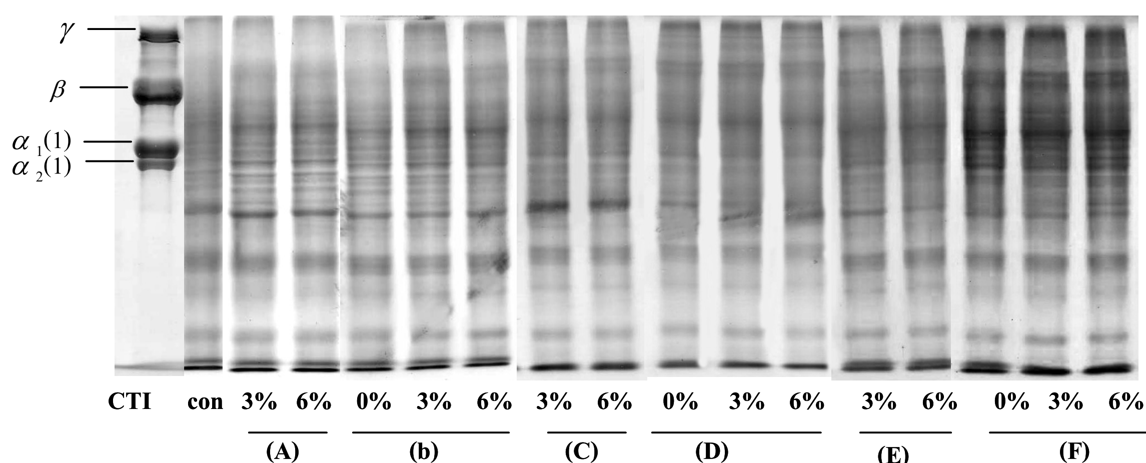


Figure 13. Protein patterns of gelatin forming solution (FFS); incorporated with seaweed extract at pH 8 (A); incorporated with seaweed extract at pH 8 and the mixture was adjusted to pH 8 (B); incorporated with seaweed extract at pH 9 (C); incorporated with seaweed extract at pH 9 and the mixture was adjusted to pH 9 (D); incorporated with seaweed extract at pH 10 (E); incorporated with seaweed extract at pH 10 and the mixture was adjusted to pH 10 (F). Numbers designate the concentration of seaweed extract (%). CTI: collagen type I from calf skin; con: control.

rendered the films with the highest TS ($p<0.05$). For EAB, film prepared from FFS (pH 8) containing seaweed extract had the increases in EAB as the amount of extract increased ($p<0.05$). The highest EAB of film was found when FFS (pH 8) containing 3% extract was used. However, no differences in EAB were noticeable between all films prepared from FFS containing extract with pHs 9 and 10, regardless of FFS pH. The result suggested that the rigid film was formed when such conditions were used. Cross-links of protein molecules via the strong bond might lead to the loss in flexibility of film. However, when seaweed extract at pH 8 was incorporated in the FFS with and without FFS pH adjustment to 8, the high EAB of film was found, particularly when FFS containing 6% seaweed extract was used. This indicated that the weak bond mainly involved in film network formation. At the alkaline pH, phenolic compounds can be converted to the quinone, and then quinone can form a dimer with amino or sulfhydryl side chains of polypeptides to form covalent C–N or C–S bonds with the phenolic ring (Strass and Gibson, 2004). When the concentrations of seaweed increased (6%), the decreases in TS were observed ($p<0.05$). Two quinones were cross-linked by themselves

at the higher concentrations (Strass and Gibson, 2004). As a consequence, protein cross-linking mediated by quinone was lowered. Additionally, self- aggregated quinone might locate between gelatin molecules, resulting in the increased spacing of cross-linked gelatin. When adjusted pH of gelatin to very alkaline pHs (9 and 10), the denaturation and dissociation of protein molecules occurred. Under these conditions, the interactions between proteins and phenolics become stronger, owing to exposure of hydrophobic protein residues that may bind to phenolics (Abugoch *et al.*, 2003). The combination of polyphenol-protein was the functions of hydrophobic bonds and hydrogen bonds. Firstly, polyphenol, which contained hydrophobic groups such as galloyl group entered into hydrophobic district of protein by hydrophobic interaction. Then phenolic hydroxyl group of polyphenol combined with polar group of protein by hydrogen bonds (Shi and Di, 2000). Probably, phenolic compounds in seaweed extract acted as cross-linker via weak bonds including hydrogen bondings or hydrophobic interactions. Thus, the mechanical properties of gelatin films were dependent on the pH of FFS. At the extreme acidic or alkaline pH values, strong electrostatic repulsion of ionized groups occurred, leading to solubilization of proteins, which is prerequisite for film formation. The degree of chain extension and the nature of sequence of amino acid residues might affect the mechanical properties of protein film (Krochta, 1997).

2.2.2.2 Light transmission and transparency of gelatin films

Transmission of UV and visible light at selected wavelength in the range of 200-800 nm of the film from bigeye snapper skin gelatin containing seaweed extract at different pHs and concentrations is shown in Table 9. Transmission in visible length (350-800 nm) of films varied from 43.14 to 76.64%. The transmission of UV light at 200 nm was in the range of 1.38 - 2.83% and transmission of 14.58 - 28.51% was found at 280 nm. The result suggested that films could prevent the lipid oxidation induced by UV light in food system. Jongjareonrak *et al.* (2005) reported that gelatin had the low content of tyrosine and phenylalanine (aromatic amino acids). In general, tyrosine and phenylalanine are well known to be sensitive chromophores, which absorb light at the wavelength below 300 nm (Li *et al.*, 2004). The aromatic amino acid content of protein material might play an important role in the UV barrier properties of protein

Table 8. Thickness and mechanical properties of gelatin film prepared from FFS incorporated with seaweed extract adjusted to different pHs at different concentrations without and with pH adjustment to various FFS pHs.

pH of extract	Seaweed extract[††] (%)	Thickness[*] (mm)	Mechanical properties[*]	
			TS (MPa)	EAB (%)
Control	0	0.029±0.005[b**]	12.12±1.32[b]	19.68±0.41[de]
pH 8	3	0.035±0.007[a]	5.66±0.74[e]	41.04±1.88[b]
	6	0.033±0.003[a]	6.39±0.77[e]	26.28±0.56[cd]
	0 (pH 8)[†]	0.031±0.005[ab]	5.66±1.11[e]	28.08±0.77[c]
	3 (pH 8)[†]	0.032±0.004[ab]	6.08±0.85[e]	45.96±1.41[b]
	6 (pH 8)[†]	0.031±0.007[ab]	4.82±0.29[e]	62.88±0.70[a]
pH 9	3	0.032±0.003[ab]	15.91±1.35[a]	23.70±0.59[cde]
	6	0.029±0.002[b]	11.04±0.25[bc]	19.80±0.30[de]
	0 (pH 9)[†]	0.029±0.002[b]	8.62±2.13[d]	23.22±0.98[cde]
	3 (pH 9)[†]	0.029±0.002[b]	9.61±2.17[cd]	17.4±0.49[e]
	6 (pH 9)[†]	0.028±0.005[b]	15.94±1.28[a]	26.64±0.28[cd]
pH 10	3	0.028±0.003[b]	16.11±2.27[a]	20.16±0.86[cde]
	6	0.029±0.002[b]	11.92±0.41[b]	24.60±0.43[cde]
	0 (pH 10)[†]	0.033±0.005[a]	8.79±1.68[d]	22.92±0.82[de]
	3 (pH 10)[†]	0.028±0.004[b]	11.86±1.48[b]	19.62±0.45[de]
	6 (pH 10)[†]	0.028±0.003[b]	16.00±1.89[a]	25.98±0.77[cd]

[*]Mean ± SD from nine determinations.

[**]Different superscripts in the same column indicate the significant differences ($p<0.05$).

[††] = based on protein content.

[†] = gelatin FFS was adjusted to the designated pHs.

films. The films prepared from other proteins including Alaska pollack surimi proteins (Shiku *et al.,* 2004) or whey protein (Fang *et al.,* 2002) offered a high UV protection, being considerably higher than in many synthetic polymer films (Shiku *et al*., 2004). In general, film incorporated with seaweed extract exhibited the decreased light transmission, compared with control film. Seaweed extract at a higher level rendered the film with higher barrier property against light transmission. This was in accordance with higher cross-link of proteins incorporated with seaweed extract at higher levels. Among all film, that prepared from FFS incorporated with 6% seaweed extract (pH8) with final pH adjustment to 8 showed the lowest UV transmission at wavelength of 280 nm.

Gelatin film showed the higher transparency value, 3.41 to 3.48, when FFS was adjusted to pH 10. The lower value represents the higher transparency. Gelatin films prepared from FFS adjusted pH to 8 and added with 6% seaweed extract was more transparent than those from FFS adjusted pHs to 9 or 10 ($p<0.05$). However, the addition of seaweed extract had no marked impact on transparency of resulting film.

2.2.2.3 Color of gelatin films

Color of gelatin films containing seaweed extract with different pHs and concentrations is shown in Table 10. Color of films was measured as lightness (L^*), greenness ($-a^*$) and yellowness (b^*). L^*-value significantly ($p<0.05$) decreased with the addition of seaweed extract, especially when the higher amount of extract was used. Films incorporated with higher concentration of seaweed extract became more yellowish. The higher greenness was found in film incorporated with higher amount of seaweed extract as indicated by the decrease in a^*-value. The result was in agreement with Cao *et al.* (2007) who reported that gelatin film incorporated with phenolic compounds (tannic acid and ferulic acid), at alkaline pH had the changes in color.

2.3 Characterizes of bigeye snapper skin gelatin film incorporated with seaweed extract

From the section 2.2, gelatin film from FFS incorporated with 6% seaweed extract (pHs 9 or 10) and adjusted pH to 9 or 10, yielded the films with the highest TS. Therefore, those films were subjected to characterization.

Table 9. Light transmission and transparency of gelatin film prepared from FFS incorporated with seaweed extract adjusted to different pHs at different concentrations without and with pH adjustment to various FFS pHs.

pH of extract	Seaweed extract[††] (%)	*Light transmission at different wavelength (nm)							*Transparency value
		200	280	350	400	500	600	800	
control	0	1.38	25.60	70.16	57.43	60.09	61.83	63.03	3.33±0.025[d**]
pH 8	3	1.54	23.47	56.73	53.40	57.65	59.49	61.02	3.23±0.020[g]
	6	1.81	19.98	51.50	53.73	59.81	61.74	63.23	3.32±0.014[de]
	0 (pH 8)[†]	1.56	20.55	43.14	51.84	54.83	56.49	57.87	3.26±0.015[f]
	3 (pH 8)[†]	2.41	23.84	63.57	54.81	59.54	61.58	63.18	3.32±0.018[de]
	6 (pH 8)[†]	1.80	14.58	54.62	50.23	56.55	58.47	60.11	3.28±0.045[f]
pH 9	3	1.74	20.55	43.44	53.91	55.26	57.80	60.46	3.29±0.025[ef]
	6	2.07	19.09	60.38	53.52	58.63	60.65	62.30	3.32±0.034[de]
	0 (pH 9)[†]	3.00	28.05	74.19	64.20	67.75	69.80	70.39	3.38±0.045[c]
	3 (pH 9)[†]	2.58	26.17	61.14	63.07	69.33	71.28	72.52	3.39±0.022[bc]
	6 (pH 9)[†]	2.07	25.58	76.07	60.77	68.95	71.19	72.82	3.40±0.019[bc]
pH 10	3	2.27	25.29	63.34	59.31	63.43	64.96	65.89	3.35±0.021[d]
	6	2.03	20.96	45.87	52.93	58.76	60.73	62.01	3.26±0.005[f]
	0 (pH10)[†]	2.83	28.29	52.78	64.06	69.60	72.89	76.64	3.42±0.018[b]
	3 (pH 10)[†]	2.15	28.51	61.27	64.99	70.12	71.60	73.39	3.41±0.017[bc]
	6 (pH 10)[†]	2.56	26.54	60.21	59.36	67.95	70.03	71.24	3.48±0.030[a]

*Mean ± SD from five determinations.

**Different superscripts in the same column indicate the significant differences ($p<0.05$).

†† = based on protein content.

† = gelatin FFS was adjusted to the designated pHs.

Table 10. Color of gelatin film prepared from FFS incorporated with seaweed extract adjusted to different pHs at different concentrations without and with pH adjustment to various FFS pHs.

pH of extract	Seaweed extract[††] (%)	Color[*]		
		L*	a*	b*
control	0	89.23±1.69[a]	-0.52±0.10[bcde]	0.64±0.90[bc**]
pH 8	3	86.36±0.36[b]	-0.57±0.15[bcde]	0.32±0.36[g]
	6	85.21±1.79[bc]	-0.77±0.04[ab]	0.72±0.45[bc]
	0 (pH 8)[†]	83.27±1.01[def]	-0.20±0.11[e]	0.39±0.10[fg]
	3 (pH 8)[†]	84.07±1.61[cd]	-0.35±0.20[cde]	0.68±0.11[bc]
	6 (pH 8)[†]	83.76±0.56[cde]	-0.82±0.80[a]	0.70±0.46[bc]
pH 9	3	85.77±0.52[b]	-0.42±0.08a[bcd]	0.39±0.18[fg]
	6	86.24±0.97[b]	-0.80±0.03[a]	0.45±0.16[cde]
	0 (pH 9)[†]	82.74±1.62[def]	-0.47±0.15[bcde]	0.40±0.14[fg]
	3 (pH 9)[†]	83.18±1.48[def]	-0.69±0.40[bcd]	0.72±0.12[bc]
	6 (pH 9)[†]	83.24±1.32[def]	-0.72±0.16[bc]	1.13±0.25[a]
pH 10	3	81.79±0.50[ef]	-0.72±0.08[bc]	0.69±0.07[bc]
	6	82.15±1.23[ef]	-0.80±0.12[a]	0.80±0.12[b]
	0 (pH 10)[†]	86.38±0.39[b]	-0.30±0.26[de]	0.41±0.11[def]
	3 (pH 10)[†]	82.22±0.99[ef]	-0.41±0.31[bcde]	0.51±0.24[bcd]
	6 (pH 10)[†]	82.80±1.31[def]	-0.42±0.14[bcde]	0.60±0.19[bc]

[*]Mean ± SD from five determinations.

[**]Different superscripts in the same column indicate the significant differences ($p<0.05$).

[††] = based on protein content.

[†] = gelatin FFS was adjusted to the designated pHs.

2.3.1 Mechanical and physical properties

Mechanical properties, water vapor permeability and thickness of gelatin films without and with seaweed extract addition under different conditions are shown in Table 11. All gelatin films had similar TS and thickness ($p>0.05$). EAB of gelatin films incorporated with seaweed extract at pHs 9 and 10 was approximately 2-fold greater than that of the control. The adjustment of pH of FFS to 9 and 10 most likely caused the stretching or unfolding of gelatin molecules, in which gelatin with the longer chains could form the network effectively. Also, oxidized phenolic compounds in seaweed extract might link the gelatin to gain the higher chain length. Thus, the incorporation of seaweed extract could improve both TS and EAB of resulting film. Rheological characteristics (viscoelasticity and gel strength) and chemical/structural properties (amino acid composition, molecular weight distribution, and triplex helix formation) of gelatins from the skin of different fish species were responsible for the differences in behavior (Gómez-Guillén *et al.,* 2002).

Decreases in water vapor permeability of gelatin films incorporated with 6% seaweed were observed, when compared with control film,regardless of final pH of FFS used for film preparation. Phenolic compounds, either in reduced or oxidized form, might enhance the cross-linking of gelatin. Increasing reticulation of the network could decrease the free volume of the polymeric matrix and increase the tortuosity of the pathway of the water molecules through the network, thus decreasing diffusion rate of water molecules through the films (Cao *et al.,* 2007).

Table 11. Physical and mechanical properties of gelatin films incorporated with seaweed extract at different pHs.

Film samples	TS[*] (MPa)	EAB[*] (%)	WVP[**] ($x10^{-10} gm^{-1}s^{-1}Pa^{-1}$)	Thickness[*] (µm)
Control	10.04 ± 0.78[a]	12.51 ± 2.04[b]	1.28 ± 0.17[a†]	29.73 ± 2.01[a]
6% SE, pH 9	11.43 ± 1.11[a]	25.98 ± 2.34[a]	0.99 ± 0.12[b]	30.62 ± 3.34[a]
6% SE, pH 10	10.92 ± 1.94[a]	23.88 ± 1.34[a]	0.89 ± 0.15[b]	29.51 ± 1.98[a]

[*] Mean ± SD from nine determinations.

[**] Mean ± SD from five determinations.

[†] Different superscript in the same column indicate significant differences ($p<0.05$).

SE: Seaweed extract

2.3.2 Moisture content and color

Moisture content and color of gelatin film incorporated with 6% seaweed at different pHs and the control film are shown in Table 12. Similar moisture content was found in all films, ranging from 25.12 to 25.97%. Thus, the addition of seaweed extract had no influence on moisture content of resulting film (p>0.05). Additionally, incorporation of seaweed extract had no impact on L*-value of resulting film, regardless of final pH of FFS used for film preparation (p>0.05). Nevertheless, the greenness and yellowness of the film incorporated with seaweed extract increased, as indicated by the decrease in *a**-value and the increase in *b**-value respectively, compared with the control film. Pigments remaining in seaweed extract most likely contributed to the changes in colors of resulting films. Chlorophylls, the pigments responsible for the characteristic green color of plants, result in color changes in food (Koca *et al.,* 2006).

Table 12. Moisture content and color of gelatin films incorporated with seaweed extract at different pHs.

Treatment	Moisture content* (%)	L*†	a*†	b*†
Control	25.12±0.67[a**]	88.51±0.27[a]	-1.45±0.15[b]	2.10±0.09[a]
6% SE, pH 9	25.97±1.30[a]	88.45±0.38[a]	-1.77±0.07[a]	3.41±0.15[c]
6% SE, pH 10	25.48±1.68[a]	88.74±0.39[a]	-1.81±0.06[a]	3.21±0.10[b]

*Mean ± SD from tripicate determinations.

†Mean ± SD from five determinations.

**Different superscripts in the same column indicate the significant differences (p<0.05).

SE: Seaweed extract.

2.3.3 Light transmission and film transparency

Transmission of UV and visible light at selected wavelength in the range of 200-800 nm of gelatin film incorporated with 6% seaweed extract at different pHs is shown in Table 13. Light transmission of all films tested was negligible at 200 nm and low at 280 nm. Films from bigeye snapper and brownstripe red snapper skin gelatin were reported to exhibit a high absorption to light in the UV range (200-280 nm) (Jongjaroenrak *et al.,* 2006). The films obtained with other protein systems such as Alaska pollack surimi proteins (Shiku *et al.,* 2004) or whey protein (Fang *et al.,* 2002) also offered the UV protection. In general, film incorporated with seaweed extract

showed the more light transmission in visible range (350-800 nm) than the control. With pH adjustment to alkaline pH, the unfolding of gelatin was more intense. As a result, the uniform network could be formed and the light could pass through such a network more easily. No differences in transparency of gelatin films incorporated with and without seaweed extract were found, irrespective of pH of FFS used for film preparation ($p > 0.05$).

Table 13. Light transmission and transparency of gelatin films incorporated with seaweed extract at different pHs.

Film samples	Wavelength (nm)							Transparency value*
	200	280	350	400	500	600	800	
Control	1.42	27.57	56.29	59.57	62.26	64.03	65.25	3.32 ± 0.01[a**]
6% SE, pH 9	2.06	26.98	57.42	62.96	71.30	73.36	74.82	3.38 ± 0.01[a]
6% SE, pH 10	2.58	27.01	61.78	63.75	70.95	73.10	74.36	3.39 ± 0.01[a]

* Mean ± SD from five determinations.

** Different superscripts in the same column indicates the significant differences ($p<0.05$).

SE: Seaweed extract.

2.3.4 Film solubility and protein solubility

Film solubility and protein solubility of gelatin films incorporated without and with seaweed extract at different pHs are shown in Table 14. The incorporation of seaweed extract into gelatin film resulted in the lower film solubility in comparison with film without seaweed extract addition (control) ($p<0.05$). The lower solubility observed in gelatin films suggested that the protein in gelatin underwent more aggregation, leading to the formation of larger molecular weight aggregates as evidenced by the lessened protein solubility. For film solubility, more cross-linked protein network, more likely entrapped the glycerol used as plasticizer in the film. As a consequence, glycerol was less leached out in the water. Therefore, the film became more resistant to solubilization, which can be beneficial for further applications.

Table 14. Film solubility and protein solubility of gelatin films incorporated with seaweed extract at different pHs.

Film samples	Film solubility* (%)	Protein solubility* (%)
Control	86.93±0.43[a]	74.06±2.27[a**]
6% SE, pH 9	71.67±2.11[b]	66.14±1.98[b]
6% SE, pH 10	69.84±0.26[b]	62.82±2.33[b]

* Mean ± SD from five determinations.

** Different superscripts in the same column indicates the significant differences ($p<0.05$).

SE: Seaweed extract**.**

2.3.5 Surface characteristics

SEM micrographs of the surface and cross-section of gelatin films incorporated with 6% seaweed extract at different pHs are illustrated in Figure 14. The control film had the smooth surface both top and bottom, while those incorporated with 6% seaweed possessed the rougher surface, regardless of pHs. The cross-section of gelatin film incorporated with seaweed extract was rougher with some discontinuous zone. The higher roughness might be governed by the increasing bondings between phenolic compounds and protein strand via covalent and non-covalent bondings. These discontinuous zones could be related to the formation of channels during the process of drying. This might be associated with the increase in EAB of film incorporated with seaweed extract (Table 11). Carvalho and Grosso (2004) found that gelatin based film modified with transglutaminase, glyoxol and formaldehyde showed a loss of fibrillar orientation with apparent decrease in free volume.

2.3.6 Hydrolysis by protease

The protein digestibilities of gelatin films containing 6% seaweed extract at different pHs are presented as the degree of hydrolysis (DH) (Table 15). DH of all gelatin films hydrolyzed by α-chymotrypsin increased as the hydrolysis time increased ($p<0.05$). For the same hydrolysis time, the highest DH was found in the control. Gelatin films incorporated with 6% seaweed extract at pH 9 had the higher hydrolysis than the counterpart with pH 10 ($p<0.05$). The result suggested that the incorporation of seaweed extract resulted in the increased resistance of films to enzymatic hydrolysis. The greater

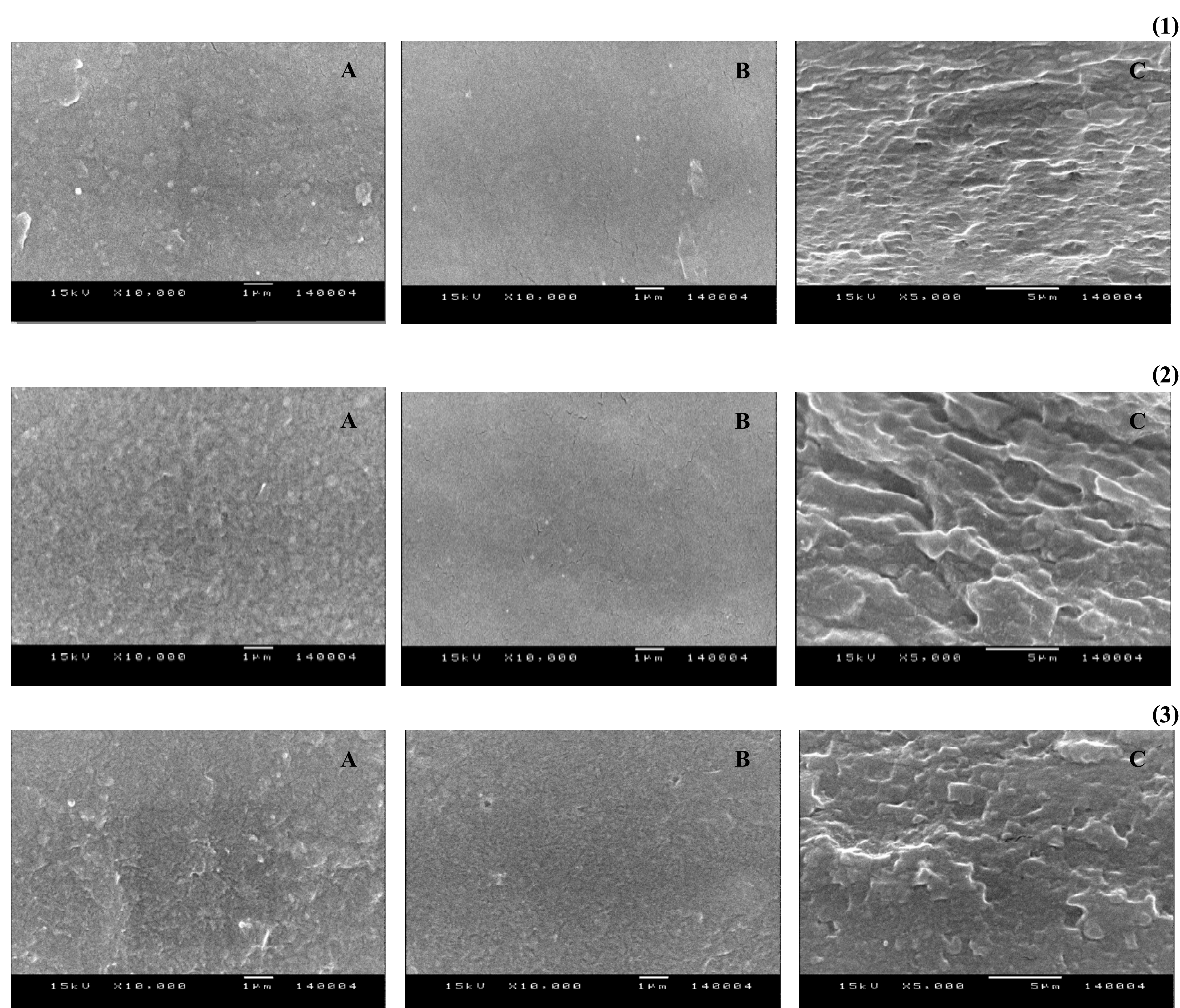


Figure 14. SEM micrographs of gelatin films. Upper surface (A), lower surface (B) and cross section (C). Control film (without seaweed extract) (1), film incorporated with 6% seaweed extract; pH 9 (2) and film incorporated with 6% seaweed extract, pH 10 (3).

resistance toward digestion by α-chymotrypsin of film prepared at pH 10 in the presence of seaweed extract was probably because the phenolic compounds in the extract might undergo the oxidation more effectively than at pH 9. Therefore, quinone could be formed to a higher extent and the cross-linking via non-disulfide covalent bond was more pronounced. Apart from α-chymotrypsin, other digestive proteolytic enzyme also involved in the digestion of proteins in the digestive system (Shiku *et al.,* 2003). Jongjareonrak *et al.* (2006) reported the protein digestibility of gelatin film from bigeye snapper skin and brownstripe red snapper skin by α-chymotrypsin ranging from 64 to

69%. Therefore, bigeye snapper skin gelatin films were easily digested and can used as biodegradable films.

Table 15. Degree of hydrolysis (%) of gelatin films incorporated with seaweed extract at different pHs by α- chymotrypsin.

Film samples	Hydrolysis times (min)*			
	30	60	90	120
Control	53.56 ± 0.53[a**]	61.22 ± 1.40[a]	70.02 ± 0.20[a]	70.68 ± 1.00[a]
6% SE, pH 9	40.97 ± 1.13[b]	59.76 ± 1.40[a]	60.76 ± 0.53[b]	63.89 ± 0.53[b]
6% SE, pH 10	40.24 ± 1.20[b]	53.43 ± 0.40[b]	55.23 ± 0.73[c]	55.89 ± 1.00[c]

* Mean ± SD from triplicate determinations.

** Different superscripts in the same column indicate the significant differences ($p<0.05$).

SE: Seaweed extract.

2.3.7 Protein solubility in various solvents

Protein solubility of gelatin films incorporated without and with seaweed extract at pH 9 or 10 in various solvents is shown in Table 16. Solubility of gelatin film incorporated with seaweed extract was lower than that of the control for all solvents used. When S1 was used, the lower solubility was found in film incorporated with seaweed extract for both pHs ($p<0.05$). For S2, alkaline pH of solvent (pH 8) might facilitate the dissolvation of film network associated with the increased repulsive force of protein molecules under alkaline condition (pH 8). It was noted that the higher solubility was found in film with pH 10 than pH 9 ($p<0.05$), when S2 was used. Under acidic and alkaline conditions, protein molecules in film-forming solutions are partially unfolded due to the protein denaturation, and their hydrophobic groups are exposed (Kristinsson and Hultin, 2003). When S3 (20 mM Tris, pH 8.0 containing 1% (w/v) SDS) and S4 (20 mM Tris, pH 8.0 containing 1% (w/v) SDS and 8 M urea) were used, the increase in solubility were noticeable in all films. The result indicated the presence of H-bond and hydrophobic interaction of protein in the film matrix. Shi and Di (2000) reported that the formation of phenolic-protein was governed in part by hydrophobic interaction and hydrogen bonds. Firstly, polyphenol, which contained hydrophobic groups, entered into hydrophobic district of protein by hydrophobic interaction. Then phenolic hydroxyl

group of polyphenol combined with polar group of proteins by hydrogen bonds.

Additionally, some of phenolic compounds were converted to quinone under alkaline condition. Strass and Gibson (2004) reported that quinone reacted with amino or sulfhydryl side chains of polypeptides to form covalent C-N or C-S bond. When S5 was used, a slight increase was observed. However, incomplete solubilization by S5 indicated the presence of non-disulfide covalent bond in the film matrix, particullaly for film incorporated with seaweed extract.

Table 16. Protein solubility (%)[*] of gelatin films incorporated with seaweed extract at different pHs.

Gelatin films	S1[†]	S2	S3	S4	S5
Control	20.52±0.98[a**]	43.29±2.70[a]	68.24±1.34[a]	87.15±2.47[a]	88.35±3.01[a]
6% SE, pH 9	10.32±1.13[b]	37.68±0.39[c]	60.81±0.63[b]	80.43±1.79[b]	83.56±3.27[b]
6% SE, pH 10	9.98±2.66[b]	40.99±0.88[b]	63.73±1.88[c]	81.88±0.97[b]	83.28±3.09[b]

[†] (S1) 0.6 M KCl; (S2) 20 mM Tris, pH 8.0; (S3) 20 mM Tris, pH 8.0 containing 1% (w/v) SDS; (S4) 20 mM Tris, pH 8.0 containing 1% (w/v) SDS and 8 M urea; (S5) 20 mM Tris, pH 8.0 containing 1 % (w/v) SDS, 8 M urea, and 2 % (v/v) *β*-mercaptoethanol.

[*] Mean±SD from triplicate determinations.

[**]Different superscripts in the same column indicate significant differences ($p<0.05$).

SE: Seaweed extract.

2.3.8 Thermal properties

Transition temperature (T_{max}) and transition enthalpy (ΔH) of gelatin films incorporated without and with seaweed extract at pH 9 and 10 are shown in Table 17. Two major peaks were found in gelatin films. Transition temperature of all gelatin films were in the range of 57.82 - 59.41°C and 170.54 – 178.96°C. Transition temperature indicated the temperature causing the disruption of the polymer interaction formed during film preparation. For transition enthalpy, the highest enthalpy of the first transition was found in the control treatment. For the first transition, slightly higher T_{max} was found in film incorporated with 6% seaweed extract (pH 10). Thus, the enhanced cross-linking induced by phenolic compound in seaweed extract might contribute to lower molecular mobility. The transition enthalpy of proteins increases with the chain rigidity and the

intensity of both inter- and intramolecular interactions, including hindrance to internal rotation along the macromolecular chain (Berreto *et al.,* 2003). Jongjareonrak *et al.* (2006) reported that transition temperature of bigeye snapper skin observed from endothermic transition with 50% glycerol was 61.08 J/g. For second transition, gelatin film incorporated with 6% seaweed extract (pH 10) exhibited the highest T_{max} (178.96°C); however it required the lowest enthalpy (129.91 J/g). The increase in the transition enthalpy of melting was due to the increase in cross-linking mediated by the reticulation agent. A reduction in the number of hydrogen bonds with a simultaneous increase in the extent of covalent cross-linking resulted in the increase in the thermal stability of melting (de Carvalho and Grosso, 2004). The amount of imino acids showed the direct positive correlation to the thermal stability of protein via hydrogen bond (Sikorski *et al.,* 1984). The integrity and molecular weight of protein chains contribute to the network structure of films obtained (Shiku *et al.,* 2004).

Table 17. Transition temperatures and enthalpy of gelatin films incorporated with seaweed extract at different pHs

Films samples	1st Transition*		2nd Transition*	
	Transition temperature (°C)	Transition enthalpy (J/g)	Transition temperature (°C)	Transition enthalpy (J/g)
Control	59.30±0.51^{a**}	18.24±0.46^{a}	170.54±1.79^{b}	154.70±0.84^{a}
6% SE, pH 9	57.82±0.61^{b}	9.44±0.25^{b}	171.04±1.74^{b}	144.57±3.03^{b}
6% SE, pH 10	59.41±0.71^{a}	9.93±1.04^{b}	178.96±0.79^{a}	129.91±3.33^{c}

* Mean ± SD from triplicate determinations.

** Different superscripts in the same column indicate the significant differences ($p<0.05$).

SE: Seaweed extract**.**

2.4 Moisture sorption isotherm

A moisture sorption isotherm of gelatin films is depicted in Figure 15. Moisture sorption isotherms presented a sigmoidal shape in common with those of most foods and hydrophilic film. Moisture content of all gelatin films increased slowly at low Aw (0.18-0.64) and increased rapidly at Aw of 0.64-0.90. Gelatin films prepared from FFS

incorporated with seaweed extract with pHs 9 and 10 showed a higher adsorption rate than the control film. At pH 9 or 10, the protein became more charged, thereby enhancing water adsorption. Physical and barrier properties of hydrophilic protein films can also be significantly influenced by the moisture concentration in the film (Gennadios *et al.*, 1993; Gontard *et al.*, 1993). Due to their inherent hydrophilic nature, protein-based films tend to absorb large quantities of water at elevated relative humidity (RH) conditions (Jangchud and Chinnan, 1999). Moreover, unreacted hydroxyl groups of phenolic could combine with water. Phenolic compounds in gelatin film incorporated with seaweed extract could play a role in absorbing water. Cho and Rhee (2002) reported that under given RH conditions, films with higher glycerol ratio absorbed more moisture with higher adsorption rate, and films with higher plasticizer contents exhibited higher equilibrium moisture contents.

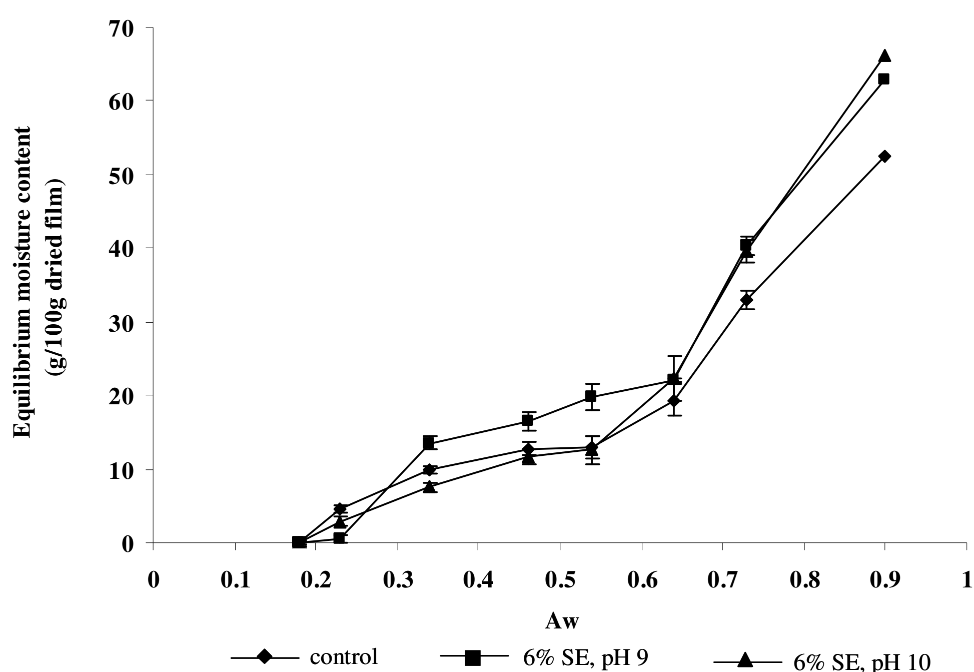


Figure 15. Moisture sorption isotherms at 28-30°C of gelatin films incorporated without and with seaweed extract at different pHs. SE: Seaweed extract. Bars represent standard deviation from triplicate determinations.

2.5 Changes of gelatin films during storage

2.5.1 Changes in mechanical properties

Mechanical properties of gelatin films incorporated without and with seaweed extract stored at 54% RH at room temperature (28-30°C) are shown in Figure 16. Increase in TS and decrease in EAB were observed when storage time increased ($p<0.05$). The result suggested the formation of more rigid structure by polymer arrangement and cohesion within the film matrix. For the control film, TS markedly increased after 1 week of storage time; however, no changes were observed during week 3 and 4 ($p>0.05$). The decrease in EAB of the control film was in agreement with the increase in TS. This indicated that the control film became more rigid with increasing storage time. Cao *et al.* (2007) prepared bovine bone gelatin film by addition of 10 mg tannic acid/g gelatin and adjusting pH of FFS to 9 prior to casting. After storage at RH of 50±3% at 25°C for 90 days, the mechanical strength of gelatin film increased by 11.53%, compared to that of new prepared film. Due to the lower compactness of the control film, the aggregation was more pronounced as the storage time increased. On the other hand, the network of film incorporated with seaweed extract had been cross-linked. Therefore, a lesser change in term of molecular aggregation or rearrangement was found in the film incorporated with seaweed extract.

2.5.2 Changes in color and transparency

L*, a* and b*-values of gelatin-base film incorporated without and with seaweed extract at different pHs during storage at 54% RH and room temperature (28-30°C) are shown in Figure 18. No changes in L*-value were found in all films during the storage of 4 weeks ($p>0.05$). Within the first week of storage film incorporated with seaweed extract at pH 10 showed the increase in *a**-value but the decrease in *b**- value. Conversely, the control film had the decrease in *a**-value but the increase in *b**-value.

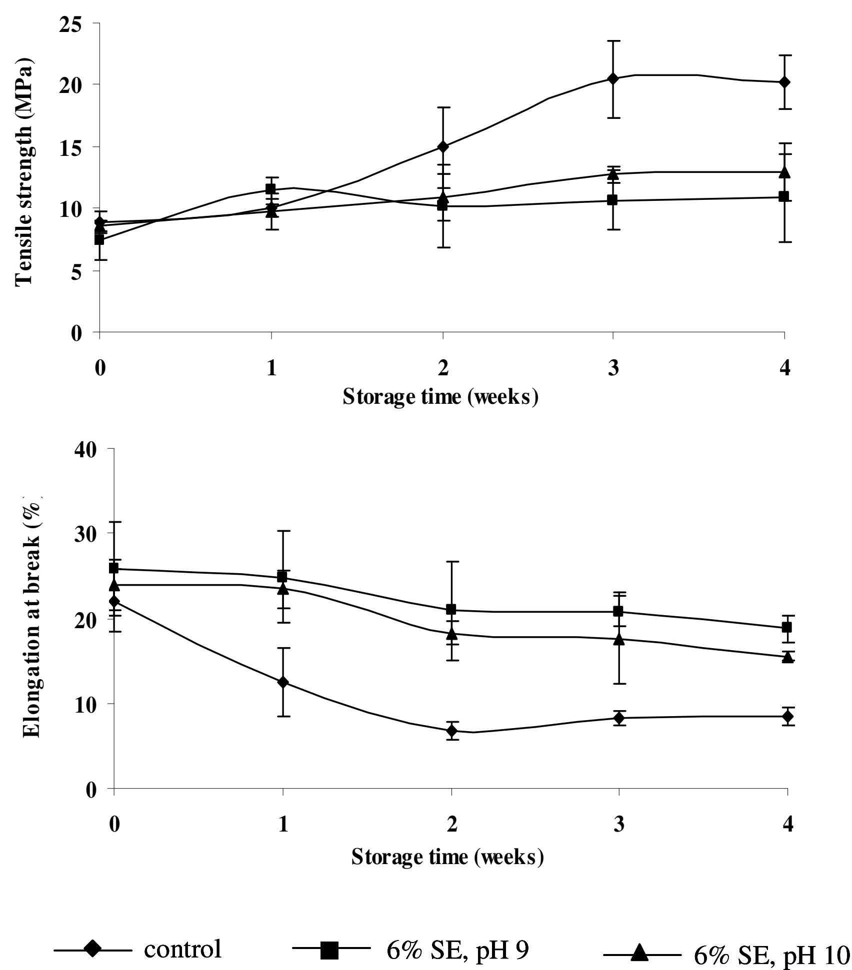


Figure 16. Changes in tensile strength and elongation at break of gelatin films incorporated without and with seaweed extract at different pHs during storage at room temperature (28-30°C). Bars represent the standard deviation from nine determinations.

The transparency value of gelatin films incorporated without and with seaweed extract during storage at room temperature (28-30°C) for 4 weeks is shown in Figure 17. The lower transparency value represents the higher transparent. Control film had the increase in transparency value during the storage up to 4 weeks, whereas films incorporated with seaweed extract at pH 9 or 10 had no changes in transparency value. The result indicated that the control film became less transparent as the storage time increased. Lipid retained in the gelatin, ever at low amount, could undergo oxidation, resulting in the formation of carbonyl compounds, which reacted with amino group of gelatin via Maillard reaction reaction as the storage time increased.

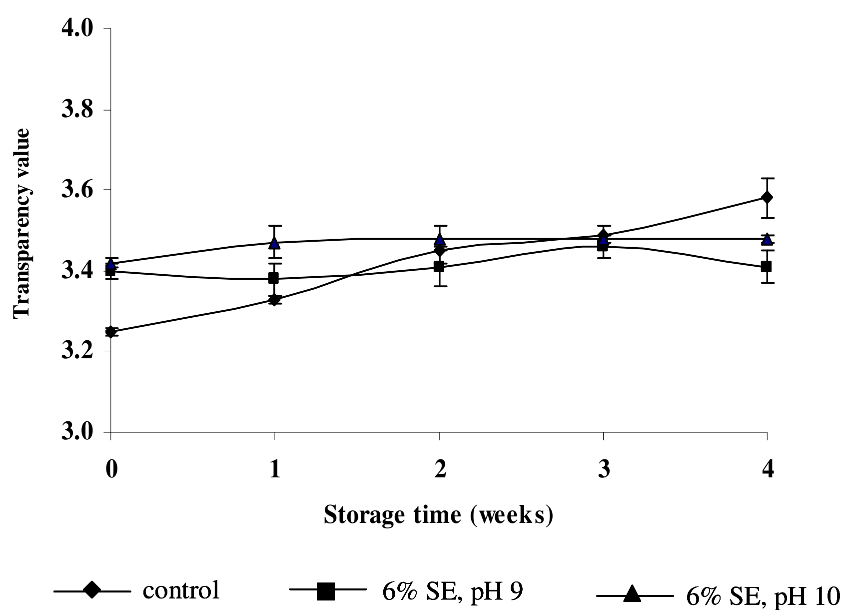


Figure 17. Changes in transparency value of gelatin-based films incorporated without and with seaweed extract at different pHs during storage at room temperature (28-30°C). Bars represent the standard deviation from five determinations.

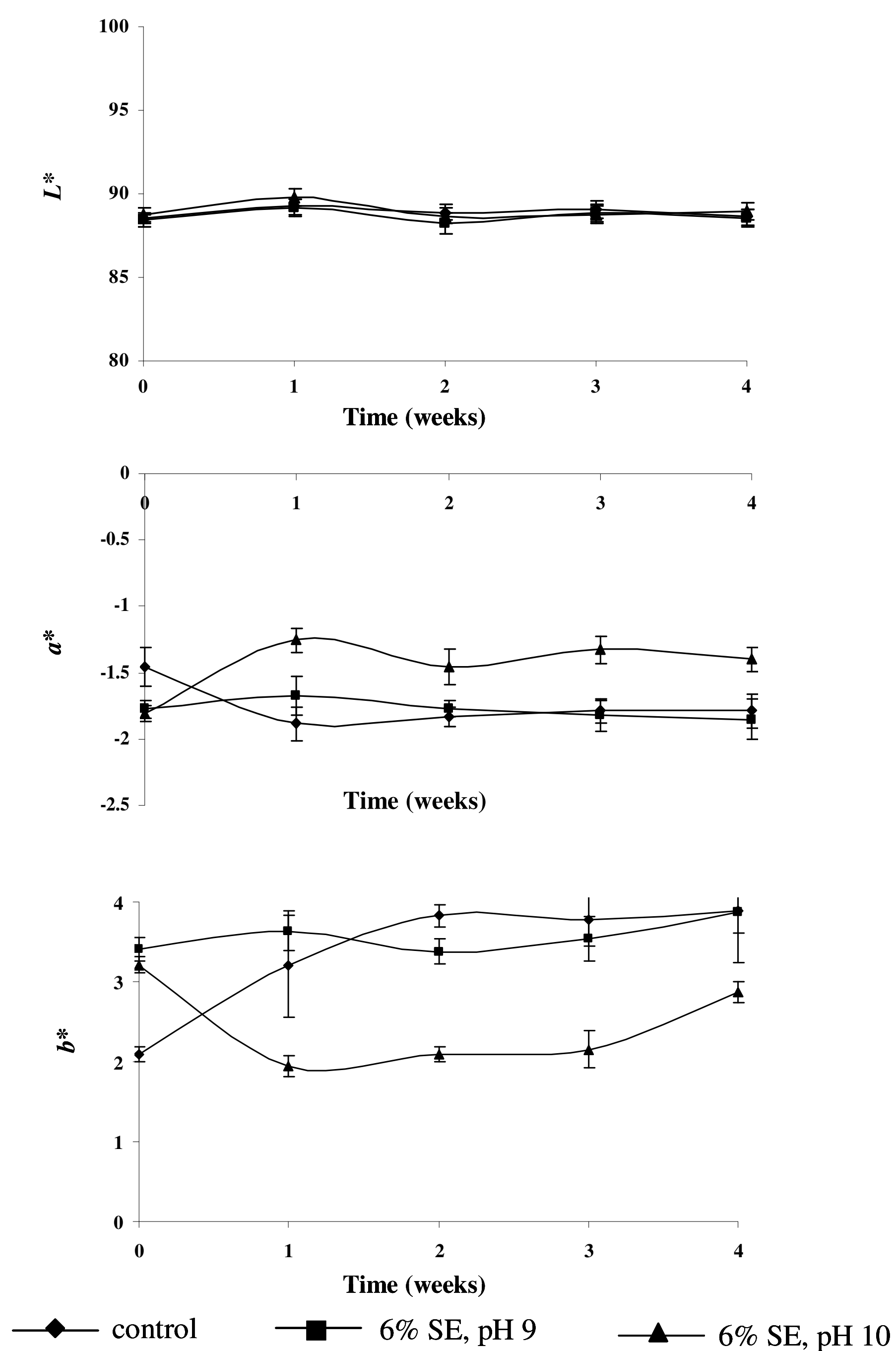


Figure 18. Changes in L*, a* and b*-values of gelatin films incorporated without and with seaweed extract at different pHs during storage at room temperature (28-30°C). Bars represent the standard deviation from five determinations.

2.5.3 Changes in film solubility and protein solubility

Film solubility and protein solubility of gelatin films incorporated without and with seaweed extract at pH 9 or 10 during storage at room temperature (28-30°C) are shown in Figure 19. In general, solubility of all films continuously decreased with increasing storage time ($p<0.05$). As previously described (section 2.3.5), gelatin films incorporated with seaweed extract had the lower film solubility, compared with the control film. Films incorporated with seaweed extract also had the decreased protein solubility. From the result, the decreases in protein solubility observed in all films suggested that the protein in film underwent more aggregation, leading to the more formation of cross-links with the larger molecular weight (Orliac *et al.*, 2002).

2.5.4 Changes in water vapor permeability

Water vapor permeability of gelatin films incorporated with seaweed extract at pH 9 or 10 slightly decreased throughout the storage time ($p<0.05$) (Figure 20). Marked decreased in water vapor permeability of gelatin film incorporated with seaweed extract (pH 10) was observed within the first week of storage ($p<0.05$). Generally, gelatin films incorporated with seaweed extract had the lower water vapor permeability, compared with the control film. The phenolic compounds in seaweed extract might facilitate the cross-linking of gelatin, in which the reactive groups, especially anionic groups, were not available for water binding.

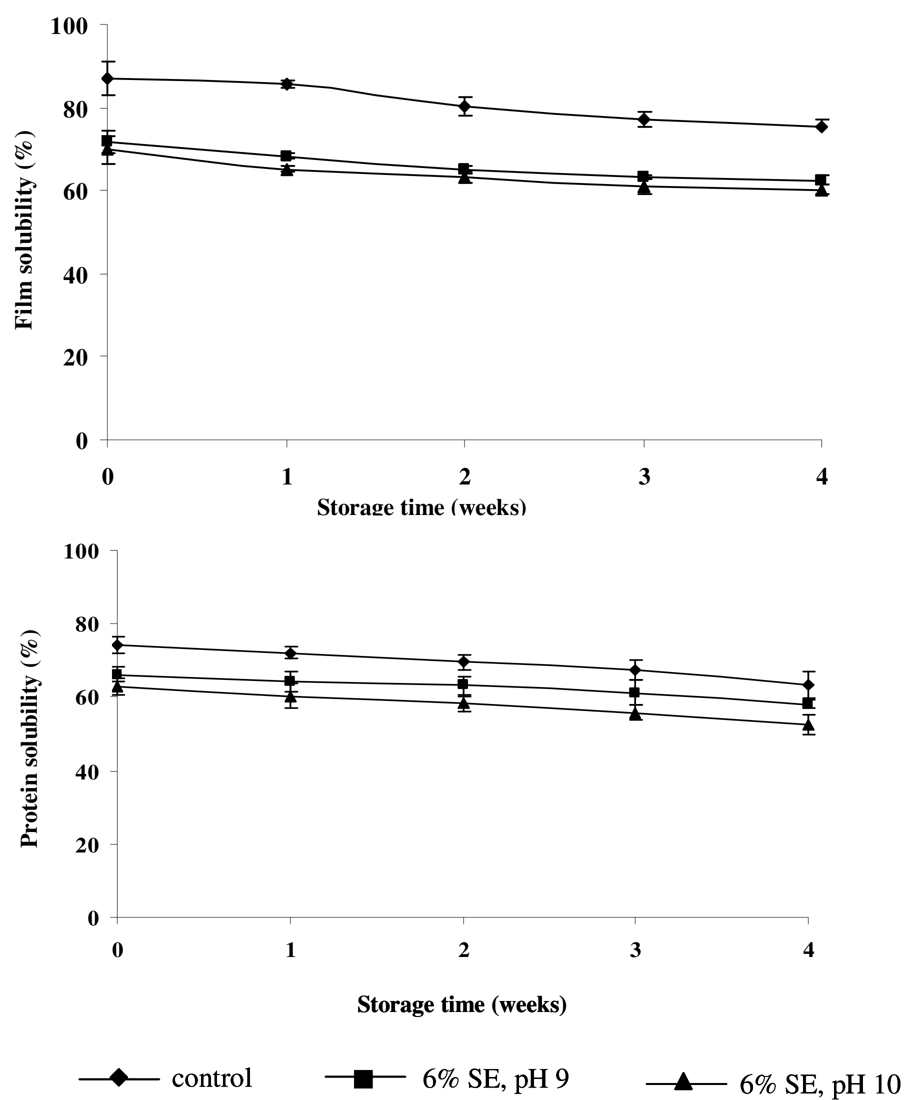


Figure 19. Changes in film solubility and protein solubility of gelatin films incorporated without and with seaweed extract at different pHs during storage at room temperature (28-30°C). Bars represent the standard deviation from five determinations.

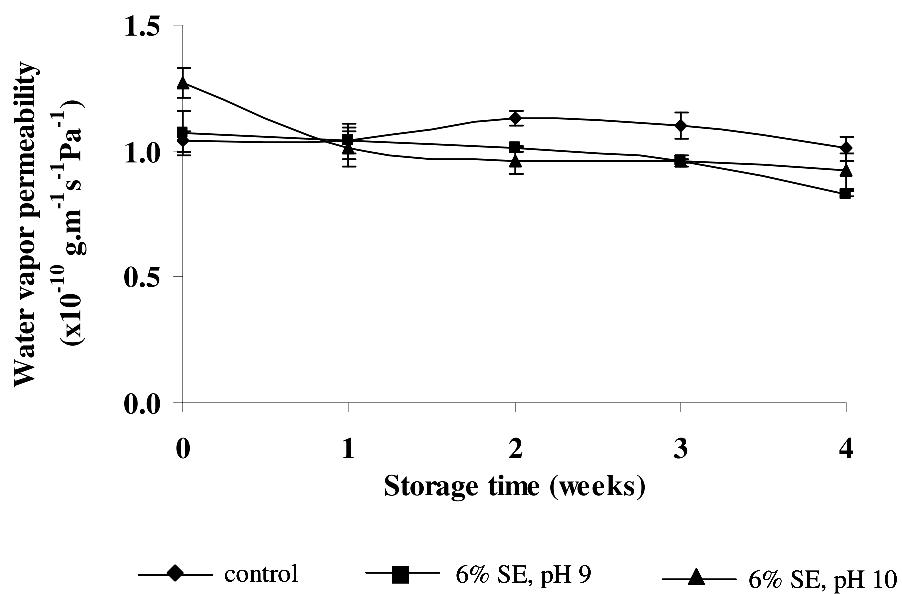


Figure 20. Changes in water vapor permeability of gelatin films incorporated without and with seaweed extract at different pHs during storage at room temperature (28-30°C). Bars represent the standard deviation from five determinations.

CHAPTER 4

CONCLUSION

1. Methanolic extracts of brown seaweeds, *T. ornata* and *S. polycystum,* could be a source of natural antioxidants, which could retard lipid oxidation in different model systems, regardless of chlorophyll removal. The extracts could inhibit *S. aureus,* but were not able to inhibit the growth of *B. subtilis, S. enteritidis* and *A. niger.*

2. Bigeye snapper skin gelatin film incorporated with 6% seaweed extract at pH 9 or 10 had the improved mechanical and physical properties as indicated by enhanced cross-linking induced by oxidized phenols. Films obtained had the lower protein solubility and were resistant to hydrolysis by protease and thermal transition.

3. Films incorporated with seaweed extract underwent the mechanical changes to a lessen extent during extended storage. However, all films became darker and more yellowish at the lower rate when seaweed extract was added. Apart from the improvement of mechanical properties, seaweed extract could increase the stability of films during the extended storage.

SUGGESTION

1. Identification of phenolic compound in the seaweed extract should be performed.

2. Further application of developed film containing seaweed extracts should be carried out to prevent lipid oxidation in foods.

3. Gelatin-based films incorporated with other seaweed extract including red seaweed and green seaweed should be studied.

REFERENCES

Abbott, I.A. 1995. Taxonomy of Economic Seaweeds: reference to some Pacific species, Volume V: California Sea Grant College, Uni. Calif. La Jolla, California.

Abugoch, L.E., Martínez, E.N. and Añón, M.C. 2003. Influence of extracting solvent upon the structural properties of amaranth (*Amaranthus hypochondriacus*) glutelin. J. Agric. Food Chem. 51: 4060–4065.

Aewsiri, T., Benjakul, S., Visessanguan, W. and Tanaka, M. 2008. Chemical compositions and functional properties of gelatin from pre-cooked tuna fin. Int. J. Food Sci. Technol. 43: 685-693.

Akoh, C.C. and Min, D.B. 1998. Food lipids: Chemistry, Nutrition and Biotechnology. *In* Lipid-Based Synthetic Fat Substitutes (Akoh, C.C and Min, D.B. ed.). p. 559-588. Marcel Dekker. New York, USA.

Ang, J.P.O. 1986. Analysis of the vegetation structure of a *Sargassum* community in the Philippines. Mar. Ecol-Prog. Ser. 28: 9-19.

AOAC. 1990. *Official Method of Analytical Chemists.* 15th Ed., Association of Official Analytical Chemists, Arlington, VA.

AOAC. 2000. Official Methods of Analysis, 17th ed. Association of Official Chemists. Gaithersberg, Maryland, USA.

Arvanitoyannis, I.S., Psomiadou, E., Nakayama, A., Aiba, S.S. and Yamamoto, N. 1997. Edible films made from gelatin, soluble starch and polyols, Part 3. Food Chem. 60: 593-604.

Arnao, M.B., Cano, A. and Acosta, M. 2001. The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chem. 73: 239-244.

Arnesen, J.A. and Gildberg, A. 2002. Preparation and characterisation of gelatine from the skin of harp seal (*Phoca groendlandica*). Bioresource Technol. 82: 191–194.

Arnesen, J.A. and Gildberg, A. 2007. Extraction and characterisation of gelatine from Atlantic salmon (*Salmo salar*) skin. Bioresource Technol. 98: 53-57.

Asghar, A. and Henrickson, R.L. 1982. Chemical, Biochemical, Functional and Nutritional Characteristics of Collagen in Food Systems. *In* Advances in Food Research. Vol. 28 (Chichester, C.O., Mrata, E. M. and Schweigert, B.S. ed.). p. 237-273. Academic Press. London.

ASTM. 1989. Annual Book of ASTM Standards. American Society for Testing and Materials. Philadelphia.

Athukorala, Y., Lee, K. W., Song, C.B., Ahn, C.B., Shin, T.S., Cha, Y.J., Shahidi, F. and Jeon, Y.J. 2003. Potential antioxidant activity of marine red alga *Grateloupia filicina* extracts. J. Food Lipids. 10: 251-265.

Aungtonya, C. and Liao, L.M. 2002. Marine flora (alga and sea grasses) in the reference collection of the Phuket Marine Biology Center, Thailand. Phuket. Marine Biology Center Resource. 64: 65-8.

Babin, H. and Dickinson, E. 2001. Characterization of gelatin based films modified with transglutaminase, glyoxal and formaldehyde. Food Hydrocolloids. 15: 271-276.

Barreto, M. and Meyer, J.J.M. 2006. Isolation and antimicrobial activity of a lanosol derivative from *Osmundaria serrata* (Rhodophyta) and a visual exploration of its biofilm covering. S. Afr. J. Bot. 72: 521-528.

Bateman, J.F., Lamandé, S.R. and Ramshaw, J.A.M. 1996. Collagen Superfamily. *In* Extracellular Matrix: Molecular Components and Interactions (Comper, W. D. ed.). p. 22-27. Harwood Academic Publishers. UK.

Baydar, N.G., Ozkan, G. and Sagdis, O. 2005. Total phenolic contents and antimicrobial activity of grape (*Vistis vinfera* L.) extracts. Food Control. 15: 335-339.

Bell, A.E., 1989. Gel Structure and Food Biopolymers. *In* Water and Food Quality. (Hardman, T.M. ed.). p. 251-275. Elsevier Applied Science. London.

Benjakul, S. and Morrissey, M. 1997. Protein hydrolysates from Pacific whiting solid wastes. J. Agric. Food Chem. 45: 3423-3430.

Bennamara, A., Abourriche, A., Berrada, M., Charrouf, M., Chaib, N., Boudouma, M. and Garneau, F. X. 1999. Methoxybifurcarenone: an antifungal and antibacterial meroditerpenoid from the brown alga *Cystoseira tamariscifolia.* Phytochemistry. 52: 37-40.

Bergman, I. and Loxley, R. 1963. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. Anal. Chem. 35: 1961-1965.

Bertan, L.C., Tanada-Palmu, P.S., Siani, A.C. and Grosso, C.R.F. 2005. Effect of fatty acids and 'Brazillian elemi' on composite films based on gelatin. Food Hydrocolloids. 19: 73-82.

Beuchat, L.R. 1994. Antimicrobial Properties of Spices and Their Essential Oils. *In* Natural Antimicrobial Systems and Food Preservation. (Dillon, V.M. and Board, R.G. ed.). p. 167-180. CAB International. Wallingford.

Bigi, A. Cojazzi, G. Panzavolta, S. Rubini, K. and Roveri, N. 2001. Mechanical and thermal properties of gelatin film at different degrees of glutaraldehyde crosslinking. Biomaterials. 22: 763-768.

Bigi, A., Cojazzi, G., Panzavolta, S., Roveri, N. and Rubini, K. 2002. Stabilization of gelatin films by crosslinking with genipin. Biomaterials. 23: 4827-4832.

Binsi, P.K., Shamasundar, B.A., Dileep, A.O., Badii, F. and Howell, N.K. 2009. Rheological and functional properties of gelatin from the skin of Bigeye

snapper (*Priacanthus hamrur*) fish: influence of gelatin on the gel-forming ability of fish mince. Food Hydrocolloids. 23: 132-145.

Bruno, M. and Moresi, M. 2005. Interrelation between the transient functions of Bologna sausage using Friedrich and Heymann theory. J. Texture Stud. 36: 1-24.

Burjandze, T.V. 1979. Hydroxyproline content and location in relation to collagen thermal stability. Biopolymers. 18: 931-936.

Burtin, P. 2003. Nutritional value of seaweeds. Electron. J. Agric. Food Chem. 2: 498–503.

Cahyana, A.H., Shuto, Y. and Kinoshita, Y. 1992. Pyropheophytin a as an antioxidative substance from the marine alga, Arame (*Eisennia bicyclis*). Biosci. Biotechnol. Biochem. 56: 1533-1535.

Cao, I.M. and Chang, K.C. 2001. Edible films prepared from water extract of soybeans. J. Food Sci. 67: 1449-1454.

Carvalho, R.A., Sobral, P.J.A., Thomazine, M., Habitante, A.M.Q.B Giménez, B., Gómez-Guillén, M.C. and Montero, P. 2008. Development of edible films based on differently processed Atlantic halibut (*Hippoglossus hippoglossus*) skin gelatin. Food Hydrocolloids. 22: 1117-1123.

Chambi, H. and Grosso, L. 2006. Edible films produced with gelatin and casein cross-linked with transglutaminase. Food Res. Int. 39: 458-466.

Chan, E.W.C., Lim, Y.Y. and Omar, M. 2007. Antioxidant and antibacterial activity of leaves of Etlingera species (Zingiberaceae) in Peninsular Malaysia. Food Chem. 104: 1586-1593.

Chandini, S.K., Ganesan, P. and Bhaskar, N. 2008. *In vitro* antioxidant activities of three selected brown seaweeds of India. Food Chem. 107: 707-713.

Chawla, S.P., Venugopol, V. and Nair, P.M. 1996. Gelation of proteins from washed muscle of threadfin bream (*Nemipterus japonicus*) under mild acidic conditions. J. Food Sci. 61: 362-371.

Chen, C.W. and Ho, C.T. 1995. Antioxidant properties of polyphenols extracted from green and black tea. J. Food Lipids. 2: 35-46.

Chen, J.H. and Ho, C.T. 1997. Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. J. Agric. Food Chem. 45: 2374-2378.

Chen, F. 1996. High cell density culture of microalgae in heterotrophic growth. Trends Biotechnol. 14: 421-426.

Chen, F., Li, H.B., Wong, R.N.S., Ji, B. and Jiang, Y. 2005. Isolation and purification of the bioactive carotenoid zeaxanthin from the microalga *Microcystis aeruginosa* by high-speed counter-current chromatography. J. Chromatography A. 1064: 183-186.

Cheow, C.S., Norizah, M.S., Kyaw, Z.Y. and Howell, N.K. 2007. Preparation and characterization of gelatins from the skins of sin croaker (*Johnuis dussumieri*) and shortfin scad (*Decapterus macrosoma*). Food Chem. 101: 386-391.

Chew, Y.L., Lim, Y.Y., Omar, M. and Khoo, K.S. 2008. Antioxidant activity of three edible seaweeds from two areas in South East Asia. LWT-Food Sci. Technol. 41: 1067-1072.

Chiang, Y.M., Chuang, D.Y., Wang, S.Y., Kuo, Y.H., Tsai, P.W. and Shyur, L.F. 2004. Metabolite profiling and chemopreventive bioactivity of plant extracts from *Bidens pilosa*. J. Ethnopharmacol. 95: 409–419.

Chiou, B.S., Avena-Bustillos, R.J., Shey, J., Yee, E., Bechtel, P.J., Imam, S.H., Glenn, G.M. and Orts, W.J. 2006. Rheological and mechanical properties of cross-linked fish gelatins. Polymer. 47: 6379-6386.

Chipley, J.R. 1974. Effects of 2,4-dinitrophenol and N,N'-dicyclohexylcarbodiimide on cell envelope-associated enzymes of *Escherichia coli* and *Salmonella enteritidis*. Microbiol. 10: 115-120.

Cho, S.M., Gu, Y.S. and Kim, S.B. 2005. Extracting optimization and physical properties of yellow fin tuna (*Thunnus albacares*) skin gelatin compared to mammalian gelatin. Food Hydrocolloids. 19: 221-229.

Cho, S.Y. and Rhee, C. 2002. Sorption characteristics of soy protein films and their relation to mechanical properties. LWT-Food Sci. Technol. 35: 151-157.

Choi, W.S. and Han, J.H. 2002. Film-forming mechanism and heat desnaturation effects on the physical and chemical properties of pea-protein-isolate edible films. J. Food Sci. 67: 1399–1406.

Chopra, I. and Greenwood, D. 2001. Antibacterial agents: basis of action. *In* Encyclopedia of life sciences. (Battista, J. Ed.). Nottingham, UK.

Cowan, M.M. 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12: 564-582.

Cuq, B., Aymard, C., Cuq, J.L. and Guilbert, S. 1995. Edible packaging films based on fish myofibrillar proteins: formulation and functional properties. J. Food Sci. 60: 1369-1374.

Cuq, B., Gontard, N. and Guilbert, S. 1998. Protein as agricultural polymers for packaging production. Cereal Chem. 75: 1-9.

Cuq, B. 2002. Formation and Properties of Fish Myofibrillar Protein Films and Coating. *In* Protein-Based Films and Coatings. (Gennadious, A. ed.). p. 213-232. CRC Press. New York.

Cuvelier, M.E., Richard, H. and Berset, C. 1992. Comparison of the antioxidative activity of some acid-phenols: structure-activity relationship. Biosci. Biotechnol. Biochem. 56: 324-325.

Davison, M.P. 1993. Parebens and Phenolic Compounds. *In* Antimicrobial in Foods 2nd ed. (Davison, P.M. and Banen, A.L., ed.). p 263-306. Marcel Dekker. New York.

Dawidowicz, A.L., Wianowska, D. and Baraniak, B. 2006. The antioxidant properties of alcoholic extracts from *Sambucus nigra* L. (antioxidant properties of extracts). LWT-Food Sci. Technol. 39: 308-315.

de Carvalho, R.A. and Grosso, C.R.F. 2004. Characterization of gelatin based films modified with transglutaminase, glyoxal and formaldehyde. Food Hydrocolloids. 18: 717–726.

Decker, A.E. 1998. Antioxidant Mechanism. *In* Food Lipid: Chemistry, Nutrition, and Biotechnology. (Akoh, C.C and Min, D.B., ed.). p. 397-448. Marcel Decker. New York.

Decker, E.A. and Crum, A.D. 1993. Antioxidant activity of carnosine in cooked ground pork. Meat Sci. 34: 245-253.

Dejong, G.A.H. and Koppelamn, S.J. 2002. Transglutaminase catalyzed reactions: impact on food applications. J. Food Sci. 67: 2798-2806.

de Silva, S.M.S., Gamage, S.K.T., Savitrikumar, N. and Balasubramanium, S. 1982. Antibacterial activity of extract from the brown seaweed *Stoechospermum marginatum.* Phytcochemistry. 21: 944-945.

Di, P.P., Chico, B., Villalonga, R., Mariniello, L., Damiao, A.E., Masi, P. and Porta, R. 2006. Chitosan-whey protein edible films produced in the absence or presence of transglutaminase: analysis of their mechanical and barrier properties. Biomacromolecules 7: 744-749.

Donohue, J.T.M., Tuma, D.J. and Sorell, M.F. 1983. Acetaldehyde adducts with proteins: binding of [^{14}C] acetaldehyde to serum albumin. Arch. Biochem. Biophys. 220**:** 239–246.

Duarte, M.C.T., Figueira, G.M., Sartoratto, A., Rehder, V.L.G. and Delarmelima, C. 2005. Anti-*Candida* activity of Brazilian medicinal plants. J. Ethnopharmacol. 97: 305–311.

Dziezak, J.D. 1986. Antioxidants: The ultimate answer to oxidation. Food Technol. 40(9): 94-102.

Ely, R., Supriya, T. and Naik, C.G. 2004. Antimicrobial activity of marine organisms collected off the coast of South East India. J. Exper. Marine Biol. Ecol. 309: 121-127.

Endo, Y., Usuki, R. And Kaneda, T. 1985. Antioxidant effects of chlorophyll and pheophytin on the auto-oxidation of oils in dark. I. Comparison of the inhibitory effects. J. Am. Oil Chem. Soc. 62: 1375-1378.

Faleiro, M.L., Miguel, M.G., Ladeiro, F. Venâncio, F. Tavares, R., Brito, J.C., Figueiredo, A.C., Barroso, J.G. and Pedro, L.G. 1993. Antimicrobial activity of essential oils isolated from *Portuguese endemic* species of Thymus. Lett. Appl. Microbiol. 36: 35-40.

Fang, Y., Tung, M.A., Britt, I.T., Yada, S. and Dalgleish, D.G. 2002. Tensile and barrier properties of edible films made from whey proteins. J. Food. Sci. 67: 188-193.

FAO. 2006. Year Book of Fishery Statistics, vol. 98/1&2. Food and Agricultural Organisation of the United Nations, Rome.

Fennema, O.R. 1996. Lipids. *In* Food Chemistry (3rd ed.). p. 321-429. Marcel Dekker. New York.

Fernández-Díaz, M.D., Montero, P. and Gómez-Guillén, M.C. 2003. Effect of freezing fish skins on molecular and rheological properties of extracted gelatin. Food Hydrocolloids. 17: 281-286.

Ferruzzi, M., Bohm, V., Courtney, P. and Schwartz, S. 2002. Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assay. J. Food Chem. Toxicol. 67: 2589-2595.

Flodin, C. and Whitfield, F.B. 1999. 4-Hydroxybenzoic acid: a likely precursor of 2,4,6-tribromophenol in *Ulva lactuca*. Phytochemistry. 51: 249-255.

Frankel, E.N. 1985. Chemistry of Auto-Oxidation: Mechanism, Products and Flavor Significance. *In* Flavor Chemistry of Fats and Oils. (Min, D.B. and Smouse, T.H. ed.) p. 1–37. Champaign, IL.

Friedman, M., Kim, S. V., Lee, S.J., Han, G.P., Han, J.S., Lee, K.R. and Kozukue, N. 2005. Distribution of catechins, theaflavins, caffeine, and theobromine in 77 teas consumed in the United States. J. Food Sci. 70: 550-559.

Gennadios, A., McHugh, T.H., Weller, C.L. and Kroachta, J.M. 1994. Edible Coatings and Film Based on Proteins. *In* Edible Coatings and Films to Improve Food Quality. (Kroachta, J. M., Baldwin, E. A. and Nisperos-Carriedo, M. ed.). p. 201-277. Technomic Publishing Co., Inc. Lancaster, PA.

Gennadios, A. and Weller, C.L. 1990. Edible films and coating from wheat and corn protein. Food Technol. 44(10): 63-69.

Gennadios, A. and Weller, C.L. 1993. Modification of physical and barrier properties of egg albumin films. J. Food Sci. 61: 585-589.

Gennadios, A., Honda, A., Froning, G.W., Weller, C.L. and Hanna, M.A. 1998. Physical properties of egg white-dialdehyde starch films. J. Agric. Food Chem. 46: 1297-1302.

Giese, J. 1996. Antioxidants: tools for preventing lipid oxidation. Food Technol. 50(11): 73-81.

Gilsenan, P.M. and Ross-Murphy, S.B. 2000. Rheological characterization of gelatins from mammalian and marine sources. Food Hydrocolloids. 14: 191-195.

Gómez-Guillén, M.C., Sarabia, A.I., Solas, M.T. and Montero, P. 2001. Effect of microbial transglutaminase on the functional properties of megrim (*Lepidorhom busboscii*) skin gelatin. J. Sci. Food Agric. 81: 665-673.

Gómez-Guillén, M.C., Turnay, J., Fernandez-Diaz, M.D., Ulmo, N., Lizarbe, M.A. and Montero, P. 2002. Structural and physical properties of gelatin extracted from different marine species : a comparative study. Food Hydrocolloids. 16: 25-34.

Giménez, B., Gómez-Guillén, M.C. and Montero, P. 2005.The role of salt washing of fish skins in chemical and rheological properties of gelatin extracted. Food Hydrocolloids. 19: 951-957.

Giménez, B., Gómez-Guillén, M.C. and Montero, P. 2005a. Storage of dried fish skins on quality characteristics of extracted gelatin. Food Hydrocolliods. 19: 958-963.

Gontard, N., Guilbert, S. and Cuq, J.L. 1993. Water and glycerol as plasticizer affect mechanical and water vapor barrier properties of edible wheat gluten film. J. Food Sci. 58: 206-211.

Gordon, M.H. 2001. The Mechanism of Antioxidant Action *in vitro. In* Food Antioxidants. (Hudson, B.J.F. ed.). p. 1-18. Elsevier Applied Science. London.

Grossman, S. and Bergman, M. 1992. “Process for the production of gelatin from fish skins”. US Patent. 5,093,474. March, 3, 1992.

Gudmundsson, M. and Hafsteinsson, H. 1997. Gelatin from cod skins as affected by chemical treatments. J. Food Sci. 67: 37-39.

Guilbert, S., Gentard, N. and Cuq, B. 1996. Technology and applications of edible protective films. Pack. Techol. Sci. 8: 339-346.

GülÇin, Ì., Sat, G.İ., Beydemir, ş., Elmastaş, M. and Küfrevioğlu, Ö.İ. 2004. Comparison of antioxidant activity of clove (*Eugenia caryophylata* thumb) buds and lavender (*Lavandula stoechas* L.). Food Chem. 87: 393-400.

Hagerman, A.E., Riedl, K.M., Jones, G.A., Sovik, K.N., Ritchard, N.T. and Hartzfeid, P.W. 1998. High molecular weight plant polyphenolics Tannins) as biological antioxidants. J. Agric. Food Chem. 46: 1887-1892.

Harrington, W.F. and Venkateswara, R.N. 1970. Collagen structure in solution. I. kinetics of helix regeneration in single-chain gelatins. Biochemistry. 9(19): 3714-3724.

Haug, I.J., Draget, K.I. and Smidsrod, O. 2004. Microcapsules and emulsions containing low bloom gelatin and method of making and using thereof. Food Hydrocolloids. 18: 203-213.

He, Y. and Shahidi, F. 1997. Antioxidant activity of green tea and its catechins in a fish meat model system. J. Agric. Food Chem. 45: 4262-4266.

Helcke, T. 2000. Gelatin, the food technologist's friend or foe. Int. Food Ingredi. 1: 6-8.

Heo, S.J., Park, E.J., Lee, K.W. and Jeon, Y.J. 2005. Antioxidant activities of enzymatic extracts from brown seaweeds. Biores. Technol. 96: 1613-1623.

Hermansson, A.M. 2007. Physico-chemical aspects of soy proteins structure formation. J. Texture Stud. 9: 33-58.

Hernandez-Munoz, P., Villalobos, R. and Chiralt, A. 2004. Effect of thermal treatments on functional properties of edible films made from wheat gluten fractions. Food Hydrocolloids. 18: 647-654.

Hill, C. 1995. Bacteriocins: Natural Antimicrobials From Microorganisms. *In* New Methods of Food Preservation. (Gould, G.W. ed.). p. 22-39. Blackie Academic and Professional. London.

Hoshina, C., Tomita, K. and Shioi, Y. 1998. Antioxidant activity of chlorophylls: Its structure-activity relationship. Photosyn. Res. 4: 3281–3284.

Intarasirisawat, R., Benjakul, S., Visessanguan, W., Prodpran, T., Tanaka, M. and Howell, N. K. 2007. Autolysis study of bigeye snapper (*Priacanthus macracanthus*) skin and its effect on gelatin. Food Hydrocolloids. 21: 537-544.

Iwata, K., Ishizaki, S., Honda, A. and Tanaka, M. 2000. Preparation and characterization of edible films from fish water-soluble proteins. Fish. Sci. 66: 372-378.

Jadhav, S.J., Nimbalker, S.S., Kulkami, A.D. and Madhavi, D.L. 1996. Lipid Oxidation in Biological and Food Systems. *In* Food Antioxidants. (Madhavi, D.L. *et al*., ed.). p. 5-64. Marcel Dekker. New York.

Jamilah, B. and Harvinder, K.G. 2002. Properties of gelatins from skins of fish-black tolapia (*Oreochromis mossambicus*) and red tilapia (*Oreochromis nolitica*). Food Chem. 77: 81-84.

Jangchud, A. and Chinan, M.S. 1999. Peanut protein film as affected by drying temperature and pH film forming solution. J. Food Sci. 64: 153-157.

Johnson, I.T. 2001. Polyphenolic compounds: Interactions with the gut and implications for human health. Curr. Med. Chem. 8: 1245-1255.

Johnston-Banks, F. A. 1990. Gelatin. *In* Food Gels. (Harris, P. ed.). p. 233-289. Elsevier Applied Science. London.

Jones, N.R. 1997. Used of gelatin in edible products. *In* The Science and Technology of Gelatin. (Ward, A.G. and Courts, A. ed.) p. 366-394. Academic Press. London.

Jongjareonrak, A., Benjakul, S., Visessanguan, W. and Tanaka, M. 2005. Isolation and characterization of collagen from bigeye snapper (*Priacanthus macracanthus*) skin. J. Sci. Food Agric. 85: 1203-1210.

Jongjareonrak, A., Benjakul, S., Visessanguan, W., Prodpran, T. and Tanaka, M. 2006.Characterization of edible films from skin gelatin of brownstripe red snapper and bigeye snapper. Food Hydrocolloids. 20: 492-501.

Jongjareonrak, A., Benjakul, S., Visessanguan, W. and Tanaka, M. 2006a. Skin gelatin from bigeye snapper and brownstripe red snapper: chemical compositions and effect of microbial transglutaminase on gel properties. Food Hydrocolloids. 20: 1216-1222.

Kester, J.J. and Fennema, O.R. 1986. Edible film and coatings: A review. Food Technol. 40(12): 47-59.

Kim, S.J. and Ustunol, Z. 2001. Solubility and moisture sorption isotherms of whey-protein-based edible film as influenced by lipid and plasticizer incorporation. J. Agric. Food Chem. 49: 4388-4391.

Kim, Y.S., Park, W.P. and Choi, Y.J. 2003. New approaches for the effective recovery of fish proteins and their physicochemical characteristics. Fish. Sci. 69: 1231-1239.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. 2005. Characterization of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*). Food Chem. 89: 363-372.

Knobloch, K., Pauli, A., Iberal, B., Weis, N. and Weigand, H. 1989. Antibacterial activity and antifungal properties of essential oil components. J. Essent. Oil Res. 1: 119–128.

Koca, N., Burdurlu, H. S. and Karadeniz, F. 2006. Effect of pH on chlorophyll degradation and colour loss in blanched green peas. Food Chem. 100: 609-615.

Kolodziejska, I., Kaczorowski, K., Piotrosia, B. and Sadowska, M. 2004. Modifications of properties of gelatin from skins of Baltic cod (*Gadus morhua)* with transglutaminase. Food Chem. 86: 203-209.

Kolodziejska, I., Skeierka, E., Sadowska, M., Kolodziejski, W. and Niecikowska, C. 2008. Effect of extracting time and temperature on yield of gelatin from different fish offal. Food. Chem. 107: 700-706.

Koleva, I.I., Linssen, J.P.H., Beek, T.A.V., Evstatieva, L.N., Kortenska, V. and Handjieva, N. 2003. Antioxidant activity screening of extracts from Sideritis species (*Labiatae*) grown in Bulgaria. J. Sci. Food Agric. 83: 809-819.

Kranl, K., Schlesier, K., Bitsch, R., Hermann, H., Rohe, M. and Böhm, V. 2005. Comparing antioxidative food additives and secondary plant products use of different assays. Am. J. Clin. Nutri. 93: 171-175.

Kristinsson, H.G. and Hultin, H.O. 2003. Effect of low and high pH treatment on the functional properties of cod muscle proteins. J. Agric. Food Chem. 51: 5103-110.

Kristjansson, M.M., Guthmundsdottir, S., Fox, J.W. and Bjarnason, J.B. 1995. Characterization of collagenolytic serine proteinase from the Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. 100: 707-717.

Krochta, J.M. and DeMulder-Johnston, C. 1977. Edible and biodegradable polymer films; challenges and opportunities. Food Technol. 51(2): 61-74.

Kuda, T., Tsunekawa, M., Goto, H. and Araki, Y. 2005. Antioxidant properties of four edible algae harvested in the Noto Peninsuka, Japan. J. Food Comp. Anal. 96: 625-633.

Kumaron, A. and Karunakaran, R.J. 2007. In vitro antioxidant properties of methanol extract of five *Phollanthus* species from India. LWT-Food Sci. Technol. 40: 344-352.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature. 227: 680-685.

Ledward, D.A. 1986. Gelation of gelatin. *In.* Functional Properties of Food Macromolecules. (Mitchell, J.R. and Ledward, D.A. ed.). p 171-201. Elsevier Applied Science. London.

Lewmanomont, K. 1978. Some edible algae of Thailand, Kasetsart Journal. 12: 119-129.

Lewmanomont, K. 1995. Algea in Thai. Office of Environmental Policy and Planning, Bangkok.

Lewmanomont, K. and Ogawa, H. 1995. Common Seaweeds and Seagrasses of Thailand. Integrated Promotion Technology Co. Ltd., Bangkok.

Lee, K.Y., Shim, J. and Lee, H.G. 2004. Mechanical properties of gellan and gelatin composite films. Carbohyd. Polym. 56: 251-254.

Leong, L.P. and Shui, G. 2002. An investigation of antioxidant capacity of fruits in Singapore markets. Food Chem. 76: 69-75.

Leuenberger, B.H. 1991. Investigation of viscosity and gelation properties of different mammalian and fish gelatins. Food Hydrocolloids. 5: 353-361.

Li, H.B., Cheng, K.W., Wong, C.C., Fan, K.W., Chen, F. and Jiang, Y. 2007. Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. Food Chem. 102: 771-776.

Li, B., Liu, B.L., Gao, L.Z. and Chen, H.L. 2004. Studies on bullfrog skin collagen. Food Chem. 84: 65-69.

Liang, H.C., Chang, W.H., Lin, K.J. and Sung, H.W. 2003. Genipin-crosslinked gelatin microspheres as drug carrier. J. Biomed. Res. 65: 271-282.

Liang, Q., Quian, H. and Yao, W. 2005. Identication of a vonoids and their glycosides by high-performance liquid chromatography with electrospray ionization masss pectrometry and with diode array ultraviolet detection. Eur. J. Mass Spectrom. 11: 93-101.

Lim, S.N., Cheung, P.C.K., Ooi, V.E.C. and Ang, P.O. 2002. Evaluation of antioxidative activity of extracts from a brown seaweed *Sargassum siliquastrum.* Food Chem. 50: 3862-3866.

Liu, H.Y., Li,D. and Guo, S.D. 2008. Extraction and properties of gelatin from channel catfish (*Ietalurus punetaus*) skin. LWT-Food Sci. Technol. 41: 414-419.

Lobban, C.S. and Harrison, P.J. 1994. Seaweeds. *In* Seaweed Ecology and Physiology. p. 1-5. Cambridge University Press. New York.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 16: 265-275.

Mahmoud, R. and Savello, P.A. 1993. Solubility and hydrolyzability of films produced by transglutaminase catalytic cross-linking of whey protein. J. Dairy Sci. 76: 29–35.

Maisuthisakul, P., Pasuk, S. and Ritthiruangde, P. 2008. Relationship between antioxidant properties and chemical composition of some Thai plants. J. Food Comp. Anal. 21: 229-240.

Mayachiew, P. and Devahastin, S. (2008). Antimicrobial and antioxidant activities of Indian gooseberry and galangal extracts. LWT-Food Sci. Technol. 41: 1153-1159.

Mayakun, J. and Prathep, A. 2005. Seasonal variations in diversity and abundance of macroalgae at Samui Island, Surat Thani Province, Thailand. Songklanakarin J. Sci. Technol. 27: 653-663.

McHugh, T.H. and Krochta, J.M. 1994. Water vapor permeability properties of edible whey protein-lipid emulsion films. J. AOAC Int. 71: 307-312.

Meller, K.S. and Krochta, J.M. 1997. Oxygen and aroma barrier properties of edible films: a review. Trend Food Sci. Technol. 8: 228-237.

Meier, E., Kunz, G., Haller, 0. and Arnheiter, H. 1990. Activity of rat Mx proteins against a rhabdovirus. J. Virol. 64: 6263-6269

Mendis, E., Rajapakse, N. and Kim, S.K. 2005. Antioxidant properties of a radical scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. J. Agric. Food Chem. 53: 581-587.

Montero, P., and Gomez-Guillen, M.C. 2000. Extracting conditions for megrim (*Lepidorhmbus boscii*) skin collagen affect functional properties of the resulting gelatin. J. Food Sci. 65: 434-438.

Morales, F.J. and Jimennez-Perez, S. 2001. Free radical scavenging capacity of Maillard reaction products as related to color and fluorescence. Food Chem. 72: 119-125.

Moure, A., Cruz, J.M., Franco, D., Dominguez, J.M., Sineiro, J. and Dominguez, H. 2001. Natural antioxidants from residual sources. Food Chem. 72: 145-171.

Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N. and Suzuki, N. 2001. Characterization of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). Food Chem. 72: 425-429.

Nagai, T. and Yukimoto, T. 2003. Preparation and functional properties of beverages made from sea algae. Food Chem. 81: 327-332.

Nagayama, K., Iwamura, Y., Shibata, T., Hirayama, I. and Nakamura, T. 2002. Bacteriocidal activity of phlorotannins from the brown alga *Ecklonia kurome.* J. Antimicrob. Chemoth. 50: 889-893.

Nalinanona, S., Benjakula, S., Visessanguanb, W. and Kishimura, H. 2008. Improvement of gelatin extraction from bigeye snapper skin using pepsin-aided process in combination with protease inhibitor. Food Hydrocolloids. 22: 615-622.

Namiki, M. 1990. Antioxidant/antimutagens in food. Crit. Rev. Food Sci. Nutr. 29: 273-300.

Newman, D.J., Cragg, G.M. and Snader, K.M. 2003. Natural products as source of new drugs over the period 1981-2002. J. Nat. Prod. 66: 1022-1037.

Norland, R.E. 1990. Fish Gelatin. *In* Advances in Fisheries Technology and Biotechnology for Increased Profitability. (Voight, M.N. and Botta, J.K. ed.). p. 325-333. Technomic Publishing. Lancaster, PA.

Oh, J.H., Wang, B., Field, P.D. and Aglan, H.A. 2004. Characteristics of edible films made from dairy proteins and hydrolysate cross-linked with transglutaminase. Int. J. Food Sci. Technol. 39: 287-294.

Olde Damink, L.H.H., Dijkstra, P.J., Van Luyn, M.J.A., Van Wachem, P.B., Nieuwenhuis, P. and Feijen, J. 1995. Glutaraldehyde as a crosslinking agent for collagen-based biomaterials. J. Mater. Sci-Mater. Med. 6: 460-472.

Orliac, O., Roilly, A., Silvestre, F. and Rigal, L. 2002. Effects of additives on the mechanical properties, hydrophobicity and water uptake of thermo-moulded films produced from sunflower protein isolate. Polymer. 43: 5417-5425.

Osawa, T. 1994. Novel Natural Antioxidants for Utilization in Food and Biological System. *In* Postharvest Biochemical of Plant Food-Material in the Tropics. (Uritani, I. *et al*., ed.). p. 241-251. Japan Scientific Societies Press. Japan.

Owasu-Apenten, R.K. 2002. Evaluation of a sulphydryl–disulphide exchange index (SEI) for whey proteins-β-lactoglobulin and bovine serum albumin. Food Chem. 83: 541-545.

Ozsoy, N., Can, A., Yanardag, R. and Akev, N. 2008. Antioxidant activity of *Smilax excelsa* L. leaf extracts. Food Chem. 110: 571-583.

Patil, R.D., Dalev, P.G., Mark, J.E., Vassileva, E. and Fakirov, S. 2000. Biodegradation of chemically modified gelatin films in lake and river waters. J. Appl. Polym. Sci. 76: 29–37.

Piotrowska, B., Sztuka, K., Kołodziejska, I. and Dobrosielska, E. 2008. Influence of transglutaminase or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) on the properties of fish-skin gelatin films. Food Hydrocolliods. 22: 1362-1371.

Pokorny, J. 1991. Natural antioxidants for food use. Trend Food Sci. Technol. 2: 223-227.

Prathep, A. 2005. Spatial and temporal variations in diversity and percentage cover of macroalgae at Sirinart Marine National Park, Phuket province, Thailand. Sci. Asia. 31: 225-233.

Prindle, R.F. 1983. Phenolic Compounds. *In* Disinfection, Sterilization, and Preservation. (Block, S.S. ed.). p. 197-224. Philadelphia, PA.

Rabe, T. and Staden, J.V. 1997. Antibacterial activity of South African plants used for medicinal purposes. J. Ethnopharmacol. 56: 81-87.

Rajalakshmi, D. and Narasimhan, S. 1996. Food Antioxidants: Sources and Method of Evaluation. *In* Food Antioxidants. (Madhavi, D.L. *et al*., eds.). p. 65-158. Marcel Dekker. New York.

Rajapakse, N., Mendis, E., Jung, W.K., Je, J.Y. and Kim, S.K. 2005. Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. Food Res. Int. 38: 175-182.

Reed, J.D. 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. J. Anim. Sci. 73: 1516-1528.

Rhim, J.W., Gennadios, A., Fu, D., Weller, C.L. and Hanna, M.A. 1999. Properties of ultraviolet irradiated protein films. LWT-Food Sci. Technol. 32: 129-133.

Robinson, H.W. and Hodgen, C.G. 1940. The biuret reaction in the determination of serum protein. I. A study of the condition necessary for the production of the stable color which bears a quantitative relationship to the protein concentration. J. Biol. Chem. 135: 707-725.

Sadowska, M., Kolodziejska, I. and Niecikowska, C. 2003. Isolation of collagen from the skin of Baltic cod (*Gadus morhua*). Food Chem. 81: 257-262.

Sakanaka, S., Tachibana, Y., Ishihara, N. and Juneja, L.R. 2004. Antioxidant activity of egg-yolk protein hydrolysates in a linoleic acid oxidation system. Food Chem. 86: 99-103.

Salas, J.J., Williams, M., Harwood, J.L. and Sánchez. 1999. Lipoxygenase activity in olive (*Olea europaea*) fruit. J. Am. Oil Chem. Soc. 76: 1163-1168.

S´anchez, C.S., Gonz´alez, A.M.T., Garc´ıa-Parrilla, M.C., Granados, J.J.Q., Garc´ıa de la Serrana, H.L. and Mart´ınez, M.C.L. 2007. Different radical scavenging tests in virgin olive oil and their relation to the total phenol content. Anal. Chem. Acta. 593: 103-107.

Santoso, J., Yoshie-Stark, Y. and Suzuki, T. 2004. Anti-oxidant activity of methanol extracts from Indonesian seaweeds in an oil emulsion model. Fish. Sci. 70: 183-188.

Shahidi, F. and Wanasundara, P.K.J.P.D. 1992. Phenolic antioxidants. Crit. Rev. Food Sci. Nutr. 32: 67-103.

Shellhammer, T.H. and Krochta, J.M. 1997. Whey protein emulsion film performance as affected by lipid type and amount. J. Food Sci. 62: 390-394.

Shi, B. and Di, Y. 2000. Plant Polyphenols. p. 134-138. Science Press. Beijing.

Shiku, Y., Hamaguchi, P.Y. and Tanaka, M. 2003. Effect of pH on the preparation of edible films based on fish myofibrillar. Fish. Sci. 69: 1026-1032.

Shiku, Y., Hamaguchi, P.Y. Benjakul, S., Visessanguan, W. and Tanaka, M. 2004. Effect of surimi quality on properties of edible film based on Alaska pollack. Food Chem. 86: 493-499.

Siriwardhana, N., Lee, K.W., Kim, S.H., Ha, J.W. and Jeon, Y.J. 2003. Antioxidant activity of *Hizikia fusiformis* on reactive oxygen species scavenging and lipid peroxidation inhibition. Food Sci. Technol. Int. 9: 339-346.

Sakata, K., Yamamoto, K. and Watanable, N. 1992. Antioxidative Compounds from Marine Organisms. *In* Food Phytochemicals for Cancer Prevention. (Ho, C.T. Osawa, T., Huang, M.T. and Rose, R.T. ed.). p. 165-182. American Chemical Society. Washington, USA.

Seo,Y., Park, K. E., Kim, Y.A., Lee, H.J., Yoo, J.S., Ahn, J.W., Lee, B.J. 2006. Isolation of tetraprenyltoluquinol from the brown alga *Sargassum thumbergii*. Chem. Pharm. Bull. 54: 1730-1733.

Slinkkard, K. and Singleton, V.L. 1997. Total phenol analyses: automation and comparison with manual methods. Am. J. Ecol.Vittic. 28: 49-55.

Smith, J.E., Hunter, C.L. and Smith, C.M. 2002. Distribution and reproductive characteristics of nonindigenous and invasive marine algae in the Hawaiian Islands. Pac. Sci. 56: 299-315.

Sobral, P.J.A., Mennegalli, F.C., Hubinger, M.D. and Roques, M.A. 2001. Mechanical, water vapor barrier and thermal properties of gelatin based edible films. Food Hydrocolloids. 15: 423-432.

Stahnke, L.H. 1995. Dried sausages fermented with *Staphylococcus pylorus* at different temperatures and different ingredient levels- part II volatile components. Meat Sci. 41: 193-209.

Stansby, G. 1987. Gelatin Gels. *In* Advanced in Meat Research. Vol. IV. (Pearson, A.M., Dutson, T.R. and Bailey, A.J. ed.). p. 209-222. Nostrand Reinhold. New York.

Stewart, H.L. 2004. Ontogenetic changed in buoyancy, breaking strength, extensibility and reproductive investment in a drifting macroalga *Turbinatria ornata* (Phaeophyta). J. Phycol. 42: 43-50.

Stiger, V. and Payri, C.E. 1999. Spatial and temporal patterns of settlement of the brown macroalgae*Turbinaria ornata* and *Sargassom mangarevense* in a coral reef on Tahiti. Mar. Ecol. Prog. Ser. 191: 91-100.

Strauss, G. and Gibson, S.M. 2004. Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. Food Hydrocolliods. 18: 81-89.

Stuchell, Y.M. and Krochta, J.M. 1994. Enzymatic treatment and thermal effects on edible soy proteins films. J. Food Sci. 59: 1332-1337.

Surak, J.G. and Singh, R.G. 2006. Butylated hydroxyanisole (BHA) induced changes in the synthesis of polar lipids and in the molar ratio of tetrahymanol to polar lipids in tetrahymena pyriformis. J. Food Sci. 45: 1251-1255.

Tabata, H., Katsube, T., Tsuma, T., Ohta, Y., Imawaka, N. and Utsumi, T. 2008. Isolation and evaluation of the radical-scavenging activity of the antioxidants in the leaves of an edible plant, *Mallotus japonicus.* Food Chem. 109: 64-71.

Tachakittirungrod, S., Okonogi, S. and Chowwanapoonpohn, S. 2007. Study on antioxidant activity of certain plants in Thailand: Mechanism of antioxidant action of guava leaf extract. Food Chem. 103: 381-388.

Tanaka, M., Chiu, W.K., Nagashima, Y. and Taguchi, T. 1992. Inhibitory effect of the Maillard reaction products towards lipid oxidation. J. Tokyo Univ. Fish. 79: 135-141.

Tepe, B., Donmez, R., Unlu, M., Candan, F., Daferera, D., Vardar-Unlu, G., Polissiou, M. and Sokmen, A. 2004. Antimicrobial and antioxidative activities of the essential oils and methanol extracts of *Salvia cryptantha* (Monbret et Aucher ex Benth.) and *Salvia multicaulis* (Vahl). Food Chem. 84: 519-525.

Thies, C., 1995. Complex Coacervation. *In* How to Make Microcapsules, Combined Lecture and Laboratory Manual. (Thies, C. ed.). p. 1-43. St Louis.

Torres, J.A. 1997. Edible Films and Coatings from Proteins. *In* Food Proteins and their Applications. (Damodaran, S. and Paraf, S. eds.). p. 467-507. Marcel and Dekker. New York.

Usuki, R., Suzuki, T., Endo, Y. and Kaneda, T.K. 1984. Residual amounts of chlorophylls and pheophytins in refined edible oils. J. Biol. Chem. 48: 991-994.

Walker, J.R.K. 1994. Antimicrobial Compounds in Food Plants. *In* Natural Antimicrobial Systems and Food Preservation. (Dillon, V.M. and Board, R.G. ed.). p. 181-204. CAB International. Wallingford.

Wanasundara, U.N. and Shahidi, F. 1998. Antioxidant and pro-oxidant activity of green tea extracts in marine oils. Food Chem. 63: 335-342.

Whysner, J., Wang, C.X., Zang, E., Iatropoulos, M.J. and Williams, G.M. 1994. Dose response of promotion of butylated hydroxyanisole in chemically Initiated tumors of the rat fore stomach. Food Chem. Toxicol. 32: 215-222.

Williams, C.A. and Harborne, J.B. 1977. The leaf flavonoids of Zingiberales. Biochem. Syst. Ecol. 5: 221-229.

Wong, D.W.S. 1989. Proteins. *In* Mechanism and Theory in Food Chemistry. (Wong, N.P. ed.). p. 90-96. Van Nostrand Reinhold. New York, USA.

Wu, H.C., Chen, H.M. and Shiau, C.Y. 2003. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). Food Res. Int. 36: 949-957.

Yamaguchi, T., Takamura, H., Matoba, T. and Teara, J. 1998. HPLC method for evaluation of the free radical-scavenging activity of food by using 1,1-diphenyl-2-picrylhydrazyl. Biosci. Biotechnol. Biochem. 62: 1201-1204.

Yan, X.J., Li, X.C., Zhou, C.X. and Fan, X. 1996. Preservation of fish oil rancidity by phlorotannins from *Sargassum kjellmanianum.* J. Appl. Psychol. 8: 201-203.

Yan, X., Nagata, T. and Fan, X. 1999. Antioxidative activities in some common seaweeds. Plant Food Hum. Nutr. 52: 253-262.

Yang, R., Li, C., Lin, Y., Peng, G., She, Z. and Zhou, S. 2006. Lactones from a brown alga endophytic fungus (No. ZZF36) from the South China Sea and their antimicrobial activities. Bioorg. Med. Chem. Lett. 16: 4205-4208.

Yen, G. and Hsieh, P. 1995. Antioxidative activity and scavenging effects on active oxygen of xylose-lysine Maillard reaction products. J. Sci. Food Agric. 67: 415-420.

Yildirim, M. and Hettiarachchy, N.S. 1998. Properties of films produced by cross linking whey proteins and 11S globulin using transglutaminase. J. Food Sci. 63: 248-252.

Yoshie, Y., Wang, W., Petillo, D. and Suzuki, T. 2000. Distribution of catechins in Japanese seaweeds. Fish. Sci. 66: 998-1000.

Yoshie, Y., and Suzuki, T. 2000. Polyphenolic Compounds from Seaweed: Distribution and First Approach to Find Out Useful Effect as Antioxidant. *In* Proceeding of JSPS-DGHG International Symposium, Bogor, Indonesia. 426-431.

Yoshimura, Y., Iijima, T., Watanabe, T. and Nakazawa, H. 1997. Antioxidative effect of Maillard reaction products using glucose-glycine model system. J. Agric. Food Chem. 45: 4106-4109.

APPENDIX

ANALYTICAL METHODS

1. Determination of moisture content (AOAC, 2000)

Method

1. Dry the empty dish and lid in the oven at 105°C for 3 h and transfer to dessicator to cool. Weigh the empty and lid.
2. Weigh about 3 g of sample to the dish. Spread the sample to the uniformity.
3. Place the dish with sample in the oven. Dry for 3 h at 105°C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried sample.

Calculation

$$\text{Moisture content (\%)} = \frac{(W1 - W2) \times 100}{W1}$$

where W1 = weight (g) of sample before drying
W2 = weight (g) of sample after drying

2. Determination of protein content (AOAC, 2000)

Reagents

1. Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) with 1 part of copper sulphate ($CuSO_4$)
2. Sulfuric acid (H_2SO_4)
3. 40% NaOH solution (w/v)
4. 0.2 N HCl solution
5. 4% H_3BO_3 solution (w/v)
6. Indicator solution: Mix 100 ml of 0.1% methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)

Method

1. Place sample (0.5-1.0 g) in digestion flask.
2. Add 5 g Kjedahl catalyst and 20 ml of conc. H_2SO_4
3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently unit frothing ceases. Boil briskly until solution clears.
4. Cool and add 60 ml distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid and 5-7 indicator in receiver. Rotate flask to mix content thoroughly; then heat until all NH_3 is distilled.
6. Remove receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

Calculation

$$\text{Protein content (\%)} = \frac{(A\text{-}B) \times N \times 1.4007 \times 6.25}{W}$$

where
- A = volume (ml) of 0.2 N HCl used sample titration
- B = volume (ml) of 0.2 N HCl used in blank titration
- N = normality of HCl
- W = weight (g) of sample
- 14.007 = atomic weight of nitrogen
- 6.25 = the protein-nitrogen conversion factor for fish and its by-products

3. Determination of ash content (AOAC, 2000)

Method

1. Place the crucible and lid in the furnace at 550°C overnight to ensure that impurities on the surface of crucible are burned off.
2. Cool the crucible in the desiccator (30 min).
3. Weigh the crucible and lid to 3 decimal places.
4. Weigh about 5 g sample into the crucible. Heat over low Bunsen flame with lid half covered. When fumes are no longer produced, place crucible and lid in furnace.

5. Heat at 550°C overnight. During heating, do not cover the lid. Place the lid after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
6. Weigh the ash with crucible and lid when the sample turns to gray. If not, return the crucible and lid to the furnace for the further ashing.

Calculation

$$\text{Ash content (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

4. Determination of fat content (AOAC, 2000)

Reagent

1. Petroleum ether

Method

1. Place the bottle and lid in the incubator at 105°C overnight to ensure that weight of bottle is stable.
2. Weigh about 3-5 g of sample to paper filter and wrap.
3. Take the sample into extraction thimble and transfer into soxhlet.
4. Fill petroleum ether about 250 ml into the bottle and take it on the heating mantle.
5. Connect the soxhlet apparatus and turn on the water to cool them and then switch on the heating mantle.
6. Heat the sample about 14 h (heat rate of 150 drop/min).
7. Evaporate the solvent by using the vacuum condenser.
8. Incubate the bottle at 80-90°C until solvent is completely evaporated and bottle is completely dried.
9. After drying, transfer the bottle with partially covered lid to the desiccator to cool. Reweigh the bottle and its dried content.

Calculation

$$\text{Fat content (\%)} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

5. Hydroxyproline content (Bergman and Loxley, 1963)

Reagents

1. 6 N HCl
2. Oxidant solution (the mixture of 7% (w/v) chlororamine T and acetate/citrate buffer, pH 6 at a ratio of 1:4 (v/v))
3. Ehlich's reagent solution (the mixture of solution A (2 g of *p*-dimethylamino benzaldehyde in 3 ml of 60% (v/v) perchloric acid (w/v))
4. Isopropanol
5. Hydroxyproline standard solution (400 ppm)

Method

Sample preparation:

1. Weigh about 0.1-2.0 g sample (depending on type of sample) into screw cap tube.
2. Add 6 N HCl into the sample at the ratio of 1 : 10 (solid/acid, w/v).
3. Heat at 110°C for 24 h in oil bath.
4. Clarify the hydrolysate with activated carbon and filter through Whatman No. 4 filter paper.
5. Neutralize the filtrate with 10 M NaOH and 1 M NaOH to obtain the pH 6.0-6.5

Hydroxyproline determination:

1. Transfer 0.1 ml of the neutralized sample into a test tube and add 0.2 ml of isopropanol then mix well.
2. Add 0.1 ml of oxidant solution and mix well.
3. Add 1.3 ml of Ehrlich's reagent solution.
4. Heat the mixture at 60°C for 25 h in the water bath and then cool for 2-3 min in running water.
5. Add isopropanol at ratio of 3 : 13 (mixture/isopropanol, v/v) and mix well.
6. Read absorbance at 558 nm.
7. Plot the standard curves and calculate the unknown.

Table: Experimental set up for the hydroxyproline's assay

Tube number	Water(µL)	400 ppm Hydroxyproline (µL)	Effective Hydroxyproline Concentration (ppm)
1	100.0	0.0	0
2	97.5	2.5	10
3	95.0	5.0	20
4	92.5	7.5	30
5	90.0	10.0	40
6	87.5	12.5	50
7	85.0	15.0	60
8	0.0	0.0	unknown

6. Biuret method for quantitation of protein (Robinson and Hodgen, 1940)

Reagents

1. Biuret reagent: combine 1.50 g $CuSO_4.5H_2O$, 6.00 g sodium potassium tartrate, and 500 ml distilled water in a beaker and stir, add while stirring 300 ml of 10% NaOH (w/v), transfer to plastic bottle for storage.
2. Distilled water
3. Standard reagent: 10 mg/ml bovine serum albumin (BSA)

Method

1. To each of seven disposable cuvette, add the following reagents according to the table.
2. Add 2.0 ml of the biuret reagent to each tube, and mix well.
3. Incubate the mixture at room temperature for 30-45 min, and then read the adsorbance of each tube at 540 nm.
4. For tube 1-5, plot the absorbance at 540 nm as a function of effective BSA concentration and calculate the best fit straight line from data. Then, using the average absorbance for the three sample of unknown read the concentration of sample from the plot.

Table: Experimental set up for the Biuret's assay

Tube number	Water (μL)	10 mg/ml BSA (μL)	Effective BSA Concentration (mg/ml)
1	500	0	0
2	400	100	2
3	300	200	4
4	200	300	6
5	100	400	8
6	0	500	10
7	0	0	unknown

7. Lowry's procedure for quantitation of proteins (Lowry *et al.,* 1951)

Reagents

1. A: 2% sodium carbonate in 0.1 N NaOH
2. B: 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate
3. C: 2 N Folin-Ciocalteu's phenol reagent + distilled water (ratio of 1 : 1)
4. D: 50 ml reagent A + 1 ml reagent B
5. Standard reagent: Bovine serum albumin (BSA) at concentration of 1 mg/ml

Method

1. To each of eight disposable cuvette, add the following reagents according to the table.
2. Add 2 ml reagent D to each of the standards and unknown tube and then vortex immediately.
3. Incubate precisely 10 min at room temperature.
4. Add 0.2 ml reagent C (previously dilute 1 : 1 with distilled water) and vortex immediately.
5. Incubate 30 min at room temperature (sample incubated longer than 60 min should be discarded).
6. Read absorbance at 750 nm.
7. Plot standard curves and calculate the unknown.

Table: Experimental set up for the Lowry's assay

Tube number	Water (µL)	1 mg/ml BSA (µL)	Effective BSA Concentration (mg/ml)
1	200	0	0
2	180	20	0.1
3	160	40	0.2
4	140	60	0.3
5	100	100	0.5
6	60	140	0.7
7	0	200	1.0
8	0	0	unkhown

8. Thiobarbituric acid-reactive substance (TBARS) of lecithin liposome system (Frankel *et al.*, 1997)

Reagent

1. TBA solution (15% TCA/ 0.375% TBA/ 0.025N HCl)
2. 0.2 % BHT

Method

1. Mix liposome sample (1 ml) with 20 µl of butylated hydroxytoluene (0.2%) and add 2 ml of TBA solution into the mixture.
2. Heat the mixtures for 10 min in a boiling water bath (95-100°C) to develop pink color.
3. Cool with tap water and centrifuge for 20 min at 5,500 × g.
4. Read the absorbance of the supernatant containing the pink chromogen at 532 nm and express TBARS as mg MDA/ml liposome.

9. Electrophoresis (SDS-PAGE) (Laemmli, 1970)

Reagent

1. 30% Arylamide-0.8% bis Acrylamide
2. Sample buffer: Mix 30 ml of 10% of SDS, 10 ml of glycerol, 5 ml of β-mercaptoethanol, 12.5 ml of 50 mM Tris-HCl, pH 6.8, and 10 mg bromophenol blue. Bring the volumn to 100 ml with distilled water. Divide into 1 ml aliquots, and store at -20 °C.
4. 2% (w/v) Ammonium persulfate

5. 1% (w/v) SDS
6. TEMED (*N,N,N'N'*- tetramethylethylenediamine)
7. 0.5 M Tris-HCl, pH 6.8
8. 1.5 M Tris-HCl, pH 8.8
9. Electrode buffer: Dissolve 3 g of Tris-HCl, 14.4 g of glycine and 1 g of SDS in distilled water. Adjust to pH 8.3. Add distilled water to 1 liter to total volume.
10. Staining solution: Dissolve 0.05 g of Coomassie blue R-250 in 15 ml methanol. Add 5 ml of glacial acetic and 80 ml of distilled water.
11. Destaining solution: 30% methanol-10% glacial acetic acid

Method

Pouring the running gel:

1. Assemble the minigel apparatus according to the manufacture's detailed instructions. Make sure that the glass and other components are rigorously clean and dry before assembly.
2. Mix the separating gel solution by adding as defined in following Table.
3. Transfer the separating gel solution using a Pasture pipettes to the center of sandwich is about 1.5 to 2 cm from the top of the shorter (front) glass plate.
4. Cover the top of the gel with a layer of distilled water by squiting the distilled water against the edge of one of the spacers. Allow the resolving gel to polymerize fully (usually 30-60 min).

Pouring the stacking gel:

1. Pour off completely the layer of isobutyl alcohol.
2. Prepare a 4% stacking gel solution by adding as defined in Table.
3. Transfer stacking gel solution to tickle into the center of the sandwich along an edge of the one of the spacers.
4. Insert comb into the layer of stacking gel solution by placing one corner of the comb into the gel and slowly lowering the other corner in. Allow the attacking gel solution to polymerize 30 to 45 min at room temperature.

Table: Experimental set up for running and stacking gel

Reagents	7.5% running gel	4% stacking gel
30% Acrylamide-bis	2.500 mL	0.665 mL
1.5 M Tria-HCl buffer, pH 8.8	2.500 mL	-
0.5 M Tris-HCl buffer, pH 6.8	-	1.25 mL
Distilled water	4.845 mL	3.00 mL
10% SDS	10 µL	50 µL
2% Ammonium persulfate	50 µL	25 µL
TEMED	5 µL	3 µL

Sample preparation:

1. Weigh 3 g of gelatin and completely dissolve with distilled water in a final volume of 30 ml.
2. Centrifuge at 3,500*xg* for 5 min at ambient temperature and collect supernatant.

Loading the gel:

1. Dilute the protein to be 4:1 (v/v) with sample buffer in microcentrifuge tube and boil for 1 min at 100°.
2. Remove the comb without tearing the edge of the polyacrylamide wells.
3. Fill the wells with electrode buffer.
4. Place the upper chamber over the sandwich and lock the upper buffer chamber to the sandwich. Pour electrode buffer into the lower buffer chamber. Place the sandwich attached to the upper buffer chamber into the lower chamber.
5. Fill the upper buffer chamber with electrode buffer so that the sample wells of the stacking gel are filled with buffer.
6. Use a 10-25 µL syringe with a flate-tipped needle; load the protein sample into the wells by carefully applying the sample as a thin layer at the bottom of the well.
7. Fill the remainder of the upper buffer chamber with additional electrode buffer.

Running the gel:

1. Connect the power supply to the anode and cathode of the gel apparatus and run at 50 V and 150 V.
2. After the bromophenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

Disassembling the gel:

1. Remove the upper buffer chamber and the attached sandwich.
2. Orient the gel so that the order of the sample well is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels. Carefully slide the spacers out from the edge of the sandwich along its entire length.
3. Insert a spatula between the glass plates at one corner where the spacer was and gently pry the two plates apart.
4. Remove the gel from the lower plate. Place the plate with the gel attached into the shallow dish of fixing agent or dye and swishing the plate.

Staining the gel:

1. Plate the gel in a small plastic box and cover with the staining solution. Agitate slowly for 3 h. or more on a rotary rocker.
2. Pour off the staining solution and cover the gel with a solution of destaining solution I. Agitate slowly for about 15 min.
3. Pour off the destaining solution I and replace with flesh solution. Repeat until the gel is clear except for the protein bands.