

Appendix 1

Table 1 Changes in breaking force of surimi added with different cryoprotectants during frozen storage at -18°C for 12 weeks.

Frozen storage (weeks)	Treatments			
	control	8%trehalose	The blends	commercial
0	848.63±53.76 ^{aD}	841.00±20.07 ^{aC}	845.36±31.67 ^{aC}	840.93±22.87 ^{aC}
1	741.94±50.93 ^{aC}	867.60±14.57 ^{bC}	875.86±28.35 ^{bD}	858.50±18.79 ^{bD}
2	721.90±11.37 ^{aC}	886.56±39.62 ^{cC}	859.20±48.45 ^{bcC}	835.16±33.48 ^{bCD}
4	720.26±12.86 ^{aB}	880.67±19.57 ^{bC}	881.21±41.78 ^{bC}	791.93±41.75 ^{bBC}
6	620.67±42.23 ^{aB}	744.33±35.78 ^{bB}	755.07±47.92 ^{bAB}	743.63±42.05 ^{bAB}
8	609.90±24.19 ^{aAB}	722.90±18.40 ^{bAB}	715.56±42.99 ^{bA}	750.33±38.39 ^{bAB}
10	585.43±41.18 ^{aAB}	753.36±30.45 ^{bB}	724.28±13.51 ^{bAB}	722.67±18.72 ^{bA}
12	492.00±28.42 ^{aA}	762.83±7.85 ^{bB}	789.20±37.94 ^{bA}	763.96±41.83 ^{bA}

Mean±SD from triplicate determination.

Different letters in the same row indicate the significant difference ($p<0.05$) Different letters (capital) in the same column indicate the significant difference ($p<0.05$)

Table 2 Changes in deformation of surimi added with different cryoprotectants during frozen storage at -18°C for 12 weeks.

Frozen storage (weeks)	Treatments			
	control	8%trehalose	The blends	commercial
0	12.53±0.38 ^{aD}	12.58±0.52 ^{aC}	12.30±0.82 ^{aC}	12.48±0.78 ^{aC}
1	8.96±0.21 ^{aC}	11.88±0.53 ^{bBC}	12.65±0.22 ^{bC}	12.93±0.58 ^{bC}
2	6.88±0.49 ^{aB}	11.69±0.75 ^{bBC}	11.79±0.26 ^{bBC}	10.90±0.76 ^{bAB}
4	7.04±0.72 ^{aBC}	11.147±0.51 ^{bAB}	11.72±0.53 ^{bBC}	11.20±0.26 ^{bB}
6	6.93±0.78 ^{aB}	12.16±0.23 ^{cC}	11.68±0.07 ^{bcABC}	11.37±0.12 ^{bB}
8	6.40±0.38 ^{aB}	10.63±0.29 ^{bAB}	10.38±0.02 ^{bAB}	10.10±0.74 ^{bA}
10	6.16±0.41 ^{aB}	10.15±0.36 ^{bA}	11.59±0.45 ^{cABC}	10.27±0.23 ^{bA}
12	5.85±0.15 ^{aA}	10.27±0.52 ^{bA}	11.37±0.21 ^{bABC}	10.06±0.28 ^{bA}

Mean±SD from triplicate determination.

Different letters in the same row indicate the significant difference ($p<0.05$)

Different letters (capital) in the same column indicate the significant difference ($p<0.05$)

Table 3 Changes in breaking force of surimi added with different cryoprotectants and subjected to multiple freeze-thaw cycles

Freeze thaw cycle	Treatments			
	control	8%trehalose	The blends	commercial
0	845.36±24.58 ^{aD}	855.42±37.56 ^{aB}	850.25±15.41 ^{aB}	872.11±39.41 ^{aB}
1	802.06±11.16 ^{aD}	795.70±19.55 ^{aB}	801.33±36.65 ^{aB}	842.16±34.14 ^{aB}
2	663.76±24.89 ^{aC}	680.96±27.62 ^{abA}	735.66±41.11 ^{cdA}	751.26±39.51 ^{dA}
4	568.60±30.93 ^{aB}	665.43±45.37 ^{bA}	655.03±41.87 ^{bA}	648.76±20.69 ^{bA}
6	380.90±15.29 ^{aA}	633.69±9.09 ^{bA}	671.23±14.25 ^{cA}	628.23±19.54 ^{bA}

Mean±SD from triplicate determination.

Different letters in the same row indicate the significant difference ($p<0.05$)

Different letters (capital) in the same column indicate the significant difference ($p<0.05$)

Table 4 Changes in deformation of surimi added with different cryoprotectants and subjected to multiple freeze-thaw cycles.

Freeze-thaw cycles	Treatments			
	control	8%trehalose	The blends	commercial
0	12.22±0.49 ^{aE}	12.35±0.37 ^{aD}	11.94±0.47 ^{aC}	12.24±0.31 ^{aD}
1	11.23±0.35 ^{aD}	12.23±0.52 ^{aD}	12.22±0.16 ^{aC}	12.07±0.36 ^{aD}
2	8.53±0.40 ^{bC}	10.61±0.28 ^{aC}	10.59±0.24 ^{aB}	10.20±0.21 ^{aC}
4	5.10±0.31 ^{bB}	9.45±0.14 ^{aB}	9.90±0.34 ^{aB}	9.15±0.31 ^{aB}
6	4.28±0.13 ^{bA}	7.43±0.17 ^{aA}	8.11±0.36 ^{bA}	7.73±0.46 ^{abA}

Mean±SD from triplicate determination.

Different letters (small) in the same row indicate the significant difference ($p < 0.05$)

Different letters (capital) in the same column indicate the significant difference ($p < 0.05$)

Appendix 2

The level quality of gel surimi from bigeye snapper

grade	Breaking force value (g)	Deformation (mm.)
B (Lowest quality)	<400	6
A	400-500	8
AA	500-600	10
SA	600-800	12
SSA (highest quality)	>800	14

Source: Man A Frozen Food Co, Ltd., Songkhla, Thailand.

Appendix 3

Analytical Methods

1. Determination of ATPase (Benjakul *et al.*, 1997)

Reagents

1. 0.6 M KCl, pH 7.0
2. 0.5 M Tris-maleate, pH 7.0
3. 0.1 M CaCl₂
4. 0.1 M MgCl₂
5. 0.01 M EGTA
6. 15 % Trichloroacetic acid
7. Distilled water
8. 20 mM ATP

Method

1. Pipette 0.5 ml of natural actomyosin solution (NAM) (2.5-4 mg/ml in 0.6 M KCl, pH 7.0)
2. Add the following solution as shown in the table

Solution (μL)	Determination			
	Ca ²⁺ -ATPase	Mg ²⁺ -ATPase	Mg ²⁺ -Ca ²⁺ -ATPase	Mg ²⁺ -EGTA-ATPase
1. 0.5 M Tris-maleate, pH 7.0 (keep in 4 °C)	300	300	300	300
2. 0.1 M CaCl ₂	500	0	5	0
3. 0.1 M MgCl ₂	0	100	100	100
4. 0.01 M EGTA	0	0	0	250
5. NAM (1.5-3.0 mg/ml)	500	500	500	500
6. Distilled water	3450	3850	3845	3600
7. 20 mM ATP	250	250	250	250

3. Incubate for 5-10 min at 25 °C
4. Stop reaction using 2.5 ml chilled 15% trichloroacetic acid.
5. Centrifuge at 6,500 rpm for 5 min.
6. Measure inorganic phosphate in the supernatant.
7. Blank:
 - NAM + Tris maleate + water (500+300+3950 μL)
 - Add 2.5 ml, 15% TCA
 - Add 250 μL , 20 mM ATP
 - Measure inorganic phosphate

2. Determination of total sulfhydryl content (Benjakul *et al.*, 1997)

Reagents

1. 0.1% 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)
2. 0.2 M Tris-HCl buffer, pH 6.8 (containing 8M urea, 2% SDS, and 10 mM EDTA)

Method

1. Mix actomyosin (1 ml, 4 mg/ml) with 9 ml of 0.2 M Tris-HCl.
2. Take 4 ml-aliquot of the mixture and add with 0.4 ml of 0.1% DTNB solution. Incubate the mixture at 40 °C for 25 min.
3. Measure the absorbance at 412 nm with spectrophotometer.
4. Prepare a blank by replacing the sample with 0.6 M KCl, pH 7.0.
5. Calculate SH content from the absorbance using the molar extinction coefficient of 13,600 $\text{M}^{-1}\text{cm}^{-1}$ and express as $\text{mol}/10^5 \text{ g protein}$.

3. Determination of disulfide bond (Thannhauser *et al.*, 1987)

Reagents

1. 5,5-dithiobis-2-nitrobenzoic acid (Ellman's reagent)
2. Na_2SO_3
3. glycine
4. sodium sulfite
5. EDTA

- NTSB preparation

1. Dissolve 100 mg of Ellman's reagent (0.235 mmol) in 10 ml of 1 M Na_2SO_3 .
2. Adjusted pH to 7.5.
3. Incubate the bright red solution at 38 °C, and bubble the oxygen through it with a gas dispersion tube until the color turns to be yellow.
4. Store this stock solution for up to 1 year at -20 °C.

- NTSB assay solution

The NTSB assay solution is prepared from the stock solution by diluting it 1:100 with a freshly prepared solution that is 2 M in guanidine thiocyanate, 50 mM in glycine, 100 mM in sodium sulfite, and 3 mM EDTA. The pH should be adjusted to 9.5. The assay solution is used directly to measure disulfide bond concentrations and is stable for up to 2 weeks when stored at room temperature.

Method

1. Add 10-200 μL of protein solution into 3 ml of the NTSB assay solution.
2. Incubate the reaction mixture in the dark for 25 min.
3. Measure the absorbance at 412 nm against a blank.
4. Calculate disulfide bond concentration from the absorbance using the molar extinction coefficient of $13,900 \text{ M}^{-1}\text{cm}^{-1}$ and express as $\text{mol}/10^6 \text{ g}$ protein.

4. Determination of surface hydrophobicity (Benjakul *et al.*, 2000)

Reagents

1. 10 mM phosphate buffer, pH6.0 containing 0.6 M NaCl
2. 8 mM 1-anilinonaphthalene-8-sulphonic acid (ANS) in 0.1 M phosphate buffer, pH7.0

Method

1. Prepare actomyosin in 10 mM phosphate buffer, pH 6.0 containing 0.6 M NaCl and dilute to 0.1, 0.2, 0.3, 0.5, 0.9 mg/ml protein using the same buffer.
2. Incubate the diluted protein (2 ml) at 20 °C for 10 min.
3. Add 10 μ L of 8 mM ANS in 0.1 M phosphate buffer, pH 7.0.
4. Measure the fluorescence intensity of ANS-protein conjugates using a spectrofluorometer at excitation wavelength 374 nm and emission wavelength 485 nm.

Calculation

Protein hydrophobicity was calculated from initial slopes of plots of relative fluorescence intensity vs. protein concentration using linear regression analysis. The initial slope was referred to as SoANS.

5. Determination of solubility in 0.6 M KCl (Benjakul and Bauer, 2000)

Reagents

1. 0.6 M KCl, pH 7.0
2. 50% Trichloroacetic acid
3. 10% Trichloroacetic acid
4. 0.5 M NaOH

Method

1. Weigh 2 g sample.
2. Add 18 ml of 0.6 M KCl (pH 7.0) and homogenize for 30 s.

3. Stir the homogenate at room temperature (25-27 °C) for 4 h, and centrifuge at 12,000xg for 20 min at 4 °C.
4. To 10 ml of the supernatant, add cold 50% (w/v) trichloroacetic acid to obtain the final concentration of 10%.
5. Wash the precipitate with 10% trichloroacetic acid and solubilize in 0.5 M NaOH.
6. Determine the protein content using the Biuret method.

6. pH determination (Benjakul *et al.*, 1997)

Method

1. Weigh 5 g of sample.
2. Add 10 volumes of distilled water (w/v).
3. Homogenize for 2 min
4. Measure pH using pH meter.

7. Measurement of expressible moisture (Hasegawa, 1987)

Method

1. Place the sample (X g) between 2 filter paper on top and three filter paper on the bottom.
2. Increase the pressure to 5 kg/cm² within 1 sec.
3. Maintain at 10 kg/cm² constant pressure for 2 min, then remove the sample, and weigh the pressed sample (Z).

Calculation

$$\text{Expressible moisture (\%)} = \frac{(X-Z) \times 100}{X}$$

8. **Biuret method quantitation of protein (Copeland *et al.*, 1994)**

Reagents

1. Biuret reagent: combine 1.50 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 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 eight disposable cuvette, add the following reagents according to the table.
2. To tubes 6-8, 50 μL of protein sample were added, mix the contents of each tube well by using the closed end of the capillary tube as stirring rod.
3. Add 2.0 ml of the biuret reagent to each tube, and mix well.
4. Incubate the mixture at room temperature for 30-45 min, then read the absorbance of each tube at 540 nm
5. 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	450	0	unknown
7	450	0	unknown
8	450	0	unknown

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

Reagents

1. Protein molecular weight standards
2. 30% Acrylamide-0.8% bis Acrylamide
3. 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 5-10 mg Bromophenol blue (enough to give dark blue color to the solution). Bring the volume to 100 ml with distilled water. Divide into 1 ml aliquots, and stored at -20°C .
4. 2% (w/v) Ammonium persulfate
5. 1%(w/v) SDS
6. TEMED (N,N,N',N'-tetramethylenediamine)
7. 0.5 M Tris-HCl, pH 6.8
8. 1.5 M Tris-HCl, pH 8.8
9. 0.1 M EDTA

10. 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.
11. Staining solution: Dissolve 0.04 g of Coomassie blue R-250 in 100 ml of methanol. Add 15 ml of glacial acetic acid and 85 ml of distilled water.
12. Destaining solution I: 50% methanol-7.5% glacial acetic acid
Destaining solution II: 5% methanol-7.5% glacial acetic acid

Method

Pouring the separating 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 separation gel solution by adding, as defined in following Table.
3. Transfer the separating gel solution using a Pasteur pipette 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 isobutyl alcohol alcohol by gently squirting the isobutyl alcohol 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 trickle into the center of the sandwich along an edge of 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 stacking gel solution to polymerize 30 to 45 min at room temperature.

Reagents	10% running gel	4% stacking gel
30% Acrylamide-bis	1.167 ml	0.4 ml
1.5 M Tris-HCl buffer, pH 8.8	0.875 ml	-
0.5 M Tris-HCl buffer, pH 6.8	-	1.0 ml
10% SDS	0.35 ml	0.3 ml
Distilled water	0.7585 ml	0.9 ml
0.1 M EDTA	-	0.8 ml
2% Ammonium persulfate	0.35 ml	0.4 ml
TEMED	6 μ L	5 μ L

Sample preparation:

1. Weigh 3 g of sample and homogenize with 5% (w/v) SDS in a final volume of 30 ml
2. Incubate the mixture at 85 °C for 1 h.
3. Centrifuge at 3,500xg 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 micro-centrifuge tube and boil for 1 min at 100 °C.
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.
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. Place the gel in a small plastic box and cover with the staining solution. Agitate slowly for 1 h or more on 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 cover the destaining solution II. Discard destaining solution and replace with fresh solution. Repeat until the gel is clear except for the protein bands.