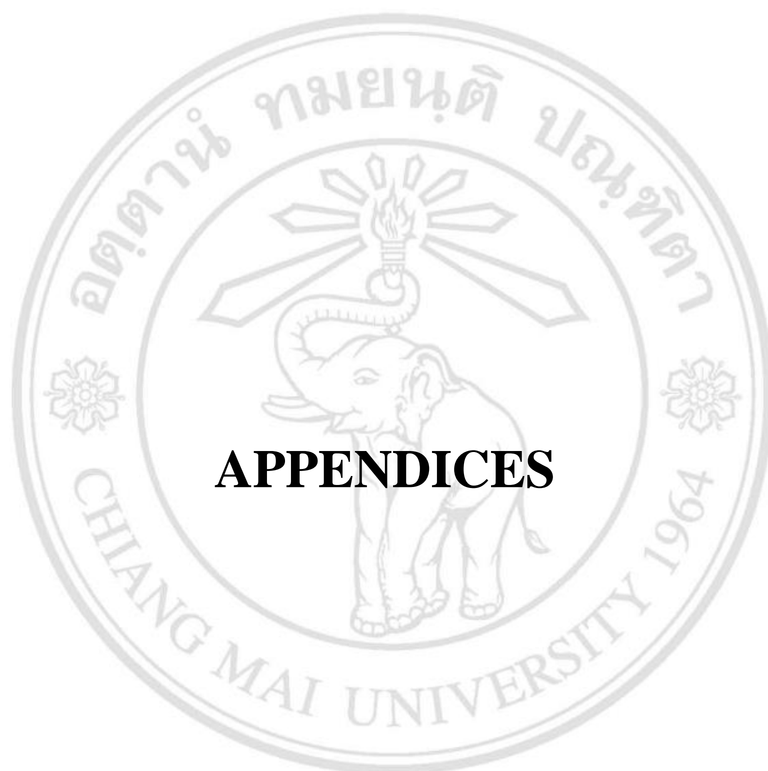

APPENDICES

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APPENDIX A

REAGENTS AND SOLUTIONS

1. Gelatin Extraction and Gelatin Hydrolysate Preparation

1.1 0.8 M Sodium hydroxide solution

Dissolve 32 g of sodium hydroxide with 800 mL of distilled water. Adjust the final volume of solution to 1000 mL with distilled water and keep in a brown bottle at room temperature.

1.2 0.5% (v/v) Hydrochloric acid solution

Add 5 mL of 37% hydrochloric acid into 200 mL of distilled water. Adjust the solution to final volume of 1000 mL with distilled water and keep in a brown bottle at room temperature.

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1.3 0.1 M Phosphate buffer, pH 6.0-8.0

For buffer pH 6.0, dissolve 12.14 g of sodium phosphate monobasic dehydrate and 3.22 g of di-sodium hydrogen phosphate dehydrate with 800 mL of distilled water. Adjust pH of buffer solution to 6.0 and make to final volume of 1000 mL.

For buffer pH 7.0, dissolve 5.84 g of sodium phosphate monobasic dehydrate and 15.47 g of di-sodium hydrogen phosphate dehydrate with 800 mL of distilled water. Adjust pH of buffer solution to 7.0 and bring to final volume of 1000 mL.

For buffer pH 8.0, dissolve 0.94 g of sodium phosphate monobasic dehydrate and 24.97 g of di-sodium hydrogen phosphate dehydrate with 800 mL of distilled water. Adjust pH of buffer solution to 8.0 and make to final volume of 1000 mL.

All adjusted buffer solution is transfer into a brown bottle and keep at room temperature.

2. Degree of Hydrolysis Determination

2.1 0.2125 M Phosphate buffer, pH 8.2

Dissolve 2.55 g of sodium phosphate monobasic dehydrate in 90 mL of distilled water. Adjust to pH 8.2 and make up volume to 100 mL with distilled water. Keep buffer in a brown bottle at room temperature.

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2.2 0.01% TNBS solution

Mix 10 μL of TNBS stock solution with distilled water and make up to 100 mL final volume. Keep the solution in brown bottle at 4°C.

2.3 0.1 M Sodium sulfite solution

Dissolve 1.26 g of sodium sulfite in 80 mL of distilled water. Make the final volume into 100 mL and keep in a brown bottle at room temperature.

2.4 6 N Hydrochloric acid solution

Slowly add 49.27 mL of 37% hydrochloric acid into a volumetric flask contained 25 mL of distilled water. Adjust to 100 mL final volume with distilled water. Transfer the solution to brown bottle and keep at room temperature.

2.5 6 N Sodium hydroxide solution

Dissolve 24 g of sodium hydroxide with distilled water and adjust to final volume of 100 mL. Keep the solution in brown bottle at room temperature.

3. Determination of Bioactivities

3.1 4 mM Trolox stock solution

Dissolve 10 mg of trolox with 10 mL of absolute ethanol. Keep the solution in brown bottle at 4°C.

3.2 7 mM ABTS solution

Dissolve one tablet of ABTS (10 mg) with 2.60 mL of distilled water in a brown bottle. ABTS solution is freshly prepared for each use.

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3.3 2.45 mM Potassium persulfate solution

Dissolve 0.017 g of potassium persulfate in 25 mL of distilled water and keep in a brown bottle at 4°C until used.

3.4 10 mM TPTZ in 40 mM hydrochloric acid solution

Dissolve 31 mg of TPTZ with 10 mL of 40 mM hydrochloric acid solution (0.328 mL of 37% in final volume of 100 mL of distilled water). Transfer solution into a brown bottle and keep at 4°C until used.

3.5 20 mM Ferric chloride solution

A 0.054 g of ferric chloride is dissolved in 10 mL of distilled water. The solution is transferred to a brown bottle and keeps at 4°C until used.

3.6 300 mM Acetate buffer solution, pH 3.6

Dissolve 0.31 g of sodium acetate trihydrate in a beaker contained 1.6 mL of glacial acetic acid. Make the volume up to 80 mL with distilled water and adjust pH of the solution to 3.6. Add distilled water until final volume of 100 mL is obtained. The solution is kept in a brown bottle at 4°C until used.

3.7 20 mM Ferrous sulfate solution

Dissolve 0.556 g of ferrous sulfate in 100 mL of distilled water. Keep the solution in brown bottle at 4°C until used.

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3.8 50 mM Ferrozine stock solution

Dissolve 0.25 g of ferrozine with 10 mL of distilled water. Keep the solution in brown bottle at 4°C until used.

3.9 2 mg/mL Linoleic acid in absolute ethanol

Weight 0.2 g of linoleic acid in brown bottle and mix with 100 mL of absolute ethanol. Keep the solution at 4°C until used.

3.10 0.2 M Potassium phosphate buffer, pH 7.0

Dissolve 12 g of sodium phosphate monobasic dehydrate with 450 mL of distilled water. Adjust to pH 7.0 and make up the volume to 500 mL of distilled water. Keep the buffer in brown bottle at room temperature.

3.11 30% Ammonium thiocyanate solution

Dissolve 30 g of ammonium thiocyanate with 100 mL of distilled water. Keep in brown bottle at 4°C until used.

3.12 10 mM Phosphate buffer, pH 7.0, with 0.5 M sodium chloride

Dissolve 0.12 g of sodium phosphate monobasic dehydrate with 80 mL of DI water. Add 2.79 g of sodium chloride and adjust the pH to 7.0. Make the final volume to 100 mL and transfer the buffer into brown bottle to keep at 4°C.

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3.13 50 mM Tris with 300 mM sodium chloride

Dissolve 0.605 g of Tris with 80 mL of DI water, followed by adding 1.647 g of sodium chloride to the solution. Adjust the pH to 8.3 and make up to 100 mL final volume. Keep the buffer in brown bottle at 4°C.

3.14 6 mM HHL solution in Tris with sodium chloride

Dissolve 26 mg HHL (Hippuryl-L-histidyl-L-leucine hydrate) in 10 mL of Tris buffer solution.

3.15 1.0 M Hydrochloric acid solution

Mix 4.106 mL of 37% hydrochloric acid with distilled water. Adjust the final volume to 50 mL. Keep the solution in brown bottle at room temperature.

4. Purification with Gel Filtration and Ion Exchange Chromatography

4.1 20 mM Sodium acetate buffer, pH 4.0

Mix 1.14 mL of glacial acetic acid with 900 mL of distilled water. Adjust the pH to 4.0 and make up to 1000 mL final volume. Keep the solution in brown bottle at room temperature.

4.2 20 mM Sodium acetate buffer, pH 4.0, with sodium chloride 0.2-1.0 M

Mix 1.14 mL of glacial acetic acid with 900 mL of distilled water. Add 11.48, 23.18, 34.89, 46.60 and 58.32 g of sodium chloride to the solution for 0.2, 0.4, 0.6, 0.8 and 1.0 M of sodium chloride, respectively. Adjust the pH to 4.0 and make up to 1000 mL final volume. Keep the solution in brown bottle at room temperature.

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APPENDIX B

STANDARD CALIBRATION CURVE AND CALCULATIONS

B-1 Determination of Protein Concentration

Protein determination was performed using Bradford assay. Bovine serum albumin (BSA) was used as a standard protein. The Bradford reagent was prepared by dissolving 50 mg of coomassie brilliant blue G-250 in 50 ml of 95% ethanol, followed by adding 100 ml of 85% (w/v) phosphoric acid. Slowly add the acid solution into 850 ml of distilled water and stir until the dye completely dissolve. Keep the dye solution in brown bottle at 4°C. The solution was filtered through Whatman no.1 paper just before use to remove the precipitates. The sample (200 µL) was mixed with 4.8 mL of Bradford reagent and incubated 5 min at room temperature. The absorbance was measured at 595 nm. The protein standard calibration curve was used to determine the protein in the sample. The calculation was performed as following. The linear equation of the calibration curve was: $y = 0.0055x$; where y was the absorbance of the sample at 595 nm and x was the amount of BSA referring to protein quantities.

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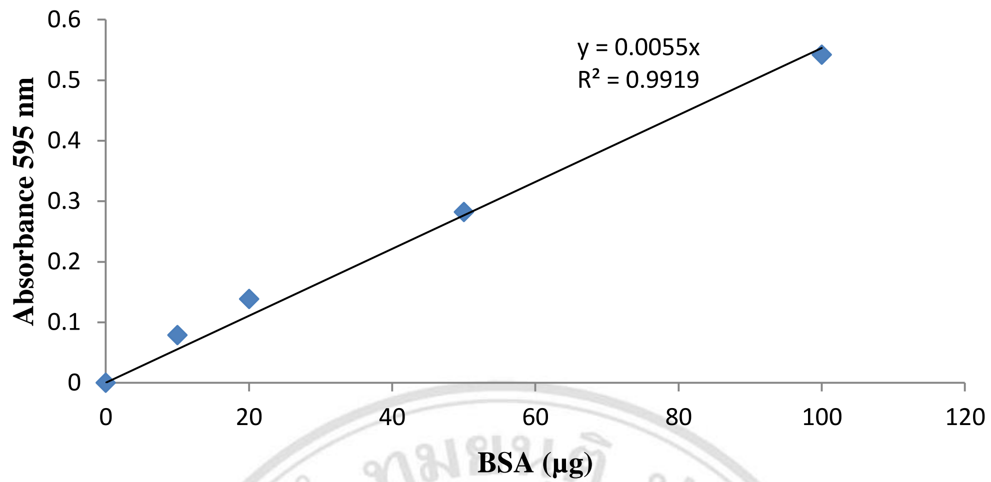

Figure B-1 Standard protein calibration curve

For example; the absorbance of trypsin hydrolysate was 0.066.

Therefore; 0.066 = 0.0055x

x = (0.066)/(0.0055)

x = 12.06 µg/ 200 µL

Calculated in the form of mg/mL;

x = (12.06 µg/ 200 µL) × (1000 µL/1 mL)

x = 60.30 µg/mL × (1 mg/1000 µg)

x = 0.060 mg/mL

Therefore, trypsin hydrolysate had protein concentration as 0.060 mg/mL

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B-2 Trolox Standard Calibration Curve of ABTS Radical Scavenging Assay

The trolox stock solution (4 mM) was diluted into different concentration ranged from 100 to 600 µM. The evaluation of TEAC value was carried out by comparing the inhibition percentage of sample with the standard calibration curve (Figure B-2). The calculation was done as following.

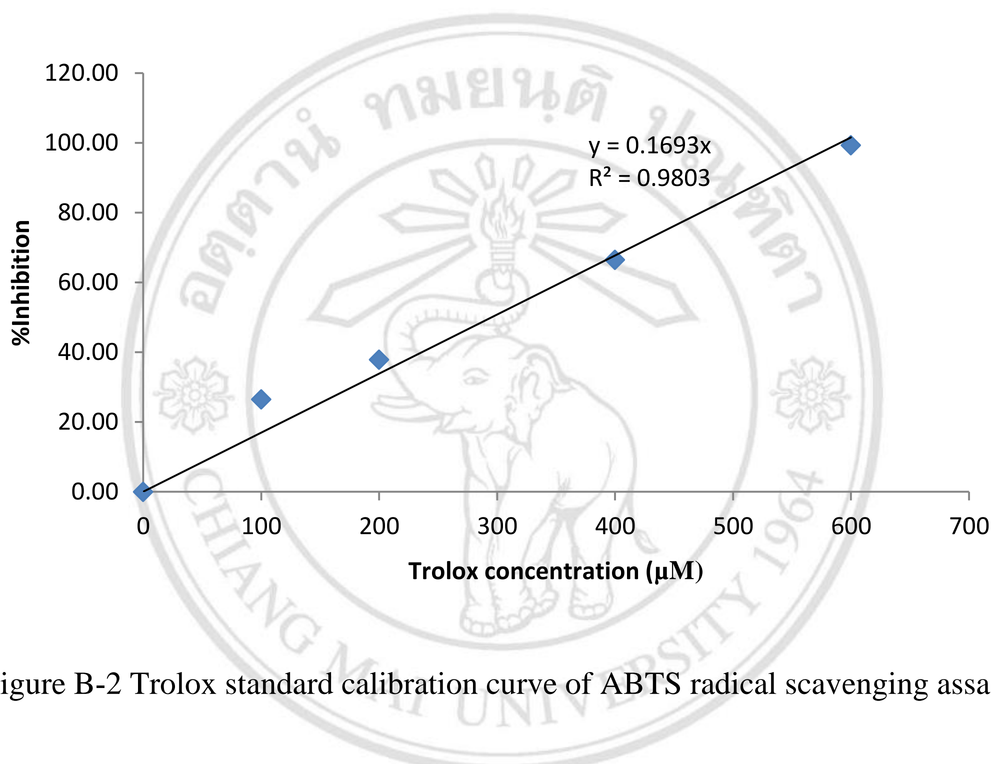

Figure B-2 Trolox standard calibration curve of ABTS radical scavenging assay

The linear equation of the standard calibration curve was: y = 0.1693x; where y was the inhibition percentage and x was the trolox concentration (µM). The inhibition percentage was calculated from following equation:

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$$\%\text{inhibition} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

where A_{sample} was the absorbance of mixture with the presence of the sample or trolox and $A_{control}$ was the absorbance of mixture without sample or trolox.

For example; the inhibition percentage of bromelain hydrolysate was 48.642%.

Therefore; $48.642 = 0.1693x$

$x = (48.642)/(0.1693)$

$x = 287.31$ μM trolox/20 μL of sample

The protein concentration of bromelain hydrolysate was 0.067 mg/mL

$x = (287.31$ μM trolox/20 μL$) \times (1000$ μL/1 mL$)$

$x = (14365.2$ μM trolox/mL$) \times (1$ mL/0.067 mg$)$

$x = 214412.18$ μM trolox/mg peptide $= 214.41$ mM trolox/mg peptide

Therefore 1 mg peptide in bromelain hydrolysate could exhibit the antioxidant activity in ABTS radical scavenging assay equivalent to 214.41 mM trolox.

B-3 FRAP Assay Standard Calibration Curve

Concentration of trolox in this assay had ranged from 20-200 μM, which prepared from diluting the 4 mM trolox stock solution. The FRAP value was determined by comparing the absorbance of sample with the standard calibration curve (Figure B-3). The calculation was done as following. The linear equation of the standard calibration curve was: $y = 0.0049x$; where y was the absorbance at 593 nm and x was the trolox concentration (μM).

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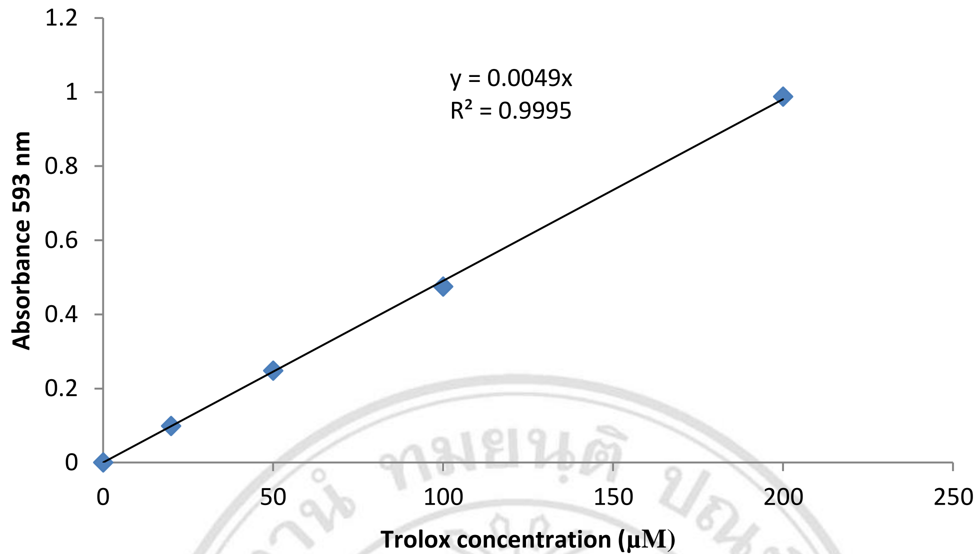

Figure B-3 Trolox standard calibration curve of FRAP assay

For example; the absorbance at 593 nm of flavourzyme hydrolysate was 0.111.

Therefore; $0.111 = 0.0049x$

$x = (0.111)/(0.0049)$

$x = 22.61$ µM trolox/100 µL of sample

The protein concentration of flavourzyme hydrolysate was 0.053 mg/mL

$x = (22.61$ µM trolox/100 µL$) \times (1000$ µL/1 mL$)$

$x = (226.1$ µM trolox/mL$) \times (1$ mL/0.053 mg$)$

$x = 4265.6$ µM trolox/mg peptide $= 4.266$ mM trolox/mg peptide

Therefore 1 mg peptide in flavourzyme hydrolysate could exhibit the antioxidant activity in FRAP assay equivalent to 4.266 mM trolox.

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APPENDIX C

AMINO ACIDS, ONE AND THREE LETTER CODES

Table C Single- and three-letter codes of amino acids

Amino acid	Three letter code	Single letter code
Alanin	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glutamine and glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S

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Table C Single and three letter codes of amino acids (Continued)

Amino acid	Three letter code	Single letter code
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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APPENDIX D

PROTEIN PATTERNS OF THE LOW MW FRACTION OF EACH HYDROLYSATE USING TRIS-TRICINE SDS-PAGE

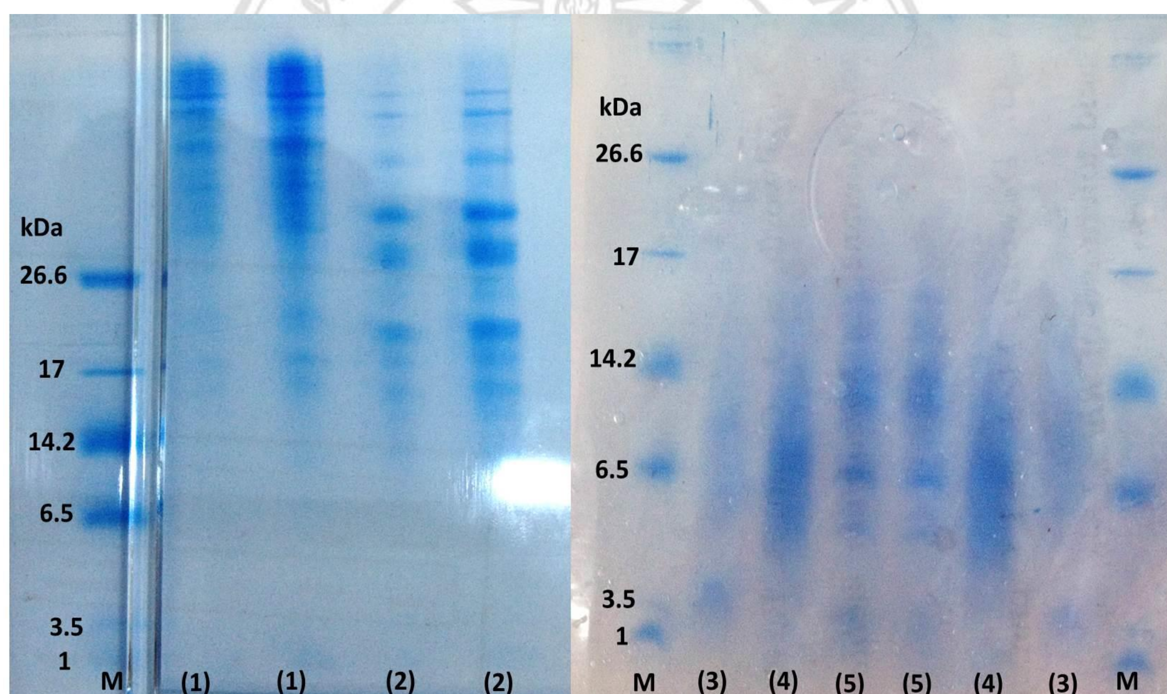

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Figure D Protein patterns of each hydrolysate from Tris-Tricine SDS-PAGE; (1) Alcalase, (2) Neutrase, (3) Bromelain, (4) Papain, and (5) Flavourzyme hydrolysate. Lane M is the lane of ultra-low range molecular weight marker (MW 1,060-26,600 Da) (Sigma-Aldrich, USA).

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Publications	Choonpicharn S, Jaturasitha S, Rakariyatham N, Suree N, Niamsup H (2015) Antioxidant and antihypertensive activity of gelatin hydrolysate from Nile tilapia skin. J Food Sci Technol 52:3134-3139

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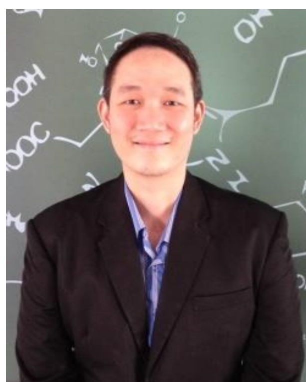
Choonpicharn S, Tateing S, Jaturasitha S, Rakariyatham N, Suree N, Niamsup H (2015) Identification of bioactive peptide from Oreochromis niloticus skin gelatin. J Food Sci Technol (DOI 10.1007/s13197-015-2091-x)



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