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Effect of pretreatments on chemical compositions of mince from Nile tilapia (*Oreochromis niloticus*) and fishy odor development in protein hydrolysate

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Abstract

Fish protein hydrolysates (FPH) have gained increasing attention as nutritious fish products. Lipid oxidation associated with fishy odor in FPH limits its utility. Thus, an appropriate pretreatment of fish mince prior to hydrolysis by lowering pro-oxidants and lipid substrates could tackle such a problem. Different pretreatments of Nile tilapia minces including (1) washing (W), (2) washing and membrane removal (W-MR), and (3) washing/membrane removal followed by acid or alkaline solubilization (W-MR-Ac or W-MR-Al) were conducted prior to hydrolysis. During the hydrolysis process, degree of hydrolysis (DH) and chemical changes were monitored. Color and sensory properties of milk fortified with hydrolysates prepared from mince without and with pretreatment were also determined. Among the pretreated mince samples, W-MR-Al contained the lowest remaining myoglobin and heme iron contents and also showed the lowest total lipid and phospholipid contents ($P < 0.05$). When mince and W-MR-Al were hydrolyzed using Alcalase for up to 120 min, higher DH were found in W-MR-Al. Furthermore, lower peroxide values, thiobarbituric acid reactive substances, and non-heme iron contents of hydrolysates from W-MR-Al were also observed ($P < 0.05$). When FPH powder from mince and W-MR-Al (0.3% to 0.5%) were fortified in milk, the former yielded a lower likeness score ($P < 0.05$) at all levels used. The addition of the latter up to 0.5% had no effect on likeness of all attributes, compared with control (without FPH). An appropriate pretreatment of mince was a promising approach to lower fishy odor problem, caused by lipid oxidation in FPH.

Keywords: Nile tilapia, Lipid oxidation, Fishy odor, Protein hydrolysate, Fortification

Background

Lipid oxidation is a great concern to the food industry because it can cause an adverse effect on flavor, odor, texture, and nutritional value of muscle-based foods (Liang and Hultin 2005a). Even though lean fish are traditionally used for fish processing with a consistent high quality owing to their negligible lipid contents and heme pigments, lipid oxidation still takes place in lean fish and their products. Phospholipid membranes with a high content of unsaturated fatty acids are believed to be the key substrate for lipid oxidation (Liang and Hultin 2005b). In addition, membranes also have a

large surface area and thus to contact with pro-oxidants in the aqueous phase around membranes (Liang and Hultin 2005a). Moreover, heme proteins in the raw material can also become oxidized, thereby promoting lipid oxidation and development of unpleasant odor/flavor (Raghavan et al. 2008).

Nile tilapia is popular in Southeast Asia and other counties like China, India, etc., owing to its white flesh and delicacy. In general, it is sold as whole fish or as fillets. To add more value, hydrolysis processes have been developed to convert fish protein into the potent bioactive peptides. Nevertheless, one problem connected to protein hydrolysate from fish flesh is the presence of pro-oxidants such as heme proteins and unstable lipid substrates (Raghavan and Kristinsson 2008). Oxidation of lipids is associated with fishy odor development which can make hydrolysates unsuitable for use as food supplements. Yarnpakdee et al. (2012) reported that fishy smell in protein hydrolysate from Nile tilapia mince was mainly caused by lipid oxidation, especially when fish which were not fresh were used. To tackle such a problem, pretreatments of mince prior to enzymatic hydrolysis, in which both pro-oxidants and lipids are removed or reduced, are required.

Protein isolate with a low amount of undesirable compounds has been successfully prepared (Raghavan et al. 2008; Halldórsdóttir et al. 2011). In brief, fish muscle proteins (myofibrillar and sarcoplasmic proteins) are solubilized at acidic or alkaline pH (pH approximately 2.5 or 11, respectively) and undesirable contaminants are separated from soluble proteins by centrifugation. Subsequently, soluble proteins are precipitated at their isoelectric points (pH approximately 5.5) (Hultin and Kelleher 2000). Hydrolysates from brownstripe red snapper protein isolate showed negligible fishy odor, compared with that prepared directly from fish mince (Khantaphant et al. 2011). As a consequence, a wider range of applications for FPH can be achieved. Since the protein hydrolysate from Nile tilapia mince had the low acceptability associated with fishy odor development, the appropriate pretreatment, which was able to remove lipids effectively prior to hydrolysate preparation, could be a promising means to tackle such a problem. However, no information regarding the pretreatments of mince from Nile tilapia and their effect on fishy odor in the resulting protein hydrolysate has been reported. The objectives of this study were to elucidate the effect of pretreatments on the removal of pro-oxidants and phospholipid membranes from Nile tilapia mince and to monitor lipid oxidation and fishy odor development during a hydrolysis process.

Methods

Chemicals

Alcalase 2.4 L (E.C. 3.4.21.62) was obtained from Novozyme (Bagsvaerd, Denmark). 2,4,6-trinitrobenzenesulfonic acid, bathophenanthroline disulfonic acid, sodium dodecyl sulfate (SDS) and 1,1,3,3-tetramethoxypropane were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid, sodium nitrite, ferrous chloride, and iron standard solution were obtained from Merck (Darmstadt, Germany). Disodium hydrogen phosphate, sodium dihydrogen phosphate and 2-thiobarbituric acid and cumene hydroperoxide were procured from Fluka (Buchs, Switzerland). Methanol, acetone, chloroform, and ammonium thiocyanate were obtained from Lab-Scan (Bangkok, Thailand). All chemicals were of analytical grade.

Fish samples

Fresh Nile tilapia (*Oreochromis niloticus*) with a weight of 0.5 to 0.8 kg/fish were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were transported in ice with a fish/ice ratio of 1:2 (*w/w*) to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 30 min.

Preparation of mince with different pretreatments

Preparation of mince

Whole fish were washed and flesh was separated manually. Flesh was minced to uniformity using Moulinex AY46 blender (Group SEB, Lyon, France) in a walk-in-cold room (4°C). The mince obtained was placed in polyethylene bags and kept in ice not longer than 2 h before use.

Preparation of washed mince

Mince was homogenized with five volumes of cold distilled water (2 to 4°C) using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min. The homogenate (4°C) was stirred for 15 min, followed by centrifugation at 9,600 × *g* for 10 min at 4°C using a Beckman Coulter centrifuge Model Avant J-E (Beckman Coulter, Inc., Fullerton, CA, USA). The washing process was repeated twice. The sample obtained was referred to as 'washed mince, (W)'.

Preparation of mince with membrane removal

Prior to membrane separation, the mince was subjected to washing as previously described. Membranes were then removed from washed mince by treatment with Ca^{2+} and citric acid according to the method of Liang and Hultin (2005) with a slight modification. Washed mince sample was homogenized with nine volumes of cold solution including 8 mM $CaCl_2$ and 5 mM citric acid, using a homogenizer at a speed of 11,000 rpm for 2 min. After continuous stirring for 60 min (4°C), the sample was centrifuged at 4,000 × *g* for 15 min at 4°C, and the mince obtained was referred to as 'washed mince with membrane removal (W-MR)'.

Preparation of protein isolate with membrane removal

To prepare the protein isolate, acid and alkaline solubilization processes were used as described by Rahavan and Hultin (2009) with a slight modification. The W-MR sample was homogenized with nine volumes of cold distilled water (2 to 4°C) at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH either 3.0 or 11.0. The mixtures were kept on ice for 60 min. Homogenates were then centrifuged at 5,000 × *g* for 10 min at 4°C. Acid and alkaline soluble fractions were collected and adjusted to pH 5.5 to precipitate the myofibrillar proteins. The mixture was then centrifuged at 10,000 × *g* for 20 min. The pellet obtained was referred to as 'acid and alkaline solubilized protein isolate with membrane removal followed by acid or alkaline solubilization (W-MR-Ac and W-MR-Al), respectively.

Analyses

All prepared mince samples were subjected to following analyses:

Determination of pH

All samples were subjected to pH measurement as described by Benjakul et al. (1997). The samples were homogenized with ten volumes of deionized water (*w/v*) at a speed of 11,000 rpm for 1 min. pH of homogenate was measured using a Model Docu-pH Meter (Sartorius AG, Gottingen, Germany).

Determination of myoglobin content

Myoglobin content was determined by a direct spectrophotometric measurement (Chaijan et al. 2005). Sample (2.0 g) was mixed with 20 ml of cold 40 mM phosphate buffer (pH 6.8), and the mixture was homogenized at 13,500 rpm for 10 s. The mixture was centrifuged at 3,000 × *g* for 30 min at 4°C, and the supernatant was filtered through a Whatman filter paper No.1 (Whatman International Ltd., Maidstone, England). The absorbance of the supernatant was read at 525 nm using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Myoglobin content was calculated from the molar extinction coefficient of 7.6×10^{-3} and a molecular weight of 16,110 dalton (Gomez-Basauri and Regenstein 1992). The myoglobin content was expressed as mg/100 g dry sample.

Determination of heme and non-heme iron content

Heme iron content was determined as per the method of Cheng and Ockerman (2004) with a slight modification. To a ground sample (2 g), 9 ml of acid acetone (90% acetone, 8% deionized water, and 2% HCl, *v/v/w*), were added. The mixture was macerated with a glass rod and allowed to stand for 1 h at room temperature. The extract was filtered with a Whatman No. 42 filter paper (Whatman International Ltd., Maidstone, UK), and the absorbance was read at 640 nm against an acid acetone using a spectrophotometer. Heme iron content was calculated with the factor of 0.00882 μg/μg hematin using the following formula:

$$\text{Heme iron (mg/100 g dry sample)} = \text{total pigment (ppm)} \times 0.00882$$
$$\text{where total pigment (ppm)} = A_{640} \times 680.$$

Non-heme iron content was determined according to the method of Schricker et al. (1982). The ground sample (1.0 g) was transferred into a screw cap test tube and 50 μl of 0.39% (*w/v*) sodium nitrite were added. Thereafter, 4 ml of 40% trichloroacetic acid and 6 M HCl (ratio of 1:1 (*v/v*), prepared freshly) were added. The tightly capped tubes were placed in an incubator shaker (W350, Memmert, Schwabach, Germany) at 65°C for 22 h and then cooled at room temperature for 2 h. The supernatant (400 μl) was mixed with 2 ml of the non-heme iron color reagent (prepared freshly). After vortexing using a Vortex-Genie2 mixer (Scientific Industries, Bohemia, NY, USA) and standing for 10 min, the absorbance was read at 540 nm. The color reagent was prepared by mixing a 1:20:20 ratio (*w/v/v*) of: (1) bathophenantroline (0.162 g dissolved in 100 ml of double deionized water with 2 ml thioglycolic acid); (2) double deionized water; (3) saturated sodium acetate solution.

The non-heme iron content was calculated from an iron standard curve. The iron standard solutions ($Fe(NO_3)_2$ in HNO_3) with concentrations ranging from 0 to 5 ppm were used. The concentration of non-heme iron was expressed as mg/100 g dry sample.

Determination of lipid content

Lipid content was determined by a Soxhlet apparatus according to the method 920.39B of AOAC (2000). Lipid content was expressed as g/100 g dry sample.

Determination of phospholipid content

The phospholipid content was determined by measuring phosphorus according to the method of Suzuki and Suyama (1985) with a slight modification. To the samples (0.6 to 0.8 g), 20 ml of 4 M NaOH were added and mixed vigorously. The samples were heated in a boiling water bath (90 to 95°C) for 30 min and then cooled at room temperature for 1 h. The mixture was mixed with 20 ml of 4 M HCl for neutralization. The supernatant (0.2 ml) was mixed with 2 ml of phosphate reagent (ammonium molybdate solution: malachite green, 1:3 *v/v*). The mixture was then incubated at room temperature for 30 min. The absorbance was measured at 620 nm. Disodium hydrogen phosphate solutions with concentrations of 0 to 15 μg/ml were used for a standard curve preparation. A factor of 25 was used for converting phosphorus content to phospholipid based on an average molecular weight of phosphatidyl choline divided by atomic weight of phosphorus (Sigfusson and Hultin 2002). The phospholipid content was expressed as mg/100 g dry sample.

Determination of peroxide value

The peroxide value (PV) was determined according to the method of Richards and Hultin (2000) with a slight modification. Ground sample (4.5 to 5.5 g) was homogenized at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, *v/v*). The homogenate was then filtered using a Whatman No.1 filter paper. To 7 ml of filtrate, 2 ml of 0.5% NaCl were added. The mixture was vortexed at a moderate speed for 30 s, followed by centrifugation at 3,000 × *g* for 3 min at 4°C using a refrigerated centrifuge to separate the sample into two phases. To the lower phase (3 ml), 2 ml of cold chloroform/methanol (2:1) mixture, 25 μl of 30% (*w/v*) ammonium thiocyanate and 25 μl of 20 mM iron (II) chloride were added. The reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. The blank was prepared in the same manner, except distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0.5 to 2 ppm. PV was expressed as mg cumene hydroperoxide/kg dry sample.

Determination of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust (1978). A ground sample (0.5 g) was homogenized with 2.5 ml of a solution containing 0.375% (*w/v*) thiobarbituric acid, 15% (*w/v*) trichloroacetic acid, and 0.25 M HCl. The mixture was heated in a boiling water bath (95 to 100°C) for 10 min to develop a pink color, cooled with running tap water, and centrifuged at 3,600 × *g* at 25°C for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane at concentrations ranging from 0 to 10 ppm. TBARS was calculated and expressed as mg malonaldehyde/kg dry sample.

Determination of trimethylamine content

Trimethylamine (TMA) content was determined according to the method of Conway and Byrne (1933). A ground sample (5 g) was mixed with 20 ml of 4% (*w/v*)

trichloroacetic acid and homogenized at a speed of 11,000 rpm for 1 min. The homogenate was filtered using a Whatman No. 4 filter paper. In the outer ring, formaldehyde (10% *v/v*) (1 ml) was added to the filtrate (1 ml) to fix ammonia present in the sample. To initiate the reaction, saturated K_2CO_3 (1 ml) was mixed with the prepared sample to release TMA. TMA was trapped in 1 ml of the inner ring solution (1% boric acid (*w/v*) containing the Conway indicator). The Conway unit was incubated at 37°C for 60 min. The titration of the inner ring solution was performed using 0.02 M HCl, and the amount of TMA was calculated. TMA content was expressed as mg N/100 g dry sample.

SDS-polyacrylamide gel electrophoresis

Protein patterns were determined by SDS-polyacrylamide gel electrophoresis using a 4% stacking gel and a 10% running gel according to the method of Laemmli (1970). Samples (3 g) were solubilized in 27 ml of 5% SDS. The mixture was homogenized for 1 min at a speed of 13,000 rpm and incubated at 85°C for 1 h to solubilize all proteins. Proteins (15 μg), determined by the Biuret method (Robinson and Hogden 1940), were loaded onto the gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-PROTEAN II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (*w/v*) Coomassie Brilliant Blue R-250 (Sigma, St Louis, MO, USA) in 50% (*v/v*) methanol and 7.5% (*v/v*) acetic acid and destained with 50% (*v/v*) methanol and 7.5% (v/v) acetic acid for 12 min, followed by 5% (*v/v*) methanol and 7.5% (*v/v*) acetic acid for 3 h. A wide range molecular weight marker was used to estimate the molecular weight.

Pretreated mince with the lowest pro-oxidant and lipid contents was selected for the hydrolysis study.

Effect of selected pretreatment on composition and properties of protein hydrolysate

Changes during hydrolysis

To study the impact of pretreatment on changes in protein hydrolysates, mince and W-MR-Al (100 g) were mixed with distilled water (2 to 4°C) using a sample/water ratio of 1:4 (*w/v*) to obtain a final protein concentration of 2% (*w/v*). The mixtures were adjusted to pH 8.0 and were then pre-incubated at 50°C for 20 min prior to enzymatic hydrolysis using Alcalase. The hydrolysis reaction at 50°C was initiated by adding Alcalase at a level of 1% (*w/w*). Samples were taken at different times during hydrolysis (0, 10, 20, 30, 60, 90, and 120 min), and the reactions were terminated by heating the sample in boiling water for 10 min. The obtained protein hydrolysates were then subjected to analyses of non-heme iron content, PV, and TBARS as previously described. Degree of hydrolysis (DH) of hydrolysates was also determined as per the method of Benjakul and Morrissey (1997).

Properties and the use of protein hydrolysates

After 2 h of hydrolysis, the reaction mixtures were heated for 10 min in boiling water to terminate the hydrolytic reaction. The mixture was then centrifuged at 2,000 × *g* at 4°C for 10 min. The supernatant obtained was lyophilized using a freeze-drier (Model Duratop™ lP/Dura Dry™ lP, FTS® System, Inc., Stone Ridge, NY, USA). The lyophilized fish protein hydrolysates produced from mince and W-MR-Al were referred to as 'FPH_{mince}' and '$FPH_{W\text{-}MR\text{-}Al}$', respectively. The resulting hydrolysates were subjected to color measurement and were fortified in low-fat milk.

Color measurement

The color of both hydrolysate powders was measured by a colorimeter (ColorFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system. L^*, a^*, b^*, ΔE^*, and ΔC^* representing lightness, redness/greenness, yellowness/blueness, total difference of color, and the difference in chroma, respectively, were reported. ΔE^* and ΔC^* were calculated as follows:

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

where ΔL^*, Δa^*, and Δb^* are the differentials between color parameter of the samples and the color parameter of the white standard ($L^* = 92.82$, $a^* = -1.24$, $b^* = 0.50$)

$$\Delta C^* = C^*_{\text{sample}} - C^*_{\text{standard}}$$

$$\text{where } C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

Preparation of low fat milk fortified with protein hydrolysates

Low fat milk, containing 1% milk fat (Foremost, Frieslandfoods Co., Ltd., Samutprakan, Thailand), was purchased from a local supermarket, Hat Yai, Thailand. FPH_{mince} and $FPH_{W\text{-}MR\text{-}Al}$ were added to the milk at different levels (0.3, 0.4, and 0.5%) and mixed well. The resulting milks (25 to 26°C) were subjected to sensory evaluation.

Sensory evaluation

A likeness evaluation of low fat milk fortified with and without protein hydrolysates was performed by 30 untrained panelists at the ages of 22 to 30, who were regular milk consumers. The assessment was conducted for color, fishy odor, fishy flavor, and overall likeness using a 9-point hedonic scale: 1, dislike extremely; 5, neither like nor dislike; 9 like extremely (Meilgaard et al. 2007). For fishy odor/flavor likeness score, the lower likeness score indicate the stronger fishy odor/flavor.

Statistical analysis

Experiments were run in triplicate using three different batches of samples. Data were subjected to analysis of variance. Comparison of means was carried out by Duncan's multiple range tests. The *t*-test was used for pair comparison (Steel and Torrie 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

Results and discussion

Characteristics of mince with different pretreatments

Chemical compositions

Mince from Nile tilapia subjected to various pretreatments showed varying compositions as presented in Table 1. Pretreatments included washing the mince, washing the mince with W-MR and preparing acid- and alkaline-aided protein isolate from the washed mince with W-MR-Ac and W-MR-Al. Different samples had the varying pH (5.41 to 6.57), depending on pretreatments used. Mince and washed mince showed similar pH (6.35 to 6.57). The lower pH was found in W-MR sample (5.92). Membrane removal process using citric acid more likely resulted in the decrease in pH. For both

Table 1 Chemical compositions of Nile tilapia mince after different pretreatments

Parameters	M	W	W-MR	W-MR-Ac	W-MR-Al
pH	6.35 ± 0.02b*,**	6.57 ± 0.10a	5.92 ± 0.18c	5.41 ± 0.02d	5.55 ± 0.01e
Myoglobin content (mg/g dry sample)	5.34 ± 0.50a	2.58 ± 0.36b	0.95 ± 0.11c	0.41 ± 0.16d	0.11 ± 0.01d
Heme iron content (mg/100 g dry sample)	4.68 ± 0.25a	2.94 ± 0.07b	2.57 ± 0.23c	0.84 ± 0.06d	0.74 ± 0.34d
Non-heme iron content (mg/100 g dry sample)	2.25 ± 0.25a	1.68 ± 0.16b	1.57 ± 0.36b	0.89 ± 0.16c	0.59 ± 0.03c
Lipid content (g/100 g dry sample)	1.66 ± 0.60a	1.49 ± 0.26a	0.70 ± 0.15b	0.27 ± 0.05bc	0.10 ± 0.05c
Phospholipid content (mg/100 g dry sample)	13.52 ± 0.23a	7.00 ± 0.20b	5.30 ± 0.27c	2.69 ± 0.16d	2.35 ± 0.05d
PV (mg cumene hydroperoxide/kg dry sample)	28.89 ± 2.02a	22.43 ± 0.36b	15.32 ± 1.86c	8.09 ± 0.12d	4.42 ± 0.64e
TBARS (mg MDA/kg dry sample)	10.58 ± 0.07a	8.42 ± 0.06b	5.63 ± 0.04c	4.52 ± 0.12c	1.79 ± 0.07d
TMA content (mg N/100 g dry sample)	ND***				

*Mean ± SD ($n = 3$).
** Different letters within the same row indicate the significant differences ($P < 0.05$).
***ND, not detectable.

W-MR-Ac and W-MR-Al samples, the lowest pHs (5.41 to 5.55) were found and were similar to pH used for protein precipitation (5.5).

No TMA was detected in Nile tilapia mince and all pretreated mince samples. This result indicated that Nile tilapia, which was cultured in a freshwater farm, did not contain trimethylamine-N-oxide (TMAO), a compound found in marine fish for osmoregulation. TMAO can be reduced to TMA, a fishy odor compound (Gram and Huss 1996). As a result, it could be presumed that the fishy odor developed in Nile tilapia mince was not mainly due to the formation of TMA.

The washing process reduced myoglobin by 51.7%, compared with that found in mince. However, more myoglobin was removed when the membrane removal process was implemented ($P < 0.05$). The lowest myoglobin contents were found in W-MR-Ac and W-MR-Al samples ($P < 0.05$). Pretreatment of washed mince with $CaCl_2$ and citric acid might enhance the removal of myoglobin by increasing the polarity of myoglobin, in which it could be leached out more easily. When the solubilization process via acid or alkaline pH adjustment was used, the dissociation of protein complexes might occur. This could enhance the liberation of myoglobin from the muscle. The reduction in heme proteins, myoglobin and heme iron contents, not only improved the color of mince, but also increased its oxidative stability. Total lipids and phospholipids in the washed mince were also significantly lowered by 10.2% and 48.2%, respectively. Similar results were also observed by Tongnuanchan et al. (2011) who noted that lipid content of washed red tilapia mince was deceased by 14.4%, in comparison with that found in mince. Washing is a process, which can remove lipids and undesirable materials such as blood, pigment and odorous substances (Rawdkuen et al. 2009). Nevertheless, lipoproteins or membrane phospholipids associated with other muscle proteins might not be leached out easily. Additionally, some lipids were solidified at low temperatures during the washing process. This might lead to less removal of those lipids.

It was found that W-MR had marked decrease in lipids and phospholipids, compared with mince. $CaCl_2$ and citric acid used for membrane removal might disconnect the linkage between membranes and cytoskeleton protein, which linked together via

electrostatic interaction. Ca^{2+} as well as citric acid might interact with those components, especially the phospholipids membrane, thereby providing the charge or polarity to those components. As a consequence, they were more likely soluble in the aqueous phase. As a result, the releases of the phospholipid membranes from attached cytoskeletal were facilitated due to the loosened structure. In addition, Ca^{2+} could interact with the polar head of phospholipid to form a calcium-phospholipid complex. In addition, citric acid might play a role as a binding agent for the basic amino acid residues of cytoskeletal proteins, thereby competing with the acidic phospholipids of membranes (Hrynets et al. 2011). Hrynets et al. (2011) reported that addition of 6 or 8 mmol/l of citric acid resulted in the substantial removal of lipid and pigments from mechanically separated turkey meat. Vareltzis et al. (2008) reported that the presence of 10 mmol/l $CaCl_2$ and 5 mmol/l citric acid during acid or alkaline aided protein isolation significantly improved lipid reduction in mussel proteins.

When the pH-shift methods were applied in mince with membrane removal, much more lipids and phospholipids were removed. The dissociation of protein complexes might provide a larger surface, in which both citric acid and $CaCl_2$ could help in removing phospholipids more effectively. Total lipid in W-MR-Ac and W-MR-Al decreased by 83.7% and 94.0%, compared with that found in mince, while phospholipids decreased by 80.1% and 82.6%, respectively. During pretreatment, not only undesirable materials, especially lipids and heme proteins, were removed but also lipid oxidation products were eliminated as indicated by the lower PV and TBARS values. The lowest PV and TBARS were found in the W-MR-Al sample. Higher PV and TBARS values were found in W-MR-Ac compared with W-MR-Al ($P < 0.05$). The result suggested the greater susceptibility of acid treated protein isolate toward oxidation. Raghavan and Hultin (2009) reported that cod protein isolate prepared using the acid process was significantly more susceptible to lipid oxidation than using the alkaline process. Among various pro-oxidative constituents of muscle tissue, phospholipid membrane, and heme proteins are mainly involved in oxidative deterioration (Liang and Hultin 2005a; Raghavan and Hultin 2009; Thiansilakul et al. 2011). Thus, the alkaline solubilization process was more appropriate than the acid solubilization process since lipid oxidation could be minimized under alkaline condition.

Protein patterns

Protein patterns of different pretreated minces are depicted in Figure 1. Myosin heavy chain (MHC), actin, tropomyosin, and troponin were found in fish mince. After washing or membrane separation, some sarcoplasmic proteins or some cytoskeletal proteins which interact with the membrane phospholipids might be removed. As a result, the myofibrillar proteins became concentrated as indicated by the increased band intensity of MHC, actin, tropomyosin, and troponin, while some protein bands with molecular weight of 49.8, 39.3, and 37.0 kDa disappeared. No marked differences in protein patterns were observed in W-MR compared to those found in isolates. However, a slight degradation of MHC band was obtained in W-MR-Ac. It was also found that band intensity of all myofibrillar proteins increased in W-MR-Ac and W-MR-Al, when compared with other treatments. When proteins became charged at alkaline or acidic conditions, protein repulsion occurred, thereby solubilizing the protein. When pH is adjusted to 5.5, the myofibrillar proteins are precipitated, while several sarcoplasmic

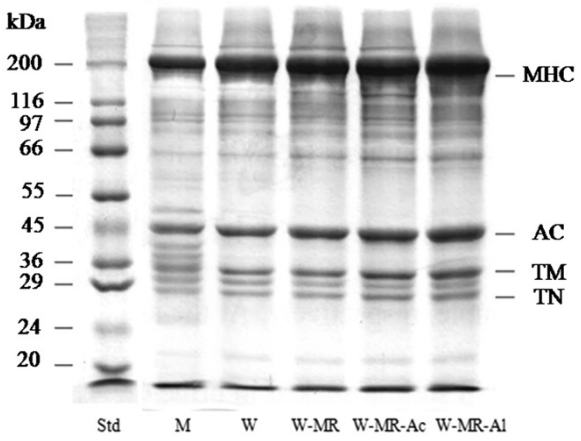

Figure 1 Protein patterns of Nile tilapia mince before and after different pretreatments. M, mince; W, washed mince; W-MR, washed mince with membrane removal; W-MR-Ac W-MR-with acid solubilization; W-MR-Al, W-MR-with alkaline solubilization. MHC, myosin heavy chain, AC, actin; TM, tropomyosin; TN, troponin.

proteins or cytoskeleton proteins remained in the supernatant. As a consequence, myofibrillar proteins were more concentrated as evidenced by the increase in band intensity. Since W-MR-Al sample had the highest myofibrillar protein content with the lowest lipids, pro-oxidants as well as lipid oxidation products, it was chosen for preparation of protein hydrolysate.

Changes in DH and compositions of protein hydrolysate during hydrolysis

Changes in DH

Hydrolysis of mince and W-MR-Al by Alcalase as a function of time is shown in Figure 2. Degree of hydrolysis (DH) of protein hydrolysates increased as hydrolysis time increased ($P < 0.05$). DH is known to have a great impact on several properties of protein

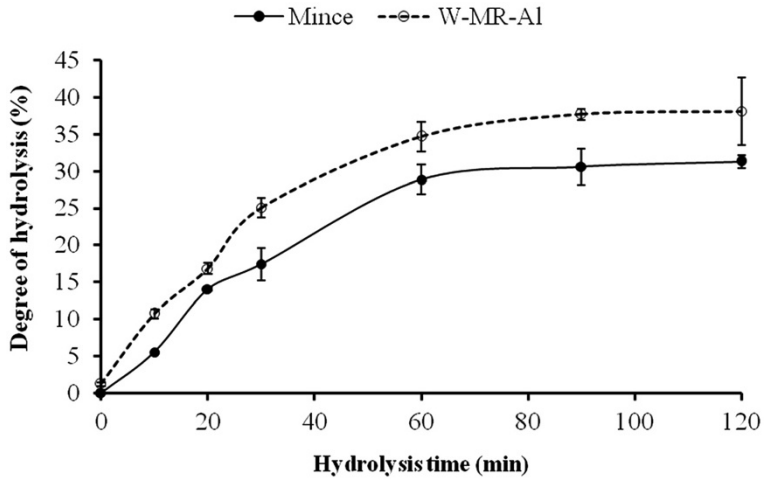

Figure 2 Changes in DH of Nile tilapia mince and W-MR-Al during hydrolysis with Alcalase. Bars represent the standard deviation ($n = 3$).

hydrolysates (Adler-Nissen 1979; Abdalla and Roozen 1999). Rapid hydrolysis was observed within the first 60 min, indicating that a large number of peptide bonds were hydrolyzed (Shahidi et al. 1995). Thereafter, the hydrolysis rate was decreased, mainly due to a decrease in available hydrolysis sites in substrate, enzyme autodigestion, and/or product inhibition (Kristinsson and Rasco 2000). At the same time of hydrolysis, the hydrolysates obtained from W-MR-Al possessed a higher DH than did those derived from mince ($P < 0.05$). The result suggested that the W-MR-Al sample was more susceptible to cleavage by Alcalase. This was possibly due to more exposure of peptide bonds of the protein isolates, where Alcalase could cleave the peptides more effectively. Alcalase has several advantages toward hydrolysis including (1) broad specificity (2) availability (3) inexpensive price and (4) resistance to gastrointestinal hydrolysis (Ovissipour et al. 2009; Tiengo et al. 2009; Zhu et al. 2010).

During alkaline solubilization, the repulsion between protein molecules results in the dissociation of actomyosin complex. The liberated peptide chains and looser protein structures were more prone to hydrolysis by Alcalase. Thus, the configuration of protein was another important factor governing the rate of hydrolysis, thereby affecting the properties of hydrolysate obtained.

Changes in non-heme iron content

Changes in non-heme iron content in hydrolysates from mince and W-MR-Al during hydrolysis were monitored as depicted in Figure 3a. An increase in non-heme iron content was observed with increasing hydrolysis time for both samples ($P < 0.05$). It was suggested that the disruption of the porphyrin ring of heme protein remaining in both samples more likely occurred during the hydrolysis process at 50°C. Chaijan et al. (2005) noted that the destabilization of the heme-globin complex occurs under harsh conditions such as very acidic pH and high temperature, leading to the release of free iron called 'non-heme iron'. Thiansilakul et al. (2011) found that heating could weaken the porphyrin ring with the subsequent iron released. Released iron might act as a pro-oxidant, which is able to enhance lipid oxidation. Furthermore, Kristensen and Andersen (1997) revealed that heated heme iron had a more significant effect on pro-oxidative activity than did heated free iron. Regardless of hydrolysis time, less non-heme iron was found in the hydrolysate from W-MR-Al than in hydrolysate from mince ($P < 0.05$). Since water soluble components, including heme proteins, were largely removed by washing in combination with membrane removal and alkaline solubilization process, non-heme iron content was also reduced. The result was in agreement with the low amount of myoglobin and heme iron retained in W-MR-Al (Table 1). Therefore, the pro-oxidant present during hydrolysis can be lowered by combined pretreatments of protein substrate via washing, membrane removal, followed by alkaline solubilization.

Changes in PV and TBARS

Lipid oxidation of hydrolysates produced from mince and W-MR-Al expressed as PV and TBARS values is shown in Figure 3b,c, respectively. For hydrolysates produced from mince, a continuous increases in PV was found up to 60 min of hydrolysis process, followed by a gradual decrease up to the end of hydrolysis ($P < 0.05$), suggesting that the hydroperoxides formed were decomposed to other compounds. However,

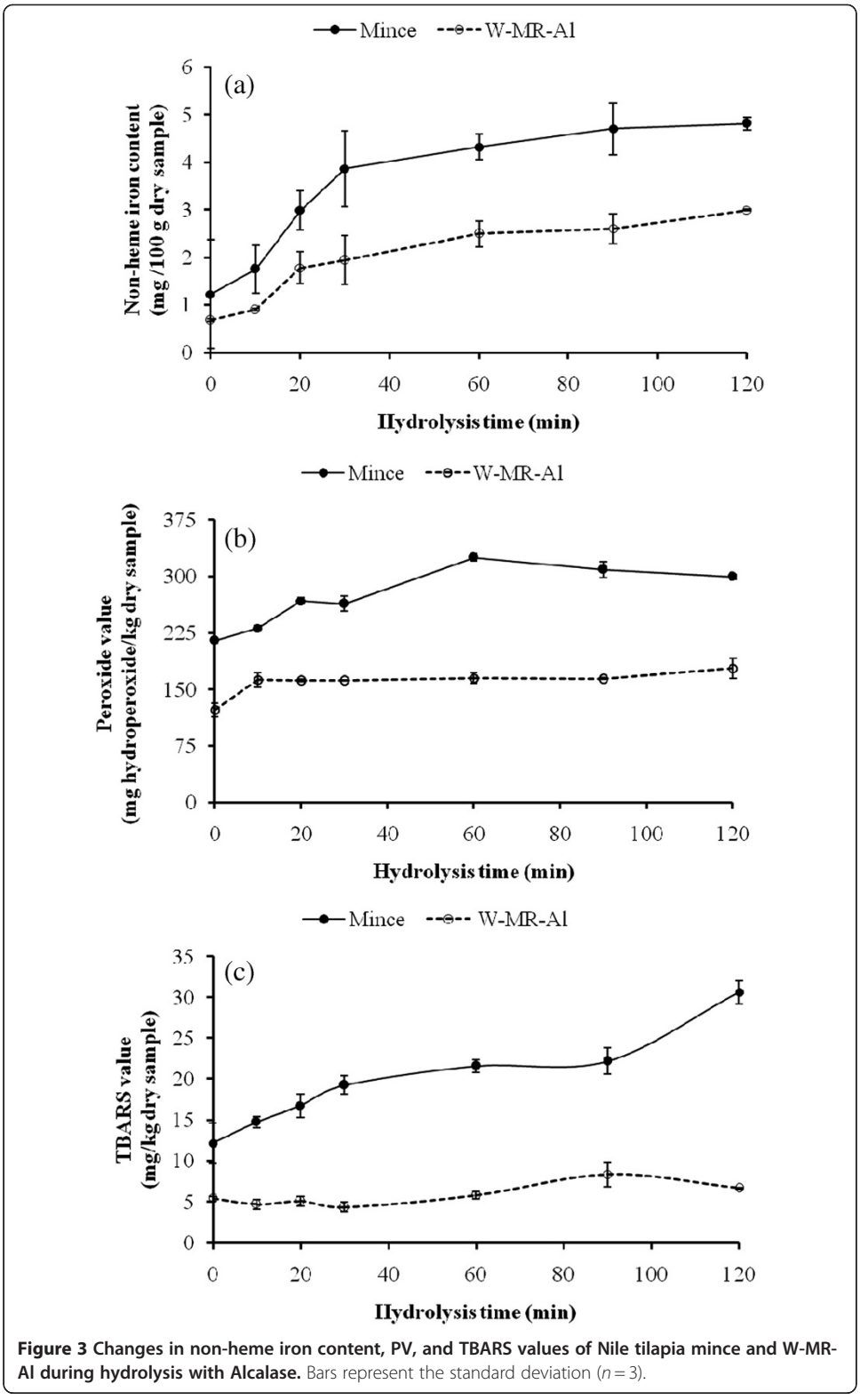

Figure 3 Changes in non-heme iron content, PV, and TBARS values of Nile tilapia mince and W-MR-Al during hydrolysis with Alcalase. Bars represent the standard deviation ($n = 3$).

no changes in PV were observed in hydrolysates from W-MR-Al during 10 to 120 min of hydrolysis ($P > 0.05$). The result demonstrated that lipid oxidation took place in mince at a higher extent during hydrolysis. This was more likely due to the presence

of more lipids and pro-oxidants in mince, compared with W-MR-Al. Apart from myoglobin, hemoglobin in fish muscle also serves as an effective catalyst of lipid oxidation (Apte and Morrissey 1987). Heme dissociation, heme destruction and iron released play a role in lipid oxidation of fish muscle (Gandemer 1999; Hultin 1994). Generally, washing/membrane removal in combination with the alkaline solubilization process could yield hydrolysate with negligible lipid oxidation.

TBARS values of both hydrolysates increased as hydrolysis time increased ($P < 0.05$) (Figure 3c). The result reconfirmed that lipid oxidation took place during hydrolysis, particularly when mince was used as the substrate. A much higher increase in TBARS of hydrolysates produced from mince was observed, and this was more likely related to the increase in non-heme iron content during extended hydrolysis (Figure 3a). For hydrolysates produced from W-MR-Al, a slight increase in TBARS was observed after 60 min of hydrolysis ($P < 0.05$). TBARS tended to decrease at 120 min, possibly caused by a loss of low-molecular-weight decomposition products. Adduction of aldehyde with other compounds, especially proteins, might also contribute to the lowered TBARS. Even though a large amount of lipid as well as pro-oxidant could be removed, the lipid oxidation of hydrolysate produced from W-MR-Al still occurred to some extent. Some lipoproteins might be co-precipitated with myofibrillar proteins during isoelectric precipitation (Kristinsson et al. 2005). Thus, W-MR-Al could yield hydrolysate with lower lipid oxidation products.

Color and fortification of protein hydrolysate powders in low fat milk

Color of freeze-dried hydrolysates produced from mince and W-MR-Al is presented in Table 2. All hydrolysates exhibited a slight creamy yellowish color. There were differences in L^* (lightness), b^* (yellowness), ΔE^* (total color difference), and ΔC^* (color intensity difference) between $FPH_{W\text{-}MR\text{-}Al}$ and FPH_{mince}. Higher L^* and lower b^* values were observed in $FPH_{W\text{-}MR\text{-}Al}$, compared with those of FPH_{mince} ($P < 0.05$). This was in accordance with the lower ΔE^* and ΔC^* in the former. Generally, myoglobin and hemoglobin have been known to be responsible for color characteristics of fish flesh or their product. The pretreatment of mince via washing/membrane removal in combination with alkaline solubilization could remove or significantly reduce those pigments, leading to lower amounts of colored compounds retained in the resulting mince. Moreover, lipid oxidation causes poor visual appearance and a yellowish discoloration via the Maillard reaction. Yarnpakdee et al. (2012) noted that yellow discoloration of hydrolysate produced from an extended storage Nile tilapia muscle was mainly caused by lipid

Table 2 Color parameters of protein hydrolysates prepared from Nile tilapia mince and W-MR-Al

Parameters	FPH_{mince}	$FPH_{W\text{-}MR\text{-}Al}$
L^*	91.80 ± 0.26b*	93.40 ± 0.30a
a^*	−1.12 ± 0.09a	−0.85 ± 0.06a
b^*	5.37 ± 0.58a	2.98 ± 0.25b
ΔE^*	4. 98 ± 0.51a	2.59 ± 0.17b
ΔC^*	4.15 ± 0.57a	1.77 ± 0.26b

Values are given as mean ± SD ($n = 3$).
*Different letters within the same row indicate the significant differences ($P < 0.05$).

Table 3 Likeness score of milk fortified with protein hydrolysate from Nile tilapia mince and W-MR-Al

Attributes	Control[a] (w/o hydrolysate)	FPH_{mince} (%)			$FPH_{W\text{-}MR\text{-}Al}$ (%)		
		0.3	0.4	0.5	0.3	0.4	0.5
Color	8.25 ± 0.75a[*,**]	8.17 ± 0.85a	7.83 ± 1.00a	8.03 ± 0.98a	8.21 ± 0.77a	8.00 ± 0.85a	8.07 ± 0.86a
Fishy odor	7.87 ± 0.82a	7.68 ± 0.80a	7.45 ± 1.06a	6.44 ± 1.93b	7.81 ± 0.83a	7.62 ± 1.17a	7.50 ± 1.38a
Fishy flavor	7.70 ± 0.75a	7.46 ± 0.90ab	6.84 ± 1.51b	6.18 ± 1.89c	7.67 ± 0.96a	7.52 ± 1.28a	7.33 ± 1.14ab
Taste	7.63 ± 0.72a	7.24 ± 1.50ab	6.72 ± 1.58bc	6.07 ± 1.85c	7.45 ± 1.18ab	7.07 ± 1.27ab	7.18 ± 1.79ab
Overall	7.57 ± 0.69a	7.17 ± 1.42a	6.46 ± 1.72b	6.22 ± 1.90b	7.43 ± 1.19a	7.24 ± 1.30a	7.28 ± 1.65a

Values are given as mean ± SD ($n = 3$).
[**]Score are based on a 9-point hedonic scale (1: Dislike extremely, 5: Neither like nor dislike, 9: Like extremely).
[*]Different letters within the same row indicate the significant differences ($P < 0.05$).

oxidation and could be inhibited by antioxidant (Trolox and EDTA) incorporation. In the present study, the lower lipid oxidation occurred in hydrolysate from W-MR-Al. Thus, discoloration caused by browning reaction might be retarded.

When low-fat milk was fortified with both hydrolysates at different levels (0.3% to 0.5%), color, fishy odor, fishy flavor, and overall likeness scores were evaluated as shown in Table 3. For color likeness, there was no difference among all milk samples tested ($P > 0.05$). The milk fortified with $FPH_{W\text{-}MR\text{-}Al}$ had no changes in fishy odor or flavor, taste, and overall likeness scores when the levels were added up to 0.5% ($P > 0.05$). This indicated that $FPH_{W\text{-}MR\text{-}Al}$ could be fortified in milk at a high level without detrimental effect on sensory property. For the sample fortified with FPH_{mince}, no difference in fishy odor likeness score was observed when added at up to 0.4% ($P > 0.05$). However, FPH_{mince} at a level of 0.4% resulted in a decrease in fishy flavor, taste, and overall likeness score ($P < 0.05$), indicating the stronger fishy flavor presented in milk. The result suggested that FPH was a major source of fishy flavor detected in milk. Fishy odor/flavor in FPH_{mince} correlated with higher lipid oxidation, PV, and TBARS values, (Table 1). Sohn et al. (2005) reported that the offensive odor detected in fish flesh was directly related with the formation of secondary lipid oxidation products. Therefore, $FPH_{W\text{-}MR\text{-}Al}$ could serve as a nutritive ingredient with some bioactive activities and could be supplemented without the adverse effect on sensory properties.

Conclusions

Pretreatment of Nile tilapia mince played a significant role in the reduction of prooxidants and lipids, especially neutral lipids and membrane phospholipids. Washing, along with a process to remove membranes prior to alkaline solubilization, was very effective in preparing a substrate for protein hydrolysate production, in which fishy odor and taste could be significantly lowered. FPH prepared from the appropriate pretreatment could be fortified in low fat milk at a level up to 0.5% or higher. This suggests that FPH could be widely used in other food systems.

Abbreviations

AC: actin; DH: degree of hydrolysis; FPH: fish protein hydrolysates; FPH_{mince}: fish protein hydrolysates produced from mince; $FPH_{W\text{-}MR\text{-}Al}$: fish protein hydrolysates produced from W-MR-Al; M: mince; MHC: myosin heavy chain; PV: peroxide values; SDS: sodium dodecyl sulfate; SPSS: Statistical Package for Social Science; TBARS: thiobarbituric acid reactive substances; TM: tropomyosin; TMA: trimethylamine; TN: troponin; W: washed mince; W-MR: washed mince with membrane removal; W-MR-Ac: washing/membrane removal/acid solubilization process; W-MR-Al: washing/membrane removal/alkaline solubilization.

Competing interests
The authors declare no competing financial interests.

Authors' contributions
SB formulated the hypothesis and designed the studies. SY carried out the experimental and analyzed the data. SB and SY prepared the manuscript. HK participated in the discussion and correction of the manuscript. All authors read and approved the final manuscript.

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