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The effects of pretreatments on antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper (*Lutjanus vitta*)

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ABSTRACT

Different pretreatments of mince from brownstripe red snapper (*Lutjanus vitta*) including 1) washing; 2) membrane separation; 3) washing followed by membrane separation and 4) membrane separation followed by washing were conducted prior to hydrolysis. Among the resulting minces, that subjected to membrane separation with subsequent washing (MS/W) contained the lowest remaining myoglobin content, phospholipid content, heme iron and non-heme iron contents ($p < 0.05$) and showed the lowest TBARS values throughout 9 days of storage at 4 °C in the presence and absence of 0.15 mol L^{-1} cupric acetate ($p < 0.05$). When hydrolysates from 1) mince, 2) MS/W and 3) protein isolate from MS/W (PI) with different degree of hydrolysis (DH) (20, 30 and 40%) were prepared using proteases from pyloric caeca of brownstripe red snapper, antioxidative activities determined by DPPH, ABTS radical scavenging activities, ferric reducing antioxidant power and metal chelating activity varied with hydrolysates and DH. Antioxidative activities increased with increasing DH up to 40% ($p < 0.05$). At all DH tested, hydrolysate prepared from MS/W exhibited the highest antioxidative activities determined by all assays, compared to those from mince and PI ($p < 0.05$). Hydrolysate from MS/W with 40% DH had the molecular weight lower than 6.5 kDa as determined by SDS-PAGE. In liposome oxidation system, the addition of hydrolysate from MS/W resulted in the lower TBARS, compared with the control throughout the incubation period of 48 h at room temperature (25–28 °C). Therefore, fish mince with membrane separation followed by washing was the most appropriate source for production of hydrolysate possessing antioxidative activity with the lowered amount of lipids and pro-oxidants.

1. Introduction

Numerous fish protein hydrolysates have been shown to have antioxidative activities such as gelatin hydrolysate from the skin of Alaska pollack (Kim et al., 2001), protein hydrolysate from the frame of yellowfin sole (Jun, Park, Jung, & Kim, 2004), protein hydrolysate from the muscle of round scad (Thiansilakul, Benjakul, & Shahidi, 2007a) and yellow stripe trevally (Klompong, Benjakul, Kantachote, & Shahidi, 2007). One critical problem of hydrolysate preparation from these proteinaceous sources is the presence of pro-oxidants such as heme proteins and unstable lipid substrates (Raghavan & Kristinsson, 2008). As a consequence, the desirable antioxidative activity can be lowered and the hydrolysate may exhibit the pro-oxidative activity to some extent.

Fish muscle contains myoglobin and other heme proteins, which in turn become the major pro-oxidants in muscle (Chaijan, Benjakul, Visessanguan, & Faustman, 2005; Thanonkaew, Benjakul, Visessanguan, & Decker, 2006). Furthermore, phospholipids, major components of cell membranes, are believed to readily prone to oxidative deterioration due to their highly unsaturated fatty acid composition (Borst, Visser, Kouptsova, & Visser, 2000). In fish, especially lean fish, phospholipids make up most of the lipids of the cell (Liang & Hultin, 2005a). The presence of these pro-oxidants and lipids could decrease the stability of protein hydrolysates and may limit their use in food systems (Raghavan & Kristinsson, 2008). Removal of these compounds could therefore alleviate such problems occurring in hydrolysate from fish muscle.

Brownstripe red snapper (*Lutjanus vitta*), a lean fish, is one of the main raw materials for surimi production in Thailand (Khantaphant & Benjakul, 2008). Apart from being processed into surimi, its flesh can be used as the raw material for production of protein hydrolysate with bioactivities. To produce protein hydrolysate from brownstripe red snapper with high antioxidative activity and negligible pro-

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oxidant or lipids susceptible to oxidation, the appropriate pretreatment of muscle by washing and/or membrane separation before hydrolysis should therefore taken into consideration.

Pyloric caeca of various fish has been reported to be the important source of trypsin such as arabesque greenling (Kishimura, Hayashi, Miyashita, & Nonami, 2006), New Zealand hoki (Shi, Marshell, & Simpson, 2007) and bluefish (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007). Recently, protease from pyloric caeca of brownstripe red snapper has been shown to hydrolyze fish skin gelatin effectively (Khantaphant & Benjakul, 2008). Along with the pretreatment, proteases from pyloric caeca of brownstripe red snapper could be used as the potential aid for the preparation of protein hydrolysate with antioxidative activity, in which the new value-added product of health benefit could be produced. The objectives of this study were to elucidate the effect of washing and membrane separation on the removal of pro-oxidant and membranes and to produce protein hydrolysates from pretreated mince of brownstripe red snapper with the lowered pro-oxidative activity using proteases from its pyloric caeca.

2. Materials and methods

2.1. Chemicals

2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 4, 6-tripyridyl-triazine (TPTZ), 1,1,3,3-tetramethoxypropane, bathophenanthroline disulfonic acid and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid (ferrozine) were purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid (TBA) and potassium persulfate were obtained from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA), iron standard solution and sodium nitrite were procured from Merck (Darmstadt, Germany). Sodium sulfite and ammoniumthiocyanate were obtained from Riedel-deHaën (Seelze, Germany). All chemicals were of analytical grade.

2.2. Preparation of mince with different pretreatments

2.2.1. Mince preparation

Brownstripe red snapper, stored in ice and off-loaded approximately 3 days after capture, were purchased from a dock in Songkhla province, Thailand. Fish were transported in ice with the fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, whole fish were washed and only flesh was separated manually. Flesh was minced to uniformity using a Moulinex AY46 blender (Group SEB, Lyon, France). The mince obtained was placed in polyethylene bags and kept in ice not longer than 2 h before use.

2.2.2. Preparation of washed mince

Mince was homogenized with five volumes of cold distilled water using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min, followed by stirring at 4 °C for 15 min prior to centrifuging at 9600 × *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'.

2.2.3. Preparation of mince with membrane separation

Membrane was removed from mince by treatment with Ca^{2+} and citric acid according to the method of Liang and Hultin (2005b) with a slight modification. Mince was homogenized with nine volumes of cold 8 mmol L^{-1} $CaCl_2$ solution in the presence of 5 mmol L^{-1} citric acid using a homogenizer at a speed of 11,000 rpm for 2 min. After continuous stirring for 60 min at 4 °C, the sample was centrifuged at 4000 × *g* for 15 min at 4 °C and the mince obtained was referred to as 'membrane separated mince; MS'.

2.2.4. Preparation of membrane separated/washed mince and washed/membrane separated mince

Minces subjected to washing prior to membrane separation (W/MS) and to membrane separation followed by washing (MS/W) were also prepared. All samples were subjected to analyses.

2.3. Analyses

2.3.1. Determination of myoglobin content

Myoglobin content was determined by direct spectrophotometric method (Chaijan et al., 2005). Sample (2.0 g) was mixed with 20 mL of cold 40 mmol L^{-1} phosphate buffer (pH 6.8), followed by homogenization at 13,500 rpm for 10 s. The mixture was centrifuged at 3000 × *g* for 30 min at 4 °C and the supernatant was filtered through Whatman filter paper No. 1 (Schleicher & Schuell, Maidstone, England). The absorbance of supernatant was read at 525 nm. Myoglobin content was calculated from the molar extinction coefficient of 7.6×10^{-3} and a molecular weight of 16,110 (Gomez-Basauri & Regenstein, 1992). The myoglobin content was expressed as mg/100 g dry sample.

2.3.2. Determination of heme iron and non-heme iron contents

Heme iron content was calculated based on the content of myoglobin, which contains iron at a level of 0.35 g/100 g (Gomez-Basauri & Regenstein, 1992). Heme iron content was expressed as mg/100 g dry sample.

Non-heme iron content was determined according to the method of Chaijan et al. (2005). Sample (1.0 g) was weighed and transferred into a screw cap test tube and 50 mL of 3.9 g L^{-1} sodium nitrite and 4 mL of a mixture of 400 gL^{-1} TCA and 6 molL^{-1} HCl (1:1 (v/v) ratio, freshly prepared) were added. The tightly capped tubes were placed in a temperature controlled water bath (Model W350, Memmert, Schwabach, Germany) for 22 h at 65 °C followed by cooling to room temperature (25–28 °C) for 2 h. The supernatant (400 μL) was mixed with 2 mL of the non-heme iron color reagent, a freshly prepared mixture of bathophenanthroline disulfonic acid, double-deionized water and saturated sodium acetate solution at a ratio of 1:20:20 (w/v/v). After thorough mixing, the mixture was allowed to stand for 10 min and the absorbance was measured at 540 nm. The non-heme iron content was calculated from the iron standard curve using iron standard solution (0–2 mg L^{-1}). The non-heme iron content was expressed as mg/100 g dry sample.

2.3.3. Determination of phospholipid content

Phospholipid content was determined based on the direct spectrophotometric measurement of complex formation between phospholipids and ammonium ferrothiocyanate as described by Stewart (1980). Lipids were extracted from the sample by the method of Bligh and Dyer (1959). Thereafter, lipids (20 μL) were dissolved in chloroform to a final volume of 2 mL. One mL of thiocyanate reagent (a mixture of 0.10 mol L^{-1} ferric chloride hexahydrate and 0.40 mol L^{-1} ammoniumthiocyanate) was added. After thorough mixing for 1 min, the lower layer was removed and the absorbance at 488 nm was measured. A standard curve was prepared with phosphatidylcholine (0–0.1 g L^{-1}). The phospholipid content was expressed as mg/100 g dry sample.

2.4. Occurrence of lipid oxidation in mince with different pretreatments

Mince with different pretreatments was mixed with sodium azide as an antimicrobial agent to obtain a final concentration of