Food Chemistry 212 (2016) 213–224

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Tuning the pH-shift protein-isolation method for maximum hemoglobin-removal from blood rich fish muscle

Mehdi Abdollahi [a,b,*], Sofia Marmon [a,c], Manat Chaijan [a,d], Ingrid Undeland [a]

[a] Department of Biology and Biological Engineering-Food and Nutrition Science, Chalmers University of Technology, SE 412 96 Gothenburg, Sweden
[b] Department of Seafood processing, Faculty of Marine Sciences, Tarbiat Modares University, P.O. Box 46414-356, Noor, Iran
[c] Department of Plant Breeding, Swedish University of Agriculture, P.O. Box 101, SE 230 53 Alnarp, Sweden
[d] Department of Agro-Industry, School of Agricultural Technology, Walailak University, Thasala, Nakhon Si Thammarat 80161, Thailand

ARTICLE INFO

Article history:
Received 31 October 2015
Received in revised form 18 April 2016
Accepted 26 May 2016
Available online 27 May 2016

Keywords:
Acid
Alkaline
pH-shift
Hemoglobin
Heme proteins
Fish

ABSTRACT

A main challenge preventing optimal use of protein isolated from unconventional raw materials (e.g., small pelagic fish and fish by-products) using the pH-shift method is the difficulty to remove enough heme-pigments. Here, the distribution of hemoglobin (Hb) in the different fractions formed during pH-shift processing was studied using Hb-fortified cod mince. Process modifications, additives and pre-washing were then investigated to further facilitate Hb-removal. The alkaline pH-shift process version could remove considerably more Hb (77%) compared to the acidic version (37%) when proteins were precipitated at pH 5.5; most Hb was removed during dewatering. Protein precipitation at pH 6.5 improved total Hb removal up to 91% and 74% during alkaline and acid processing, respectively. Adding phytic acid to the first supernatant of the alkaline process version yielded 93% Hb removal. Combining one prewash with phytic acid at pH 5.5 followed by alkaline/acid pH-shift processing increased Hb removal up to 96/92%.

1. Introduction

Increasing demand for seafood products, in parallel with global population growth and traditional fish supply decline, have caused great interest to use underutilized resources, like fish processing byproducts, small fatty pelagic fishes, and by-catch as protein sources (Gehring, Gigliotti, Moritz, Tou, & Jaczynski, 2011). However, recovering proteins from these complex raw materials, while retaining their functionality, has been difficult due to high heme protein and lipid content, high susceptibility to lipid oxidation, and difficulties in removing impurities (e.g., bones, scales, connective tissues, and so on) which results in colour and oxidation problems (Kristinsson & Liang, 2006).

Acid and/or alkaline solubilization followed by isoelectric precipitation, also called pH-shift processing, since its first development (Hultin and Keller, 1999; Hultin et al., 2003), has been successfully recognized as a promising technique for protein recovery from unconventional complex aquatic raw materials, including whole dark muscle fatty fish (Marmon & Undeland, 2010), gutted fish (Marmon & Undeland, 2010; Taskaya, Chen, & Jaczynski, 2009) fish processing by-products (Yi-Chen Chen & Jaczynski, 2007; Shaviklo, Thorkelsson, Arason, & Sveinsdottir, 2012) blue mussels (Vareltzis & Undeland, 2012) and whole krill (Chen, Tou, & Jaczynski, 2009). The process involves selectively extracting proteins from homogenized raw material in water using a high (>10.5) or a low (<3.5) pH to solubilize the muscle proteins. Application of centrifugation facilitates removal of solubilized proteins from high and low density unwanted material. The solubilized protein are then recovered by isoelectric precipitation (usually pH 5.5) and dewatered by centrifugation or filtration. However, one of the main challenges that still prevents an optimal use of the protein isolates is the difficulty to remove enough heme-pigments (hemoglobin, Hb, myoglobin, Mb) to make the colour of the isolates appealing and prevent their pro-oxidative effects.

Heme-pigments, including Hb and Mb, are believed to be the main pro-oxidants in muscle foods. Presence of blood and/or residual heme-proteins in fish muscle, or products thereof, can affect several quality parameters, such as whiteness, microbial growth and lipid oxidation (Chaijan, Benjakul, Visessanguan, & Faustman, 2005; Richards & Hultin, 2002; Undeland, Kristinsson, & Hultin, 2004a). Conversion of reduced Hb/Mb to oxidised forms, i.e. metHb/metMb, leads to a greyish-brown colour of fish muscle and is also related to lipid oxidation. Both oxidised forms have shown strong lipid pro-oxidative capacity according to several

* Corresponding author at: Department of Biology and Biological Engineering-Food and Nutrition Science, Chalmers University of Technology, SE 412 96 Gothenburg, Sweden.
E-mail addresses: abdollahi.mkh@gmail.com, khozaghi@chalmers.se (M. Abdollahi).

http://dx.doi.org/10.1016/j.foodchem.2016.05.165
0308-8146/

mechanisms, such as hydroperoxide decomposition and ability to act as free radicals following conversion to ferryl/perferryl forms (Baron, Skibsted, & Andersen, 2002). In this way, the content of heme proteins in fish muscle and fish products have shown to correlate strongly with the degree of lipid oxidation during handling, processing and subsequent storage of fish (Maqsood & Benjakul, 2011; Richards & Hultin, 2002).

Moreover, it has been found that Hb and Mb removal efficacy decreases with increasing degree of autoxidation caused by ice storage (Chaijan et al., 2005) and pH reduction (Thongraung, Benjakul, & Hultin, 2006; Ingrid Undeland, Kristinsson, & Hultin, 2004b). It has been found that the large pH changes during the pH-shift can also affect proxidative properties of Hb. Kristinsson and Hultin (2004a) showed that at acidic pH (pH 1.5–3), Hb unfolds, oxidizes and aggregates when it is readjusted to pH 5.5 or 7 and thus it is easily recovered with other muscle protein during centrifugation. The same group also found that after low pH treatment, washed cod muscle, fortified with Hb, became slightly more susceptible to lipid oxidation, while alkaline treatment slightly protected the muscle from lipid oxidation mediated by trout Hb (Kristinsson & Hultin, 2004b). Furthermore, it was shown that for channel catfish, a lower level of Hb obtained with the alkali-aided process led to an isolate with significantly lower TBARS values, compared to an isolate made with the acid-aided process, despite a similar reduction in lipids (Kristinsson, Theodore, Demir, & Ingadottir, 2005). Despite these observations, no study has systematically recorded the distribution of Hb between the fractions formed during the pH-shift processing. Also, no studies are available where the pH shift process has been actively tuned to minimize Hb/heme-protein content of the final isolate. Approaches used so far to improve the quality of produced protein isolate including additives like antioxidants (Raghavan & Hultin, 2009; Ingrid Undeland, Hall, Wendin, Gangby, & Rutgersson, 2005), citric acid and calcium chloride (Vareltzis, Hultin, & Autio, 2008) and ethanol (Marmon, Liljelind, & Undeland, 2009) before or during the pH-shift process. All with the aim to improve the quality of the final isolate or increase removal of lipids and toxic materials.

Thus, the present study was aimed to map the distribution of Hb in the different fractions formed during alkaline and acid pH-shift processing of blood rich fish raw materials. In an ideal situation, as much Hb as possible would precipitate into the sediment formed in the first step of the pH-shift process, and would stay soluble in the supernatant of the second step of the process (Fig. 1). This is since both of these fractions are removed and would thus minimize Hb of the final protein product. To reach this goal, new strategies and additives (Fig. 1.) were tested to further facilitate Hb-removal during the different steps of the pH shift process. Protein recovery as well as moisture content of protein isolate were studied along with Hb-removal as a function of different process changes and additions. Hb-enriched cod mince or Hb-enriched buffer were used as models in the different experiments.

2. Materials and methods

2.1. Chemicals and reagents

Bovine Hb, SDS, zinc sulfate, phytic acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, Tris-HCl, bovine serum albumin and Drabkin's reagent were purchased from Sigma–Aldrich (St. Louis. MO, USA). Hydrochloric acid (HCl) and acetone were purchased from Fluka (Bunchs, Switzerland).

2.2. Bleeding of trout

Fish blood was obtained from female trout (*Oncorhynchus mykiss*) with an average weight of 1–1.2 kg kept at the Department of Zoology, Gothenburg University (Sweden). The fish were kept in 2 m^3 tanks with aerated freshwater (~10 °C) from the departmental re-circulation system. The fish were kept under a 12:12 photoperiod and fed commercially available trout pellets. The trout was killed with a blow to the head. Blood was immediately drawn from the caudal vein using evacuated blood collection tubes pretreated with lithium heparin (BD Vacutainer, Plymonth, UK) and put on ice until preparation (Rowley, 1990). The experiments are covered by permit 89/2013 from the regional animal ethics committee in Gothenburg.

2.3. Hemolysate preparation

Hemolysate was prepared according to the method of Fyhn et al. (1979) by adding 4 vol of ice cold saline (1.7% NaCl in 1 mM Tris, pH 8) to the heparinized blood. Mixing was done gently by turning the covered test tube 10 times. The mix was centrifuged (Wifug, Type X-1, Stockholm, Sweden) at 700*g* at 4 °C for 10 min, after which the supernatant was removed. Three washes of the red blood cells were then performed with 10 vol (based on whole blood) of saline. Again, mixing was done in a gentle manner, and each wash followed by 10 min centrifugations as above. Finally the cells were lysed in 3 vol of 1 mM Tris pH 8 for 1 h on ice. After this, 1/10 vol of 1 M NaCl was added before centrifugation to aid in stromal removal. Centrifugation was done at 28,000*g* for 15 min at 4 °C (Sorvall® Superspeed RC-5C Plus, Kendro Laboratory Products, Stockholm, Sweden). Following centrifugation the supernatant was collected, put in vials and stored in a −80 °C freezer until needed. Scanning of the hemolysate revealed that Hb was mostly in the oxy-form (>90%). Hb in the hemolysate was quantified using Drabkin's method (Drabkin, 1950).

2.4. Studies in liquid model system

2.4.1. Preparation of a Hb-fortified liquid model system and studying Hb changes as a function of pH

In order to mimic what will happen with Hb during the first step of the pH-shift process, a liquid model was developed to allow for screening studies. A 4.1 μM solution of Hb was prepared by adding 800 μl of the hemolysate (513 μM) to 100 ml of 15 mM phosphate buffer at pH 7, which is similar to what will happen when 1 part of dark muscle fish containing in average 50 μM Hb is used in the pH-shift method, comprising 9 parts of water. Then, the pH of the solution was adjusted to values between 1.5 and 12 using 1 mM HCl or NaOH. With 0.5 pH-unit intervals, 4 ml samples were taken for analysis of Hb precipitation. After 10 min ice storage, the samples were centrifuged at 8500*g* for 20 min and as an index of Hb-precipitation the amount of protein in the supernatant was measured using the Lowry method (Lowry, Rosebrough, Farr, Randall, & Others, 1951) using bovine serum albumin as a standard.

To study the effect of pH readjustment on Hb precipitation and Hb spectral changes, in both the alkaline and acidic pH-shift process versions, pH was readjusted to 4.8, 5.5 and 6.5 after 30 min of ice storage. Samples were also scanned with a spectrophotometer (Cary 60 UV–vis, Agilent technologies, Santa Clara, USA) to see how pH would affect Hb structure and redox stability during the pH-shift process.