Characterization of Proteins Isolated from Catfish Frame Using Acid and Alkaline Solubilization for Supplementing in Gelatin Film

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***Abstract*—The frame of catfish is considered as waste and should be utilized maximally in order to meet the zero-waste fishery industry. Protein isolation using a pH-shifted methods was applied by adjusting the pH of fish frame to be either 3 or 12. Thereafter, the soluble proteins were recovered by centrifugation and lyophilization to be powder denoted as acid protein isolate and basic protein isolate. Characterization of these proteins powder reveal that basic protein isolate had higher water holding and fat binding capacities than that did by acid protein isolate. The solubility of both protein powders in water was observed comparably. However, solubility of acid protein isolate was higher than basic protein isolate, when 0.6 M NaCl was used as the solvent. The secondary structural changes were clearly noticed on the FT-IR, especially at the amide band (I, II, and III). The acid protein isolate and BPI solution were incorporated in filming solution constituted of gelatin. The film substituted with acid protein isolate exhibited higher in thickness and solubility than another one. However, basic protein isolate incorporation in gelatin film could improve tensile strength as well as water vapor permeability. The results suggested that isolation of protein from catfish frame could be possible by pH-shifted method and isolated protein could be used to produce edible film, especially gelatin film.**

***Keywords*—catfish frame, by-products, protein isolate, gelatin film, pH-shifted method**

I. Introduction

Catfish is one of economic species and is widely aqua cultured in wet land throughout the Asian countries [1]. This fish production has been sold as either the fresh cut and processed products such as dried and frozen fillets. Through this process, fish has to be eviscerated and trimmed out the undesirable parts as waste. This results in a burden for waste management as well as an increase in the production cost. This waste accounts for almost 50%, including skin, bone, fin, head, gut etc. However, this waste might be the low-cost raw materials, if it can be used in the continued industries according to the zero-waste production. In order to utilize efficiently, isolation and characterization of potential components from waste, particularly fish frame, are necessary and should be investigated.

Muscle proteins from fish frame could be recovered by pH-shift method, which based on acid and alkaline solubilization [2]. After fish muscle is solubilized at low or high pH, the soluble proteins are separated by means of centrifugation. This facilitates elimination of insoluble materials, including bones, skin, and connective tissue. Thereafter, the soluble proteins are collected and recovered by isoelectric precipitation. This can be done by adjusting the pH of protein solution to the pI prior to precipitation. The precipitated proteins provide desirable functional properties [2, 3]. Moreover, isolation of protein by this method could reduce fat content resulted in the stable products such as protein gel and edible film. Edible film from gelatin is wildly used commercially in food industries due to the practical properties and cost worthy. However, manipulation the properties by adding other ingredients has been extensively studies in order to explore for the advantage over gelatin film.

Proteins isolated from pH-shifted method have been introduced into film forming solution and changes in color of muscle protein film has been reported [4]. This method could overcome coloration of muscle protein film from fish flesh. However, isolation muscle proteins from by-products from fishery industries for being the ingredient in edible film has not been reported especially from catfish.

Therefore, the aim of this study was to isolate proteins left over in the frame of catfish after processing by acid and alkaline solubilization. Characteristics of these protein isolates were investigated and the potential for supplementing in gelatin film were also evaluated.

II. Materials and Methods

A. Materials

Fish frame from catfish with an average weight of 200–300 g/fish were obtained from the local market, Nong Khai, Thailand. They were transported to the laboratory in the school of applied science, Faculty of Interdisciplinary Studies, Khon Kaen University Nong Khai Campus within 30 min. Those bone were aliquoted for 100 g each before storing in the freezer (−20 °C) prior using as sample throughout the study.

B. Isolation of Protein from Fish Frame

The method to isolate proteins from fish frame was adapted from previous method [2]. The fish frame samples were finely ground before mixing with cold de-ionized water (DI-water) at a ratio of 1:3 (w/v). The mixture was homogenized at 13,000 rpm for 3 min using a homogenizer (KA-Werke GmbH & Co. KG, Staufen, Germany). The homogenate (pH 6.3) was adjusted to pH 3 or pH 11 using 0.5 M of HCl or NaOH, respectively. The pH treated solution was centrifuged at 10,000×g for 10 min at 4 °C prior collecting the supernatant, and referred as acidic, and basic protein isolates (API and BPI), respectively. The API and BPI solution were kept under frozen condition at −40 °C prior to lyophilization using freeze-drier (beta1-8LSCplus, Christ, Osterode am Harz,

Germany). Lyophilized API and BPI powders were kept in desiccator throughout the study.

C. Characterization of Protein Isolates

1) Fourier Transform Infrared Spectroscopy (FT-IR)

API or BPI powders (1 mg) were placed on the surface of the diamond crystal cell of an Attenuated Total Reflectance (ATR) crystal accessory (INVENIO-S, Bruker Optics GmbH & Co. KG, Germany). The samples were scanned 32 times from 400–4000 cm^{-1} with a resolution of 4 cm^{-1}. The spectra were then analyzed in the amide I and amide II spectral region (1500 to 1700 cm^{-1}) using the OPUS software program.

2) Water Holding Capacity (WHC)

WHC of PI powder was carried out according of the method described by [5]. Dried SP powder (200 mg) was weighed into pre-weighed microcentrifuge tubes. Subsequently, 400 µL DI-water was added with small increments under continuous stirring. After being thoroughly wetted, the mixture was centrifuged at 2000×g for 10 min and water in the supernatant was discarded. The weight of microcentrifuge and sediment was recorded. WHC was expressed as the weight gained per unit weight of dried powder. The mean value was reported based on 3 replications.

3) Fat Absorption Capacity (FAC)

Dried PI powder (200 mg) was weighed into the pre-weighed microcentrifuge tube and thoroughly mixed with 1 mL of canola oil. The mixture was centrifuged at 1600×g for 10 min. Thereafter, the supernatant was carefully inverted on to a paper towel for 10 min to remove the unabsorbed oil. The tube containing pellets was weighed and FAC was expressed as the ratio of absorbed oil to dried sample weight as described previously [5].

4) Water solubility

To each dried PI sample (10 mg), 1 mL DI-water was added before mixing and shaking for 2 min at room temperature. The mixture was centrifuged at 2000 rpm for 10 min. Thereafter, the supernatant was collected to determine protein content by Biuret method [6]. The solubility was expressed as the protein content in supernatant.

D. Formation of Gelatin Film

API or BPI solutions (pH 3 or pH 11) was mixed with glycerol (final concentration in filming solution of 1.0%) before stirring for 10 min at room temperature. Then, commercial gelatin powder was calculated to make up the protein content, based on total solid in total film forming solution, to be 5.0%. The calculated amount of gelatin was mixed with DI-water (10 mL) and stirred for 10 min at 60 °C until completely dissolved. Thereafter, protein isolate solutions and gelatin solution were mixed together in order to obtain film forming solution. This solution was poured into plate (Paisan Superlene, Co., Ltd., Bangkok, Thailand) for approximately 50 mL/plate. Then, all plates were dried at 50 °C for 24 h. The dried films were peeled off and stored in a desiccant jar for further characterizations.

E. Characterization of Gelatin Film with API or BPI

1) Thickness

The thickness of film was measured using a digital micrometer (Mitutoyo, Model ID-C112PM, Japan). The random locations for 10 points per each film were determined. The averaged values with standard error were reported.

2) Moisture content

The moisture content of the film was determined using a Moisture Balance (Mettle-Toledo GmbH, Model HE73 Greifensee Switzerland). The averaged values with standard error were reported.

3) Solubility

A piece of film (20×20 mm) was cut and dried in an oven at 105 °C for 24 h in order to obtain the initial film weight (Wi). The piece of film was then placed into a flask filled with 50 mL of DI-water prior shaking at a speed of 2000 rpm for 24 h at ambient temperature. Thereafter, the film sample was taken to re drying process again at the same condition to get the final Weight of film (Wf). Film solubility was determined based on weight loss of film as follows equation:

$$Solubility\ (\%) = \left(\frac{w_i - w_f}{w_i}\right) \times 100$$

4) Color

Color values of film were measured using the Hunter color meter (Color reader, CR-10, Minolta, Japan). Color values were reported as Hunter L*, a*, and b* for lightness, redness, and yellowness, respectively. The averaged values with standard error were reported.

5) Mechanical properties

Film samples were cut into 10×70 mm and tempered at Relative Humidity (RH) 50% for 40 h. These samples were therefore analyzed for tensile strength and elongation according to ASTM D882 [7]. The tip or sample end was attached the test probe of a tax analyzer (Model TA-XT plus, Stable Micro Systems, USA) with the distance between the grips of 150 mm. The pull speed was set at 10 mm/min. The elongation was expressed as percentage. In addition, the tearing force (FMAX) was recorded for calculating the tensile strength by dividing with cross-sectional area according to this equation:

$$TS = \frac{F_{MAX}}{A}$$

where F_{MAX} = Maximum tearing force of the film.
A = Thickness x width of film.

F. Statistical Analysis

A significant difference at confidential level (p <0.05) was considered for mean values, which was based on the ANOVA analysis using the PASW statistics 16 (SPSS Inc., Chicago IL, USA).

III. Results and Discussion

A. Functional Properties of PI

1) Solubility

The solubility of protein is a key factor in determining its functional properties. The PI powders after lyophilization were partly solubilized regardless the pH value during isolation (Table 1). Lyophilization, a process in which a substance is frozen and then the water is removed under

vacuum, may alter the natural structure of PI, making it less able to interact with water molecules and difficult to reconstitute. This would explain that why solubility of lyophilized PI could be reconstituted for approximately 40% regardless the pH of isolation. The low solubility of SP powder may hinder its ability to perform certain functions and food applications. Normally, the isoelectric point (pI) of fish protein is around pH 5.2–5.5, the proteins have no net charge. Adjusting protein solution to pH that far away from pI value resulting in fully solubilization due to charged repulsion. At pH values below this range, the proteins become positively charged, while that at pH values above this one, they become negatively charged. However, drying of proteins while they fully solubilized as the charged molecules resulted in less solubility after reconstitution to the same condition. Hardly solubilized might be due to the structural changes during freezing and drying processes of lyophilization. A reduction of solubility was mainly due to secondary structural changes resulting in refolding/aggregation could be observed in sarcoplasmic proteins from threadfin bream [5]. It can be seen that solubilization of PI was not affected by NaCl although fish muscle proteins have been classified as salt soluble proteins. This suggested that structural changes up on pH adjustment following by lyophilization were likely to be irreversible denaturation.

Table 1. The solubility (%) of protein isolates

Protein isolates	Solvents	
	DI-water	NaCl (0.6 M)
API	40.80±0.87[a]	17.81±0.15[a]
BPI	39.97±1.46[b]	13.69±0.73[b]

Mean ± SE was averaged from 2 replications.
a–b Different letters within the same column indicate statistically different at p <0.05.

2) WHC and FAC

The WHC of PI samples exhibited higher value than that of dried egg white (1.68 g/g sample) as reported previously [8], regardless pH treatments. In addition, alkaline treatment resulted in an increase this value to be 4.73 g/g sample. This suggested that alkaline treatment not only solubilized myofibrillar protein attached with fish bone but also induced protein unfolding. Such changes allowed more void spacing matrixes up on lyophilization. Therefore, more water could be trapped in the BPI as seen in Table 2. Thus, more water molecules would be more interacted with protein molecules, resulting in an increase WHC.

Fat has ability to bind with nonpolar or hydrophobic amino acids, presenting in protein structures via hydrophobic interactions. The PI powders showed higher FAC than that of other proteins reported previously [9]. It can be seen that FAC increased when protein was isolated by alkaline condition (Table 2). This behavior was corresponded with that of SP treated at alkaline solution [5]. This suggested that acid could induced more structural changes of proteins to the different extent than did by alkaline. Finally, conformational and structural changes of PI induced by alkaline would likely promote either WHC or FAC.

Table 2. The water holding and fat absorption capacities (%) of protein isolates

Protein isolates	Functional properties	
	WHC (%)	FAC (%)
API	3.12±0.42[b]	6.95±0.02[b]
BPI	4.73±0.16[a]	7.12±0.16[a]

Mean ± SE was averaged from 2 replications.
a–b Different letters within the same column indicate statistically different at p <0.05.

B. Structural Characteristics of PI

FT-IR spectra analysis of protein isolate showed the prominent bands of the amide I and amide II (Fig. 1). The BPI showed the prominent bands of C-O stretching at the amide I region around 1637 cm^{-1}.

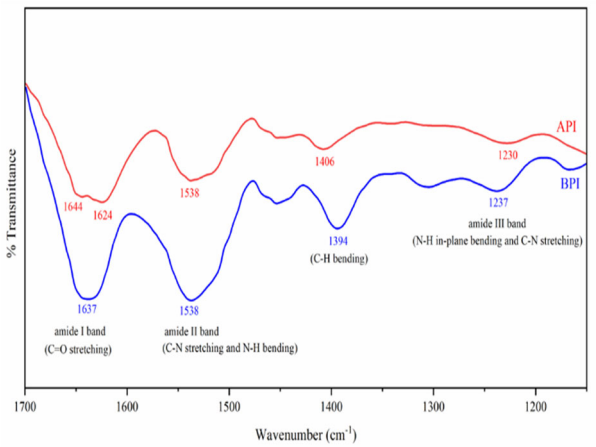

Fig. 1. FT-IR spectra of proteins isolated by acid and alkaline solubilization (API@ pH 3 and BPI@pH 11).

The C-H stretching and C-O bending were observed at amide II region at the wave number of about 1538 cm^{-1}. The observation of these predominant amide I and II are considered to β-sheet [10]. It can be seen that the amide I of API were slight shifted to be 1644 and 1624 cm^{-1}, while the amide II was shown at 1538 cm^{-1}. The wavenumber at 1394 cm^{-1} in BPI is contributed from C-H bending of the amino acid side chains. This band was slight shifted to be 1406 in API. This suggested that secondary structure of API was changes more drastically than that of BPI. The greater denaturation of API than that of BPI would explain why BPI showed a superior character regarding the WHC and FAC (Table 2).

C. Characteristic of Gelatin Film with PI

1) Thickness

The film produced in this experiment exhibited a thickness in the ranges of 0.15 mm (Table 3). This was similar to that of polylactic acid/gelatin film, which measured 0.179 mm [11]. However, these values were higher than that reported in the myofibrillar/sarcoplasmic protein film [4]. This might be due to the different solid content in the filming solution. The thickness of API/gelatin film was slightly higher than BPI/gelatin film. This might be due to the different rearrangement of soluble solid in the film matrix, which governs by structural changes of protein molecules. Structural changes of protein influenced the interactions of

the components in the film-forming film solution, resulting in different thickness [12].

2) Moisture content

The edible films exhibiting low moisture content would be able to show a potential for being food packaging. This property allows the film for protecting food from moisture loss during storage. The PI/gelatin film exhibited a low moisture content at about 12% (Table 3). These films were categorized as having low moisture levels since their moisture content was below 14% [13]. It can be seen that the moisture content values of film were not different regarding the isolation process. This suggested that PI having similar water absorption ability when incorporated in the gelatin film although they exhibited slightly different WHC (Table 2). This phenomenon may be attributed to interactions between gelatin and other constituents. This would limit the availability of hydrophilic sites to interact with water [11].

3) Solubility

Solubility of film is relied on the structural integrity of the film, the hydrophobicity of the plasticizer, and the degree of protein denaturation. This property plays a crucial role for application of this film in food package. High solubility film could be consumed instantly with wrapped food. This might be useful for design edible sack for packing seasoning in the instant food products. All film samples showed lower solubility than reported previously [14]. In addition, API/gelatin film exhibited higher solubility than that did by BPI/gelatin film (Table 3). This might be due to higher solubility of API than BPI (Table 1). This suggested that solubility of proteins incorporated in film forming solution governs the solubility of gelatin film. Therefore, isolation of protein from fish bone by acid treatment would be more possible for application. This was based on the solubility and moisture content in the film.

Table 3. The thickness of gelatin film with protein isolates

Protein isolates in gelatin film	Physicochemical properties		
	Thickness (mm)	Moisture content (%)	Solubility (%)
API	0.15±0.02[a]	12.44±0.68[a]	17.66±2.37[a]
BPI	0.14±0.01[b]	12.90 ±0.78[a]	13.16±4.08[b]

Mean ± SE was averaged from 2 replications.
a–b Different letters within the same column indicate statistically different at p <0.05.

4) Color value

Color and appearance of film plays a crucial role on presenting the food package and should be evaluated for potential in food application. The results in Table 4 showed that API/gelatin film showed higher in lightness than that of BPI/gelatin film. In addition, the lower yellowness and redness were also observed. This suggested that film form by protein isolated with acid treatment exhibited whither or brighter than did by alkaline treatment. This might be due to different degree of denaturation of residue myoglobin resulting in different of color. High degree of denaturation of myoglobin residue resulted in whither protein powder were observed in sarcoplasmic protein [15]. Based on color measurement, API showed more potential for showing food appearance, while storing in the package.

Table 4. The color of gelatin film with protein isolates

Protein isolates in gelatin film	Color values		
	*L**	*a**	*b**
API	82.27±1.77[a]	0.67±0.11[b]	16.73±2.70[b]
BPI	74.77±1.59[b]	0.81±0.49[a]	30.79±2.28[a]

Mean ± SE was averaged from 5 replications.
a–b Different letters within the same column indicate statistically different at p <0.05.

5) Mechanical properties

Tensile strength value of API/gelatin was found at about 8 MPa and could not be classified as resistant film. Normally, the resistant film exhibits the tensile strength value above 20 MPa [11]. However, the behavior of resistant film was observed when BPI was used in film forming recipe and the tensile strength was shown at about 31 MPa (Table 5). Higher tensile strength might be due to the higher ability of BPI to interact with non-polar molecules as indicated by higher FAC (Table 2). This characteristic would more facilitates the interactions of protein molecules and plasticizer (glycerol) during film drying. High tensile strength of BPI/gelatin film would be able to protect food products against tearing force, while it has been used as a package. The result suggested that alkaline isolation of protein exhibited high feasibility for this application.

Table 5. The mechanical properties of gelatin film with protein isolates

Protein isolates in gelatin film	Mechanical properties	
	Tensile strength (MPa)	Elongation (%)
API	8.08±2.7[b]	216.64±13.7[b]
BPI	31.49±1.1[a]	228.72±17.5[a]

Mean ± SE was averaged from 2 replications.
a–b Different letters within the same column indicate statistically different at p <0.05.

The elongation of film, which indicated an ability to extend the film, showed such a high value over 200%. In addition, this value for BPI/gelatin was higher than that of API/gelatin films (Table 5). This data strongly suggested the feasibility of using BPI to incorporated in gelatin film rather than API. This strategy would yield the stronger and more flexible film. The alkaline residue might be left over in the film might not affected, if the food grade of chemicals is selected. Moreover, selection of alkaline food might be one of the strategies for most compatible packages and food products.

IV. Conclusion

Proteins left over in the frame of catfish could be isolated successfully by acid and alkaline solubilization. Their secondary structural changes were induced by acid to the greater extent than did by alkaline. High water holding and fat binding capacities of protein isolated by alkaline solubilization improved mechanical properties when it was supplemented in gelatin film. However, high solubility of protein isolated by acid solubilization improved film solubility, when it is used in gelatin film. The acid isolation was thus suitable for producing soluble film, while the suitability of alkaline isolation might be the production of resistant film. Therefore, isolation protein by acid and alkaline solubilizations would be the promising method to recovery proteins from fishery by-products for application in edible film.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

Bung-Orn Hemung conducted the experiments, analyzed data regarding the characterization of protein isolates and gelatin film, and organizing the manuscript; Chonticha Masusai evaluated the changes in structure of PI by FT-IR. All authors had approved the final version.

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