Properties of Tilapia Bone Powder and Its Calcium Bioavailability Based on Transglutaminase Assay

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***Abstract*—This paper was about the properties of tilapia (*Oreochromis niloticus*) bone powder and the bioavailability of its calcium based on the transglutaminase assay by the following procedures: Firstly, tilapia frames were soaked in 0.8 N NaOH at 90 °C for 1 h. The bone residues were autoclaved at 121 °C, 350 g.cm^{-2} for 60 min before drying/grinding to obtain the tilapia bone powder. Moisture, crude fat, and protein contents of the powder were found 2.46 ± 0.03, 5.82 ± 0.04, and 14.81 ± 0.33 %, respectively. The highest component was the ash content, which was found to be 75.83 ± 0.12 %. The color values were 98.08, 16.79, and 79.46 for *L*, *a*, and *b* values, respectively. Secondly, the solubility of tilapia bone powder was tested at the ratio of powder:water of 1:4 and the value was found 9.38 ± 0.07%. Thirdly, soluble ash was determined to obtain the Ca^{2+} content and found to be 116.56 mg.L^{-1} as assessed by inductively coupled plasma mass spectrometry. At last, the tissue transglutaminase (tTGase) assay was introduced to analyze for the bioavailability of soluble Ca^{2+}. This reaction is based on the requirement of Ca^{2+} for full activation of tTGase. Soluble Ca^{2+} (162 nM) could activate the crude tilapia tTGase (0.42 mg) to catalyze the incorporation of monodansylcadaverine (MDC) for 11.28 nmole into dimethylated casein (2 mg) at 37°C within 10 min. An increase in MDC incorporation was observed when Ca^{2+} in the reaction was increased.**

***Index Terms*—Tilapia, fish bone powder, soluble calcium, transglutaminase.**

I. Introduction

Tilapia (Oreochromis niloticus) was originally found in Africa. Tilapia is the good source of protein and also it is popular among commercial fisheries [1]. It can be fed by algae or plant-based feed. Therefore, the cost of production can be reduced practically. China is the largest tilapia producer country followed by Egypt [2]. The annual production around the world is about 1,500,000 tones with the approximately value of 1.8 billion U. S. dollars, which is similar to total value of salmon and trout.

The whole tilapia can be processed into skinless and boneless fillets. The yield of these processes has been reported in the range of 30-37 %, depending on fish size and trimming process [3]. Thereafter, the left parts of fish have been turned to be waste or by-products. The left part contains bone, skin, protein, scale, fat, and blood. In the left part, bone is the main component consisting of 10-15% of total fish biomass [3]. Bone is also the source of important minerals: sodium, phosphorous, and calcium. Among them, calcium ion (Ca^{2+}) is important for development of human bone and teeth particularly in infant. Utilization of fish bone can be a natural source of Ca^{2+} for being food ingredient and Ca^{2+} supplementary. It would be the strategy to maximally utilize fish resource as well as to effectively reduce the waste from fishery industry.

As far as the bioavailability of Ca^{2+} in the supplementary has been concerned, the bioavailability should be tested. It can be performed based on the enzymatic reaction. This is because Ca^{2+} is the cofactor for several enzymes, including tissue transglutaminase.

Tissue transglutaminase (tTGase) is Ca^{2+}-dependent enzyme in the transferase category, found in animal tissue such as in mammalian and fisheries. It catalyzes the acyl transfer reaction from glutamyl peptide into primary amines or lysyl peptide. This reaction results in the incorporation of amine into protein substrate. The activity of tTGase has been assayed based on the incorporation of synthetic amines, cadaverine or dansyl cadaverine, into protein substrate, dimethylated casein. Since the reaction is dependent on available Ca^{2+}, this reaction could be used to evaluate the bioavailability of soluble Ca^{2+}. Therefore, the objective of this study was to produce and characterize tilapia bone powder. In addition, the bioavailability of Ca^{2+} in the tilapia bone powder was also investigated, which is based on the activity assay of crude tTGase from tilapia muscle.

II. Materials and Methods

A. Fish Sample

Tilapia was bought from local market at Nong Khai province, Thailand. Live fish (0.3 kg per each) were transferred to the Food Science Laboratory, School of Food Science, Faculty of Applied Science and Engineering, Khon Kaen University, Nong Khai Campus. Fish samples were eviscerated manually. Fish fillets were used for crude tTGase extraction and the main frames were collected for bone preparation. All samples were kept under vacuum package, stored at -20 °C until used. Monodansyl cadaverine (MDC) and dithiothreiol (DTT), calcium chloride ($CaCl_2$) and *N,N'*-dimethylated casein were from Sigma Chemicals (St. Louis, MO, USA). Other chemicals were of analytical grade.

B. Fish Bone Preparation

The main frame of fish were soaked in NaOH solution (0.8%) with the ratio of frame:NaOH of 1:2 at 90 °C for 1 h. Thereafter, the bone residues were rinsed with de-ionized water (DI-water) and then they were dried in the oven drying at 100 °C overnight (≈12 h). Size reduction of dried bone samples was performed by using the homogenizer at 15,000

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rpm for 4 min (AM8-Nihonseiki Kaisha, Japan). The bone powder was kept under vacuum condition at -20 °C until characterization.

C. Fish Bone Characterization

Proximate Analysis: Fish bone was analyzed for protein content based on total nitrogen content by Kjeldahl method (ISO 5983-1997). Moisture content was performed gravimetrically after oven drying at 105 °C for 12 h (AOAC, 2000). Crude fat was analyzed using Soxhlet extraction using petroleum ether as a solvent (AOCS, Ba 3-38). Ash content was performed using dry ashing by combustion sample at 550°C for 16 h (AOAC, 2000).

Color Measurement: Fish bone powder was analyzed for the color values using colorimeter (CR-10, Minolta Co. Ltd., Japan). The color values were reported as the *L*, *a*, *b*.

Solubility: Tilapia bone powder was mixed with DI-water at the ratio of powder:water of 1:4 and stirred at room temperature overnight. The mixture was filtered through filter paper (No. 1, Whatman). The retentate was dried before weighing to obtain the insoluble particle. Subsequently, the solubility of bone powder was calculated accordingly.

Ca^{2+}Content: The content of Ca^{2+} was determined using wet ashing followed by inductively coupled plasma mass spectrometry (ICP-MS). Tilapia bone powder was digested with nitric acid (0.1 M) at 90 °C for 2 h. The digested sample was determined for Ca^{2+} content using ICP-MS (7500ce, Agilent Technology, Tokyo, Japan).

D. Bioavailability of Ca2+ Based on tTGase Assay

*Crude tTGase Extraction***:** Tilapia muscle was mixed with 3 volumes of extraction buffer (10 mM NaCl, 50 mM Tris-Cl, 2 mM DTT) and homogenized for 2 min. The homogenate was centrifuged at 10,000 g for 30 min and supernatant was filtered through the filter paper. The filtrate was used as crude tTGase and protein content was assayed using dye binding method (Bradford).

Determination of tTGase Activity: The activity was tested based on incorporation of MDC into DMC according to the method described by Takagi (1986) with slight modification. The reaction contained 2 mg DMC, 15 μL MDC, 70 mM Tris-Cl, pH 7.5, 3 mM DTT, and 100 μL of crude tTGase (0.42 mg protein). The presence of standard Ca^{2+} and soluble Ca^{2+} from fish bone were tested at 10 μM along with negative control (without Ca^{2+}). The reaction was done at 37 °C for 10 min. Then, the incorporation of MDC into DMC was estimated by fluorescent spectrophotometer (RF-1501, Shimadzu, Kyoto, Japan). The excitation and emission wavelengths were at 350 and 480 nm, respectively. The unit of enzyme activity was calculated as the amount of enzyme that catalyzes the incorporation of MDC (1nmol) into described amount of DMC within 1 min at 37 °C.

III. Results and Discussion

A. Proximate Analysis

Tilapia bone powder was analyzed and showed the small amount of moisture content. This suggested that the drying process is performed perfectly. It has been reported that water molecules are not included into bone tissue but bound weakly at the surface of the bone [4]. Therefore, it could be removed almost completely during oven drying. The moisture content in the bone powder was found at the low level as the data shown in Table I. According to the low moisture content, the bone powder would be stable even at room temperature. In addition, the agglomeration/aggregation to be the cake would not occur during the storing bone powder. Besides the physicochemical changes, low moisture content also allows the fish bone powder to be resisted for microbial deterioration. This is because moisture content at 2% is not sufficient for microbial growth. This stable form provides the easy/safety way to use the fish bone powder as the ingredient in many applications.

It has been reported that lipid could be filled in the bone, especially the main bone of fish frame, which contains the junction of many pieces of bone [5]. In addition, those lipids could not be gotten rid off easily, because those lipids are complexes, and hardly remove by just soaking the bone in the alkaline solution [6]. According to this information, there is the possibility to observe fat residue in the fish bone powder. Indeed, crude fat content in the bone tissue was almost 6% (Table I). The lipid content in fish bone was reported to be in the range of 1-27% [7]. Lipid content in the bone powder from mackerel was approximately 47%, while the lipid contents in cod and saithe both were about 1.4% [3]. The lipid content in bone is correlated with body fat in each fish species and large (old) fish normally contains high fat. Tilapia is considered as a low fat fish species, it contains low fat even found in the adult stage. Fish lipid is likely unsaturated fatty acid. Fatty acid profile in fish bone powder from several species revealed that the content of unsaturated fatty acids was almost 80% [3]. Normally, the unsaturated fatty acids are susceptible to oxidative degradation (oxidation). However, tilapia bone powder supposes to get the less risk in autoxidation since there is a little fat content.

Protein content in tilapia bone powder was approximately 14%. This value for the bone powder from cod, saithe, blue whiting, salmon, trout, herring, mackerel were found in the range of 26-41% [3]. Therefore, the protein content in tilapia bone powder was much lower than in other fish species. This might be due to the different preparation procedures. In the previous study, fish meat was removed from fish bone by only boiling in the hot water, while this study was done by the hot alkaline solution. Therefore, alkaline solution would be more effective to solubilize and leach out more meat tissue and proteins from the bone. However, the alkaline solution was not effective enough to get rid of protein completely since some of protein still left in the bone powder. Normally, proteins participated in the bone are categorized into stroma protein [8]. This stroma protein is resistant to both acid and alkaline solutions. Collagen associated amino acids, glycine, proline, and hydroxyproline, were found in the bone powder from blue whiting, herring, and mackerel [3]. Protein content in the bone is increased as fishes grow up.

The major component in the bone powder was ash content, which was found to be 75%. The ash in bone powder from several fish species could be found up to 40% [3]. The ash in tilapia bone powder was much higher than that reported previously. This suggested that the bone preparation was important to get the high purity of bone component. Application of alkaline solution was the practical method to

remove organic materials particularly protein. The ratio of ash/protein is the important criteria to indicate the bone mineralization, associated with hardness of the bone. This value for salmon bone was lower than 1.00 [3]. The ash/protein ratio of tilapia bone powder was 5.35, which is much higher than that reported previously. This suggested that not only the bone mineralization but also the preparation method could affect this value. In addition, this value could be used to indicate the purity of bone powder. Therefore, alkaline aid solubilization could be the practical method to produce fish bone powder with high purity.

TABLE I: MAJOR COMPONENT OF TILAPIA BONE POWDER

Proximate parameter	Content (%)
Moisture	2.46 ± 0.03
Crude fat	5.82 ± 0.04
Protein	14.81 ± 0.33
Ash	75.83 ± 0.12

Mean ± SE was estimated from 3 replications.

B. Color

The color of tilapia bone powder was shown visibly as the white powder and the *L* value was about 98 as shown in Table II. The color of fish bone powder is correlated well with the organic residues presenting in the powder. High content of organic compounds resulted in the less lightness of the powder. The white powder was more preferred to fortify into several food products rather the dark powder. Fish bone powder was used as supplementary ingredient in the skimmed milk [9]. However, the changes in color might be possible since the bone powder contains amino acids. The browning reaction occurs through the non-enzymatic reaction. Therefore, the suitable storage condition for tilapia bone powder should be at low temperature and vacuum condition.

TABLE II: COLOR VALUE OF TILAPIA BONE POWDER

Color	Value
L	98.08 ± 0.04
a	16.79 ± 0.06
b	79.48 ± 0.09

Mean ± SE was estimated from 3 replications.

C. Solubility and Ca2+ Content

Usually, the ash is the indicator of mineral content in the sample. Tilapia bone powder showed high ash, it supposed to have high mineral content. However, the mineral uptake of human body depends not only on ash content but also on the solubility and availability to absorb. Therefore, the soluble mineral has been considered as the quality of ash. Meanwhile, it is the good indicator of ash for health. Feeding fish with soluble Ca^{2+} promoting the mineralization of salmon was reported [10]. The bone powder of tilapia was not solubilized completely (Table III). This suggested that all mineral components in the powder could not be absorbed. The solubility of mineral in the bone powder may be associated with several factors including fish species and fish age. If the bone of old fish is mineralized completely, the bone would be hard. In contrast, small fish species are often eaten as a whole. Then, Ca^{2+} from fish bone is soluble and is absorbed readily into human body. Fish species have the different bone structures: cellular and acellular bone structures. The cellular bone from fish species in the family of Salmonadae is less strained than in acellular fish bone. This is because of the lower surface to volume ratio in cellular fish bone [11]. Thus, Ca^{2+} from acellar bone could be more available when compared to the cellular bone fish. The availability of Ca^{2+} from tilapia bone powder was expected because this fish is classified into the acellular bone.

Although the soluble ash was observed at the low level, the Ca^{2+} content in solution was also observed (Table III). The Ca^{2+} is considered as the macro mineral in the bone. Normally, it complexifies with phosphorous in the form of hydroxyappatite ($Ca_5(PO_4)OH_2$) [7]. Therefore, bone tissue is the important depot for storage of calcium and phosphates and also is essential in the regulation of plasma concentrations of these minerals [12]. It would be hypothesized that Ca^{2+} in the tilapia bone could be solubilized to be the soluble Ca^{2+}. However, the utilization of soluble Ca^{2+} is also an interesting point. There are several documents reporting the availability of Ca^{2+} residue in fish bone powder. Those studies have been tested with the complicated system, like the rat and human body model [13]. The absorption and retention of Ca^{2+} was correlated well with a reduction of bone loss[14]. In contrast, the simple reaction to test the bioavailability of Ca^{2+} would be more convenient and should be developed.

TABLE III: SOLUBILITY AND SOLUBLE CALCIUM ION CONTENT IN TILAPIA BONE POWDER

Quality attribute	Content
Solubility (%)	9.38 ± 0.07
Soluble calcium content ($mg.L^{-1}$)	116.56 ± 0.15

Mean ± SE was estimated from 2 replications.

D. tTGase Activity

Ca^{2+} is one of major minerals, serving as the cofactor for several enzymes in the metabolic pathway. The tTGase is one of enzyme, requiring Ca^{2+} for full activation. Ca^{2+} is needed to induce conformational changes of the enzyme molecule to be prompt for catalyzing the reaction. The tTGase assay has been developed and Ca^{2+} has to be included for 5 mM [15]. It has been reported that tTGase bound with Ca^{2+} could remove the controlling residue, covering the active site. Therefore, the reaction could be preceded. This fact would be the basic background for developing the bioavailability test of Ca^{2+} from fish bone. Since the availability of Ca^{2+} is important for activation of tTGase, the bioavailability of Ca^{2+} from fish bone could be evaluated. It can be seen that tTGase could not catalyze the incorporation of MDC into DMC in the absence of Ca^{2+} (Table IV). However, the tTGase activity increased instantly when soluble Ca^{2+} was added for 162 nM. This result clearly demonstrated that the presence of Ca^{2+} from tilapia bone powder at this level is enough to activate crude tTGase from tilapia. This was the evidence that soluble Ca^{2+} from tilapia bone powder is also available for biochemical reaction. The activation ability of this soluble Ca^{2+} was comparable to that of standard Ca^{2+} ($CaCl_2$) since the comparable activity was found when soluble Ca^{2+} was replaced by standard Ca^{2+}.

E. Effect of Ca2+ on tTGase Activity

It has been reported that the active site of tTGase contains

cysteine as the catalytic residue. This site is susceptible to form disulfide bond with another cysteine, resulting in the enzyme inactivation. However, this disulfide linkage is hampered by tyrosine. Thus, the enzyme inactivation is minimized in the physiological condition [16]. Ca^{2+} is required to maintain the appropriate conformation of residues at the active site of tTGase [17]. The results in Table V showed that the activity of crude tTGase from tilapia was increased when Ca^{2+} was added more. This suggested that increasing soluble Ca^{2+} resulted in the higher availability. It has been reported that the Ca^{2+} sensitive of tTGase is varied from different spices. The purified tTGase from tilapia could be fully activated by addition of Ca^{2+} up to 1 mM [18]. The Ca^{2+} of 0.5 and 1 mM could be enough to activate liver tTGase from red sea bream and threadfin bream, respectively [19-20]. Since soluble Ca^{2+} from tilapia bone powder could activate crude tTGase activity, therefore, it could be available for biochemical reaction.

TABLE IV: ACTIVITY OF TRANSGLUTAMINASE ACTIVATED BY SOLUBLE CALCIUM FROM FISH BONE

Treatment	tTGase Activity (U/mg)
Negative control (without Ca^{2+})	0.01 ± 0.002
Soluble Ca^{2+} from tilapia bone (162 nM)	9.71 ± 0.04
Positive control (162 nM of standard Ca^{2+})	10.75 ± 0.08

Mean ± SE was estimated from 2 replications.

TABLE V: ACTIVITY OF TRANSGLUTAMINASE ACTIVATED BY SOLUBLE CALCIUM FROM FISH BONE

Soluble Ca^{2+} from tilapia bone (nM)	tTGase Activity (U/mg)
0	0.01 ± 0.002
162	11.28 ± 0.04
810	20.71 ± 0.09
1000	45.82 ± 0.13

Mean ± SE was estimated from 2 replications.

IV. CONCLUSION

Tilapia bone powder was produced successfully and the major component was ash. It showed the white powder and contained less moisture and fat contents. The solubility of bone powder was found at low level but the soluble Ca^{2+} residue could activate the crude tTGase. Therefore, it would be available for biochemical activity.

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