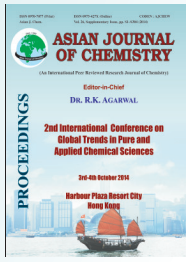
Asian Journal of Chemistry; Vol. 26, Supplementary Issue (2014), S103-S106

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.19024



Characteristics of Tilapia Bone Powder and Calcium Extraction by Acid Solution

MANOP SRIUTTHA[1], MALLIKA CHANTARANGSEE[1] and BUNG-ORN HEMUNG[1,2,*]

[1]Faculty of Applied Science and Engineering, Khon Kaen University, Nong Khai Campus, Nong Khai 43000, Thailand
[2]Indo-China Country International Trade and Economic Research Sector, Khon Kaen University, Nong Khai Campus, Nong Khai 43000, Thailand

*Corresponding author: E-mail: bunhem@nkc.kku.ac.th, hemung3@gmail.com

Published online: 24 December 2014; AJC-16448

Tilapia bone powder was prepared by hot water and alkaline treatments, autoclaved, dried and ground to be powders. Fourier-transform infrared spectroscopy indicated that tilapia bone powders consisted of inorganic matters, especially hydroxyapatite. The proximate analysis revealed that organic compounds, protein and fat were presented but they could be removed more effectively in alkaline treatment than that in water. Microstructure showed the corrosive surface of the sample treated with alkaline, while the compact surface was found in another one. Therefore, the alkaline-treated bone was selected for calcium extraction by different acids and the calcium content was estimated by the redox titration. Based on the extractability and feasibility in food application, an acetic acid was selected to evaluate the suitable concentration and extraction time. The results turned to be at 0.25 M for 48 h. When, the calcium from tilapia bone powder was extracted at this condition, the calcium content was 2,376 mg/L, as quantified exactly by the inductively coupled plasma-optical emission spectroscopy. The extracted calcium could activate the calcium-dependent enzyme, transglutaminase, suggesting the availability for biological reaction.

Keywords: Calcium, Fish bone, Transglutaminase, Acid solutions.

INTRODUCTION

Tilapia (*Oreochromis niloticus*) is the fresh water fish species and it is produced commercially through an aquaculture around the world with the annual production equal to trout and salmon[1]. Tilapia is currently processed to be either food or non-food products such as a frozen fillet or leather[2]. Through those processes, tilapia frame must be removed and it is considered as waste or by-products accounting for up to 15 % of the fish weight. Fish bone ash is the major component in such waste and consists of 34-36 %[3]. However, it is currently used to make fish bone meal for feeding although it is the good source for important mineral, especially calcium and phosphorous[4]. The potential of fish bone as a source of mineral supplement was documented[5]. In addition, a calcium capsule containing tilapia bone powder has been developed for being the calcium supplementary[2]. Generally, calcium in fish bone powder is mainly found as the hydroxyappatite (HA), which is hardly solubilized[3]. The bioavailability of the fish bone capsule might be limited. Thus, extraction of calcium from fish bone powder is necessary for fulfilling the nutritional implication.

Application of the pulsed-electric-field-assisted extraction was reported as an effective method to solubilize calcium from the bone[6]. However, this technique has not yet been applied commercially. It seems like acid extraction is more feasible since it provides high extraction rate[7]. However, extraction of calcium from fish bone has not been investigated, while the extraction of calcium from pig bone and egg shell has been performed. Thus, extraction of calcium from fish bone by acid solution should be optimized. This would lead to the strategy for recovery calcium from waste as mineral source. In addition, the bioavailability of soluble calcium should also be evaluated for gaining the health benefit. The bioavailability test of calcium from fish bone could be performed in either *in vitro* or *in vivo* systems[8,9]. The potential of soluble calcium as cofactor for enzyme is represented the bioavailability as well. The activating ability of soluble calcium from fish bone toward the transglutaminase, calcium-dependent enzyme, was documented[1]. This breakthrough provided the simple method to evaluate the bioavailability of calcium extracted from fish bone. Therefore, the objectives of this study were:

(1) to characterize the tilapia bone powder.

(2) to optimize the extraction of calcium by acid solutions.

(3) to evaluate the bioavailability of soluble calcium based-on the transglutaminase assay.

EXPERIMENTAL

Tilapia bone preparation and characterization: Fish bone powder was prepared according to the method described previously with slight modifications[1]. The main frame of tilapia was soaked in hot water and alkaline solution (0.8 % NaOH, w/v) at a ratio of bone to solution of 1:2 (w/v) for 1 h. The bone samples were rinsed with trapped water, autoclaved at 121 °C (350 g cm^{-2}) for 1 h and dried overnight in an oven drier at 105 °C. The dried samples were ground into powder and sieved through a sieving mesh (38 µm) and used as water-treated bone and alkaline-treated bone powders, respectively.

Chemical compositions were analyzed for moisture, crude protein, crude fat and total ash contents by the standard method from American Official Analytical Chemists[10].

The bone powder was mixed with KBr at the ratio of 1:40 and pressed into pellet before determining the vibrational spectra by the fourier-transform infrared (FT-IR) spectroscopy (Spectrum one 60045, Perkin Elmer, England). The spectra were recorded as the average from 32 scans in the wave number from 4000-450 cm^{-1}. Microstructure at 5,000 magnifications was analyzed by the scanning electron microscopy (SEM) using S-3000N (Hitachi, Japan) after gold coating.

Optimization of calcium extraction: The extractability of calcium from tilapia bone powder (an alkaline-treated bone) was evaluated using sulfuric acid, hydrochloric acid, nitric acid, acetic acid and citric acid at 0.25 M. The bone powder was mixed with each acid solution at the ratio of bone: acid at 1:20 (w/v) for 1 h. The calcium was extracted by shaking at room temperature at the speed of 200 rpm. The clear solution was filtered through filter paper (Whatman® No. 1) before precipitating into calcium oxalate. Then, the oxalate was solubilized by sulfuric acid. Finally, the soluble calcium was quantified roughly by the redox titration as described previously[11]. The highest extraction yield was considered as 100 % and results was expressed as normalized extractability.

The bone powder was mixed with an acetic acid at different concentrations (0.03, 0.06, 0.12, 0.25, 0.50 and 1.00 M). Extraction process was set at the same shaking speed, shaking time, ratio of powder: acetic acid and temperature. Extractability was recorded after quantifying the calcium content in solution by redox titration and expressed as normalized value as described above.

The bone powder was used to extract calcium by acetic acid at 0.25 M for different times (1, 3, 6, 12, 24, 48 and 72 h) by setting the extraction process as described above. Calcium content was also quantified by redox titration before expressing the data as normalized value.

Bioavailability of extracted calcium from tilapia bone: Tilapia bone powder (alkaline-treated bone) was used to extract calcium by an acetic acid (0.25 M) for 48 h at room temperature. The extracted solution was adjusted to pH 7. The exact concentration of calcium was determined by the inductive coupled plasma-optical emission spectroscopy (Optima 4300 DV; Perkin Elmer Instruments; Norwalk, CT, USA). The emission wavelength for calcium was recorded at 317.933 nm and the calcium content in the extract was 2,376 mg/L. This solution was used to activate the crude transglutaminase from tilapia muscle to catalyze the incorporation of monodansyl cadaverine (MDC) into the dimethylated casein (DMC) as details for determining the transglutaminase activity.

The tilapia muscle (10 g) was homogenized with 90 mL of an extraction buffer (10 mM NaCl, 50 mM *tris*-Cl, pH 7.5). The homogenate was centrifuged at 10,000 g for 0.5 h at 4 °C. The supernatant was filtered through the filter paper (Wattman® No. 1) and the filtrate was used as the crude transglutaminase.

The cocktail reaction for transglutaminase activity contained dimethylated casein (2 mg/mL), *tris*-Cl, pH 7.5 (70 mM), dimethylated casein (15 M) and 100 L of crude transglutaminase. The reaction was incubated at 37 °C for 10 min and stopped by addition of an ammonium sulfate to the final concentration of 400 mM. The fluorescence intensity of reaction was monitored at excitation and emission wavelengths of 350 and 480 nm, respectively.

Statistical analysis: The data were analyzed by multiple comparison tests with one-way ANOVA (SPSS, 16.0). The difference was considered at $P < 0.05$.

RESULTS AND DISCUSSION

Chemical compositions: The chemical composition of bone powder was affected by the different methods, applying to remove the organic matters. Moisture content was still found in the bone sample although it appeared as the dried powder. This suggested that water molecule could be re-absorbed after drying process. Due to the similar preparation method, this value was comparable to that reported previously[1]. However, it was much lower than that reported for cod, saithe, blue whiting, salmon, trout, herring and mackerel because organic substances were removed by different methods from our study[12]. It can be seen that the sample treated with alkaline showed higher moisture content than another sample (Table-1). This suggested alkaline-treated powder adsorbed water to the greater extent than that of another one. Thus, the reaction sites for chemical reaction on bone particle were more available when it is treated with alkaline.

Protein content in sample treated with alkaline was observed at the lower level when compared with that treated with water. It has been established that collagen was the major protein found in the bone matrix[13]. Alkaline treatment allowed collagen to be eliminated to the greater extent than water. Softening of connective tissue from fish bone under alkaline condition has been reported[14]. However, the collagen could not be removed completely as evidenced by presenting of protein in the sample. This might be because the collagen is imbedded inside of the mineral crystal as model proposed previously[13]. Application of alkaline also provided more benefits by getting rid of fat since a reduction of crude fat had been observed (Table-1). This indicated that the organic matters could be removed more effectively by alkaline solution. Consequently, total ash content was increased and it is observed as the major component. Therefore, tilapia bone powder showed a potential to be the source of natural mineral, especially calcium.

TABLE-1
COMPOSITION OF TILAPIA BONE POWDER TREATED WITH WATER AND ALKALINE SOLUTION

Composition (%)	Treatment	
	Water	Alkaline
Moisture	1.82 ± 0.16[b]	2.67 ± 0.26[a]
Crude fat	6.96 ± 0.11[a]	3.12 ± 0.04[b]
Crude protein	21.46 ± 0.20[a]	17.77 ± 0.20[b]
Total ash	65.00 ± 0.14[b]	70.16 ± 0.03[a]

Different letters in the same row indicate the statistic difference ($P < 0.05$) between columns

Fourier-transform infrared spectroscopy: The FT-IR spectra of fish bone powder showed the peaks corresponding to the vibration of several functional groups. The peak at wave number of 560 cm^{-1} was reported to be the vibration of an inorganic phosphate[14,15] (PO_4^{2-}). It was found in both bone powders. The O-H stretching was observed at wave number of 3423 cm^{-1}. This suggested that calcium present in the bone is in the form of hydroxyapatite. The apatite from cod and sier fish (*Thynnus thynnus*) has also been characterized[4,16]. The vibration of organic groups through the C-H stretching at 1451 and 1419 cm^{-1} were generated from CH_3 and CH_2, respectively. The signal for organic compounds was also observed at wave number of 2923 cm^{-1}. This suggested that the organic compounds were presented in the sample, corresponding to the observation of fat and protein (Table-1). The signal at 2340 cm^{-1} was observed only in sample treated with alkaline. This was reported to be the N-H stretching of organic compounds[16]. Therefore, that organic compounds might be modified by alkaline treatment. The collagen in the bone was softened by alkaline treatment[2]. These results suggested that alkaline may solubilize collagen from the bone resulting in a reduction of protein content.

Scanning electron microscopy: The microstructure of bone particle showed the smooth and compact structure when the bone was prepared by soaking in water. However, the rough and corrosive structures were observed when sample was treated with alkaline (Fig. 1). The alkaline solution solubilized collagen[1], imbedded at the bone surface, increasing porosity of the bone. The corrosive surface of tilapia bone powder was also documented after alkaline treatment[2]. This may explain why the alkaline-treated sample could re-adsorb water to the greater extent than that without alkaline. Fish bone particle with high porosity could enable calcium to be extracted easily when comparing with the compact surface. Therefore, the alkaline treatment was selected to prepare the fish bone powder for calcium extraction.

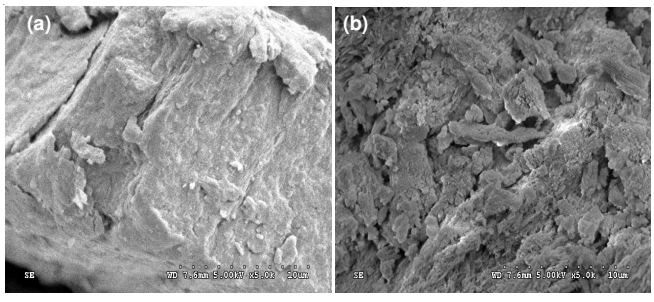


Fig. 1. Microstructure of tilapia bone powder treated with water (a) and alkaline (b)

Optimization of calcium extraction: The strong acid and weak acids were selected to evaluate the efficiency for calcium extraction from the tilapia bone powder. The results clearly showed that all acids could be used to extract calcium from the bone (Table-2). However, the hydrochloric acid and acetic acid seemed to show the highest potential. Hydrochloric acid was used to digest organic compounds prior to determine mineral components[17]. However, application of hydrochloric acid in the food system is quite limited. In contrast, acetic acid has been applied regularly in food system in the form of vinegar. Bone softening by curing in an acetic acid resulted in calcium liberation[18]. Therefore, acetic acid was selected to be the suitable acid for studying the effect of concentration on the extractability.

TABLE-2
CALCIUM EXTRACTION FROM TILAPIA BONE POWDER AS AFFECTED BY ACID TYPES

Acid type	Normalized extractability (%)
Sulfuric acid	63.16 ± 0.24[bc]
Hydrochloric acid	98.21 ± 0.32[a]
Nitric acid	71.80 ± 0.31[b]
Acetic acid	100.00 ± 0.43[a]
Citric acid	60.65 ± 0.41[bc]

Different letters among rows indicate the statistic difference ($P < 0.05$)

An increase of acetic acid concentration up to 0.25 M resulted in higher extractability (Table-3). However, further increasing of calcium extractability was not observed although the concentration of an acetic acid was increased until 1.00 M. Therefore, the suitable concentration was considered at 0.25 M. In addition, using lower acid concentration would be beneficial for further application.

TABLE-3
EFFECT OF ACETIC ACID CONCENTRATION ON CALCIUM EXTRACTABILITY FROM TILAPIA BONE POWDER

Acetic acid concentration (M)	Normalized extractability (%)
0.03	51.80 ± 0.21[d]
0.06	79.11 ± 0.32[bc]
0.12	86.91 ± 0.73[b]
0.25	100.00 ± 1.04[a]
0.50	79.40 ± 0.83[bc]
1.00	91.47 ± 0.41[b]

Different letters among rows indicate the statistic difference ($P < 0.05$)

Extraction of calcium by acetic acid at 0.25 M from 1-48 h provided an increase in calcium extractability (Table-4). An increase of extraction time resulted in an increasing of calcium liberation from fish bone during curing in the vinegar[19]. However, calcium extractability was not increased more significantly when the time was prolonged to 72 h. Moreover, prolonging the extraction time may promote the microbial growth due to the presentation of organic compounds. Therefore, the suitable time for calcium extraction was selected at 48 h.

Based on this study, an acetic acid at 0.25 M was used as the extracting solvent to extract calcium from fish bone. The calcium content in the extract was determined exactly by ICP-OES and found the total calcium content in the solution at 2376 mg/L. This value could be calculated at about 59.28 mM. This value was much higher than reported previously when water was to extract calcium from tilapia bone[1]. The

TABLE-4
EFFECT OF THE EXTRACTION TIME ON THE CALCIUM EXTRACTABILITY FROM TILAPIA BONE POWDER BY ACETIC ACID (0.25 M)

Extraction time (h)	Normalized extractability (%)
1	54.38 ± 0.29^{cd}
3	61.05 ± 0.43^{c}
6	54.86 ± 0.07^{cd}
12	49.98 ± 0.10^{d}
24	60.59 ± 0.45^{c}
48	94.30 ± 0.18^{ab}
72	100.00 ± 0.37^{a}

Different letters among rows indicate the statistic difference ($P < 0.05$)

bioavailability of soluble calcium was evaluated based-on the transglutaminase assay.

Bioavailability of extracted calcium from tilapia bone: Since transglutaminase is a calcium-independent enzyme, calcium ion is needed for full activation. In addition, calcium is required to add up in the reaction cocktail for determining the transglutaminase activity[20]. The activity of partially purified transglutaminase from red sea bream was not detected the presence of an ion chelator, EDTA[21]. The ability of crude transglutaminase from tilapia muscle to catalyze the amine (MDC) incorporation into the dimethylated casein was used to evaluate transglutaminase activity prior purification[22]. Therefore, availability of soluble calcium, extracted from fish bone by acetic acid (0.25 M) for 48 h, for transglutaminase would be an indicator for bioavailability. The transglutaminase activity was almost not found in sample without addition of the soluble calcium (Fig. 2). This confirmed that transglutaminase is the calcium-dependent enzyme. In the presence of 500 nM of calcium, transglutaminase activity increased markedly. This result indicated that soluble calcium from tilapia bone powder could activate transglutaminase activity. In addition, an increase in calcium concentration resulted in an increasing of transglutaminase activity. The soluble calcium extracted from tilapia bone powder by only water also activated crude transglutaminase[1]. Calcium has been reported to induce conformational changes of fish *trans*-glutaminase. The phenomena allowed the active site of enzyme, generally buried inside, to bind with substrate[23]. This suggested that the soluble calcium in this study is in the free form.

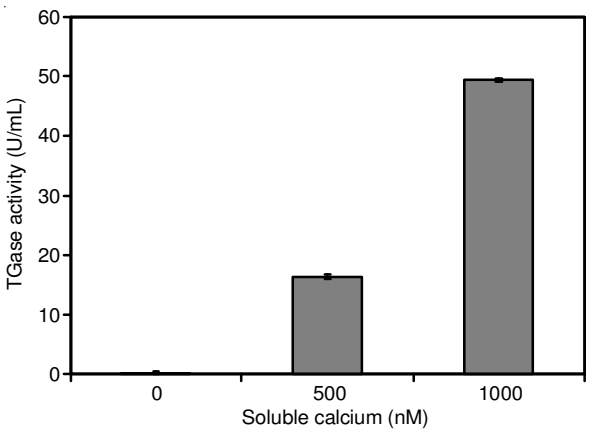


Fig. 3. Transglutaminase activity as affected by concentration of soluble calcium from tilapia bone powder extracted by acetic acid (0.25 M) for 48 h at room temperature

Conclusion

Tilapia bone powder could be prepared by removing organic compounds in alkaline solution. The alkaline treatment provided the porous surface, accelerating the calcium extraction. The acetic acid was selected as the suitable acid due to the high extractability as well as the compatibility to food applications. The suitable concentration and extraction time were optimized to be 0.25 M and 48 h. The concentration of the extracted calcium was observed at almost 60 mM when the bone was extracted at the suitable condition. Soluble calcium could activate crude transglutaminase to catalyze the incorporation of synthetic amine into modified casein, suggesting the bioavailability.

ACKNOWLEDGEMENTS

The authors are grateful to the Faculty of Applied Science and Engineering, Khon Kaen University, Nong Khai Campus, for all facilities. The special thanks are honored to Miss Alisa Phukiew and Miss Panamas Chantakul for optimization of calcium extraction. The "Indo-China Country International Trade and Economic Research Sector" was acknowledged for the supportive fund. Financial support was also partly from the Thailand Research Fund (TRF) through the Grant No. MRG5380082.

REFERENCES

1. B. Hemung, *Int. J. Biosci. Biochem. Bioinform*., **3**, 306 (2013).
2. K. Techochatchawal, N. Therdthai and S. Khotavivattana, *Asian J. Food Ag-Ind*., **2**, 539 (2009).
3. S.A. Tont, W.G. Pearcy and J.S. Arnold, *Mar. Biol*., **39**, 191 (1977).
4. C. Piccirillo, M.F. Silva, R.C. Pullar, I. Braga da Cruz, R. Jorge, M.M.E. Pintado and P.M.L. Castro, *Mater. Sci. Eng. C*, **33**, 103 (2013).
5. P. Phiraphinyo, S. Taepakpurenat, P. Lakkanatinaporn, W. Sunthornsuk and L.S. Songklanakarin, *J. Sci. Technol.*, **28**, 327 (2005).
6. Y. Yin and G. He, *Sep. Purif. Technol*., **61**, 148 (2008).
7. Y.C. Zhu, S.A. Huang, J. Bai and R.P. Liang, *Livest. Poult. Ind*., **1**, 128 (2005).
8. P.H. Luu and M.H. Nguyen, *Tap ChiKhoa Coc Va Cong Nghe*., **47**, 91 (2009).
9. M. Malde, S. Bugel, M. Kristensen, K. Malde, I. Graff and J. Pedersen, *Nutr. Metab*., **7**, 61 (2010).
10. J.N. Richardson, M.T. Stauffer and J.L. Henry, *J. Chem. Educ*., **80**, 65 (2003).
11. J.M. Tennyson and R.S. Winlers, Chapter Editors, Fish and Marine Products: Treatment and Preparation of Sample Procedure; in ed.: W. Howitz, Official Methods of Analysis of AOAC International 17th ed. Gaithersburg (MD): AOAC International, p. 2 (AOAC official Method 937.07; Vol. 2, Chapter 35.1.01 (2000).
12. J. Toppe, S. Albrektsen, B. Hope and A. Aksnes, *Comp. Biochem. Physiol. B*, **146**, 395 (2007).
13. C. Hellmich and F.J. Ulm, *J. Biomech*., **35**, 1199 (2002).
14. M. Ishikawa, S. Mori, H. Watanabe and Y. Sakai, *J. Food Process. Preserv*., **13**, 123 (1989).
15. K. Prabakaran and S. Sajeswari, *Trend Biomater. Artif. Organs*, **20**, 20 (2006).
16. V. Coman, R. Grecu, M. Baciut, G. Baciut, P. Prodan and V. Simon, *J. Optoelectron. Adv. Mater*., **9**, 3372 (2007).
17. G.F. Smith and H. Diehl, *Talanta*, **3**, 41 (1959).
18. C. Shimosaka, *J. Clin. Biochem. Nutr*., **31**, 9 (2002).
19. C. Shimosaka, *J. Clin. Biochem. Nutr*., **26**, 173 (1999).
20. J. Takagi, Y. Saito, T. Kikuchi and Y. Inada, *Anal. Biochem*., **153**, 295 (1986).
21. B. Hemung, E.C.Y. Li-Chan and J. Yongsawatdigul, *J. Agric. Food Chem*., **56**, 7510 (2008).
22. A. Worratao and J. Yongsawatdigul, *Food Chem*., **93**, 651 (2005).
23. H. Yasueda, Y. Kumazawa and M. Motoki, *Biosci. Biotechnol. Biochem*., **58**, 2041 (1994).