Process Development and Characteristics of Biocalcium from Skipjack Tuna (Katsuwonus pelamis) Eyeball Scleral Cartilage

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Abstract

Scleral cartilage biocalcium (SCBC) powder from skipjack tuna (*Katsuwonus pelamis*) eyeball scleral cartilage (STESC) was produced using hexane and isopropanol (1:1) for defatting, followed by protein removal using alcalase at different concentrations (1.5–4.5%) for various times (0-180 min). Calcined scleral cartilage (CSC) was also prepared and characterized. The lowest fat remaining in STESC was attained after 6 cycles of defatting ($P < 0.05$). The use of 1.5% alcalase for 90 min resulted in the highest soluble protein removed, while showed the lowest loss in hydroxyproline. Higher value of calcium (39.90%), phosphorus (18.97%) and L^* (87.97) were detected in the CSC powder, compared to SCBC powder (23.28%, 10.99% and 81.77, respectively) ($P < 0.05$). However, a^*, b^*, and ΔE^* of CSC powder were lower than SCBC counterpart. The mean particle sizes of both powders had a negligible difference ($P > 0.05$). XRD-diffractogram of both powders confirmed the presence of hydroxyapatite. CSC showed the loss of water, amide I, II, III, and other organic functional group peaks in Fourier transform infrared spectra. Also, smaller number and abundance of volatile compounds were found in CSC powder. STESC showed a higher calcium solubility in gastrointestinal tract than CSC ($P < 0.05$). Therefore, scleral cartilage of tuna eyeball could be used as an alternative source for producing biocalcium.

1. Statement Of Novelty

This is the first report on biocalcium from skipjack tuna eyeball scleral cartilage. Simple process including pretreatment using mixed solvents for defatting and enzymatic hydrolysis for protein removal was successfully developed.

2. Introduction

Calcium is a significant element for human bone and teeth. Moreover, calcium is involved in physiological functions such as muscle contraction, wound healing, blood flow facilitation, heart rate regulation, and nerve impulse transmission. Calcium deficiency can cause osteoporosis and reduced bone density [1]. This might be because of insufficient consumption of calcium from the regular diet. Calcium supplementation could solve calcium deficiency problem. However, pure calcium has low absorption due to calcium precipitation in phosphate salt form. The fish-bone associated with peptides could increase the solubility and bioavailability of calcium [2].

Canned tuna is the top of Thai exporting products [3]. During tuna processing, the unconsumed portions are generated and discarded by 50% [4], which have been used as feed or fertilizer. To add the value of tuna by-products, those leftovers have been converted to collagen, protein hydrolysate, fish oil, and biocalcium [5–8]. Skipjack tuna eyeball (STE) without scleral cartilage (SC) has been utilized for oil extraction [9, 10]. SC was removed before extracting the oil from eyeball tissue [9, 10]. The function of the vertebrate sclera is to support eye structure and maintain the intraocular pressure [11, 12]. Since SC has a similar function to bone matrix or cartilage, it could be utilized as an alternative biocalcium. However, the oil of eyeball tissue remains on the surface of SC. Oil-rich in polyunsaturated fatty acids (PUFAs) are

oxidized, generating secondary oxidation products such as aldehyde and ketone, which further react with nitrogenous compounds (volatile base nitrogen and free amino acid). This results in darker color mediated by Maillard reaction [13]. Alkaline treatment has been effectively applied for biocalcium preparation to remove the proteinaceous substances from the bone matrix [14]. However, the harsh conditions should be avoided. Alcalase, an alkaline protease, has been reported to have higher hydrolysis potential than other proteolytic enzymes such as pepsin and papain [15]. Therefore, fat and non-collagenous protein removing processes are still required for producing the biocalcium from SC. Moreover, the information regarding the amount and types of calcium from scleral cartilage is still lacking. This study aimed to prepare and characterize the biocalcium from skipjack tuna eyeball scleral cartilage (STESC), the leftover from tuna eyeball oil extraction.

3. Materials And Methods

3.1 Chemicals, solvents and enzyme

The analytical grade chemicals used for analyses were acquired from Sigma (St. Louis. MO, USA). Organic solvents were obtained from Rci Labscan (Bangkok, Thailand). Alcalase (EC 3.4.21.62) (2.4 unit/g) from *Bacillus licheniformis* was procured from Siam Victory Chemicals Co, Ltd. (Bangkok, Thailand).

3.2 Skipjack tuna eyeball scleral cartilage (STESC) preparation

Skipjack tuna (*Katsuwonus pelamis*) eyeballs were provided from Chotiwat Manufacturing Company, Ltd. (Songkhla, Thailand). Eyeballs from tuna head, manually took out by the factory staff, were transferred into the polyethylene bag and brought in ice to the laboratory. After arriving at the laboratory, eyeballs were rinsed with running tap water. STESC was manually separated from eyeball tissue and kept in a zip lock bag and frozen at -20°C until use.

3.3 Chemical compositions of STESC

STESC was blended to obtain uniformity and subjected to proximate analysis using AOAC method [16].

3.4 Pre-treatment of STESC

3.4.1 Effect of defatting for different cycles on fat removal of STESC

STESC was cut in a small size (less than 1 × 1 cm^2). Thereafter, STESC was submerged in 10 volumes of mixed solvents (hexane: isopropanol; 1:1) with continuous stirring at 250 rpm for 1 h using an overhead stirrer (Model RW 20n, IKA-Werke GmbH & CO.KG, Staufen, Germany) at room temperature. The mixed solvents were discarded and an equal volume of mixed solvents was added. After each defatting cycle, the sample was taken and determined for fat content using the Soxhlet extraction method as guided by

AOAC [16]. The number of defatting cycles yielding the sample with the lowest fat content was selected for further study.

3.4.2 Effects of enzyme concentration and hydrolysis time on protein removal of STESC

Enzymatic protein removal process of STESC was done following the procedure of Idowo et al. [17] with some modification. Firstly, defatted STESC (50 g) was transferred into 1 L-beaker. Thereafter, distilled water (DW) (500 mL) was added to obtain the ratio of water and sample at 10:1 (v/w). The mixtures were agitated using an overhead stirrer at 150 rpm for 2 min. pH of mixtures was adjusted to 8.0 using 0.1 M HCl or 0.1 M NaOH. Subsequently, the mixtures were incubated in a temperature-controlled water bath (Model W350, Memmert, Schwabach, Germany) at 60°C for 15 min. Subsequently, alcalase was added at different concentrations (1.5, 3.0 and 4.5%, w/w protein). Enzymatic deproteinization was performed with continuous stirring. At designated times (0, 15, 30, 60, 90, 120, 150 and 180 min), the mixture (2 mL) was taken and enzyme was inactivated by submerging in hot water (90°C) for 15 min. Afterward, 5% sodium dodecyl sulfate (85°C) was added into the mixtures at a ratio of 1:1 (v/v) and incubated at 85°C for 30 min to solubilize all proteins. The mixture was then centrifuged using a centrifuge (Model Allegra 25R, Beckman Coulter, CA, USA) (4,000 × g, 15 min). The supernatant was collected and examined for degree of hydrolysis (DH) [18], soluble protein content (SPC) by biuret method [19] and hydroxyproline content (HC) [20]. The enzyme concentration and digesting time yielding highest SPC with lowest HC were selected for biocalcium preparation.

3.5 Preparation of scleral cartilage biocalcium (SCBC) and calcined scleral cartilage (CSC)

For SCBC, the pretreated STESC was dried with laboratory scale rotary dryer with air velocity of 1.5 m/s at 50°C for 5 h. A high speed grinder (Model HC-500Y, Damai, Zhejiang, China) was used to obtain the coarse particle sample. To produce CSC, SCBC was calcined using a laboratory muffle furnace (Model 320, P Nabertherm, Bremen, Germany) at 900°C for 6 h. Both SCBC and CSC were ground into fine particles using a lab planetary ball mill (Model PM 100, Retsch GmbH, Haan, Germany). To obtain the particle sizes less than 75 μm, the fined particles were sieved with the aid of a sieving machine. The powders were then characterized.

3.6 Characterization of SCBC and CSC powders

3.6.1 Chemical components

The chemical components of both samples, including moisture, protein, fat and ash contents were analyzed using the method of AOAC [16].

3.6.2 Hydroxyproline contents (HCs)

HCs of the samples were determined following the methods tailored by Bergman and Loxley [20]. HC was expressed as μg/g sample.

3.6.3 Calcium (Ca) and phosphorus (P) contents

An inductively coupled plasma – optical emission spectrometer (ICP-OES) (Model AVIO 500, Perkin Elmer, MA, USA) was used for determination of Ca and P in both powder samples [21]. The contents of Ca and P were expressed as a percentage. Moreover, mole ratio of Ca and P was also computed.

3.6.4 Color

A Hunterlab Colorflex EZ colorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA) was used for determination of color values of both powder samples and reported as lightness (*L**), redness/greenness (*a**), and yellowness/blueness (*b**). The differences in color (Δ*E**) between obtained powder samples and white plate standard were computed as shown in the equation below.

$$\Delta E^{*}=\sqrt{\left(\Delta \mathrm{L}^{*}\right)^{2}+\left(\Delta \mathrm{a}^{*}\right)^{2}+\left(\Delta \mathrm{b}^{*}\right)^{2}}$$

3.6.5 Particle size

The particle sizes of both powder samples were analyzed as explained by Wijayanti et al. [22]. Briefly, powder sample (0.5 g) was mixed with 5 mL of distilled water. The mixture was then subjected to analysis using a laser particle size analyzer (LPSA, LS 230, Coulter, CA, USA). The particle size was expressed as mean particle size ($D_{4/3}$).

3.6.6 X-ray diffraction (XRD) pattern

The powder sample was transferred to a sample holder. The sample holder was then placed in the XRD chamber. The diffractogram of both sample powders was determined by an X-ray diffractometer (Model Empyrean, PANalytical, EA Almero, Netherlands) equipped with Cu K-α anode using wavelength at 0.154 nm. An angular range of 4–90° with time/step of 70.125 s under voltage operating at 40 kV and potency of 30 mA was used for scanning conditions. The crystallinity was calculated based on the ratio of the areas of the crystalline peaks to total area using the following equation.

$$\backslash \% \ \mathrm{Crystallinity}=\left[\frac{\mathrm{A\,(peak)}}{\mathrm{A\,(total)}}\right] \times 100$$

where A(peak) = Total area of all crystalline peaks

A(total) = Total area of all crystalline and amorphous peaks

3.6.7 Fourier transform infrared (FTIR) spectra

A Bruker FTIR spectrometer (INVENIO S, Ettlingen, Germany) installed with attenuated total reflectance (ATR) diamond compartment was used. A powder sample (0.5 g) was transferred on the surface of

diamond ATR and held using a sample holder. MIR range (4000 – 400 cm^{-1}) with 32 scans at a resolution of 4 cm^{-1} was used and spectra were recorded. Bruker's OPUS version 8.5 software (Bruker Co. Billerica, MA, USA) was used for data processing and evaluation.

3.6.8 Volatile compounds

Volatile compounds of both powder samples were analyzed using a headspace-solid phase microextraction-gas chromatography-electron ionization/mass spectrometry (HS-SPME-GC-EI/MS) [23]. Volatile compounds were identified using Wiley 10 and NIST14 mass spectral libraries, based on the combination between retention time and mass spectra. Match factor over 90% of volatile compounds involving aldehyde, ketone, alcohol, and acid were selected and reported as an abundance of peak area.

3.6.9 Solubility of Ca in *in vitro* simulated gastrointestinal tract system

To determine Ca bioavailability of both powder samples, the simulated gastrointestinal tract model system (GIMs) was applied [24]. Briefly, powder sample (75 mg) was transferred to 150 mL-conical flask. Thereafter, 50 mL of 5mM HCl-KCl buffer (pH 1.5) with 2.5 mL of pepsin solution (0.04 g/mL) in the same buffer was added and the mixture was incubated for 60 min at 37°C to mimic the stomach condition. Subsequently, the duodenal condition was applied by augmenting the pH to 6.8 using 1 M of $NaHCO_3$. To the digest, 0.25 mL of pancreatin (0.08 g/mL) and 0.25 mL of bile extract (0.04 g/mL) in 10 mM of Tris-HCl buffer (pH 8.2) were added and further incubated at 37°C for 3 h. The mixture was then submerged in boiling water for 10 min. The mixture was made up to 75 mL using DW and centrifuged at 7000 × g for 10 min. The supernatant was analyzed for Ca content using ICP-OES as explained above. The solubility of Ca in both powder samples was calculated and expressed as % relative to total calcium in the initial sample.

3.7 Statistical Analysis

Preparation process and characterization of all biocalcium samples were done in triplication. A completely randomized design (CRD) was implemented throughout the study. Analysis of variance (ANOVA) was conducted and Duncan's multiple range test were applied for mean comparison [25]. SPSS statistics for windows version 17.0 (New York, NY, USA) was used for statistical analysis.

4. Results And Discussion

4.1 Chemical composition of STESC

Moisture, protein, fat and ash contents of STESC were 36.35 ± 3.29, 17.55 ± 1.05, 15.55 ± 1.13 and 27.15 ± 3.29%, respectively. Tuna is the pelagic fish, which migrate between the top and bottom of the ocean rapidly. Eyeball scleral functions as structural support and maintains the intraocular pressure [11]. The sclera is composed of connective tissue and supported by the bone and/or cartilage [26]. Therefore, the main component of STESC is mineral followed by protein and fat, respectively. To avoid the undesirable

color and flavor of biocalcium, fat should be removed by the simple and effective method before further preparation steps.

4.2 Effect of defatting for different cycles on fat removal of STESC

Fat contents of STESC before and after defatting for various cycles are shown in Table 1. Fat content in STESC was 24.44%. Defatting for different cycles resulted in defatted STESC with variable fat levels. The efficacy of mixed solvents in fat removal from STESC increased as the defatting cycles increased ($P < 0.05$). The lowest fat content was found when defatting was carried out for 6 cycles ($P < 0.05$). Mixed solvent (hexane and isopropanol) reduced fat content by 99.63% after 6 cycles of defatting process, compared to that of initial STESC ($P < 0.05$). Normally, non-polar solvents such as hexane can penetrate to the low polar matrix to a higher extent than the polar matrix [27]. On the other hand, the polar solvents are used to extract polar components such as phospholipid and free fatty acids [27]. Therefore, the mixed solvents including hexane and isopropanol could effectively remove fat from STESC. Thus, fat could be almost completely removed by the mixed solvents with 6 cycles.

Table 1
Fat contents of skipjack tuna eyeball scleral cartilage (STESC) and STESC defatted for different cycles

Sample	Fat content (%)		
STESC	24.44	±	0.21a
DSTESC-1*	6.00	±	0.37b (75.43%)**
DSTESC-2	2.04	±	0.33c (91.65%)
DSTESC-3	1.33	±	0.28d (94.57%)
DSTESC-4	0.79	±	0.15de (96.76%)
DSTESC-5	0.54	±	0.04e (97.79%)
DSTESC-6	0.09	±	0.02f (99.63%)
Data are presented as mean ± SD (n = 3).			
*Number represents defatting cycle			
**Values in parenthesis represent the percent reduction of fat relative to the initial value. Different lowercase letters in the same column denote significant differences ($P < 0.05$).			
STESC : skipjack tuna eyeball scleral cartilage			
DSTESC : defatted skipjack tuna eyeball scleral cartilage			

4.3 Effects of enzyme concentration and hydrolysis time on protein removal

Degree of hydrolysis (DH) of protein in defatted STESC as affected by alcalase concentration and hydrolysis time is shown in Fig. 1A. DH of all samples upsurged with augmenting hydrolysis time ($P < 0.05$). The higher DH was attained with the greater alcalase concentrations ($P < 0.05$). The highest DH was observed in the sample hydrolyzed with 4.5% alcalase at all hydrolysis time, compared with other enzyme concentrations ($P < 0.05$) Idowu et al. [17] also documented that the greater amount of alcalase yielded protein hydrolysate from salmon frames (chunk and mince) with the higher DH. DH obtained using alcalase at different concentrations were augmented up to the end of enzymatic hydrolysis (180 min) ($P < 0.05$). During the first 60 min, the hydrolysis rate of all samples hydrolyzed with alcalase at different concentrations was augmented sharply, suggesting the high numbers of peptide bonds cleaved. This might be due to available protein substrates for cleavage, in which enzyme-substrate complex could be formed effectively. Thereafter, the rate of hydrolysis was slightly increased until 180 min ($P < 0.05$).

Soluble protein content (SPC) of defatted STESC hydrolyzed with alcalase at different concentrations and various times is demonstrated in Fig. 1B. SPC was markedly augmented for all alcalase concentrations used up to 30 min. Thereafter, SPC of all samples gradually increased up to 180 min ($P < 0.05$). At the same hydrolysis time, SPC increased with increasing alcalase concentrations used. However, no difference in SPC was obtained when 1.5 and 3.0% alcalase was used from 90 to 180 min ($P > 0.05$). Under hydrolysis conditions, enzymes cleaved the protein molecules to the smaller size of peptides, and eventually amino acids [28]. Idowu et al. [17] also explained that the yield of salmon frames (chunk and mince) hydrolysate was augmented up to 24.05–26.39% when higher concentrations of enzymes (alcalase or papain) and longer digesting time from 0 to 240 min were used.

Hydroxyproline content (HC) of STESC hydrolyzed with alcalase at different concentrations and various times is shown in Fig. 1C. HC of all samples was increased during 30 min of hydrolysis. Thereafter, gradual increases in HC were noticeable up to 180 min of hydrolysis. HC of sample hydrolyzed with alcalase at 1.5% was constant from 60 to 180 min ($P > 0.05$). HC was used as the index for the released collagenous proteins from STESC. Alcalase is a member of serine proteinase, which has a broad spectrum in cleavage of peptide chains. HC was increased from 4.39 to 6.09% (w/w) after digestion time (30 min) using the alcalase for removing the tissue from the catfish head [29].

Hydrolysis of STESC using 1.5% alcalase for 90 min was selected for enzymatic deproteinization process, due to the lower loss of collagen in SC, compared to the higher level of alcalase used.

4.4 Chemical compositions of SCBC and CSC powders

SCBC and CSC powders contained moisture contents of 7.43 and 0.51%, respectively. No protein was observed in CSC powder, in which protein content of 31.33% was found in SCBC. CSC powder had a higher content of ash (98.06%), compared to that of SCBC powder (60.51%). Precooked skipjack tuna bones biocalcium had protein, fat, and ash contents of 24.26, 0.21, and 72.20%, respectively [14]. With high calcination temperature, all organic compounds in SC were removed completely. For CSC, only inorganic substances were remained, as shown by high ash content (98.06%). Also, calcined powders

from the salmon frame possessed high ash content (99.73%) after being combusted in a muffle furnace for 6 h [30].

4.5 Hydroxyproline content (HC) of SCBC and CSC powders

HC of SCBC and CSC powders are shown in Table 2. HC (443.20 μg/g) was only found in SCBC powder. This might indicate the presence of collagenous components in the SCBC powder. It was also related to the protein detected in SCBC powder. To form the cup-like shape of sclera, the structure of the eyeball is composed of connective tissue and cartilage and/or bone [26]. HC represents content of the collagenous proteins localized in the biocalcium [30]. Nevertheless, the HC was absent in the CSC powder due to the high-temperature combustion of collagenous proteins during calcination. A similar result showed that HC of calcined bone of salmon frame was not detected when burned under 900°C for 6 h [30]. Therefore, collagen has still remained in SCBC.

Table 2
Chemical compositions, hydroxyproline and mineral contents, color and particle size of SCBC and CSC

Parameters	Samples	
	SCBC	CSC
MC (%)	7.43 ± 0.05a	0.51 ± 0.01b
Protein (%)**	31.33 ± 0.36a	0.00 ± 0.00b
Fat (%)**	0.10 ± 0.02a	0.00 ± 0.00b
Ash (%)**	60.51 ± 0.06b	98.06 ± 0.12a
Hydroxyproline (ug/g)**	443.20 ± 0.13a	0.00 ± 0.00b
Ca (%)**	23.28 ± 0.36b	39.90 ± 0.26a
P (%)**	10.99 ± 0.17b	18.97 ± 0.78a
Mole ratio Ca/P	1.64	1.63
*L**	81.77 ± 0.54b	87.97 ± 0.15a
*a**	2.21 ± 0.06a	0.43 ± 0.02b
*b**	20.21 ± 0.27a	2.09 ± 0.09b
Δ*E**	22.91 ± 0.06a	5.39 ± 0.17b
$D_{4/3}$ (μm)	49.91 ± 40.30a	21.15 ± 17.55a
Data are presented as mean ± SD (n = 3). Different lowercase letters in the same row denote significant differences (P < 0.05).		
SCBC : scleral cartilage biocalcium		
CSC : calcined scleral cartilage		
**Dry weight basis.		

4.6 Mineral contents of SCBC and CSC powders

The contents of Ca and P in SCBC and CSC are shown in Table 2. The higher contents of Ca and P were related with higher ash content of CSC powder when compared to those of SCBC powder. SCBC and CSC powders had Ca/P mole ratios of 1.64 and 1.63, respectively (Table 2). Ca/P mole ratios of tricalcium phosphate (TCP) $Ca_3(PO_4)_2$ and hydroxyapatite (HAP) $(Ca_{10}(PO_4)_6(OH)_2$ are 1.50 and 1.67, respectively [31]. Also, the mole ratios of Ca/P of biocalcium and calcined bone from precooked skipjack tuna bones were 1.65 and 1.62, respectively [14]. Normally, vertebrate bone contained inorganic matter, especially HAP crystals. HAP is located in bone/cartilage and cross-linked with collagen fibrils [32]. Ca/P moles ratio of both sample powders were close to that of HAP [31].

4.7 Color of SCBC and CSC powders

Color values (*L**, *a** and *b**) of the SCBC and CSC powder are varied (Table 2). SCBC powder had a yellowish white color. It had lower *L**, but higher *a**, *b** and ΔE^* than CSC powder ($P < 0.05$). The remaining protein and lipid in SCBC powder might generate Maillard reaction products via the amine group of protein and carbonyl group of the lipid oxidation products, particularly aldehydes. This was witnessed by higher *a** and *b**-values with lower *L** value. On the other hand, the organic compounds, including protein and lipid of CSC powder were completely combusted during the calcination. As a result, no reactive processes were available for Maillard reaction. Similar results in color of biocalcium powder and calcined bone powder from skipjack tuna bones were also reported [14]. Overall, CSC powder was whiter than SCBC powder.

4.8 Mean particle size ($D_{4/3}$) and particle size distribution of SCBC and CSC powders

Mean particle size ($D_{4/3}$) representing the weighted mean value by volume, of SCBC and CSC powders were 49.91 ± 40.30 and 21.15 ± 17.55 µm, respectively (Table 2). Nevertheless, no difference in $D_{4/3}$ was noticed between both samples ($P > 0.05$). Moreover, both sample powders revealed a monomodal distribution (Fig. 2A), which represents the homogenous powder. Also, biocalcium and calcined bone powders from precooked skipjack tuna bones showed no difference in mean particle size and had a monomodal distribution of particle [14]. However, the dominant size of CSC powder was smaller than SCBC powder. The existence of moisture, protein, and fat in SCBC might contribute to the stickiness and agglomeration of powders as shown by the larger dominant size [33]. Particle size is the most significant factor affecting the properties of food products, including appearance, texture, and aroma [34]. Wijayanti et al. [35, 36] reported that particle size of biocalcium from Asian seabass bone played a profound role in mouthfeel of fortified products. The mouthfeel was associated with the acceptability or rejection of consumers.

4.9 X-Ray diffractogram of SCBC and CSC powders

The X-Ray diffractograms of SCBC and CSC are shown in Fig. 2B. SCBC powders showed the diffraction peaks, following to the crystalline phase of HAP (ICDD: 01-074-4172) at angles 10.37°, 25.95°, 31.69°, 33.07°, 39.85°, 46.66°, and 64.15°. For CSC, the diffraction peaks of HAP were also detected. However, the latter was higher in crystallinity than the former. The crystallinity of SCBC and CSC was 58.59 and 89.38%, respectively. The result confirmed that higher crystallinity and phase purity of CSC were related to a well-crystallized HAP phase when calcination was applied. Broad peaks in SCBC powder sample revealed both elastic and inelastic scattering of HAP nanocrystals [37]. Crystallinity of calcined bone was also higher than biocalcium from precooked skipjack tuna bones [14].

4.10 FTIR spectra of SCBC and CSC powders

FTIR spectra of SCBC and CSC powders are depicted in Fig. 2C. The characteristic peak representing the functional or bonding of SCBC appeared at different wavenumbers.

O-H stretching vibration representing absorbed moisture was found at 3303 cm^{-1}. Symmetric stretching vibration of CH_2 at 2923 cm^{-1}; asymmetric stretching vibration of CH_2 at 2853 cm^{-1}; C = O stretching vibration of ester bond at 1740 cm^{-1}; Amide I at 1644 cm^{-1}; Amide II at 1554 cm^{-1}, and Amide III at 1240 cm^{-1} reflected the presence of protein, especially collagen. C-O stretching vibration of carbonate (CO_3) at 1445 and 1413 cm^{-1}; P-O stretching vibration of phosphate (PO_4) at 1025 cm^{-1}; C-O bending vibration of CO_3 at 875 cm^{-1}, and P-O bending vibration of PO_4 at 600 and 565 cm^{-1} indicated the presence of minerals. The dominant peaks of PO_4, which was related to HAP, were observed for both sample powders at 1025, 600,

and 565 cm^{-1}. Moreover, hydroxide ion (OH^-) located in the lattice structure of HAP was obtained at 631 cm^{-1} [38]. The higher peak intensity of PO_4 in CSC powders coincided with a higher amount of ash and P content as shown in Table 2 when compared to SCBC powder. Absorbed water and organic compounds, including protein, lipid, and carbohydrate were removed or combusted at calcination temperature. The spectrum of CSC clearly indicated the peaks of inorganic substances of PO_4 (1025, 600, and 565 cm^{-1}), CO_3 (1445, 1413, and 875 cm^{-1}) and OH^- (631 cm^{-1}).

4.11 Volatile compounds of SCBC and CSC powders

Different volatile compounds were discovered in SCBC and CSC powders from STESC (Table 3). The dominant volatile compounds in SCBC were aldehydes, followed by ketones, alcohols, and acids, respectively. These volatile compounds are mainly secondary lipid oxidation products of PUFAs. These products have been known to be the major contributor to undesirable flavors and odors [39]. Hexanal, octanal, and nonanal were the dominant aldehydes in SCBC powder. Octanal and nonanal contribute to rancidity in meat, while hexanal is mainly formed by the oxidation of linoleic acid [39]. Volatile aldehyde compounds (butanal, heptanal, and benzaldehyde) were previously found in biocalcium from precooked skipjack tuna bones [14]. Moreover, SCBC powder was also shown to have a higher abundance of volatile ketones, alcohols, and acids than CSC powder. Ketones are generated from heat or oxidative induced degradation of PUFAs, whereas alcohols are produced by the degradation of secondary fatty acid hydroperoxide or the reduction of carbonyl compounds [40]. The major ketones were 2-nananone, 2-decanon and 1-enten-2-one. For alcohols, 1-hexanol, 2-hexen-1-ol and 2-butyl-cyclohexanol constituted as major compounds. Some acids including hexanoic acid, heptanoic acid, octanoic acid, and nonanoic acid were found in only SCBC but they were not detected in CSC. The organic substances, especially PUFAs and their oxidation products, were combusted under a high temperature of calcination. Therefore, negligible volatile compounds were present in CSC powder.

Table 3
Volatile compounds of SCBC and CSC

Volatile compounds	Peak area (abundance) × 10^7	
	SCBC	CSC
Aldehydes		
Propanal	1.31	ND
Butanal	1.18	0.26
2-Butenal	2.64	ND
2-Pentenal	6.50	ND
Hexanal	12.11	ND
2-Methyl-2-pentenal	2.99	ND
n-Heptenal	7.62	1.55
Benzaldehyde	8.60	ND
Octanal	13.88	2.53
Octenal	8.67	ND
Nonanal	17.24	7.79
2-Nonenal	6.12	ND
2-Decenal	4.76	ND
2-Undecenal	3.24	ND
Ketones		
2-Propanone	0.36	ND
1-Penten-3-one	2.33	ND
3-Methyl-2-heptanone	0.56	ND
2-Nonanone	5.43	ND
Dihydro-5-pentyl-2(3H)-furanone	0.98	ND
2-Decanone	3.73	ND
Alcohols		
1-Pentanol	2.75	ND

Caption : see Table 2

Volatile compounds	Peak area (abundance) × 10^7	
	SCBC	CSC
1-Hexanol	6.26	2.97
2-Hexen-1-ol	6.99	ND
2-Butyl-cyclohexanol	7.81	ND
2-Methyl-1-hexadecanol	0.99	ND
Acids		
Hexanoic acid	3.65	ND
Heptanoic acid	3.34	ND
Octanoic acid	5.79	ND
Nonanoic acid	4.77	ND
Caption : see Table 2		

4.12 Solubility of calcium in in vitro simulated gastrointestinal tract system (GIMs)

Solubility of calcium from SCBC and CSC powders relative to the total calcium content was 2.13 ± 0.02 and 0.58 ± 0.01%, respectively (data not shown). GIMs has been used to simulate the digestive system. The result could imply that some remaining proteins in SCBC powder helped increase solubility of calcium, which was consequently absorbed into body. Precipitation in phosphate salt form was prevented by peptides [2]. Nevertheless, CSC had no proteins like collagen associated with its crystal. Thus, it could be precipitated with ease as witnessed by very low solubility. Benjakul et al. also reported higher solubility of biocalcium from both pre-cooked tongol and yellowfin tuna bones than corresponding calcined bone [41]. Moreover, biocalcium from the salmon frame also showed higher solubility than calcium carbonate [17]. In general, the absorption of calcium at the jejunum and ileum is lower than at the stomach due to the higher intestinal bicarbonate secretion, accompanied by increased pH [42]. The calcium absorption can be increased in the small intestine by peptides in proteins as a function of calcium carrier [42]. Therefore, the solubility of calcium of SCBC powder was higher than CSC powder, more likely owing to the remaining protein that could increase the absorbability of biocalcium.

5. Conclusion

Skipjack tuna eyeball scleral cartilage (STESC) could be used as a raw material for biocalcium production. Defatting using the mixture of hexane and isopropanol for 6 cycles potentially removed fat from STESC. Enzymatic deproteinization process was successfully applied to removed protein form STESC by alcalase at 1.5% for 90 min, in which collagen loss of STESC was minimized. Both SCBC and CSC powders were rich in HAP, containing calcium and phosphorus. SCBC powder containing

collagenous proteins had higher solubility of calcium in GIMS than CSC powder. Thus, biocalcium could serve as a functional ingredient, especially for bone strengthening.

Declarations

Acknowledgement

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Data Availability

All data generated or analyzed during this study are included in this published article.

Conflict of interest

Theauthors have no relevant financial or non-financial interests to disclose.

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Figures

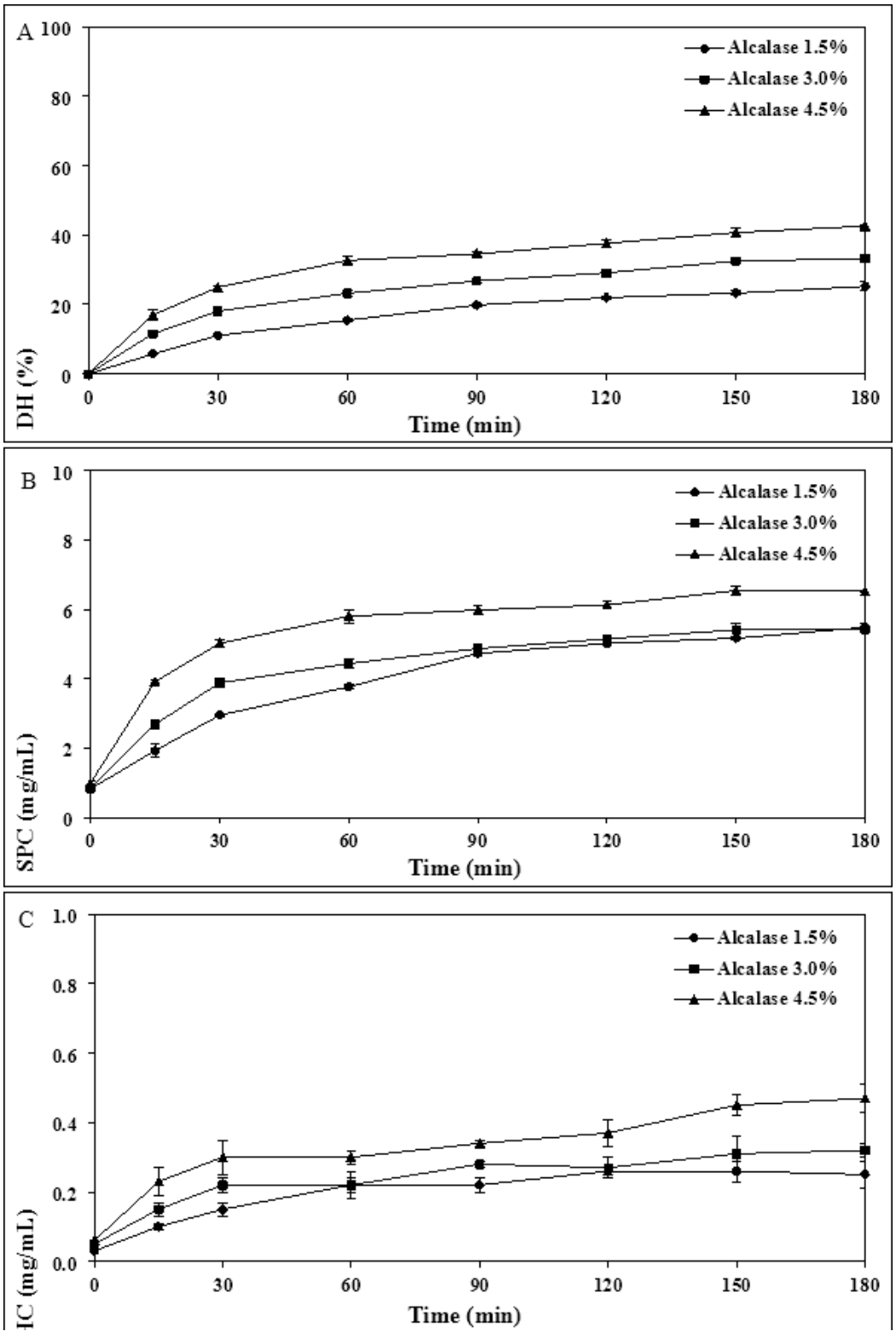
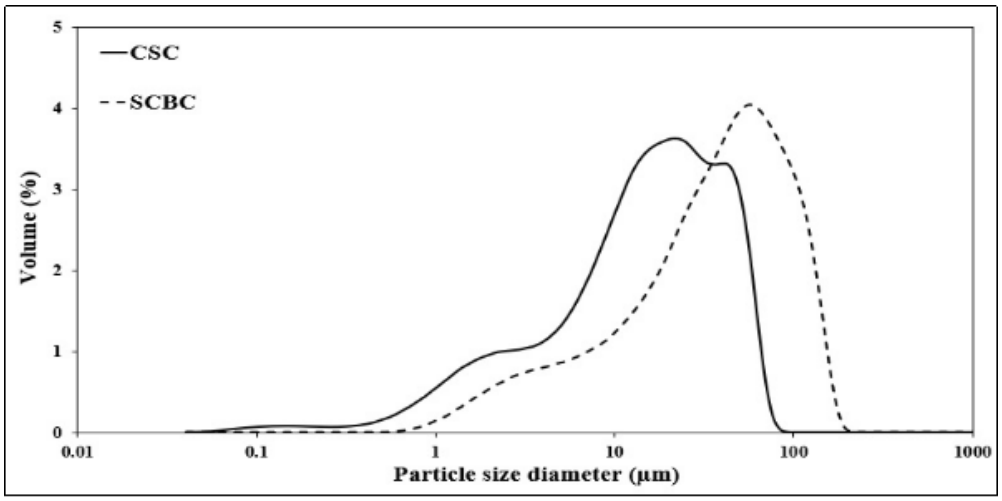
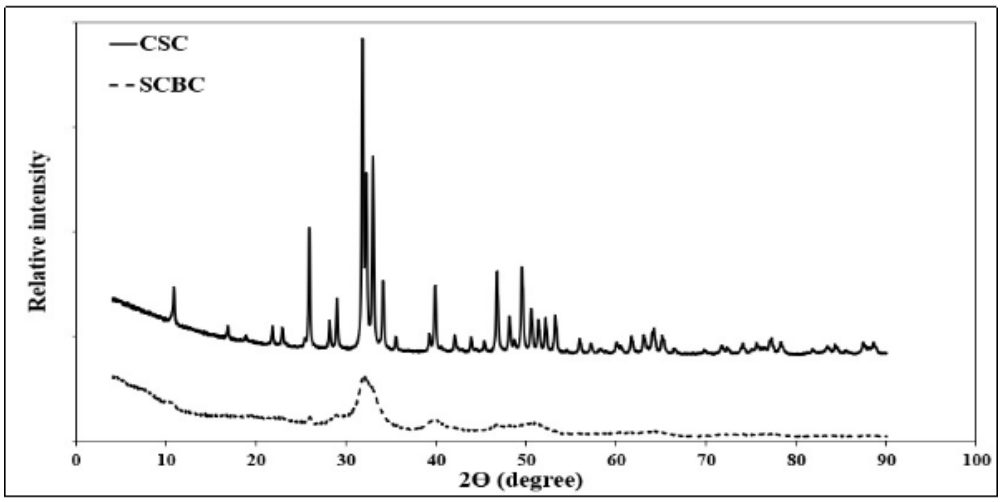


Figure 1

Degree of hydrolysis (DH) (A), soluble protein content (SPC) (B) and hydroxyproline content (HC) in hydrolysate obtained from non-collagenous protein removal process of skipjack tuna eyeball scleral cartilage using alcalase at different concentrations and times. Bars represent standard deviation (n = 3)



B



A C

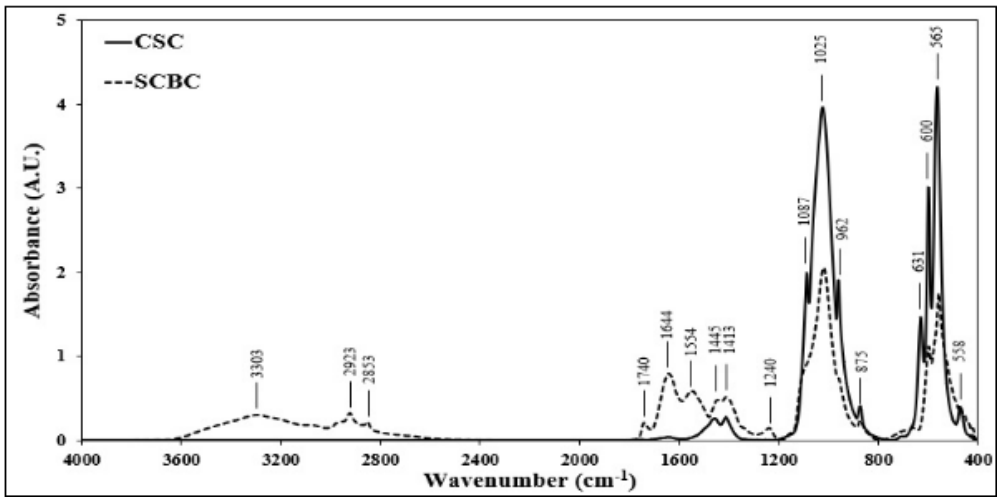


Figure 2

Particle size distribution (A), XRD diffractogram (B), and FTIR spectra (C) of scleral cartilage biocalcium (SCBC) and calcined scleral cartilage (CSC)