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The calcium-binding activity of fish scale protein hydrolysates*

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ABSTRACT

The calcium-binding activity of tilapia scale protein hydrolysates sequentially hydrolyzed by trypsin, flavor enzyme and pepsin were investigated. The hydrolysates were divided into four fractions using G-15 gel chromatography, and the F3 fraction has the higher calcium-binding activity of 196.3 mg/g. The UV-vis and the Fourier transform infrared spectroscopy (FTIR) demonstrate that the amino nitrogen atoms and the oxygen atoms belonging to the carboxylate groups are the primary binding sites for Ca^{2+}. The X-ray diffraction and scanning electron microscopy (SEM) confirmed the reaction between the peptde and calcium. The results obtained indicated that this fish scale protein hydrolysates have potential as functional foods for calcium-supplementation.

KEYWORDS

Tilapia; Fish Scale; Calcium-Binding Activity; Peptide

1. INTRODUCTION

Calcium (Ca^{2+}) is thefifth most abundant element in the earth's crust and the most abundant cation in the human [1]. The ionic form of Ca^{2+} serves as a universal intracellular messenger to modulate many processes such as neurotransmission, enzyme and hormone secretion, cell cycle regulation and programmed cell death [2,3]. The major source of calcium was provided by diet, especially milk and dairy preparations. However, calcium deficiency is widespread due to insufficient intake and diminished solubility caused by other constituents in food, like phytates, cellulose, fats, etc. [4,5]. Calcium deficiency causes hypocalcemia, bone mass loss and increment in the risk of osteoporosis development either in humans [6]. Therefore it is crucial to prevent calcium deficiency by regulating the calcium absorption and calcium solubility.

Casein phosphopeptides (CPP) enhancing the absorption of calcium has been observed by some investigators [7,8]. However, the relatively high price and lactose intolerance of CPP inevitably limits its universal application on calcium supplement. Consequently, exploiting novel calcium compositions based on other protein hydrolysates is an ideal alternative, such as hen egg yolk phosvitin, fish bone and soy protein [9-11]. Nile tilapia (*Oreochromisniloticus*) was widely distributed, and its annual aquaculture production in 2010 was 2.54 million tons according to the statistics of Food and Agriculture organization of United Nations [12]. However, the majority of the scales were dumped during the fish processing and damaged to the environment. Fish scale is rich in protein and calcium, therefore it is an ideal resource to produce calcium supplement as alternatives of CPP. The objective of this study was to evaluate calcium-binding activity of fish scale protein hydrolysates and the physical properties of peptide-calcium complex.

2. MATERIALS AND METHODS

2.1. Materials

Nile Tilapia fish scales were obtained from Shandong Meijia Group (Rizhao, China). Pepsin (porcine gastric pepsin, activity 20 units·mg^{-1} protein) was provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Trypsin (powder, porcine 1:250 > 250 USP units·mg^{-1}), Flavourzyme (from Aspergillusoryzae, >20 units·mg^{-1}), and crystalline bovine albumin, Sephadex *G*-15 were purchased from Sigma Chemical Co. (Sigma-Aldrich Inc., St. Louis, MO, USA). All other chemicals were of analytical reagent grade.

2.2. Preparation of Fish Scale Protein Hydrolysates

The fresh scale was stirred in 1.5 M NaCl solution for

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24 h and then decalcified with 0.4 M HCl. The scale was washed by deionized water (the conductivity was about 1 - 1.5 μS·cm^{-1}), then dried and smashed to powder (sieved by 80 mesh sieve). The mixture of fish scale powder and deionized water (powder:water, W:W = 1:20) was hydrolyzed by pepsin (1.5%, W/W) at pH 1.8, 40˚C for 5 h. Then 0.75% trypsin (w/w) was added for 5 h hydrolysis after pepsin was inactiviated and the pH was neutralised to 8.0. The sample was boiled for 10 min and adjusted to pH 7.0, and then flavourzyme (0.75%, w/w) was conducted further hydrolysis at 50˚C for 5 h before it was inactivated. At various time of hydrolysis, an aliquot hydrolysate was collected for calcium-binding capacity determination. The mixture and collected aliquot were centrifuged at 3000 × g for 20 min at 4˚C, filtered through 0.45 μm Millipore filters to collect soluble peptides. The peptides was freeze-dried and analyzed. The experiment was triplicated.

2.3. Isolation of Calcium-Binding Peptide by Sephadex G-15

Demineralized fish scale protein hydrolysate was loaded into Sephadex G-15 (Pharmacia, NJ, USA) on an open column (2.6 × 70 cm) at the flow rate of 0.6 mL·min^{-1}. Fractions corresponding to major peaks were collected and lyophilized immediately. All the process of chromatography was monitored at 280 nm according to the method of Jung *et al*. [13].

2.4. Preparation of Peptide-Calcium Complex

The binding reaction was performed by mixing fish scale protein hydrolysates at the concentration of 10% (w/w) with Ca^{2+} at the concentration of 60 mmol·L^{-1} under continuous stirring for 30 min at 50˚C. Thereafter, the free calcium was removed with a 100 Da molecular-weight cut-off semi-permeable membrane (Thermo Fisher Scientific Inc., Waltham, UK). The retentate was collected and lyophilized for further analysis.

2.5. Calcium-Binding Activity Analysis

Calcium-binding capacity was measured following the method reported in a previous study [14]. Demineralized samples with maximum concentration of 1000 mg·L^{-1} were mixed with 20 mM sodium phosphate buffer (pH 7.8) and 5 mM $CaCl_2$. Then the mixture was incubated at 22˚C for 30 min with continuous stirring under pH 7.8. Calcium phosphate precipitate was removed by filtrated through a 0.45 μm membrane, the calcium content of the filtrate was assayed by flame atomic absorption spectroscopy. The experiment was triplicated and means were obtained.

2.6. Fourier Transform Infrared (FTIR) and UV-Visible Spectroscopy

FTIR spectra were obtained using discs containing 0.2 mg of calcium-binding peptide or peptide-calcium complex mixed with 20 mg dried KBr powder. The spectra were recorded using an infrared spectrophotometer (Nicolet 200SXV, Thermo-Nicolet Co., Madison, WI, USA) from 4000 to 400 cm^{-1} at a data acquisition rate of 4 cm^{-1} per point. The peak signals in the spectra were analysed using Omnic 6.0 software (Thermo-Nicolet Co., Madison, WI, USA). The absorption spectra were also recorded in the 200 - 400 nm region with a Shimadzu spectrophotometer (Model UV-2550 PC) using distilled water as a reference.

2.7. X-Ray Diffraction (XRD) Analysis

XRD patterns of calcium-binding peptide and the complex were obtained using an X-ray diffractometer (Model D/MAX 2500, Rigaku International Corporation, Japan) with Cu radiation (λ = 1.54 Å) at 40 kV and 40 mA. Samples were scanned from $2\theta = 4^{\circ}$ - 90° at a scanning rate of 4˚/min. The gallery height (d-spacing distance) was determined by the peak in the XRD pattern and expressed by Bragg's equation ($\lambda = 2d\cdot\sin\theta$).

2.8. Microstructure of Calcium-Binding Peptide and the Complex

The microstructure of calcium-binding peptide and the complex were performed by a scanning electron microscope (JSM-840, JEOL Tokyo, Japan). The powder samples were sprayed and sputter-coated with gold (Sputter coater SPI-Module, PA, USA). The specimens were observed at an acceleration voltage of 25 kV.

2.9. Statistical Analysis

Experiments were conducted in triplicate. Comparison of means was performed by Duncan's test with confidence level as $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Isolation of Calcium-Binding Peptide

Tilapia scale protein hydrolyzates were separated into a Sephadex G-15 column and four major fractions (F1, F2, F3, F4) were eluted at different retention time based on their molecular weights (**Figure 1**). Of which, F3 fraction with higher calcium-binding activity of 196.3 mg/g protein was collected for further analysis (**Table 1**).

3.2. FTIR Analysis

The FTIR spectra of the calcium-binding peptide and

Table 1. The calcium-binding activity of the fractions.

Fractions	Calcium-binding activity (mg Calcium/g protein)
Crude hydrolysates	93.6 ± 3.5^{c}
F1 fractions	48.0 ± 0.2^{f}
F2 fractions	63.3 ± 1.1^{d}
F3 fractions	196.3 ± 1.8^{b}
F4 fractions	52.1 ± 1.4^{e}
Casein phosphopeptides	446.0 ± 1.2^{a}

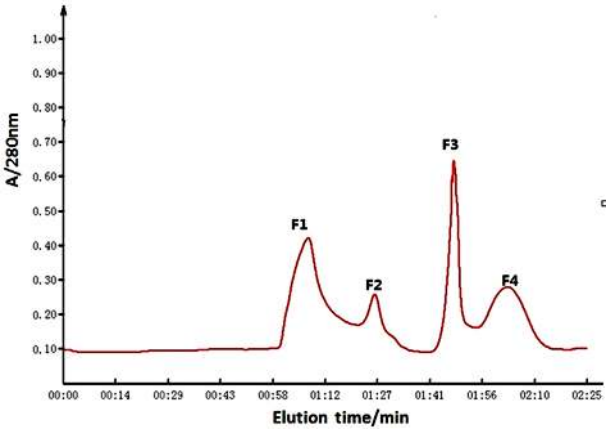

Figure 1. G-15 gel chromatography of fish scale protein hydrolysates.

the peptide-calcium complex were shown in **Figure 2**. Several absorption bands for the calcium-binding peptide in the range 525 to 849 cm^{-1} and 1034 to 1163 cm^{-1} arise from the vibration of the C-H and N-H bonds [15], and these bands were not present in the peptide-calcium complex spectra. The band (1419 cm^{-1}) for the -COO^{-} carboxylate group moved to a lower frequency (1406 cm^{-1}) in the spectrum of the peptide-calcium complex, and these vibrations were also observed by Reddy *et al*. [16] when metal ions bind to the amino acids residues in peptides, indicating the calcium binds to the fish scale peptide primarily through interactions with carboxyl oxygen and amino nitrogen atoms.

3.3. UV Scanning Analysis

As can be seen in **Figure 3**, strong absorption appeared near 200 nm and 280 nm, which could be explained by the spectral characteristics of the peptide bond and aromatic amino acids residues in the peptide [17]. In addition, the absorption intensity for the peptide-calcium complex and $CaCL_2$ is somewhat lower in the near ultraviolet region when compared with absorption intensity for the peptide alone, indicating calcium was bound by the fish scale peptide. The results were also proved by

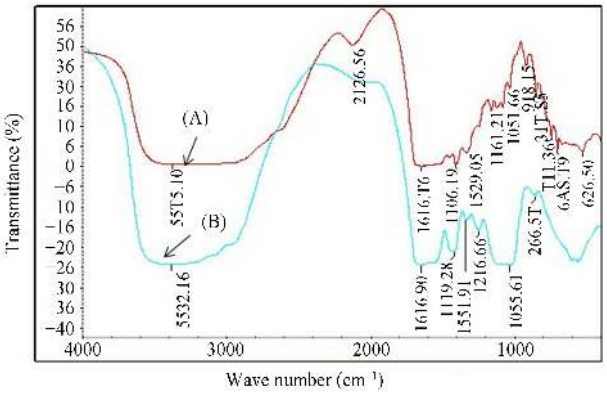

Figure 2. FTIR spectra of the calcium-binding peptide (A) (F3 Fraction) and the peptide-calcium complex (B).

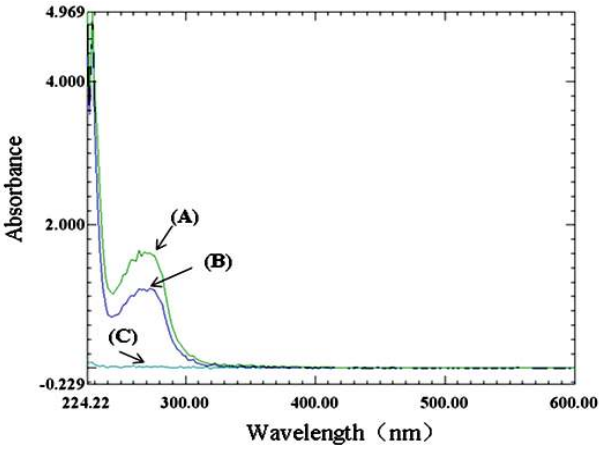

Figure 3. UV-vis absorption spectrum of the calcium-binding peptide (A) (F3 Fraction), the peptide-calcium complex (B) and $CaCL_2$(C).

Armas *et al*. [18] and Jin *et al*.[19].

3.4. X-Ray Differaction Analysis

The X-ray diffractograms of the peptide and peptide-calcium complex is shown in **Figures 4(A)** and **(B)**. The diffractogram of the peptide shows two diffraction peaks at approximately 7˚ - 8˚ and 21˚ - 23˚. The intensity of the diffraction peak at 7˚ - 8˚ in the peptide-calcium complex becomes sharp and narrow, illustrating that the presence of calcium increases the crystallinity of the complex. This phenomenon is due to the significant interaction between peptide amino acid and calcium [19].

3.5. Microstructure Analysis

The electron micrographs of peptide and peptide-calcium complex were obtained in multiples of 10,000 (**Figure 5**). The peptide shows a smooth amorphous structure, however the peptide-calcium complex shows a more fold, crystal and multi-branched structure than that of the peptide. This result could be due to the interaction between

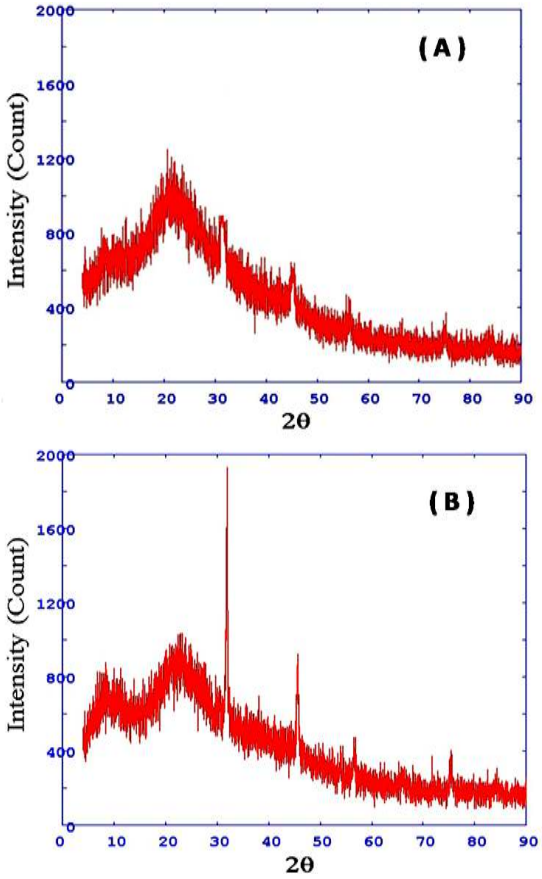

Figure 4. X-ray differaction of the calcium-binding peptide (A) (F3 Fraction) and the peptide-calcium complex (B).

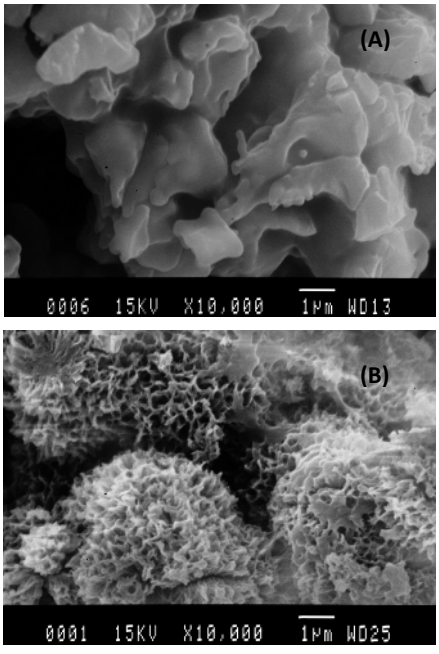

Figure 5. Microstructure of the calcium-binding peptide (A) (F3 Fraction) and the peptide-calcium complex (B).

the peptide and the calcium, and this might be associated with the sharper and narrower diffractograms of peptide-calcium complex when calcium was added (**Figure 4**).

4. CONCLUSION

The results of this study showed that enzymatic hydrolysis was the effective way to recover the calcium-binding peptide form fish scales. The F3 fraction separated by Sephadex G-15 chromatography possessed higher calcium-binding activity. The FTIR spectrum of the peptide-calcium complex indicated that the calcium interacts with the fish scale peptide, mostly via amino nitrogen atoms and the oxygen atoms of carboxylate groups. The UV-vis spectra, X-ray diffractogram and the microstructure of the peptide-calcium complex confirmed the changes of the peptide after adding the calcium, indicating the interaction between the peptide and calcium. These results indicating fish scale protein hydrolysates possess the huge potential to be an alternative for CPP as calcium supplement.

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