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Purification and characterization of caclium-binding soybean protein hydrolysates by Ca^{2+}/Fe^{3+} immobilized metal affinity chromatography (IMAC)

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ABSTRACT

Soybean protein hydrolysates (SPHs) can bind calcium in order to form soluble peptide-calcium complexes. However, amino acid composition and structural characteristics of the calcium chelating SPHs are still unclear. This study separated SPHs with calcium and iron immobilized metal affinity chromatography (IMAC), and examined the effects of SPHs with different amino acid composition on calcium binding capacity. Three fractions (F_{Fe-1}, F_{Fe-2} and F_{Fe-3}) isolated with IMAC-Fe^{3+} were shown possessing increased Glu, Gln, Lys and Pro content from F_{Fe-1} to F_{Fe-3}, and improved amount of bound calcium. Furthermore, the fractions adsorbed on IMAC-Ca^{2+} (Fe^{3+}) were separated and identified with reverse-phase (RP)-HPLC and MALDI-TOF MS/MS. The results showed that the sequence of peptides from F_{Ca-2} and F_{Fe-3} fractions was DEGEQPRPFPFP.

1. Introduction

Calcium is known as the most abundant inorganic element in the human body. Insufficient calcium uptake results in certain diseases, such as rickets in children and osteoporosis in the elderly. Various dietary components influence calcium absorption. Many animal proteins enhance calcium absorption because of the presence of special amino acids in peptides from proteolytic digestion, such as casein phosphopeptides (CPPs) and hen egg yolk phosvitin phosphopeptides (Jiang & Mine, 2000; Sato, Noguchi, & Naito, 1986). They can form soluble complexes with calcium and enhance its bioavailability. As to soybean protein, it is reported acting prominently as a calcium absorption inhibitor because of phytic acid (Heaney, Weaver, & Fitzsimmons, 1991). Meanwhile, soybean protein is an important food protein and contains a high amount of Glu and Asp possessing calcium affinity (Kumagai, Shizawa, Sakurai, & Kumagai, 1998). It was found that phytate-removed deamidated soybean globulin and its hydrolysates can enhance calcium absorption in the small intestine of rats (Kumagai et al., 2004). In the same manner, soybean protein hydrolysates (SPHs) were reported to promote calcium uptake by Caco-2 cells (Lv, Bao, Yang, Ren, & Guo, 2008).

Thus, in order to investigate the role of SPHs on calcium absorption and commercialize the production of SPHs, previous works in our laboratory have already used protease M and deamidase to prepare SPHs and established that the peptides could bind calcium in order to form soluble SPH-calcium complexes (Bao, Lv, Yang, Ren, & Guo, 2008). However, the characterization of amino acid composition and structural properties of calcium binding peptides are still unclear.

Immobilized metal affinity chromatography (IMAC) is a widely used method to isolate metal binding proteins or peptides (Porath & Olin, 1983; Storcksdieck, Bonsmann, & Hurrell, 2007). Here iron and calcium chelating IMAC was respectively used to isolate the calcium binding soybean peptides. Iron was also used as a ligand in an earlier study to purify water-soluble phosphopeptides from cheese; and peptides carrying different content of phosphorylated Ser residues were separated (Lund & Ardö, 2004).

The objective of this study was to use IMAC, reversed-phase (RP)-HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer/mass spectrometer (MALDI-TOF MS/MS) to characterize the calcium binding soybean peptides.

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Also, the relationship between amino acid composition and calcium binding abilities of SPHs was evaluated.

2. Materials and methods

2.1. Material

Defatted soybean flakes were gifts from Yihai Liangyou Co. Ltd. (Qinhuangdao, China). Protease M (product number, PRH1250748MSD; enzyme activity, 5,500 u/g) and deamidase (glutaminase, product number, P8HC161; enzyme activity, 100 u/g) were purchased from Amano Enzyme Co. Ltd. (Nagoya, Japan), Ultrafiltration membranes of molecular weight cut-offs (MWCO) of 3 and 10 kDa were products of Zhongke Membrane Technology Co. Ltd. (Beijing, China). Sepharose 6B was purchased from Pharmacia (Uppsala, Sweden). Iminodiacetic acid (IDA)-Sepharose 6B was prepared according to a previously described method (Porath & Olin, 1983). All other chemicals used were of analytical reagent grade.

2.2. Preparation of SPHs

Soybean protein isolates (SPIs) were prepared as described previously with some modifications (Sorgentini, Wagner, & Añón, 1995). SPIs were prepared from defatted soybean flakes by extraction for 1.5 h at room temperature with water adjusted to pH 8.0 with 2 mol/L NaOH (flour/water = 1:l0; w/v) and then centrifuged at 3,000*g* for 20 min. After that, the supernatant was adjusted to pH 4.5 with 2 mol/L HC1, kept for 0.5 h at room temperature, and then centrifuged (3,000*g*, 20 min). The pellet was washed with water twice to remove the soluble residues at pH 4.5, and then water was added. This dispersion was named SPIs. In order to prepare SPHs, the pH of the SPI dispersion was adjusted to pH 3.0 and denatured for 10 min in boiling water. After that, protease M was added (protein content of 20 g/L, E/S = 1:100; w/w). Enzymatic hydrolysis was then performed at 50 °C for 60 min. Following a digestion process, the dispersions were heated in boiling water for 5 min to deactivate protease M, cooled to room temperature, and afterward neutralized to pH 7.4. Subsequently, the dispersions were centrifuged at 3,000*g* for 20 min, and then glutaminase (Amano Enzyme Co. Ltd., Nagoya, Japan) was added to the resultant supernatants (*E*/*S* = 1:50; w/w) to hydrolyze at pH 7.0 in 50 °C for 180 min. After hydrolysis, the dispersions were heated in boiling water for 5 min to deactivate deamidase. Then they were cooled to room temperature and centrifuged (3,000*g*, 20 min). The supernatants were afterwards ultrafiltrated through membrane with molecular weight cutoff (MWCO) of 10 kDa using a stirred ultrafiltration cell (Millipore, Billerica, MA, USA). The permeate of peptides with molecular weight <10 kDa was collected and then ultrafiltrated with membrane of 3 kDa. After that, the retentate of peptides with molecular weight between 3 and 10 kDa was collected and diluted with distilled water, and subsequently ultrafiltrated with membrane of 3 kDa again. This process was repeated twice to remove the SPHs with molecular weight lower than that of 3 kDa. The retentate was finally collected and lyophilized. It was named as SPHs (3–10 kDa). The ultrafiltration was carried out at 4 °C.

2.3. Separation of SPHs with IMAC

IDA-Sepharose 6B (25 mL) was incubated with 30 mL of 0.2 mol/L $CaCl_2$ ($FeCl_3$) solution for 1 h. The gel was then washed with 5–6 bed volumes of distilled water. After that, 50 mmol/L pH 4.0 sodium acetic acid buffers were used to remove any loosely bound calcium (iron). The immobilized calcium (iron) column was then washed with 5–6 bed volumes of equilibrating buffer (50 mmol/L sodium acetate-acetic acid, 0.1 mol/L NaCl, pH 5.5). Twenty mg SPHs (3–10 kDa) was then loaded on the IMAC-Ca^{2+} (Fe^{3+}) column (26 × 100 mm) and washed with the equilibrating buffer. The collected peak was labeled as non-adsorbed fraction. After that, the bound peptides were eluted with gradient elution buffer: 0–0.02 mol/L Na_2HPO_4 containing 0.1 mol/L NaCl and 0.01% ammonium acetate (pH 8.1). The absorbance of the eluate was monitored at 210 nm. Regeneration of the column was achieved by washing the column with 50 mmol/L ethylenediaminetetraacetic acid (EDTA) solution (pH 4.5). Those fractions collected from the affinity column were concentrated at 4 °C using a stirred ultrafiltration cell and a 1 kDa membrane (Millipore, Billerica, MA, USA). The retentates were lyophylized and stored at −80 °C.

2.4. Amino acid composition analysis

The complete amino acid composition of peptide was determined as follows: a 1 mg portion of each sample was hydrolyzed with 6 mol/L hydrochloric acid at 110 °C for 20 h under vacuum. After that, the hydrolysates were evaporated to dryness under vacuum. The dried sample was dissolved in 200 μL of borax buffer and then was centrifuged. The resultant supernatant (100 μL) was loaded on an Agilent 1100 high performance liquid chromatograph with Zorbax Eclipse AAA column (4.6 × 150 mm, 3.5 μm) (Agilent Technologies, Palo Alto, CA, USA) at 40 °C with o-phthalaldehyde/3-mercaptopropionate/9-fluorenylmethylchloroformate pre-column derivatization and diode array detector (Evans et al., 2007).

2.5. Identification of Peptides

The fractions collected from the IMAC-Ca^{2+} (Fe^{3+}) column were applied to RP-HPLC on a protein and peptide Zorbax SB-C18 column (9.4 × 150 mm, 5 μm) (Agilent Technologies) The column was equilibrated using water with 0.1% trifluoroacetic acid (TFA) at flow rate of 1 mL/min. The linear gradient from 0–60% acetonitrile (ACN) (in 0.1% TFA) in 60 min was applied. Elution was monitored at 214 nm. The collected peaks were lyophilized. The amino acid sequence of the peptides was analyzed with a Bruker UltraflexTM III MALDI-TOF MS/MS (Bruker Daltonics, Bremen, Germany), which is operated in reflectron mode with 20 kV accelerating voltage and 23 kV reflecting voltage. A saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA was used as the matrix. One μL of the matrix solution and sample solution at a ratio of 1:1 (v/v) were applied onto the Score 384 target well. The SNAP algorithm (signal to noise ratio threshold, 5; quality factor threshold, 30) in Flex Analysis TM 3.4 was used to pick up the most prominent peaks in the mass range *m*/*z* 700–4000. The subsequent MS/MS analysis was performed in a data-dependent manner. The Mascot search engine uses mass spectrometry data to identify peptides from primary sequence databases. The program Mascot 2.1 developed by Matrix Science Ltd. (http:\\www.matrixscience.com) licensed in house <http://www.proteomics.cn> was used. Only peptides which were identified by Mascot ion scores that indicated identity or extensive homology with $p < 0.05$ were regarded as valid. For unambiguous identification of peptides, more than five peptides must be matched and the sequence coverage must be greater than 15%.

2.6. Calcium binding determination

A 10 mg portion of each lyophilized hydrolysates was dissolved in 1.5 mL of 0.02 mol/L Tris–HCl (pH 7.4), followed by a 10 min incubation at 37 °C to ensure full dissolution. After adding 1 mL of 0.06 mol/L $CaCl_2$, the solution was incubated for 30 min and