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Soybean peptide aggregates improved calcium binding capacity

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ABSTRACT

Soybean peptides are believed to have calcium binding sites as Asp and Glu on side chains. However, the effects of peptide aggregation on calcium binding capacity remain unknown. Peptides with different calcium binding capacities (F1 and F2) were separated in this study by calcium immobilized metal affinity chromatography. The calcium binding capacities and apparent molecular weight distributions of F1 and F2 were analyzed before and after the denaturation agents removal. Results showed that F2 with higher calcium binding capacity was more prone to aggregate than F1. The calcium binding capacity of both F1 and F2 decreased after urea or urea and 0.5% SDS treatments. The removal of denaturation agents resulted in peptide reaggregation and recovery of the calcium binding capacity of F2; no significant changes were found for F1. All the findings implied that aggregation of soybean peptides played an important role in calcium binding.

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1. Introduction

Calcium is the most abundant divalent cation in the human body. Insufficient calcium uptake results in certain diseases, such as rickets in children and osteoporosis in the elderly (Daengprok & Garnjanagoonchorn & Mine, 2003; Kennefik & Cashman, 2000). Preventing the precipitation of calcium through the use of certain substances can be effective to increase calcium adsorption in the body (Sato, Shindo, Gunshin, & Noguchi, 1991). Many protein hydrolysates, such as casein phosphopeptides (CPPs) and hen egg yolk phosvitin phosphopeptides, could form soluble complexes with calcium, which enhance its bioavailability (Jiang & Mine, 2000; Heaney, Weaver, & Fitzsimmons, 1991). Meanwhile, in soybean protein, deamidated soybean globulin with phytate removal and its hydrolysates could enhance calcium absorption in the small intestine of rats (Heaney et al., 1991). In the same manner, soybean protein hydrolysates (SPHs) were reported to promote calcium uptake by Caco-2 cells (Lv, Bao, Yang, Ren, & Guo, 2008). A recent study suggested that the SPHs–calcium complexes might have positive effects on bone accretion of fast growing animals (Lv, Liu, Ren, Li, & Guo, 2013).

To investigate the role of SPHs in calcium absorption and to commercialize the production of SPHs, previous works in our laboratory established that peptides could bind with calcium to form soluble SPH-calcium complexes (Bao, Lv, & Yang, 2008). The amount of bound calcium was correlated with the carboxyl group of Glu and Asp in SPHs. Moreover, primary structure results showed that peptides with high calcium binding capacity possessed Glu cluster structures (Lv, Bao, Liu, Ren, & Guo, 2013). These results indicated that the calcium binding sites of soybean peptide were carboxyl groups from side chains as in Concanavalin A and Prothrombin, instead of phosphoseryl groups that were easily found in CPPs (Hardman, Agarwal, & Freiser, 1982; Nelsestuen, 1976; Ferraretto, Signorile, & Gravaghi, 2003). In addition, these calcium binding sites usually existed in “loop” structures, such as E-F hand motif (helix-loop-helix structure) and C2 domains (Moncrief, Kretsinger, & Goodman, 1990; Zhou, Yang, & Kirberger, 2006; Sutton, Davletov, & Berghuis, 1995; Grobler, Essen, & Williams, 1996). However, the need for space structures during calcium binding remains unclear.

Peptides are hydrolysates of protein with molecular weights much smaller than those of proteins. However, peptide aggregations with high molecular weight would occur when preparing peptides with casein, lactoalbumin, or soybean protein (Creusot & Gruppen, 2007; Kuipers, Alting, & Gruppen, 2007; Nagai & Inouye, 2004; Otte, Lomholt, & Halkier, 2002). Some studies reported that calcium–peptide complexes possessed a high apparent

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molecular weight (Kretsinger, 1976). In this study, peptides with different calcium binding capacities were prepared, and the effects of their aggregation states on calcium binding were analyzed. This study aimed to understand the formation mechanism of a steady-state structure for the calcium binding of soybean peptides.

2. Material and methods

2.1. Materials

Defatted soybean meal provided by Harbin high tech (group) Co. Ltd (Harbin, China) was utilized. Protease M and Deamidase (Daiwa SD-C100S) were purchased from Amano Enzyme Co. Ltd (Nagoya, Japan). The Sepharose 6B was purchased from Pharmacia (Uppsala, Sweden). Iminodiacetic acid (IDA) was purchased from Sigma (St. Louis, MO, USA). The IDA-Sepharose 6B was prepared according to the method of Porath and Olin (Porath & Olin, 1983). All other chemicals were of analytical reagent grade.

2.2. Preparation of soybean protein hydrolysates (SPHs)

Soybean protein isolates (SPIs) were extracted as described by Lv (2009) without modification while soybean protein hydrolysates (SPHs) were further prepared according to procedure described by Bao (2008) with protease M and deamidase. The SPH solution was ultrafiltrated with membranes of molecular weight cut offs (MWCO) of 10 and 30 kDa at 4 °C. The fraction of SPH (10–30 kDa) was lyophilized.

2.3. SPH (10 kDa–30 kDa) separation by immobilized metal affinity chromatography (IMAC)

The column was packed with IDA-Sepharose 6B (50 mL) and then incubated with 0.2 mol/L $CaCl_2$ (80 mL) for calcium chelating. After washing the column with distilled water for 8 to 9 bed volumes to remove the unbound calcium, the nonspecific bound calcium was eluted by 0.05 mol/L acetic acid (HAc) buffer of pH 4.0 for 6 to 7 bed volumes. The column was then equilibrated with 0.05 mol/L NaAc-HAc buffer of pH 5.5 containing 0.1 mol/L NaCl. Then 1 mL SPH (10 kDa–30 kDa) solution (25 mg/mL) was loaded into the column, followed by elution procedure to acquire peptide fractions F1 and F2 (Lv et al., 2013b; Liu, Bao, Lv, Xu, & Guo, 2012).

2.4. Preparation of samples treated by denaturation agents

Peptides were dissolved in pH 7.4 Tris–HCl at a concentration of 10 mg/mL, and then they were treated by 6 M urea or 6 M urea and 0.5% SDS. After which the treated solution was transferred to a dialysis bag (MWCO: 500 Da, 12.5 cm × 1 cm) and dialyzed at 4 °C in denaturation agents with concentrations identical to those above. The solution outside the dialysis bag was changed every 4 h for 48 h when the dialysis was completed. The samples were reserved for the determination of calcium binding capacity and apparent molecular weight distribution.

2.5. Preparation of samples with denaturation agents removed

The peptide solution treated by 6 M urea or 6 M urea and 0.5% SDS as described in section 2.4 was transferred to a dialysis bag (MWCO: 500 Da, 12.5 cm × 1 cm) and dialyzed at 4 °C in pH 7.4 Tris–HCl to remove the denaturation agents. The solution outside the dialysis bag was changed every 4 h for 48 h when the dialysis was completed. The samples were reserved for the determination of calcium binding capacity and apparent molecular weight distribution.

2.6. Air-acetylene flame atomic absorption spectrometry to determine calcium binding capacity

Air-acetylene flame atomic absorption spectrometry (TAS-986, PuXiTongYong Co.) was used to determine the calcium contents of all the samples. The operating parameters were as follows: Current, 9.0 mA; Wavelength, 422.7 nm; Slit, 2.4 nm; Air flow, 15 min/L; Acetylene gas flow, 2.4 min/L (Liu et al., 2012).

2.7. Size exclusion (SE)-HPLC to determine apparent molecular weight distribution

The peptide samples were applied to SE-HPLC with a Waters Protein Pak-60 column (7.8 mm × 300 mm, bead size 10 μm, fractionation range 1–20 kDa). The column was equilibrated, and eluted with 30 mmol/L Tris–HCl, pH 7.4, at a 0.5 mL/min flow rate, and monitored at 214 nm. Trypsin inhibitor (20.1 kDa), lysozyme (14.4 kDa), ABI-80 (7.823 kDa), ABI-81 (5.856 kDa), and ABI-95 (3.313 kDa) (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences, Shanghai, China) were used as standard. A linear dependency of log molecular weight versus retention time (RT) was obtained.

2.8. MALDI-TOF MS/MS profile of the peptide separated by reversed-phase (RP)-HPLC

The peptide fraction collected from the IMAC-Ca^{2+} column was applied to RP-HPLC on a protein and peptide Zorbax SB-C18 column (9.4 mm × 150 mm, 5 μm) from Agilent Technologies. The column was equilibrated by 0.1% trifluoroacetic acid (TFA) solution at a flow rate of 1 mL/min. The linear gradient of 80% acetonitrile solution prepared by 0.1% TFA was applied from 0% to 55%. Elution was monitored at 214 nm. The collected fractions of peptide (F2) were lyophilized and then analyzed with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer/mass spectrometer (MALDI-TOF MS/MS; Traflex III TOF/TOF, Bruker, Germany). which is operated in reflectron mode with 20 kV accelerating voltage and 23 kV reflecting voltage. A saturated solution of a-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA was used as the matrix.

2.9. 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 lm) (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).

2.10. Molecular modeling

Molecular modeling was completed on pep-fold server and the peptide sequence used was obtained from F2 (http://bioserv.rpbs.univ-paris-diderot.fr/PEP-FOLD/). The peptide sequence was DEGEQPRPFPFP and the figure was exported by Pymol.

2.11. Statistical analysis

The means and standard deviations for each treatment group

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