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Isolation of a novel calcium-binding peptide from wheat germ protein hydrolysates and the prediction for its mechanism of combination

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ABSTRACT

To isolate a novel peptide with specific calcium-binding capacity, wheat germ protein was hydrolyzed. The hydrolysates were purified using ultrafiltration, anion-exchange chromatography, gel filtration chromatography, and reversed-phase high performance liquid chromatography. The amino acid sequence of the purified peptide was determined and confirmed to be FVDVT (Phe-Val-Asp-Val-Thr). The calcium-binding capacity of FVDVT reached 89.94 ± 0.75%, increased by 86.37% compared to the hydrolysates. The chelating mechanism between FVDVT and calcium was further investigated by Ultraviolet–Visible absorption spectroscopy, Fourier transform infrared spectroscopy, X-ray diffraction, and ^{1}H nuclear magnetic resonances spectroscopy. The results indicated that the oxygen atoms of the carboxy group and the nitrogen atoms of the amido group provided major binding sites. In addition, aspartic acid and threonine show considerable capacity for incorporating with calcium by donating electron pairs. This study provides a feasible approach to isolate calcium-binding peptides and to clarify the possible binding mechanism of calcium and peptide.

1. Introduction

Calcium is an essential mineral nutrient for humans and is closely related with intracellular metabolism, nerve conduction, bone growth, muscle contraction and cardiac function (Bass & Chan, 2006). However, inadequate calcium intake or absorption can lead to numerous diseases including osteoporosis, hypertension, colon cancer, obesity, and kidney stones (Singh & Muthukumarappan, 2008). Children, pregnant females, and the elderly are particularly affected. To overcome calcium deficiency, calcium-fortified products and calcium supplements have been commercially developed. Inorganic calcium, such as calcium carbonate and calcium chloride, is the first generation of calcium supplements. These calcium supplements are prone to the formation of calcium phosphate deposits during gastrointestinal digestion, resulting in the poor absorption and bioavailability of calcium (Bronner & Pansu, 1999). In addition, calcium carbonate may lead to intestinal side effects such as flatulence and bloating. Organic calcium, including calcium lactate and calcium gluconate, is the second generation of calcium supplements. These calcium supplements have poor therapeutic effects in clinical practice, which is attributed to their low bioavailability and low concentration of calcium. Amino acid calcium complexes are the third generation of calcium supplements and have better stability, higher bioavailability, and stronger *anti*-interference compared with the other calcium supplements (Rosado, Muñoz, López, & Allen, 1993). However, amino acid calcium complexes are gradually replaced due to their high cost and tendency to provoke fat oxidation (Guo et al., 2014). Peptide-calcium chelate can probably be a suitable alternative to facilitate and enhance calcium bioavailability. The small peptides have many merits, such as consuming little energy and accelerating transport speed, as well as being carriers that are not readily saturated in comparison to amino acids (Rerat, Nunes, Mendy, & Roger, 1988). To date, these peptides have been isolated and characterized from a variety of sources such as soybean (Bao, Lv, Yang, Ren, & Guo, 2008), shrimp (Huang, Ren, & Jiang, 2011), pollack backbone (Jung et al., 2006), hoki frame (Jung & Kim, 2007), porcine blood plasma and tilapia (Charoenphun, Cheirsilp, Sirinupong, & Youravong, 2013).

Wheat germ, a by-product of the wheat flour processing industry, is an excellent source of protein. Compared with other vegetable proteins, wheat germ protein is rich in the essential amino

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acids, especially lysine, methionine and threonine (Zhu, Zhou, & Qian, 2006a, 2006b). Enzymatic modification can be an effective means to produce biological active peptides possessing antimicrobial, antihypertensive (Zhou et al., 2013), antioxidant (Zhu et al., 2006a, 2006b) and mineral-binding activities. Compared to other bioactivities, the mineral-binding activity is associated with the amino acids in the peptide sequence and peptide molecular weight. It has been reported that wheat germ polypeptides have considerable capacity for incorporating with zinc (Zhu, Wang, & Guo, 2015), and calcium (Liu, Wang, Wang, & Chen, 2013). However, studies of the purification of specific calcium-binding peptide derived from wheat germ protein hydrolysates and the possible chelating mechanism are scarcely reported.

The purpose of this study was to isolate and identify a novel calcium-chelating peptide from wheat germ protein hydrolysates using ultrafiltration, anion exchange chromatography, gel filtration chromatography, and liquid chromatography electrospray ionization/mass spectrometry (LC-ESI/MS). Moreover, the possible chelating mode was also investigated. This finding would be of significance in utilizing the hydrolyzed peptides as calcium-binding peptide ingredients in functional foods.

2. Materials and methods

2.1. Materials

Defatted wheat germ was purchased from the Anyang Mantianxue Food Co., Ltd. (Henan, China). Alcalase 2.4 L (2.4 AU/g) was obtained from Novozymes (Bagsvaerd, Denmark). All other reagents were analytical grade products.

2.2. Extraction and hydrolysis of wheat germ protein

Wheat germ protein was extracted by dissolving the defatted wheat germ flour in distilled water (1:10, w/v). The solution was adjusted to pH 9.5 and agitated for 120 min at 40 °C. The supernatant resulting from centrifugation at 4472*g* for 15 min was adjusted to pH 4.0 to precipitate the protein. The precipitate was centrifuged again and washed with distilled water. The protein was then adjusted to pH 7.0 and freeze-dried for further research.

Wheat germ protein was hydrolyzed with alcalase following Liu's description (Liu et al., 2013). Hydrolysis parameters were as follows: protein concentration 5% (protein/deionized water, w/v), enzyme/-substrate ratio of 1:50 (w/w) at pH 8.0, 50 °C; hydrolysis for 60 min, 120 min, 180 min, 240 min, and 300 min. The alcalase was then inactivated by heating at 100 °C for 10 min, and the pH was neutralized to 7.0. The mixture was subsequently centrifuged at 4472*g* for 10 min, and the supernatant was freeze-dried as wheat germ protein hydrolysates (WGPHs). Calcium-binding capacities and molecular weights of WGPHs at different hydrolysis times (60 min, 120 min, 180 min, 240 min, and 300 min) were determined.

The degree of hydrolysis (DH) was defined as follows:

$$DH(\%) = \frac{BN_b}{\alpha M_p h_{tot}} \times 100 \quad (1)$$

where B and N_b respectively represent the amount of NaOH consumed during the proteolysis and its normality, α is the average degree of dissociation of the α-NH2 groups released during hydrolysis, M_p is the mass (g) of the protein (N × 6.25), and h_{tot} is the total number of peptide bonds available for proteolytic hydrolysis (8.3 m equiv./g).

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \quad (2)$$

where pH and pK are the values at which the proteolysis was conducted.

The molecular masses of hydrolysates were determined using a TSK gel G2000 SWXL 7.8 × 300 mm column (Tosoh, Tokyo, Japan) with an Agilent 1100 HPLC system. The process was carried out with the mobile phase (acetonitrile/water = 30:70 v/v) at a flow rate of 0.5 mL/min and monitored at 220 nm.

2.3. Calcium-binding capacity determination

Lyophilized peptides were dissolved in 0.02 mol/L Tris-HCl (pH 7.8) to a final concentration of 5.0 mg/mL, followed by a 10 min incubation to ensure full dissolution. The solution was mixed with 50 mM $CaCl_2$ and stirred at 40 °C for 60 min. After the chelating reaction, the mixture was added with absolute ethanol (9 times volume of the solution) for separating peptide-calcium chelate from free calcium due to their different solubility. Then, the mixture was centrifuged at 10000*g* at 4 °C for 15 min and washed with ethanol twice. The content of bound calcium and total calcium was assayed by atomic absorption spectrophotometer (Spectr AA-220/220Z, VARIAN Co., CA, USA) after mixed-acid digestion (HNO_3:$HClO_4$, 4:1, v/v).

$$\text{Calciumbinding capacity}(\%) = \frac{\text{amount of chelated calcium(mg)}}{\text{total amount of calcium(mg)}} \times 100 \quad (3)$$

2.4. Ultrafiltration

The initial solution was prepared by mixing the hydrolysates powder with pure water to a concentration of 15 mg/mL. The solution was first submitted to an ultrafiltration system with molecular weight cutoff (MWCO) of 3 kDa membranes. At the end of the concentration process, the permeate (<3 kDa) and the retentate (>3 kDa) were recovered. The retentate was collected as P3 (>3 kDa) for analysis. The remaining permeate constituted the filtered solution and then was passed through the 1 kDa membrane. The permeate (<1 kDa) and the retentate (1–3 kDa) were obtained and collected as P1 (<1 kDa) and P2 (1–3 kDa). The calcium-binding capacities of P1, P2, and P3 were determined.

2.5. Anion-Exchange chromatography

The lyophilized hydrolysates (P1 and P2) from ultrafiltration were dissolved in 20 mM Tris-HCl buffer (pH 7.8) and then passed through 0.45 μm filter film. The solution was loaded on the HiPrep DEAE-FF 16/10 column (1.6 × 10 cm) after the column was equilibrated with 20 mM Tris-HCl buffer (as equilibrating buffer, pH 7.8). The peak was labeled as the loosely adsorbed fraction by washing with the equilibrating buffer. Afterward, the bound peptides were eluted by gradient elution with equilibrating buffer containing 0–0.5 mol/L NaCl. The elution was at flow rate of 5 mL/min and monitored at 214 nm using an AKTA liquid chromatography system. All peak fractions were collected and dialyzed using a dialysis membrane (100 Da, Spectrum Laboratories, Inc., USA) for 48 h to remove the NaCl. The calcium-binding capacities of all fractions were determined.

2.6. Superdex peptide gel filtration chromatography

The fraction with the highest calcium-binding capacity obtained from DEAE column was collected and loaded onto a Superdex Peptide 10/300 GL column (1 × 30 cm, GE Healthcare Co., Sweden). Deionized water was used as the mobile phase with a flow rate of 0.5 mL/min. The elution was monitored at 214 nm using an AKTA liquid chromatography system. After the calcium-