Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jff

A specific peptide with calcium chelating capacity isolated from whey protein hydrolysate

Lina Zhao [a,b,☆], *Shunli Huang* [a,☆], *Xixi Cai* [a], *Jing Hong* [a], *Shaoyun Wang* [a,*]

[a] *College of Bioscience and Biotechnology, Fuzhou University, Fuzhou 350002, China*
[b] *College of Food Science, Fujian Agriculture and Forestry University, Fuzhou 350002, China*

ARTICLE INFO

Article history:
Received 21 February 2014
Received in revised form 14 May 2014
Accepted 16 May 2014
Available online

Keywords:
Whey protein hydrolysate
Calcium-chelating peptide
Purification
Structural property

ABSTRACT

A specific peptide displaying calcium-binding capacity was purified from whey protein hydrolysate. The isolation procedures included DEAE anion-exchange chromatography, Sephadex G-25 gel filtration, and reversed-phase high-performance liquid chromatography (RP-HPLC). The amino acid sequence of the peptide was determined to be Phe-Asp (FD), using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS). The calcium binding capacity of FD reached 73.34 μg/mg, and the amount increased by 116% when compared to the whey protein hydrolysate complex. The structural properties of the purified peptide were identified using fluorescence spectra, Fourier transform infrared spectroscopy (FTIR), and ^{1}H nuclear magnetic resonance (NMR) spectroscopy, respectively. The results indicated that the amido and carboxy groups of the purified peptide were transformed during chelation. The oxygen atoms of the carboxy group and the nitrogen atoms of the amido group could chelate calcium to form coordinate bonds by donating electron pairs. Furthermore, FD-Ca chelate was found to be more stable and absorbable than $CaCl_2$ under both acidic and basic conditions. Our findings suggest that the purified dipeptide Phe-Asp has the potential to be used as a calcium-binding ingredient in dietary supplements.

1. Introduction

Whey protein is a mixture of globular proteins isolated from whey, the liquid material created as a by-product of cheese production (Madureira, Pereira, Gomes, Pintado, & Malcata, 2007). With the increased production of cheese from milk, more and more whey protein was released, the reasonable exploitation of whey protein becomes particularly significant (Siso, 1996). However, whey protein is a kind of sensitive proteins whose solubility decreases in acid or heat conditions. The modification of whey protein has become an immediate area of research focus all over the world. Enzymatic modification can produce biological active peptides. As one kind of bioactive substances in functional foods, enzyme-hydrolyzed whey peptides have garnered increased attention in recent years, the effects of hydrolyzed whey peptides on human health are of great interest and are currently being investigated as a way of reducing health risk, as well as a possible supplementary treatment for several diseases (Clemente, 2000; Kim et al., 2007a; Théolier, Hammami, Labelle, Fliss, & Jean, 2013).

Calcium deficiency results in hypertension, osteoporosis and intestine cancer (Osborne et al., 1996). The intake of calcium

☆ Co-first authors.
* *Corresponding author.* Tel.: +86 591 22866375; fax: +86 591 22866278.
E-mail address: shywang@fzu.edu.cn (S.Y. Wang).
http://dx.doi.org/10.1016/j.jff.2014.05.013
1756-4646/

could increase the bone density in children and it is essential among the middle-aged and the aged to prevent osteoporosis (Cilla et al., 2011; Guénguen & Pointillart, 2000). With the increase in population of the aged throughout the world, there is a growing interest in developing calcium supplementary medicine to prevent and treat bone disease (Kim & Lim, 2004). The ionized calcium has served as main calcium supplements for human beings in recent years (Lee & Song, 2009). However, the disadvantage of ionized calcium is that it is prone to form calcium phosphate deposition in basic intestine environment (Bronner & Pansu, 1998). As a result, the bioavailability of dietary calcium is severely lowered. The organic calcium supplement including calcium-binding peptides has been becoming one of popular research topics (Narin, Benjamas, Nualpun, & Wirote, 2013). Hydrolyzed whey peptides, obtained from proteolytic digestion, have shown the considerable capacity in incorporating with divalent ions such as calcium, iron ion, etc. (Chaud et al., 2002; Kim et al., 2007b). The chelating complex chelated between whey peptides and calcium ion can promote calcium absorption in human body and therefore improve its bioavailability.

The objective of this study was to purify and characterize a highly specific calcium-binding peptide from whey protein hydrolysates. Whey protein was herein hydrolyzed, a specific calcium-binding peptide was purified, and mechanism of action was investigated. The finding would be of significance in utilizing the hydrolyzed peptides from whey protein as calcium-binding peptide ingredients in functional foods.

2. Materials and methods

2.1. *Reagents and materials*

Whey protein was purchased from Hilmar Corporation (Batch No. 20111107) (USA). Flavourzyme (2000 U/mg) and Protamex (1500 U/mg) were obtained from Novo (Novozymes, Denmark). Toyopearl DEAE-650M and Sephadex G-25 were purchased from Amersham Pharmacia Co. (Uppsala, Sweden). All reagents and chemicals were of analytical reagent and high-performance liquid chromatography (HPLC) grade.

2.2. *Preparation of whey protein hydrolysates*

Whey protein solution 5% (w/v) was denatured at 80 °C for 20 min, then the pH was adjusted to 7.0. The sample was hydrolyzed using Flavourzyme and Protamex (2:1, w/w) with a substrate:enzyme ratio of 25:1 (w/w) at 49 °C for 7 h. Hydrolysate was terminated by heating the sample in boiling water for 10 min to inactive the enzyme. The mixture was cooled to room temperature and subsequently centrifuged at 16,000 × *g* for 20 min. The supernatant, referred to as whey protein hydrolysate (WPH), was lyophilized and stored at –20 °C for subsequent purification.

2.3. *Purification of calcium-binding peptide*

A slurry of Toyopearl DEAE-650M was packed in a column (20 × 2.5 cm), then equilibrated with 5 column volume (CV) of equilibrating buffer and 20 mM Tris-HCl buffer (pH 9.0). Afterwards, 100 mg of lyophilized hydrolysates that had been through the 0.45 μm filter film was dissolved in 10 mL of the same buffer (pH 9.0) and loaded on the column. The column was washed with equilibrating buffer, the collected peak was labeled as the non-absorbed fraction. The bound peptides were eluted using a gradient elution with the same buffer containing 0–0.5 M NaCl at a flow rate of 0.5 mL/min and fraction volume was 5 mL/tube. Elution was monitored by measuring the absorbance at 214 nm. The calcium-binding abilities of all fractions were determined. The peak exhibiting the strongest ability was collected for the subsequent isolation.

The sample (200 mg) exhibiting the strongest binding ability from DEAE was dissolved in 5 mL deionized water and loaded onto a Sephadex G-25 column (100 × 2.0 cm), which had been previously equilibrated with deionized water, it was eluted with deionized water at the flow rate of 0.3 mL/min. Elution was monitored by measuring the absorbance at 214 nm. After calcium-binding capacity was determined, the fraction with the highest activity was pooled and lyophilized.

The lyophilized sample collected from the G-25 column was dissolved in approximately 30 mg/mL distilled water and purified by semi-preparation reversed-phase (RP)-HPLC on a C18 reversed-silica gel chromatograph (Gemini 5 μ C18, 250 × 10 mm; Phenomenex Inc.; Torrance, CA, USA). The injection volume was 200 μL. Elution was performed with solution A (0.05% trifluoroacetic acid (TFA) in water) and solution B (0.05% TFA in acetonitrile) with a gradient of 0–30% B at a flow rate of 4.0 mL/min for 50 min. The elution was monitored at 214 nm, and also collected for calcium binding capacity analysis. The most active fraction was chosen for analytical HPLC analysis.

Further purification was performed using an analytical C18 column. Buffers A and B were the same as those used in preparation for RP-HPLC. Runs were conducted with a liner gradient of 0–10% solvent B at a flow rate of 1 mL/min. All eluted peaks were monitored at 214 nm.

2.4. *Peptide identification by mass spectrometry*

The purified peptide was analyzed using a liquid chromatography/electrospray ionization (LC/ESI) tandem mass spectrometer (Delta Prep 4000; Waters Co.; USA) from 300 to 3000 m/z.

2.5. *Calcium-binding activity assay*

The lyophilized sample was dissolved in deionized water to a final concentration of 1.0 mg/mL. The peptide solution was mixed with the solution containing excessive $CaCl_2$ (5 mM) and superfluous 0.2 M sodium phosphate buffer (pH 8.0). The solution was stirred at 37 °C for 2 h and the pH was maintained at 8.0 using a pH meter. The calcium-binding peptide could inhibit formation of insoluble calcium phosphate through competitively combining with $CaCl_2$. The reaction mixture was centrifuged at 10,000 × *g* at room temperature for 10 min in order to remove insoluble calcium phosphate salts. The calcium content of the supernatant was determined using a colorimetric method with ortho-cresolphthalein complexone reagent (Gitelman, 1967). The absorbance at 570 nm was determined after adding the working solution to the sample. All experi-