Journal of Functional Foods 49 (2018) 333–341

Contents lists available at ScienceDirect

Journal of Functional Foods

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

Structural and functional characterization of calcium and iron-binding peptides from mung bean protein hydrolysate

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ARTICLE INFO

Keywords:
Calcium-binding peptides
Iron-binding peptides
Enzymatic hydrolysis
Ultrafiltration
Mung bean protein

ABSTRACT

This work was aimed at producing peptides containing calcium and iron-binding capabilities from mung bean protein extract after enzymatic hydrolysis. Mung bean protein hydrolysates were separated by ultrafiltration into eight fractions, and assayed for calcium and iron-binding properties. The < 5 kDa fraction was the most active and was separated by size exclusion chromatography into 4 fractions. Fraction 2 had the most active calcium and iron-binding activities, and further identified by mass spectrometry coupled with FindPept tool. Potential peptides sequences (133) were identified and 10 with high contents of leucine, isoleucine and aspartic acid were synthesized. Peptides LLLGI, AIVIL and HADAD were the best calcium binders while PAIDL was the most potent iron-binding peptide. The presence of L or I at the C- or N-terminal but not together at both terminals may have contributed to better calcium-binding ability. These peptides are potential ingredients to formulate functional foods with enhanced mineral-binding properties.

1. Introduction

Mung bean (*Vigna radiata* L. Wilczek) is widely planted in Asian countries including Thailand, Burma, Indonesia and Philippines. It has been utilized as an ingredient in various products like sprouts, flours and vermicelli (glass noodle). In vermicelli production, only the starch is extracted from mung bean seeds and utilized while the protein is removed, which makes the wastewater stream to require proper treatment (Shaheen et al., 2012). Some vermicelli manufacturers do harvest these proteins by acid precipitation from this wastewater (protein concentration 3.1%, w/v) (Sirikulchayanont, Jayanta, Pradipasena, & Miyawaki, 2007) and utilize as animal feed with low price (about 0.28–0.51 US$/kg). Approximately 8.6 kg of wastewater per kg of mung bean was produced from a vermicelli processing plant (Arunsintaweeporn et al., 2005).

Over the past few years, researcher have found that bioactive peptides from plant and animal sources produced by enzymatic hydrolysis exhibit varieties of functional properties such as anti-inflammation and mineral binding (Liu, Bao, Lv, Xu, & Guo, 2012; Lv, Bao, Liu, Ren, & Guo, 2013; Millán-Linares, Bermúdez, Yust, Millán, & Pedroche, 2014). Recent studies revealed that some small peptides released during plant protein hydrolysis possess the ability to bind minerals and enhance mineral bioavailability (Chaquilla-Quilca et al., 2016; Liu et al., 2012; Liu, Wang, Wang, & Chen, 2013; Lv et al., 2013).

Calcium is a naturally occurring essential mineral that is important for bone growth, intracellular metabolism, blood muscle contraction and structural support of skeleton (Bass & Chan, 2006; Miller, Jarvis, & McBean, 2001). Calcium deficiency can cause diseases such as rickets in children and osteoporosis in the elderly. One reason for calcium deficiency in the human body is due to the precipitation of calcium ion during pH change from acid in the stomach to a basic condition in the intestine during digestion (Bronner & Pansu, 1999). Furthermore, iron is also an important trace element in human nutrition. It is involved in many biochemical processes, including electron transfer reactions, gene regulation, binding and transport of oxygen, and cell growth. Iron deficiency can lead to diseases such as anemia, glossitis, angular stomatitis, koilonchia, blue sclera, and oesophageal webbing (Beard, 2001; Torres-Fuentes, Alaiz, & Vioque, 2012).

The bioactive properties of peptides are dependent on various factors especially the protein source and type of enzyme used for

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https://doi.org/10.1016/j.jff.2018.07.041
Received 30 March 2018; Received in revised form 12 June 2018; Accepted 14 July 2018

hydrolysis. Since, it is controlled and uses mild conditions to releases specific peptides, protease hydrolysis is often used for producing bioactive peptides. Therefore, protease selection is an important aspect to producing peptides with specific bioactive properties. Proteases that have been previously used to produce calcium-binding peptides include alcalase 2.4 L (Liu et al., 2013), protease M (Liu et al., 2012; Lv et al., 2013), and V8 protease (Chaquilla-Quilca et al., 2016). Those that have been used to produce iron-binding peptides are alcalase (Ajibola, Fashakin, Fagbemi, & Aluko, 2011; Bamdad, Wu, & Chen, 2011; Xia, Bamdad, Gänzle, & Chen, 2012) and trypsin (Zhang, Huang, & Jiang, 2012). The calcium-binding ability of peptides has been studied in Caco-2-cell and rats. Peptides from soybean protein and whey protein hydrolysates could bind calcium to form "calcium-peptide complex", exhibiting higher absorption on Caco-2-cells compared to calcium carbonate (Bao, Lv, Yang, Ren, & Guo, 2008; Xixi, Lina, Shaoyun, & Pingfan, 2015). Several researchers have reported that various sources of peptides with calcium-binding ability could improve calcium absorption in rats such as tilapia protein hydrolysates (Chen et al., 2014), soybean protein hydrolysates (Lv et al., 2013), and collagen hydrolysate (Pacific cod) (Peng, Hou, Zhang, & Li, 2017). Moreover, casein protein hydrolysate in the form of iron-peptide complex showed good iron absorption in rats compared to iron sulfate (Chaud et al., 2002).

Protein hydrolysates usually contain a mixture of peptides exhibiting different functional properties. Therefore, target bioactive peptides have to be separated from other peptides present in the protein hydrolysates in order to increase their functional capabilities. In industrial practice, ultrafiltration (UF) has been widely employed for purification, fractionation and concentration of bio-products. It has been reported that UF is able to separate bioactive peptides from protein hydrolysates of yam bean seed (Ajibola et al., 2011), barley gluten (Xia et al., 2012), soy protein (Zhang et al., 2012) and dephytinised soy protein (Zhang, Huang, & Jiang, 2014). Investigating whether mung bean can be utilized as a raw material for producing calcium and iron-binding peptides serving as ingredients of functional foods will be beneficial to the food industry. Therefore, the objective of this study was to produce and identify calcium and iron-binding peptides from mung bean enzymatic protein hydrolysates using the most suitable protease, coupled with ultrafiltration and peptide sequencing analysis.

2. Materials and methods

2.1. Materials and reagents

Dehulled mung bean was purchased from a local supermarket in Hat Yai, Songkhla province, Thailand. Bacillus licheniformis alcalase (2.4 L), Aspergillus oryzae flavourzyme (500 U/g), porcine pancreas trypsin (93 U/mg), porcine gastric mucosa pepsin (250 U/mg solid) and pancreatin from porcine pancreas (4 × USP specifications) were purchased from Sigma-Aldrich, St Louis, MO, U.S.A. All other reagents were of analytical grade and purchased from Fisher Scientific (Oakville, ON, Canada).

2.2. Extraction of mung bean protein

The preparation of mung bean protein extract (MBPE) was performed using the method described by Shaheen et al. (2012) with modifications. Dehulled mung bean seeds were rinsed with tap water and then soaked in distilled water with seed (1 kg) to water (10 L) ratio of 1:10 for 3 h. The water was discarded and warm water (50 °C) was added at seed (1 kg) to water (6 L) ratio of 1:6 and blended for 2 min. The protein was extracted with an alkaline solution (1 M NaOH, pH 9.5) for 1 h followed by centrifugation (9300*g*, 30 min, 4 °C). The supernatant was treated with acid solution (1 M HCl) until pH 4.5 was attained. The acidified mixture was centrifuged as before, the precipitate dispersed in distilled water at the ratio of 1:3 (w/v), adjusted to pH 7.0 with 1 M NaOH, and then freeze dried (Christ, Deta2-24LSC, UK). The freeze-dried sample was analyzed for proximate compositions following the method of the Association of official Analytical Chemists (Horwitz, 2000), including moisture, ash, fat, carbohydrate and protein (N × 6.25). The mung bean protein extract powder contained protein (85.22%), ash (3.67%), fat (1.47%), carbohydrate (8.65%), and moisture (1.36%).

2.3. Enzymatic hydrolysis of mung bean protein

The proteases (alcalase, flavourzyme, trypsin, pancreatin, pepsin, mixture of pancreatin and alcalase at 50/50 units, and mixture of pancreatin and flavourzyme at 50/50 units) were used for hydrolysis. Enzymes were added to freeze-dried extracts containing 10 g/L protein to obtain a final concentration of 100 units enzyme per 1 g protein. The hydrolysis condition was carried out using the optimum condition for each enzyme for maximum cleavage of MBPE. Hydrolysis using alcalase and flavourzyme were carried out at 50 °C and pH 8.0; Pepsin at 37 °C and pH 2.0 while trypsin and pancreatin were carried out at 37 °C, pH 7.0. In addition, hydrolyses using mixture of pancreatin plus flavourzyme and mixture of pancreatin plus alcalase were each carried out at 50 °C, pH 8.0. All samples were hydrolyzed in a reactor for 8 h and samples (90 mL) withdrawn at 30 min intervals, immersed in a controlled water bath (95 °C for 15 min) to stop enzyme activity. Samples were allowed to cool at room temperature, centrifuged at 10,000*g* and 4 °C for 20 min, and the supernatant stored at -20 °C for further experiments (Liu et al., 2013).

2.4. Determination of the degree of hydrolysis (DH)

The DH of mung bean protein hydrolysates (MBPHs) was analyzed using the OPA (o-phthaldialdehyde) method with slight modification of the method described by Nielsen, Petersen, and Dambmann (2001). The OPA is carried out by determining the α-amino nitrogen of peptides to estimate the percentage of cleaved peptide bonds in the protein sample. The total number of α-amino nitrogen in the sample was determined by complete acid hydrolysis using 10 mL of 6 M HCl for 1 g of sample at 110 °C for 24 h. The absorbance of this OPA reagent and hydrolysates mixture was measured at 340 nm after incubation for 20 min. The percentage DH using the following equations to calculate h (number of hydrolyzed bonds) and then DH.

2.5. Calcium-binding assay

Calcium-binding assay was performed using the following modified method (Charoenphun, Cheirsilp, Sirinupong, & Youravong, 2013). Briefly, 5 mL of 500 mg/L of MBPHs (pH 7.8) was mixed with 5 mL of 5 mM $CaCl_2$ in 20 mM sodium phosphate buffer (pH 7.8). The calcium content was determined using a pH/ION meter (2700 Series Benchtop Meters, Oakton, Singapore). The amount of bound calcium was calculated with the following formula (Charoenphun et al., 2013).

$$Calcium_B = Calcium_{Total} - Calcium_{Ub}$$

where $Calcium_B$ is amount of the calcium-binding peptides (mg/L), $calcium_{Total}$ is amount of $CaCl_2$ concentration before adding peptides (mg/L), and $calcium_{Ub}$ is amount of $CaCl_2$ concentration after mixed with peptides (mg/L). Amount of calcium ion bound to peptides was converted from mg/L to mg g^{-1} protein based on protein content of the MBPHs.

2.6. Iron binding assay

Iron binding assay was determined using the modified method of (Lee & Song, 2009). Briefly, 5 mL (1 g/L) MBPHs was mixed with 5 mL of 10 mM $FeCl_2{\cdot}4H_2O$ in 20 mM phosphate buffer (pH 7.0). The solution was stirred at 37 °C for 1 h before centrifuging (10,000*g* for 20 min at 4 °C) to remove precipitates. The amount of iron concentration in the