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Stability and antioxidative activities of casein peptide fractions during simulated gastrointestinal digestion *in vitro*: Charge properties of peptides affect digestive stability

Jing Ao [a], Bo Li [a,b,*]

[a] College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China
[b] Beijing Higher Institution Engineering Research Center of Animal Product, Beijing 100083, China

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ABSTRACT

The alcalase-treated casein hydrolysate was separated into positively charged fraction (PCF) and negatively charged fraction (NCF), which were then digested in simulated gastrointestinal juices. RP-HPLC, gel filtration chromatography, and RP-HPLC with pre-column derivatization were respectively used to analyze HPLC chromatogram, molecular weight distribution, and amino acid composition of differently charged fractions before digestion, after gastric digestion and after intestinal digestion. Trolox-equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) values were also tested to evaluate the antioxidant activities of two peptide fractions during the gastrointestinal digestion. Results showed that PCF, with high basic amino acid content, exhibited stronger degradation during digestion, releasing about 34% of free amino acids. By contrast, NCF, containing high acidic amino acids, showed better digestive stability, producing additional smaller peptides during digestion. The bioavailability of NCF was higher than PCF after gastrointestinal digestion. No significant difference in antioxidative activities was observed between NCF and its final digest ($P > 0.05$). However, the PCF digest activity was significantly lower than that of undigested PCF ($P < 0.05$). Moreover, weak polar fractions were preferentially degraded during intestinal digestion. The results suggest that acidic antioxidant peptides resistant to gastrointestinal digestion should be developed as potential functional foods or nutraceuticals.

1. Introduction

Casein is the main proteinaceous component of milk. Casein is not only a high-quality protein in milk system, primarily due to its unique amino acid composition required by growth of the neonate, but also a rich source of bioactive peptides, which play an important role in the nervous, cardiovascular, digestive and immune systems (Silva & Malcata, 2005). Many researchers have showed interest on the casein peptides, which are considered to be used as a basic compound of functional foods, nutraceuticals and dietary supplements, because of its health benefits and safety (Kim, Jang, & Kim, 2007; Kitts, 2005; Sarmadi & Ismail, 2010). Antioxidative capacity has been reported in the casein hydrolysate (Kim et al., 2007; Rival, Boeriu, & Wichers, 2000). Suetsuna, Ukeda, and Ochi (2000) separated a peptide (YFYPEL) having strong free radical scavenging activities from casein protein hydrolysate by chromatographic analyses. It was reported that the highly polar acidic domains in casein peptides, which represent the binding sites for minerals, might allow them to act as antioxidants in foods (Diaz & Decker, 2004; Miquel, Ángel Gómez, et al., 2006). García-Nebot, Cilla, Alegría, and Barberá (2011) tested cytoprotective effect of Caseinophosphopeptides (CPPs) against H_2O_2-induced oxidative stress in Caco-2 cells, and the results showed that caseinophosphopeptides protected the cells against oxidative damage by preserving cell viability, increasing GSH content, inducing catalase enzyme activity, diminishing lipid peroxidation and maintaining a correct cell cycle progression.

However, one of the greatest challenges in developing casein peptides as functional food ingredients is proving the in vivo efficacy of the bioactive components. The potential effect of casein peptides depends on their capacity to reach their target organs after oral administration. The gastrointestinal (GI) tract is known to be one of major barriers in the human body. The conditions in the GI tract, such as GI digestive enzymes and pH values in the stomach might influence the structures and functions of the peptides (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). To further exploit casein antioxidative peptides resistant to GI digestion, it is necessary to get knowledge of their stability in the GI tract. Mequel et al. studied the formation and identification of the casein phosphopeptides after simulating physiological digestion *in vitro*, and discussed the potential mineral chelating properties of CPPs identified in relation to their amino acid sequences and the presence of the phosphorylated cluster (Miquel, Alegría, Barberá, & Farré, 2006; Miquel et al., 2005; Miquel, Ángel Gómez, et al., 2006). Besides, Several studies have demonstrated the role of *in vitro*

* Corresponding author at: P.O. 294, Qinghua East Road 17, Haidian District, Beijing 100083, China. Tel./fax: +86 10 6273 6243.
E-mail address: libo@cau.edu.cn (B. Li).

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gastrointestinal digestion on the activity of the synthetic peptides with specific sequences (Quirós, Contreras, Ramos, Amigo, & Recio, 2009; Ruiz, Ramos, & Recio, 2004), and adopted methods of studying drugs. However, bioactive peptides from natural food are always complex mixture with thousands of peptides, which exert similar properties and potential interactions with each other during GI digestion. According to this, study on the change of the bioactive peptide fractions in simulated GI tract based on methodology of Proteomics or Metabolomics would be more meaningful.

Structure-activity researches have shown that the structural features, such as charge property, amino acid sequence, molecular size and hydrophobicity, may determine the bioactivity of peptides (Sarmadi & Ismail, 2010). However, few studies focused on the impact of charge property on the digestive stability. The charge property, as a character of peptide fractions, reflects their amino acid composition, and the ability of peptides to resist enzymatic attack is related to their amino acid composition due to the specificity of digestive enzymes (SDE). When peptides with different amino acid composition were taken into body orally, some specific sites in the peptide sequences should be cleaved preferentially due to SDE in GI tract. For example, peptides with proline and hydroxyproline residues are generally able to resist degradation by digestive enzymes (Segura-Campos et al., 2011); the C-terminal end of Lys and Arg residues is preferentially cleaved by Trypsin (Burrell, 1993). According to this, it is supposed that differently charged peptides may show different fate during GI digestion. The results will provide a guide for the preparation and development of oral antioxidants resistant to digestive enzymes.

The objective of this study is to evaluate the impact of charge properties of peptides on the GI tolerance of casein antioxidant peptides during simulated GI digestion. In the present study, casein hydrolysate was separated into six fractions with SP-Sephadex C-25 ion exchange chromatography, and Fraction 2 and 6 were antioxidant peptides with positive and negative charges, respectively. A two-stage *in vitro* digestion model system was used to simulate the process of human GI digestion. The digests were employed to analyze high performance liquid chromatography (HPLC) profiles, molecular weight distributions, amino acid compositions, and various antioxidative properties so as to evaluate the stability of antioxidant peptides with different electric charges during GI digestion.

2. Materials and methods

2.1. Materials and chemicals

Skimmed milk powder was purchased from Anchor, Fonterra Brands NZ Ltd., New Zealand. Alcalase® 2.4 L (endoproteinase from Bacillus licheniformis, EC 3.4.21.14, ≥2.4 AU/g) was obtained from Novozymes North America Inc. (Franklinton, NC). Trypsin (0458, 1:250, 250 NF U/mg) was obtained from AMERESCO LLC. (Solon, OH, USA). Chymotrypsin and L-glutathione reduced (GSH) were obtained from Beijing Biodee Biotechnology Co. Ltd. Pepsin (P7000, ≥250 U/mg), 2, 20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), fluorescein, phenyl isothiocyanate (PITC), triethylamine (TEA), trifluoroacetic acid (TFA), and amino acid standard were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile of chromatographic grade was purchased from Honeywell International (Morristown, NJ, USA). All other chemicals used were of analytical grade.

2.2. Extraction of casein from skimmed milk powder

A hundred grams of skimmed milk powder were mixed with 1000 mL of distilled water and placed in a water bath at 40 °C for heating. 1000 mL of sodium acetate buffer solution (0.2 M, pH 4.6), which had been preheated to 40 °C, was stirred in the skimmed milk slowly. The pH value of the solution was adjusted to 4.6 with 0.2 M acetic acid by slow stirring. After cooled to the room temperature, the suspension was centrifuged at 3000 rpm for 15 min and crude extract of casein was separated out as precipitate. The precipitate was washed three times with distilled water, and centrifuged at 3000 rpm for 10 min respectively. The precipitate was collected and dissolved in ethanol. The suspension was transfer to a Buchner funnel for vacuum filtering. The residue was lyophilized, and stored at −20 °C for further use.

2.3. Enzymatic hydrolysis of casein

The casein was hydrolyzed by Alcalase® 2.4 L. Both casein and Alcalase® 2.4 L were dissolved in 0.2 M phosphate buffer solution (pH 8.0), and the pH value of the substrate solution was adjusted to 8.0, the optimal pH value of Alcalase® 2.4 L, before initiating the hydrolysis. The hydrolysis of casein was carried out in water bath shaker at 60 °C for 4 h. The substrate concentration of the hydrolysate was 5%, and the ratio of the enzyme/casein substrate was 2%. The enzymatic hydrolysis was terminated by heating for 10 min in boiling water for inactivating the enzyme; subsequently the hydrolysate was neutralized, and then centrifuged at 10,000 ×*g* for 20 min. The supernatant was lyophilized and stored at −20 °C for future use.

2.4. Separation of antioxidant peptide fractions from casein hydrolysate

Lyophilized casein hydrolysate was diluted in buffer A (20 mM sodium acetate buffer adjusted to pH 4.0). Hydrolysate solution (1 mL) was applied on a SP-Sephadex C-25 (GE Healthcare life science) cation exchange column (2.6 × 30 cm) equilibrated with buffer A. The column was eluted with buffer A from 0 to 90 min, and then eluted with buffer A containing 0.5 M NaCl from 90 to 300 min. Elution was achieved at a flow rate of 2 mL/min, and monitored by UV absorbance at 220 nm. The fractions of 6 mL were collected, and $ABTS^{\bullet+}$ scavenging ability of each fraction was tested. The positively charged peptides and negatively charged peptides were collected respectively, and freeze-dried for further analysis.

2.5. In vitro simulated gastrointestinal digestion

In vitro gastrointestinal digestion of casein antioxidant peptide fractions with positive or negative charge was performed by modification of the method of Ruiz et al. (2004). Sample (300 mg) was first dissolved in 27 mL of distilled water, and pH was adjusted to 2.0 with HCl for pepsin hydrolysis. The simulated digestion reaction was started by adding 3 mL of pepsin solution (pH 2.0) at an enzyme: substrate (E/S) ratio of 0.5%. The mixture was incubated in a shaking incubator for 2 h at 37 °C. The pH was then adjusted to 5.3 with a saturated $NaHCO_3$ solution (0.9 M) and further to pH 7.5 with 2 N NaOH. Trypsin and chymotrypsin were added at an enzyme to substrate (E/S) ratio of 4% (w/w), and the mixture was further incubated with shaking at 37 °C for 4 h. To terminate the digestion, the test tubes were kept in boiling water for 10 min. Then the GI digests were cooled to room temperature and stored at −20 °C for further analysis.

To investigate the changes in antioxidative activity of casein peptide digests during the simulated GI digestion, aliquots of GI digests were removed at 0 (Alcalase hydrolysate), 1.0, 2.0, (switch from pepsin to trypsin and chymotrypsin) 3.0, 4.0, 5.0 and 6.0 h during the digestion *in vitro*.

2.6. High performance liquid chromatography (HPLC) analysis and determination of bioavailability

HPLC analysis of undigested samples and the GI digests was performed using SHIMADZU LC-15C system equipped with a HPLC column (ZORBAX SB-C18, 4.6 mm i.d. × 250 mm, 5 μm, Agilent Technologies, USA). The mobile phases consisted of 0.1% TFA in water (A)