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Bioavailability of peptides from casein hydrolysate *in vitro*: Amino acid compositions of peptides affect the antioxidant efficacy and resistance to intestinal peptidases

Chan Wang [a], Bo Wang [a], Bo Li [a,b,*]

[a] College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China
[b] Key Laboratory of Functional Dairy, Ministry of Education, Beijing 100083, China

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ABSTRACT

Measurements of *in vitro* bioactivity to support health benefits of bioactive compounds should be accomplished with estimates of their bioavailability to bolster nutritional significance to health claims. *In vitro* bioavailability of casein and casein peptides (casein hydrolysate and four peptide fractions) measured by the amount of peptide nitrogen is discussed. Antioxidant activities during gastrointestinal digestion and Caco-2 cell absorption were investigated as indices of peptide degradation. The antioxidant capacities of Trolox equivalent and oxygen radicals were used for assessing antioxidant efficacy of surviving peptides. Results showed that casein hydrolysate improved bioavailability compared to casein. Amino acid composition of peptides affected the resistance of peptides to digestive enzymes and intestinal peptidases. The acidic peptide fractions had higher bioavailability and a higher residual ratio of antioxidant activity. The peptides in the digest and absorbate of acidic fraction F1 with the highest bioavailability (23.14%) and the residual ratio of antioxidant activity were identified, and 12 intact, absorbed peptides (IAP) were obtained. Eleven of twelve of the IAPs were from β-Casein, and their amino acid components were rich in acidic and hydrophobic amino acids. Identification of IAPs might provide insight into the mechanism of how peptide structure provides resistance against peptidases by Caco-2 cells.

1. Introduction

Bioactive peptides are defined as specific protein fragments that have a positive effect on body functions and conditions that may affect health ultimately (Miguel et al., 2008). Peptides from various dietary sources have been shown to have a positive impact on health by functioning as opiates, immunomodulators, anticarcinogens, antimicrobials, anticariogenics, antioxidants, antithrombotic agents, and antihypertensives (Korhonen & Pihlanto, 2006; Ohsawa et al., 2008). Bioactive peptides are vital components of functional foods, a research area of high interest due to their therapeutic value and importance in the food industry (Segura-Campos, Chel-Guerrero, Betancur-Ancona & Hernandez-Escalante, 2011). Among the numerous bioactive peptides, antioxidant peptides are peptides in which researchers are interested. Antioxidant peptides have positive effects on protecting our body against oxidative damage produced by free radicals or reactive oxygen species (Zhang et al., 2009).

The physiological activity of active peptides thus far reported was evaluated directly after enzymatic preparation, separation, and purification (Kim et al., 2009; Li, Chen, Wang, Ji, & Wu, 2007). However, one of the greatest challenges when developing the nutraceutical and functional food products is proving the *in vivo* efficacy of their bioactive components. The gastrointestinal (GI) tract is known to be a major barrier for the oral administration of bioactive components. Recently, simulated gastric and intestinal digestions have been applied to evaluate gastrointestinal stability of antioxidant peptides (You, Zhao, Regenstein, & Ren, 2010). However, the barrier of intestinal absorption, which contains numerous peptidases, deserves more attention for assessing actual bioavailability. Caco-2 cell models are recognized as a tool to simulate intestinal absorption by expressing numerous brush border membrane peptidases similar to those of human intestinal epithelium (Ohsawa et al., 2008). *In vitro* GI digestion and Caco-2 cell absorption models are the most rapid tools to estimate bioavailability of a bioactive substance (Hur, Lim, Decker, & Mc-Clements, 2011).

Implementation of the potential physiological effect of bioactive peptides depends largely on its ability to remain intact until reaching the target organ after oral intake (Miguel et al., 2008). Previous research indicated that the ability of peptides to resist enzymatic attack depends partly on their amino acid composition. Peptides with proline (P) and hydroxyproline residues generally are able to resist degradation by digestive enzymes (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). In our previous research, we studied the GI digestion stability of casein-derived peptides with different charge properties, and the results showed that negatively charged fractions,

* Corresponding author at: P.O. Box 294, Qinghua East Road 17, Haidian District, Beijing 100083, China.
E-mail address: libo@cau.edu.cn (B. Li).

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which contained a high concentration of acidic amino acids, had stronger resistance to GI digestive enzymes. However, whether acidic peptides can resist peptidases following absorption has not been investigated.

Casein is the main protein in milk, and various bioactive peptides have been identified from casein hydrolysates with opiate-like (Silva & Malcata, 2005), immunomodulatory (Malinowski, Klempt, Clawin-Rädecker, Lorenzen, & Meisel, 2014), antibacterial (Tomita et al., 1991), antihypertensive (del Mar Contreras, Carrón, Montero, Ramos, & Recio, 2009), and antioxidant properties (Rival, Fornaroli, Boeriu, & Wichers, 2001). In this paper, antioxidant peptides from casein were chosen as a model of bioactive peptides for assessing its bioavailability. The casein was hydrolyzed by proteases to obtain antioxidant hydrolysates. Antioxidant peptides with different charges were separated from the hydrolysates using ion exchange chromatography. A three-stage *in vitro* model system of simulated gastric juice–intestinal juice and Caco-2 cell monolayers was used to simulate the process of human gastrointestinal digestion and absorption of peptides. Peptide nitrogen content and antioxidant activities were used as the indices for peptide degradation and bioavailability. The intact absorbed peptides (IAP) were identified from acidic fractions using nano UPLC–ESI-qTOF-MS.

2. Materials and methods

2.1. Materials

Casein (C3400), alcalase (P4860, ≥2.4 U/g), pepsin (P7000, ≥250 U/mg), pancreatin (P1750, 4× USP specifications), TNBS (P2297, picrylsulfonic acid solution), ABTS (A1888, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), L-glutathione reduced, and Trolox (238813, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (Shanghai, China). Dulbecco modified Eagle's minimal essential medium (DMEM), fetal bovine serum (FBS), non-essential amino acids (NEAA), penicillin, streptomycin, and trypsin–EDTA were products of Hyclone (Thermo Scientific). Other reagents were purchased from Beijing Chemical Reagent Co., Ltd. (Beijing, China).

2.2. Preparation of casein hydrolysate

Casein hydrolysate was prepared with alcalase, and the hydrolysis process was carried out using the method reported by Ao and Li (2013), with some modification. The casein was mixed with distilled water as a substrate for the production of protein hydrolysate. The enzymatic hydrolysis was performed at the optimal pH of 8.0. The substrate concentration of the hydrolysate was 5%, and the ratio of enzyme to substrate was 2% (*w*/*w*). The mixture was maintained at a constant pH during hydrolysis using 0.5 M NaOH and 0.5 N HCl. The hydrolysis process was carried out in a water bath shaker at 60 °C for 4 h. Inactivation of the enzymes was accomplished by heating for 10 min in boiling water and the pH was adjusted to 7.0 with 0.01 M HCl. Then, the casein hydrolysate was centrifuged at 8000 *g* for 15 min at 4 °C. The supernatant was collected, freeze-dried, and stored at −80 °C.

2.3. Separation of casein hydrolysate by SP sephadex C-25

Casein hydrolysate was further separated by cation exchange chromatography to obtain casein peptide fractions with different charges. Ion-exchange chromatography (IEC) is a versatile method for separating peptides based on exploiting differences in electrostatic charge (Kang & Frey, 2003). Lyophilized casein hydrolysate was dissolved in 20 mM sodium acetate buffer with pH 4.0 at a ratio of 1:2 (*w*/*v*) and filtered through a 0.45 μm filter. Then the filtrate (1 mL) was applied to a SP-Sephadex C-25 (GE Healthcare Life Science) cation exchange column (2.6 × 25 cm) equilibrated previously with buffer A (20 mM sodium acetate buffer with pH 4.0). The column was eluted with buffer A from 0 to 120 min, and then eluted with buffer A containing 0.5 M NaCl from 120 to 300 min at a flow rate of 2 mL/min, and the elution was monitored by UV absorbance at 220 nm. The elution profiles were produced using a HD-A chromatography data handling system (Shanghai Qingpu Huxi Instruments Factory, Shanghai, China). Fractions (F1–F4) were collected separately and concentrated using a rotary evaporator, and then the concentrated solutions were dialyzed for 12 h at 4 °C with a 500 Da molecular-weight cut-off semipermeable membrane (Beijing Chemical Reagent Co., Ltd.) to remove the NaCl in the collected solutions. During the diafiltration process, distilled water was replaced every 2 h to ensure the effect of desalination. Finally, each fraction was freeze-dried for further analysis.

2.4. Analysis of amino acid composition

Amino acid composition of peptide isolates was determined by high performance liquid chromatography using phenylthiocarbamyl (PITC), pre-column derivatization, according to the method of Vasanits and Molnár-Perl (1999), with some modifications. Triplicate samples were hydrolyzed in a glass tube fitted with a cap with 6 N HCl at 110 °C for 24 h. Amino acid analysis was conducted on a Shimadzu LC-15C HPLC system after precolumn derivatization with PITC and triethylamine. The derivative amino acid solutions were filtered through a 0.22 μm microfiltration membrane, and the filtrates were injected onto the RP-HPLC analysis system equipped with a HPLC column (ZORBAX SB-C18, 4.6 mm i.d. × 250 mm, 5 μm, Agilent Technologies, USA) and detected at 254 nm. Amino acid standard solutions were used as a standard and the contents of individual amino acids were expressed as g/100 g of the total amino acids in each sample.

2.5. Analysis of molecular weight distribution

The molecular weight distribution of casein hydrolysate was analyzed by size exclusion with a HPLC (SE-HPLC) using the Shimadzu LC-15C HPLC system (Shimadzu Scientific Instruments, Kyoto, Japan) as described by Xie, Liu, Wang, and Li (2014), with some modifications. Samples were dissolved in buffer (45% (*v*/*v*) acetonitrile containing 0.1% (*v*/*v*) trifluoroacetic acid), and aliquots of 10 μL samples (2 mg/mL) were filtered with a 0.22 μm microfiltration membrane that were loaded onto a TSK gel G2000 SWXL column (7.8 × 300 mm, TOSOH, Tokyo, Japan). Samples were eluted with the same buffer at a flow rate of 0.5 mL/min, and monitored at 214 nm. Bacitracin (1423 Da), WPWW (tetrapeptide, 674 Da), NCS (tripeptide, 322 Da), and Gly-SAR (146 Da) were used as standards to obtain a molecular weight calibration curve, which was established between retention times and logs of molecular masses of the standards.

2.6. In vitro simulated gastrointestinal (GI) digestion

Casein hydrolysate fractions were digested enzymatically with pepsin and pancreatin, according to the method of Wang, Li, Wang, and Xie (2015), with some modifications. The samples were diluted to 10 mg/mL with 0.01 mol/L HCl (pH 2.0). Using a ratio of enzyme to substrate of 1:50 (*w*/*w*), pepsin was added and the mixture was incubated in a shaking platform for 2 h at 37 °C. The pH was first adjusted to 5.3 with 0.9 mol/L $NaHCO_3$ and, subsequently, adjusted to pH 7.5 after adding 2 mol/L NaOH. Pancreatin (enzyme to substrate ratio 1:25, *w*/*w*); the mixture was incubated in a water bath for 2 h at 37 °C under constant stirring. Finally, the aliquots were submerged in boiling water for 10 min to terminate the enzymatic digestion. The pH of the digest was adjusted to 7.0 with 0.01 M HCl. Then the digest was centrifuged at 8000 *g* for 10 min at 4 °C. The supernatants were collected, freeze-dried, and stored at −80 °C. Casein and GSH were used as the control samples during the *in vitro* simulated gastrointestinal digestion.