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Non-gastrointestinal-hydrolysis enhances bioavailability and antioxidant efficacy of casein as compared with its *in vitro* gastrointestinal digest

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ABSTRACT

In this study, casein peptides prepared by alcalase-hydrolysis were originally compared with those prepared by *in vitro* gastrointestinal digestion, on oral bioavailability and *in vitro* antioxidative efficacy. Two models including simulated gastrointestinal digestion and Caco-2 cell monolayer were applied to prepare residual peptides for the bioavailability assays. Trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) were employed to detect *in vitro* antioxidant activities, while oxidative damage model of HepG2 cells was adopted to evaluate cellular antioxidant efficacy. Alcalase hydrolysate exerted stronger potentials to produce bioavailability and *in vitro* antioxidant efficacy, as compared with gastrointestinal digest. Especially, low-molecular-weight fraction of alcalase hydrolysates had excellent residual antioxidant activity by TEAC and ORAC, as well as hepatic cytoprotection against hydrogen peroxide. Enzymatic specificity of alcalase and molecular weights of peptides might play important roles on the stability of antioxidant activity during *in vitro* digestion and absorption, and producing real antioxidative efficacy *in vivo*.

1. Introduction

The formation of free radicals is unavoidable in human organisms, and the radicals are very unstable and react rapidly with other groups or substances. They are believed to play an important role in the occurrence of gastrointestinal and cardiovascular diseases, such as gastric ulcers (Sakurai, Osaka, & Yamasaki, 2005), colon cancer (Babbs, 1990), and hemolytic anemia (Jain & Hochstein, 1979). Numerous *in vitro* studies have applied trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) assays, respectively, to measure antioxidant capacities based on electron transfer and hydrogen atom transfer (Zulueta, Esteve, & Frígola, 2009). In order to obtain information more closely reflecting the *in vivo* situation, lots of cellular oxidative models have been used to assess physiological responses to various natural antioxidative candidates (Laparra, Alegría, Barberá, & Farré, 2008; Saviranta et al., 2011).

To be defined as bioactive peptides, one of the great challenges is proving their bioavailability for *in vivo* physiological functions. Survival from physiological barriers after oral administration, including gastrointestinal digestion and intestinal epithelial absorption in human tract, is the essential prerequisite for the bioactive assessment of peptides (Ekmekcioglu, 2000). Lots of compounds exerting bioactive potentials have been evaluated for their bioavailability by *in vitro* models, because of strict ethical considerations of animal studies and high costs of clinical trials (Ekmekcioglu, 2002). Furthermore, estimation of the *in vitro* bioavailability of bioactive compounds could significantly support their *in vivo* functional properties (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009).

In vitro digestion model, which is a rapid and simple way to mimic the gastrointestinal conditions, consists of simulated gastric digestion and sequential intestinal digestion (Parrot, Degraeve, Curia, & Martial-Gros, 2003). In our previous study, a simulated gastrointestinal model has been successfully validated (Zhou et al., 2009). On the other hand, Caco-2 cell model has been widely used as a predictive tool for the intestinal epithelial absorption of peptides (Cinq-Mars, Hu, Kitts, & Li-Chan, 2007). Caco-2 cell line, originally derived from human colon carcinoma, can spontaneously differentiate into intestinal epithelium under standard culture conditions (Xie, Zhou, & Li, 2012). The differentiated cells express characteristics of mature epitheliums, such as microvillus structure, tight junction at apical side, numerous brush border enzymes, and carrier-mediated transport system for di/tri peptides, which are of critical importance for absorption of intact peptides (Sambuy et al., 2005).

Bioavailability of nutritional supplements generally designates the proportion of the administered substance capable of being absorbed (Heaney, 2001). In terms of bioactive peptides, the bioavailable fractions

Abbreviations: TEAC, trolox equivalent antioxidant capacity; ORAC, oxygen radical antioxidant capacity; PN, peptide nitrogen; AH, alcalase hydrolysate; GD, gastrointestinal digest; SGD, simulated gastrointestinal digestion; BA, bioavailability; RRAA, residual ratio of antioxidant activity; RAA, remaining antioxidant activity; AAA, antioxidant amino acids.

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are mainly short peptides that survive gastrointestinal digestion and absorption (Phelan, Aherne, FitzGerald, & O'Brien, 2009). The antioxidant potentials of the bioavailable proportions are considered to be more effective on *in vivo* organisms than those of the fractions without human digestion and absorption (Ekmekcioglu, 2000; Fernández-García et al., 2009).

Casein protein is one of the important natural resources to produce antioxidant peptides (Suetsuna, Ukeda, & Ochi, 2000). The peptides are not active within the parent protein, but can be released by enzymatic hydrolysis. *In vitro* gastrointestinal digestion has been successfully utilized to prepare potential antioxidant peptides from various proteins such as oyster protein (Qian, Jung, Byun, & Kim, 2008a), giant squid muscle (Rajapakse, Mendis, Byun, & Kim, 2005) and hoke frame protein (Kim, Je, & Kim, 2007). Alcalase derived from microorganisms also has been used to enzymatically cut proteins, including tuna backbone protein (Je, Qian, Byun, & Kim, 2007), chickpea protein (Li, Jiang, Zhang, Mu, & Liu, 2008), and sunflower protein (Megías et al., 2009), into potential antioxidative peptides.

Simulated gastric and intestinal digestions have been widely applied to evaluate gastrointestinal stability of antioxidant peptides (You, Zhao, Regenstein, & Ren, 2010). The barrier of intestinal absorption, which contains numerous peptidases, also needs to be seriously considered, so as to perfectly evaluate the real bioactivity (Megías et al., 2009; Miguel et al., 2008). On the other hand, *in vitro* gastrointestinal digests of plant protein have weaker angiotensin-converting enzyme inhibitory activity than the one produced by alcalase, before and after simulated gastrointestinal digestion (Tiengo, Faria, & Netto, 2009). As far as antioxidant activity, it is not clear, whether peptides prepared by non-gastrointestinal enzymes would exhibit higher bioactive potentials than that prepared by *in vitro* gastrointestinal digestion, after gastrointestinal digestion and absorption. It has been illustrated that peptides derived from *in vitro* digest exert better resistances to gastrointestinal enzymes (Escudero, Sentandreu, Arihara, & Toldrá, 2010), which might result in a higher bioavailability. Therefore, comparison of effects of non-gastrointestinal enzymes and gastrointestinal enzymes on bioavailability and antioxidant potentials of casein is of crucial importance to assure better strategies to produce *in vivo* antioxidant efficacy.

Hence, the objectives of the study comprise (1) preparing casein hydrolysates using gastrointestinal enzymes (pepsin and pancreatin) and non-gastrointestinal enzyme (alcalase), (2) fractionating the peptides using size exclusion chromatography and determining their bioavailability, (3) stability of the corresponding fractions during simulated digestion and Caco-2 cell absorption, and (4) cellular antioxidant efficacy of the bioavailable fractions against hydrogen peroxide (H_2O_2).

2. Materials and methods

2.1. Materials

Casein (C3400), alcalase (P4860 ≥ 2.4 U/g), pepsin (P7000 ≥ 250 units/mg powder), pancreatin (P1750, 4 × USP specifications), TNBS (P2297, picrylsulfonic acid solution), ABTS (A1888, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), GSH (G4251), fluorescein (46955), AAPH (440914, 2,2′-azobis(2-methylpropionamidine) dihydrochloride), and trolox (238813, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (Shanghai, China). Vc was purchased from Beijing Aoboxing Bio-tech CO., LTD (Beijing, China). Dulbecco modified Eagle's minimal essential medium (DMEM), fetal bovine serum (FBS), non-essential amino acids (NEAA), penicillin, streptomycin, Hank's buffered saline solution (HBSS) and trypsin-EDTA were products of Hyclone (Thermo scientific). All other reagents were products of Sinopharm Chemical Reagent Co, Ltd (Shanghai, China).

2.2. Simulated gastric juice and simulated intestinal juice

The method described by Megías et al. (2009) and Zhou et al. (2009) with proper modifications was used.

Simulated gastric juice was prepared by suspending pepsin in HCl solution (pH = 2) to a final concentration of 0.0011% (w/v).

Simulated intestinal juice was captured by suspending pancreatin in NaOH solution (pH = 7.5) to a final concentration of 0.0032%.

2.3. Preparation of casein protein hydrolysates

For obtaining the alcalase hydrolysate (AH), aliquots of casein solution (4%, w/v) at pH 8.0 were hydrolyzed with alcalase at 55 °C for 4 h. The ratio of enzyme to substrate was 2% (w/w). After hydrolysis with stirring, the hydrolysate was heated in boiling water for 10 min to inactivate the enzyme. Subsequently, the pH value was adjusted to 7.0 with 0.01 M HCl. An aliquot of each AH was centrifuged at 8000 ×*g* for 15 min, and the supernatant was lyophilized for further study.

In order to prepare gastrointestinal digest (GD), the method of Samaranayaka, Kitts, and Li-Chan (2010) was applied with proper modifications. Briefly, casein protein dissolved in simulated gastric juice with substrate concentration of 4% (w/v) was incubated at 37 °C for 2 h with stirring. The pH of the solution was then adjusted to 7.5 with 1 M NaOH. An aliquot of simulated intestinal juice was then added, and the solution was incubated for 4 h at 37 °C. Enzyme activity in each assay was terminated by boiling at 100 °C for 10 min, and subsequently, the mixture was centrifuged at 8000 ×*g* for 15 min. The supernatant was lyophilized for further research.

2.4. Peptide fractionation by sephadex G-25 gel filtration

After being equilibrated with distilled water, the casein protein hydrolysate (or digest) (2 mg mL^{-1}, 1.5 mL) was loaded onto sephadex G-25 gel filtration column (1.6 × 90 cm). Each fraction was monitored at 220 nm by using HD-A chromatography data handling system (Shanghai Qingpu Huxi Instruments Factory, Shanghai, China), and collected at a flow rate of 1 mL min^{-1}.

2.5. Simulated gastrointestinal digestion

The procedure described by the published method with a proper modification (Samaranayaka et al., 2010) was applied to perform simulated gastrointestinal digestion (SGD). First, pepsin, at a ratio of enzyme to substrate of 1:35 (w/w), was added to each peptide fractions, which were at a substrate concentration of 40 mg mL^{-1} (w/v) in distilled water (pH 2.0). After the mixture was incubated at 37 °C for 2 h with shaking, the pH value was adjusted to 5.3 with a saturated $NaHCO_3$ solution. Second, pancreatin (ratio of enzyme to substrate, 1:25 w/w) was added, before the pH was modulated to 7.5 with 1 M NaOH. The mixture with shaking was incubated again for 2 h at 37 °C. Third, the mixture was heated in boiling water for 10 min to inactivate the enzymes. Each peptide digest was lyophilized and stored at −80 °C until further analysis. In all cases, solvent blanks, without peptides added, were performed.

2.6. Caco-2 cell absorption

Caco-2 cells were grown in tissue culture flasks (Nunc) (25 cm^2) in DMEM containing 2 mM L-glutamine, supplemented with 20% (v/v) FBS, 1% (v/v) NEAA, 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin. Growth medium was changed every 2–3 days. The cells used were at passages 30–40 and the culturing conditions were 37 °C in an air atmosphere of 5% CO_2 and 90% relative humidity.

For the cell permeability study, Caco-2 cells (1.5 mL of 1×10^5 cells mL^{-1}) were seeded onto cell culture inserts (0.4 μm