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Protein breakdown and release of antioxidant peptides during simulated gastrointestinal digestion and the absorption by everted intestinal sac of rapeseed proteins

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ABSTRACT

The main objective of the study was to evaluate the bioaccessibility of rapeseed soluble proteins (RSPs) by simulated gastrointestinal digestion and isolated intestine methods. In the gastric phase, the degree of hydrolysis (DH) of RSPs increased from 2.6% to 8.2%, while the surface hydrophobicity (H_0) decreased from 62.8% to 59.1%. Further digestion in the intestinal phase brought DH and H_0 to 18.2% and 50.5%, respectively. Scavenging DPPH and hydroxyl radical abilities and reducing power of RSPs increased slightly after digestion by pepsin, however, a distinct increment was achieved after subsequent digestion by trypsin. Molecular weight distributions indicated that RSPs above 3000 Da decreased significantly from 87.0% to 24.1% after digestion, revealing these fractions were more easily digested by gastrointestinal proteases than those below 3000 Da. The absorption of RSPs by intestine reached the maximum (28.6%) at the concentration of 5 mg/mL after incubation for 60 min. For the fraction below 307 Da, the absorption efficiency increased with incubation from 20 to 60 min.

1. Introduction

Rapeseed meal, a co-product after oil extraction from rapeseed, has long been considered as an important and cheap protein source for food and animal feed, due to its high protein content of 35–45%. Great efforts have been made to develop efficient methods to prepare acceptable products from rapeseed meal for human benefits (Ivanova, Chalova, Uzunova, Koleva, & Manolov, 2016; Pan, Jiang, & Pan, 2011). The bioactive properties of proteins are largely attributed to the peptides which are inactive within the sequence of the parent protein, are liberated in vitro during food processing or in vivo by the gastrointestinal digestion, displaying antioxidation, antihypertension, anticancer, anti-inflammatory and so on (Ashley et al., 2016; Kim et al., 2015; Ruiz, Ramos, & Recio, 2004; Zhou et al., 2015). Many antioxidative peptides have been prepared successfully from corn protein (Zhou et al., 2015), soy proteins (Chen, Muramoto, Yamauchi, & Nokihara, 1996), rice bran protein (Wang, Chen, Fu, Li, & Wei, 2017) and aquatic proteins (Dong et al., 2008). Nevertheless, little information is available on the application of rapeseed meal in preparation of antioxidant peptides so far.

Protein is the principal component of many food matrices and contributes to the functional and nutritional characteristics of processed food products. Different proteins under simulated gastrointestinal digestion showed different digestive characteristics (Wang, Timilsena, Blanch, & Adhikari, 2017; Wang et al., 2014). To date, it is not clear how rapeseed proteins are digested and further exert antioxidant activity in vivo. In vitro, simulated gastrointestinal digestion is extensively used since it is rapid, safe, inexpensive and does not have the same ethical restrictions as in vivo methods (Liang et al., 2012). In recent years, the method has already been developed for the bioavailability and bioactivity assessment of proteins.

In this study, proteins breakdown and release of antioxidant peptides were investigated by determination of the degree of hydrolysis (DH), surface hydrophobicity (H_0), antioxidant activities and molecular weight (MW) distributions of rapeseed soluble proteins (RSPs) during simulated gastrointestinal digestion. In addition, the absorption characteristics of RSPs by isolated everted intestinal sac was also researched, which might help us to better understand the physiological effects of RSPs as well as absorption route of bioactive peptides. The research would provide a basis for optimizing applications of RSPs in food industries to enable

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consumers to select suitable nutritional intake varieties for health benefits.

2. Materials and methods

2.1. Materials and chemicals

Rapeseed meal was obtained from Zhengda Oil Co. (Danyang, China). Crude protein content was 42.8% determined by Kjeldahl method (AOAC., 1990). *Bacillus subtilis* 10160 was purchased from China Center of Industrial Culture Collection (Beijing, China). Pepsin, trypsin, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), trinitrobenzenesulfonic acid (TNBS), ascorbic acid, bovine serum albumin, cytochrome C, aprotinin, bacitracin and L-tryptophan were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and acetonitrile used were of HPLC quality. All other chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

2.2. Preparation of RSPs

Solid-state fermentation (SSF) is performed on non-soluble materials which act as a physical support and a source of nutrient, therefore, less energy will be needed to remove water from fermented products compared with high moisture fermentation. During fermentation, insoluble proteins would be broken down by enzymes released by microorganisms, resulting in an increment of soluble proteins with various physiological functions beyond their nutritional properties. Accordingly, SSF was employed in the study to prepare RSPs based on the method of He, Jiang, Zhu, Ding, and Ma (2014).

Bacillus subtilis was activated in a LB liquid medium (prepared by dissolving 10 g tryptone, 5 g yeast extract and 5 g NaCl in 1 L distilled water and autoclaved at 121 °C for 20 min, pH 7.4) and incubated at 30 °C and 180 rpm for 24 h. After a further incubation on an agar slant medium at 30 °C for 24 h to form clear colonies, they were transferred to a refrigerator (4 °C) and used for SSF of rapeseed meal. A single colony was grown in 100 mL of LB medium and incubated at 30 °C and 180 rpm for 12 h before fermentation.

Thirty grams of rapeseed meal (milled with a mesh size of 40) was mixed with 39 mL of water and steamed at 121 °C for 15 min in an autoclave. After inoculation with a 10% (v/w) ratio of *Bacillus subtilis*, it was incubated at 31 °C for 70 h to obtain fermented rapeseed meal. RSPs were extracted with distilled water and then lyophilized. The protein content was 65.8% determined by Kjeldahl method (AOAC., 1990).

2.3. Simulated gastrointestinal digestion

The simulated digestion of RSPs was carried out by modification of the method of Chen and Li (2012) using an in vitro gastric digestion with pepsin, followed by intestinal digestion with trypsin. RSPs was redissolved in distilled water to a final protein concentration of 4% (w/v) and adjusted to pH 1.2 with 1.0 M HCl. The sample was firstly hydrolyzed with pepsin (2% w/w, protein basis) at 37 °C and 150 rpm for 4 h in a shaking water bath. Then the pH was adjusted to 7.5 with 1.0 M NaOH followed by hydrolysis with trypsin (4% w/w, protein basis) for the next 6 h. To terminate the digestion, the sample was kept in boiling water for 10 min. After cooling to room temperature, the digest was centrifuged at 11 000 rpm for 20 min.

2.4. Assay of the DH

DH of RSPs was determined using the trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979) with slight modifications, and L-leucine as a standard free amino acid. Sample solutions (0.25 mL) were pipetted into test tubes containing 2.0 mL of phosphate buffer (0.01 M, pH 8.2), then 2 mL of 0.10% (w/v) TNBS was added, followed by mixing and incubating at 50 °C for 60 min in a covered water bath. After adding 4.0 mL of 0.1 M HCl and cooling to ambient temperature, the absorbance was measured at 420 nm. The reactions on the blank and the standard solutions were carried out by replacing the sample with 1% (w/v) sodium dodecyl sulfate (SDS) and 1.50×10^{-3} M L-leucine in 1% SDS, respectively. The DH was calculated using the following formula:

$$DH(\%) = \frac{[-NH_2]_d - [-NH_2]_0}{[-NH_2]_\infty - [-NH_2]_0} \times 100$$

Where, $[-NH_2]$ indicates the concentration of free amino groups (mM) in the non-digested sample (0), or in the digested samples (d), $[-NH_2]_\infty$ indicates the concentration of total amino groups.

2.5. Assay of the H_0

H_0 of RSPs was measured according to the method given by Dorsey and Khaledi (1993). After centrifugation (10 000 rpm, 20 min), the supernatant (10 μL) was injected into a Kromasil C18 reversed-phase column (4.6 × 250 mm, 5 μm) of RP-HPLC (LC-20AT, Shimadzu Corporation, Japan). A binary gradient was used for elution with a flow rate of 1.0 mL/min and monitored at 220 nm. The solvents used were (A) water and (B) acetonitrile. Elution was as follows: time 0.0–30.0 min, linear gradient from A/B (90/10) to A/B (5/95); 30.0–33.0 min, linear gradient from A/B (5/95) to A/B (90/10); 33.0–50.0 min, elution with A/B (90/10). H_0 of RSPs was calculated as follows:

$$H_0(\%) = \left(\frac{A_2}{A_1 + A_2}\right) \times 100$$

Where A_1 and A_2 is the area of peaks appeared between 0–5 min and 5–30 min, respectively.

2.6. Antioxidative activity assay

2.6.1. DPPH radical scavenging activity

The DPPH radical scavenging activity of RSPs (digested or not) was tested using the method proposed by Zhang et al. (2011). Two milliliters of sample was added to 2 mL of DPPH ethanol solution (0.2 mM). After incubation for 30 min in the dark, the absorbance at 517 nm was measured immediately. The scavenging activity was calculated as follows:

$$\textit{DPPH radical scavenging activity}\ (\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100$$

Where A_0 is the absorbance of DPPH solution without sample, A_1 is the absorbance of the sample mixed with DPPH solution and A_2 is the absorbance of the sample without DPPH solution.

2.6.2. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the samples was determined based on a method of Li, Jiang, Zhang, Mu, and Liu (2008). The hydroxyl radical was generated through a Fenton reaction in a system containing $FeSO_4$ and H_2O_2. After mixing 1.0 mL of $FeSO_4$ (9.0 mM), 1.0 mL of H_2O_2 (8.8 mM), 1.0 mL of RSPs and 1.0 mL of salicylic acid ethanol solution (9.0 mM) and incubating at 37 °C for 30 min, the absorbance was recorded at 510 nm. The scavenging activity was calculated as follows: