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Hydrolysis kinetics and antioxidant activity of collagen under simulated gastrointestinal digestion

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ABSTRACT

The hydrolysis kinetics and antioxidant activity of collagen from Nile tilapia were investigated under simulated gastrointestinal digestion in this study. From gastric to intestinal phase, degree of hydrolysis increased from 1.15% to 16.74%, while surface hydrophobicity decreased from 21.46% to 2.06%; DPPH radical scavenging increased from 18.22% to 31.79%, but inhibition rate of linoleic acid peroxidation reached 34.63% in 40 min and then kept stability. After digestion, the collagen hydrolysates were separated into three fractions by ultrafiltration. The fraction (86.70%) with average molecular weight of 436.80 Da exhibited the highest antioxidant activity, suggesting the high digestibility and bioactivity of collagen. The fraction was further purified with multi-step chromatography and identified to be Gly-Pro-Met (303.38 Da) by UPLC-ESI-MS with IC_{50} value being 25.64 μg/mL for DPPH radical. These results may help better understanding its physiological effects and utilize it in foods and pharmaceuticals.

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1. Introduction

Collagen is a major constituent of connective tissues in vertebrates and has long been used in foods and pharmaceuticals (Wu, Fujioka, Sugimoto, Mu, & Ishimi, 2004). It has been used to improve blood circulation, arrest bleeding, and improve joint condition by reducing pain in traditional medicine (Ohara, Matsumoto, Ito, Iwai, & Sato, 2007). Recent animal experiments and human trials have also suggested that oral ingestion of collagen might have beneficial effects on skin aging (Tanaka, Koyama, & Nomura, 2009), osteoarthritis and osteoporosis (Moskowitz, 2000), rheumatoid arthritis (Trentham et al., 1993), properties of nail, hair and Achilles tendon (Matsuda et al., 2006; Minaguchi et al., 2005), etc.

Oxygen radicals are very reactive molecules, which can react with every cellular component and give rise to functional and morphologic disturbances in cells (Martínez-Cayuela, 1995). There is evidence to implicate oxygen radicals as important pathologic mediators in many human diseases and aging processes, such as skin aging (Terra et al., 2012), osteoarthritis (Davies, Guilak, Weinberg, & Fermor, 2008), osteoporosis (Xu et al., 2011), rheumatoid arthritis (Bhowmick, Chakraborti, Gudi, Moideen, & Shetty, 2008), and hair and nail damage (Fernández et al., 2011; Khengar et al., 2010), and others. Recently, the antioxidant activity of collagen peptides has been

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widely demonstrated in many different oxidative systems (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). Thus, we hypothesized that the physiological effects of collagen might be related with its antioxidant activity.

However, it is difficult to understand how collagen, a high molecular weight protein, is digested and further exerts antioxidant activity. Simulated gastrointestinal digestion is being extensively used since it is rapid, safe, inexpensive, and doesn't 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 protein, such as hen egg white lysozyme (Rao et al., 2012), amaranth protein (Delgado, Tironi, & Añón, 2011), and loach protein (You, Zhao, Regenstein, & Ren, 2010), and others. Therefore, we thought that it might also be reasonable to investigate the hydrolysis kinetics and antioxidant activity of collagen after oral administration.

In this study, the hydrolysis kinetics and antioxidant activity of collagen were investigated under the simulated gastrointestinal digestion, which may help us to better understand its physiological effects and utilize it in foods and pharmaceuticals.

2. Materials and methods

2.1. Materials

The collagen was isolated from the skin of Nile tilapia (*Oreochromis niloticus*) as in a previous report (Wang et al., 2014). The skin was pretreated by 20% (w/v) NaCl, and then digested with 0.1% (w/v) pepsin in 0.5 M acetic acid at the ratio of 1:100 (w/v) for 48 h under 4 °C. After centrifuging, the collagen was collected by salting-out precipitation with NaCl from supernatant. Simulated gastric and intestinal fluids were prepared according to the U.S. Pharmacopeial (United States Pharmacopeial Convention Council of Experts, 2007). The simulated gastric fluid was confected by dissolving 2.0 g of NaCl and 3.2 g of pepsin in 7.0 mL of HCl and sufficient water to make 1000 mL.

2.2. Simulated gastrointestinal digestion

The simulated digestion of collagen was carried out by modification of the method of Chen and Li (2012). Briefly, collagen was hydrolyzed with simulated gastric fluid at 1:20 (w/v) for 4 h under 37 °C in a shaking incubator of 150 rpm. Then, pancreatin was added at 1:100 (w/v) after adjusting the concentration of KH_2PO_4 to 0.05 M and pH to 6.8 with 1.0 M NaOH, and the mixture was further incubated for 6 h. To terminate the digestion, the samples were kept in boiling water for 10 min. Finally, the digests were cooled and centrifuged at 10,000 *g* for 15 min. The control was conducted in the same manner, but distilled water was used instead of collagen.

2.3. Assay of degree of hydrolysis (DH)

The DH of collagen was determined using o-phtaldialdehyde (OPA) method (Marambe, Shand, & Wanasundara, 2008). The average chain length (ACL) and average molecular weight (AMW) of the hydrolysates were calculated by the equation (Mišún, Čurda, & Jelen, 2008): ACL = 100/DH, AMM = ACL × 90.

2.4. Measurement of surface hydrophobicity (H_o)

The H_o was assayed according to the method of Dorsey et al. with modifications (Dorsey & Khaledi, 1993). The sample was subjected to a RP-HPLC with an Agilent Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm). The elution was performed using a liner gradient of 2–98% methanol at the flow rate of 1.0 mL/min for 30 min. The absorbance was monitored at 190 nm. The H_o was the ratio between the peaks area of 5–30 min and 0–5 min.

2.5. Determination of DPPH radical scavenging activity

The DPPH radical scavenging activity was determined as described by Hu, Xu, Chen, and Yang (2004) with slight modifications. Two milliliters of sample were added to 2 mL of 0.2 mM ethanol solution of DPPH. After shaking vigorously, the absorbance was monitored immediately at 517 nm and recorded every 5 min interval until 240 min. The scavenging rate was calculated as follows: Scavenging rate (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control and A_1 was the absorbance of the sample.

2.6. Evaluation of inhibiting peroxidation of linoleic acid

As described by Hu et al. (2004), 2 mL of sample, 2 mL of 2.51% (w/v) linoleic acid in ethanol, 4 mL of 0.05 M of phosphate buffer (pH 7.0) and 2 mL of distilled water were mixed in a vial of 10 mL and then kept at 40 °C in darkness. The peroxidation degree of linoleic acid was determined every day by ferric thiocyanate method (FTC). The inhibition rate was calculated as DPPH radical scavenging rate on the fifth day.

2.7. Separation of collagen hydrolysates by ultrafiltration

The ultrafiltration was performed on a Millipore Pellicon system. The digests were pumped to a membrane with the nominal molecular weight cut-offs (MWCO) of 1 kDa. The filtrate (F_1) was collected while the retentate was recirculated until the absorbance of filtrate at 220 nm was close to 0. Then, the retentate was subjected to another membrane with MWCO of 10 kDa and the filtrate (F_2) and retentate (F_3) were collected separately. These fractions (F_1, F_2, F_3) were then concentrated and lyophilized. The yield was obtained by comparing hydroxyproline content in these fractions with that in collagen. The hydroxyproline content was determined using the colorimetric method recommended by ISO 3496 (Anonymous, 1994).

2.8. Purification of antioxidant peptide by chromatography

The sample was loaded on a Toyopearl SP-650M column (2.5 × 30 cm) equilibrated with 0.05 M sodium acetate buffer (pH 4.5), and eluted with a linear gradient of NaCl (0–1.0 M) in the same buffer at 1.0 mL/min. Then, the potent fraction was subjected to a Sephadex G10 column (1.5 × 75 cm) eluted with distilled water at 0.5 mL/min. Subsequently, the active

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