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Purification and identification of five novel antioxidant peptides from goat milk casein hydrolysates

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ABSTRACT

The present research described the preparation, purification, and identification of antioxidant peptides from goat milk casein (GMC). Goat milk casein was hydrolyzed by using a combination of neutral and alkaline proteases to obtain goat milk casein hydrolysates (GMCH) with high antioxidant activity. After desalting by nonpolar macroporous absorption resin, GMCH was isolated and purified by gel filtration chromatography and reversed-phase HPLC, respectively, and further identified by nanoliter electrospray ionization-tandem mass spectrometry. Antioxidant activities of GMC, GMCH, and pure peptides were evaluated and compared using free radical scavenging activity, metal ion chelating ability, and anti-lipid peroxidation ability. Compared with GMC, the free radical-scavenging ability and ferrous ion-chelating ability of GMCH increased significantly. The inhibition effect of lipid peroxidation of GMCH was much stronger than that of *tert*-butylhydroquinone and phytogermine and a little lower than that of ascorbic acid. The antioxidant activity of GMCH could be attributed to the high antioxidant activity of oligopeptides, especially 5 novel oligopeptides: Val-Tyr-Pro-Phe, Phe-Gly-Gly-Met-Ala-His, Phe-Pro-Tyr-Cys-Ala-Pro, Tyr-Val-Pro-Glu-Pro-Phe, and Tyr-Pro-Pro-Tyr-Glu-Thr-Tyr, which were first observed in GMCH. The antioxidant activity of these 5 novel oligopeptides and GMCH increased 3.59 to 380 times compared with GMC, combining anti-lipid peroxidation ability of GMCH, which indicated that GMCH and its purified fractions in different stages could be used as functional food ingredients, food additives, and pharmaceutical agents in the future.

Key words: goat milk casein hydrolysate (GMCH), antioxidant peptide, isolation and purification, identification

INTRODUCTION

Chronic diseases and aging phenomena are relevant to the imbalance in free radical levels in the body. An excess of free radicals can cause lethal cellular effects by oxidizing membrane lipids, cellular proteins, DNA, and enzymes, thus shutting down cellular respiration (Urso and Clarkson, 2003). When the damage cannot be repaired promptly and accumulates to a certain extent, it often leads to diseases, such as atherosclerosis, diabetes, rheumatoid arthritis, and cancer (Halliwell, 2000; Abuja and Albertini, 2001; Collins, 2005; Hiller and Lorenzen, 2009). It is well known that lipid peroxidation occurring in food products causes rancid flavor, unacceptable taste, degradation in nutritive value, and shortening of shelf life in foods (Alférez et al., 2006; Kondyli et al., 2007). To prevent foods from undergoing deterioration and to provide protection against serious diseases, it is important to inhibit the peroxidation of lipids and the formation of free radicals occurring in the living body and foodstuffs. Lipid oxidation is inhibited by antioxidant agents.

Artificial antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, and *n*-propyl gallate, exhibit strong antioxidant activity against several oxidation systems. However, the use of artificial antioxidants in foodstuffs is restricted or prohibited in some countries because of the potential risks for the living body. Antioxidants from natural sources are receiving increased attention. Food-derived peptides have been demonstrated to be the natural antioxidants without marked adverse effects. An increasing number of food protein hydrolysates and antioxidant peptides have been found to exhibit antioxidant activity (Chen et al., 1996; Li et al., 2007; Samaranayaka and Li-Chan, 2011), especially in bovine milk casein (Phelan et al., 2009; Pihlanto, 2006, Su et al., 2012). Suetsuna et al. (2000) identified an antioxidant peptide, Phe-Tyr-Pro-Glu-Leu, from bovine milk casein by ion exchange chromatography, gel filtration chromatography (**GFC**), and HPLC, and determined that Glu-Leu was the active center of the antioxidant peptide. Goat milk casein (**GMC**) differs greatly from bovine casein in content, peptide chain length, and AA sequences (Chen et al.,

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1996; Hoelzl et al., 2005; Tomotake et al., 2006; Kondyli et al., 2007; Ceballos et al., 2009). Lee et al. (2005) obtained 3 angiotensin-converting enzyme inhibitory peptides from goat milk casein hydrolysates (**GMCH**). Although Sommerer et al. (2001) obtained 28 peptides composed of 3 to 8 AA residues from the water-soluble extract of goat cheese, the functional characteristics of these peptides were not studied. Therefore, the objective of this study was to prepare and purify antioxidant peptides from GMC and further evaluate their in vitro antioxidant properties with different methods. Finally, the sequences of several peptides with higher antioxidant activity were determined by consecutive chromatography and tandem mass spectrometry.

MATERIALS AND METHODS

Materials

The composition of raw goat milk from Xinong Saanen dairy goats was 3.46% (wt/wt) milk fat, 2.72% (wt/wt) milk protein, and 4.41% (wt/wt) lactose. The contents of DM and nonfat milk solids in raw goat milk were 11.24% (wt/wt) and 7.79% (wt/wt), respectively.

The raw goat milk (1,000 mL) was first defatted by centrifugation at 2,500 × *g* for 20 min at 4°C. Then, the skimmed goat's milk was treated by isoelectric point precipitation. The precipitate was washed 2 times with 50 m*M* acetic acid-sodium acetate buffer at pH 4.4, and centrifuged at 1,610 × g for 10 min at 4°C after each washing. The resultant precipitate was used as the wet GMC for further experiments. The protein content was 183.40 g/kg, determined by the method of Bradford (1976).

Chemicals

Four proteases, including neutral protease (EC 3.4.24.4), alkaline protease (EC 3.4.21.62), papain (EC 3.4.22.2), and trypsin (EC 3.4.21.4), were purchased from Amano Enzyme Co. (Nishiki, Nagoya, Japan). Acetonitrile and trifluoroacetic acid (**TFA**), both HPLC grade, were purchased from Fisher Co. (Fair Lawn, NJ) and Fluka Co. (Switzerland), respectively. Nonpolar macroporous absorption resin LS106 (specific surface area ≥950 m^2/g) was purchased from Xi'an LanShen Special Resin Co. (Xi'an, China); 2-deoxy-D-ribose, 2,2'-azinobis (3-ethylbenzothia-zoline-6- sulfonicacid; **ABTS**), 1,1-diphenyl-2-picrylhydrazyl (**DPPH**), phytogermine, *tert*-butylhydroquinone (**TBHQ**), and ferrozine were purchased from Sigma Co. (St. Louis, MO). Ultrapure water was obtained from a Merck Millipore water purification unit (Billerica, MA). Ascorbic acid, EDTA, and all other reagents were of analytical grade and obtained from commercial sources.

Preparation of GMCH

Wet GMC (65.43 g) was dissolved in 10 mL of 0.1 *M* NaOH and diluted by addition of 150 mL of deionized water and hydrolyzed for 3 h with single proteases at their optimum hydrolysis conditions (Table 1) in a batch stirred tank reactor. For the compound protease, GMC was hydrolyzed first for 3 h under the optimum hydrolysis conditions of a single enzyme. Then, the complex was further hydrolyzed for 3 h under the optimum hydrolysis conditions of the other enzyme. The pH was kept at a stable value by adding 0.1 *M* NaOH solution. After the hydrolysis was finished, GMCH was heated in boiling water for 10 min to inactivate the proteases to stop the reaction, cooled to room temperature, and centrifuged at 10,000 × *g* at 4°C for 20 min. The supernatant was lyophilized and stored at −20°C until used.

Determination of Antioxidant Activity and Relevant Parameters

Hydroxyl Radical-Scavenging Activity. The scavenging effect of hydroxyl radical was assayed by using the 2-deoxy-D-ribose oxidation method of Halliwell et al. (1987), Chung et al. (1997), and Zhu et al. (2006), with minor modifications. The reagents were added into a reaction tube in the following order: 0.2 mL of sample solution at various concentrations, 0.9 mL of 0.1 *M* KH_2PO_4-KOH buffer (pH 7.4), and 0.2 mL of 10 m*M* $FeSO_4$-EDTA, 0.5 mL of 10 m*M* 2-deoxy-D-ribose, and 0.2 mL of 10 m*M* H_2O_2. Solutions of $FeSO_4$-EDTA and H_2O_2 were prepared just before use. The reaction solution was incubated at 37°C for 1 h. Then, 1 mL of

Table 1. The optimum enzyme hydrolysis conditions of different proteases for goat milk casein

Parameter	Neutral protease	Alkaline protease	Papain	Trypsin
pH	7.5	8.5	6.5	7.5
Temperature (°C)	50	45	60	50
Addition of enzyme (U/g of protein)	4,000	300	4,000	2,500
Substrate concentration (g/kg)	60	60	60	60

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