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Identification of potent antioxidant bioactive peptides from goat milk proteins

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ABSTRACT

Goat milk proteins have gained increasing attention especially the bioactive peptides released from the parent proteins by digestive enzymes. Specifically, the interest in bioactives of goat milk is intensifying due to its reduced allergenicity compared to bovine milk. In this study, proteins of goat milk were fractionated into caseins (GCP) and whey proteins (GWP), hydrolyzed by pepsin and the generated peptides were examined for radical scavenging activities. The hydrolysates of whey (P-GWP) and casein (P-GCP) proteins exhibited potent superoxide anion ($O_2^{\cdot -}$) scavenging activity in a dose-dependent manner, as investigated using the natural xanthine/xanthine oxidase (X/XOD) system. The P-GWP and P-GCP dramatically quenched the $O_2^{\cdot -}$ flux but had negligible effect on the catalytic function of the enzyme, indicating specificity to scavenge $O_2^{\cdot -}$ but not oxidase inhibition. Further, both P-GWP and P-GCP were able to remarkably quench the chemical DPPH radical. Fractionation of hydrolysates by size-exclusion chromatography produced four fractions (F1-F4) from both hydrolysates, with variable $O_2^{\cdot -}$ scavenging activities. However, the slow eluting fractions (F4) of both hydrolysates and fast eluting fraction (F2) of P-GCP contained peptides with the highest scavenging activities. Peptides in the active fractions of P-GWP and P-GCP, isolated by reversed phase-HPLC, exhibited significantly strong $O_2^{\cdot -}$ scavenging activities. MALDI-TOF-MS allowed the identification of several antioxidant peptides derived from both caseins and whey proteins, with β-casein and β-lactoglobulin being the major contributors, respectively. The results demonstrate that digestion with pepsin generates multiple soluble peptides from goat milk protein fractions with remarkable ability to scavenge superoxide radicals and thus providing a fascinating opportunity for their potential candidacy as antioxidant bioactive peptides.

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1. Introduction

During the last decades, milk proteins have gained increasing attention especially the bioactive peptides released from the parent proteins by digestive enzymes. These peptides have shown to exert beneficial biological activities, including opioid, immunomodulatory, antibacterial and antioxidant activities (Correa et al., 2011; Korhonen & Pihlanto, 2007; Park, 2009; Silva & Malcata, 2005). In contrast to other dairy animals, goat is a main supplier of milk for rural regions and its importance intensifying due to allergy problem of cow milk especially among infants (Almaas et al., 2006; Park, 2009; Roncada et al., 2002; Simos et al., 2011).

Compared to cow or human milk, goat milk has distinct biological properties, such as high buffering capacity, distinct alkalinity (Park, 2009; Park & Haenlein, 2006), high digestibility due to the smaller size of its fat globules (about 3.49 μm) and higher amounts of medium and short chain fatty acids (caproic, caprilic and capric acid) which have been attributed in reduction of cholesterol in human tissues, by limiting cholesterol storage and improving its mobilization (Fahmi, Sirry, & Safwat, 1956; Haenlein, 1992). In addition, goat milk proteins are more readily digestible, and thus amino acids absorbed more efficiently than those of cow milk (Park, 2009). Medically goat milk is being recommended for neonates when human milk is lacking (Carver, 2003).

In neonates, parenteral nutrition contributes to oxidative stress, which is suspected to be a strong inducer of oxidative stress and associated diseases (Chessex, Watson, Kaczala, et al., 2010). The excessive amounts of free radicals in body can cause cellular damages such as oxidizing membrane phospholipids, damages in proteins and DNA (Lee, Koo, & Min, 2004; Li et al., 2013; Urso & Clarkson, 2003), which are

Abbreviations: GCP, goat casein proteins; GWP, goat whey proteins; P-GCP, pepsin digested-GCP; P-GWP, pepsin digested-GWP; BMP, bovine defatted milk proteins; P-GCP, pepsin digested-BMP; RP-HPLC, reversed phase high performance liquid chromatography; DPPH, 2,2-diphenyl-1-picrylhydrazyl; NBT, nitrobluetetrazolium; X/XOD, xanthine/xanthine oxidase system; CBB, coomassie brilliant blue; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry.

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associated with diseases such as diabetes, atherosclerosis, arthritis and cancer (Collins, 2005; Halliwell, 1994; Hiller & Lorenzen, 2009). Therefore, the need for natural antioxidants to protect the body from oxidative stress and the associated diseases of crucial importance (Sarmadi & Ismail, 2010). Recently, it has been reported that hexapeptides derived from human milk exerted free radical–scavenging activities *in vitro* (Miloudi et al., 2012). Therefore, it is important to assess the capacity of peptides from goat milk, as a recommended replacer for human milk, to scavenge free radicals to prevent oxidative stress.

Antioxidant peptides with potency to scavenge free radicals were identified from pepsin hydrolysate of bovine αs1-casein. Peptides from hydrolysates were isolated from bovine β-lactoglobulin digested by corolase PP (Hernández-Ledesma, Davalos, Bartolomé, & Amigo, 2005). Goat milk proteins are similar to the major cow milk proteins in their general classifications but differ in genetic polymorphisms, frequencies and contents (Grosclaude, 1995; Martin, 1993; Moatsou, Hatzinaki, Samolada, & Anifantakis, 2005; Mora-Gutierrez, Kumosinski, & Farrell, 1991). A few studies have been done to explore the antioxidant activity of goat milk proteins hydrolysates obtained by fermentation with lactic acid bacteria (Nandhini, Angayarkanni, & Palaniswamy, 2012), enzymatic hydrolysis as trypsin, subtilisin or bacterial protease (De Gobba, Espejo-Carpio, Skibsted, & Otte, 2014; Li et al., 2013) or papain (Bezerra et al., 2013). However, digestion of goat milk proteins or their fractions with pepsin, a gastrointestinal aspartic protease, has yet to be investigated and crucially needed. This study is aimed to explore the antioxidant activities of pepsin hydrolysates and the peptides generated from whey and casein protein fractions of goat milk. The xanthine/xanthine oxidase couple system and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical were employed to assess the ability of the peptides to scavenge oxygen and chemical radicals. Several antioxidant peptides were isolated from the active hydrolysates and their amino acid sequences were identified.

2. Materials and methods

2.1. Materials

Raw goat milk was collected from farms around the South Valley University (Qena, Egypt). Xanthine (X), xanthine oxidase (XOD), nitroblue tetrazolium (NBT), 2,2-Diphenyl- 1-picrylhydrazyl (DPPH), and pepsin were from Sigma-Aldrich (Tokyo, Japan). Sephacryl S-100 was product of Amersham-Pharmacia Biotech (Tokyo, Japan). All other reagents were of analytical grade.

2.2. Preparation of caseins and whey proteins

Defatted goat milk was prepared by removing fats by centrifugation of fresh goat milk at 5000 × g for 30 min at 10 °C, and after skimming the fats the milk was passed through three layers of gauze. The defatted milk was adjusted to pH 4.6 with 10% acetic acid, followed by centrifugation at 5000 × g for 10 min. The pellet "casein proteins", re-suspended in Milli-Q water, and the supernatant "whey proteins" were dialyzed against dH2O, using1000 MWCO tubes at 4 °C. These fractions were lyophilized and referred to as goat casein protein (GCP) and goat whey protein (GWP).

2.3. In vitro pepsin digestion

Both GCP and GWP dissolved in milli-Q water were adjusted to pH 3.0 with HCl. Pepsin in 1 mM HCl was added to the protein solution at enzyme-to-substrate (E/S) ratio of 1:50 (w/w). Digestion was allowed for 2 h at 37 °C with mild shaking. Reactions were immediately heated at 85 °C for 5 min then placed on ice for 5 min to irreversibly inactivate pepsin. Insoluble solids were removed by centrifugation at 3000 × g for 10 min and the resulting supernatants were adjusted to pH 7.0 then lyophilized, and referred to as pepsin digested-GWP (P-GWP) and pepsin digested-GCP (P-GCP).

2.4. Gel electrophoresis

To evaluate the protein profile after each step of processing and hydrolysis, SDS-PAGE was carried out under reducing and non-reducing conditions on 4–15% acrylamide gels, according to standard protocols (Laemmli, 1970). Protein bands were visualized with CBB.

2.5. Antioxidant activity

2.5.1. Superoxide radical ($O2^{\bullet -}$) scavenging activity

The superoxide scavenging was assessed by using the $O_2^{\bullet -}$ generating system of enzyme-coupled reaction of xanthine/xanthine oxidase (Ibrahim, Hoq, & Aoki, 2007). In the system, the superoxide ($O_2^{\bullet -}$) is generated in the process of converting xanthine (X) to uric acid by xanthine oxidase (XOD) and nitro-blue tetrazolium (NBT) is used as a probe. The scavenging capacity is assessed as competition kinetics of NBT reduction by $O_2^{\bullet -}$ and the scavenger (sample). The reduction of NBT, to form diformazan, reflecting the level of free $O_2^{\bullet -}$ anions, is measured spectrophotometrically at 562 nm.

The reaction mixture (100 μl) contained 41.4 μM NBT, 2.5 or 5 mU xanthine and various concentrations of samples in 10 mM phosphate buffer (pH 8.0) in a microtiterplate. In control reaction (Ctrl) water was added instead of sample. The reaction was initiated by adding 100 μl of 5 or 10 mU XOD. Two blanks were prepared in the same way except without the addition of XOD (SB) or both sample and XOD (CB). The kinetics of the reaction was monitored at 562 nm (37 °C) in real time every 60 sec for 20 min by Ultrospec Biotrak II microplate reader (Amersham Biosciences, Uppsala, Sweden) with on-board software and interface packet for Biochrom reader, and the kinetics were calculated for each protein concentration. Decreased absorbance of the reaction mixture containing antioxidant indicates increased $O_2^{\bullet -}$ scavenging activity. Real-time kinetics of uric acid flux was also employed in order to distinguish whether the anti-oxidative action is unique to $O_2^{\bullet -}$ scavenging or inhibition of the enzyme (XOD). The flux of uric acid was monitored at 295 nm, in a similar reaction mixture but without NBT, whereas buffer was added instead. The blank values were subtracted from each sample. The results were expressed as the rate of absorbance change, by subtracting the reading at 0 time from the subsequent readings; (∂Abs) = Abs (given time) − Abs (0 time). Results are representative of three independent experiments in triplicate wells per sample.

2.5.2. DPPH radical scavenging activity

The DPPH radical scavenging was measured by using two methods: (1) liquid-based kinetic reduction assay of DPPH, and (2) dry TLC-dot assay, where the ethanolic DPPH solution was sprayed over dried protein sample spotted on a TLC sheet.

For the liquid-based assay, it was performed as described by (Brand-Williams, Cuvelier, & Berset, 1995) based on the reduction of the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radicals by antioxidants thus decreasing absorbance at 562 nm. The reaction mixture contained 41.4 μM DPPH in 100 μl ethanol in a microtiterplate. The reaction was initiated by adding 100 μl of samples (at concentrations 200 μg/ml). In control reaction (Ctrl) water was added instead of protein. The kinetics of the scavenging reaction was monitored at 562 nm (37 °C) in real time every 60 s for 20 min by Ultrospec Biotrak II microplate reader with on-board software and interface packet for Biochrom reader. The results were expressed as the rate of absorbance change, as above mentioned, of two independent experiments in triplicate wells per sample.

The TLC-dot assay was employed to investigate the radical scavenging activity of water soluble peptides, which might not dissolve in ethanol in the liquid-based assay (Nia et al., 2004). Briefly, protein samples were spotted (6 μl) on a 5 cm × 7.5 cm thin layer chromatography

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