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Original Article

Cytotoxic and antioxidant capacity of camel milk peptides: Effects of isolated peptide on superoxide dismutase and catalase gene expression

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ABSTRACT

Peptides from natural sources such as milk are shown to have a wide spectrum of biological activities. In this study, three peptides with antioxidant capacity were identified from camel milk protein hydrolysate. Pepsin and pancreatin were used for hydrolysis of milk proteins. Ultrafiltration and reverse-phase high-performance liquid chromatography were used for the concentration and purification of the hydrolysate, respectively. Sequences of the three peptides, which were determined by matrix-assisted laser desorption/ionization time-of-flight spectrophotometry, were LEEQQQTEDEQQDQL [molecular weight (MW): 1860.85 Da, LL-15], YLEELHRLNAGY (MW: 1477.63 Da, YY-11), and RGLHPVPQ (MW: 903.04 Da, RQ-8). The 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to evaluate the cytotoxicity of these chemically synthesized peptides against HepG2 cells. *In vitro* analysis showed antioxidant properties and radical scavenging activities of these peptides on 2,2-diphenyl-1-picrylhydrazyl, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)+, O^{2-}, and OH^{-} free radicals. HepG2 cells were treated with YY-11 peptide for 48 hours, and the expression of superoxide dismutase and catalase genes was examined using real-time polymerase chain reaction. The results revealed a significant increase in the expression of superoxide dismutase and catalase genes in treated HepG2 cells.

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1. Introduction

Free radicals are produced in a wide range of biological and chemical systems [1]. Reactive oxygen species (ROS) including superoxide anions, hydroxyl radicals, nitric oxide radicals, and peroxyl radicals are various forms of free radicals that are produced as byproducts of cellular respiration in mitochondria [2]. Free radicals have dual functions, in which they can play a role in signaling pathways and defense responses against pathogens, but excessive free radicals can damage biomolecules such as DNA, proteins, and lipids and eventually

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cause oxidative stress [3]. Under normal conditions, ROS can be neutralized by the enzymatic and nonenzymatic mechanisms in the body; however, increases in the amount of ROS in the body will lead to an imbalance between free radicals and antioxidants, which finally leads to oxidative stress [2]. A variety of diseases such as cancers are in association with oxidative stress [4]. Owing to the harmful effects of free radicals and oxidative stress in the body, prevention of these reactions seems necessary [5]. Body cells have effective strategies to prevent DNA damage induced by free radicals [6]. Antioxidant enzymes such as glutathione peroxidase, superoxide dismutase (SOD), and catalase (CAT) are part of defense mechanisms against oxidative stress and are able to inhibit ROS rapidly [7]. Levels of these enzymes increase in oxidative stress conditions to prevent possible damage; however, in some cases, the amount of endogenous antioxidants is not enough to inhibit free radicals and an external source of antioxidants is required [8]. Some synthetic antioxidant compounds such as butylated hydroxytoluene and butylated hydroxyanisole, despite their use in medicine, have adverse side effects on the body [9]. Therefore, researches have focused on the identification and extraction of antioxidant compounds from natural sources. Peptides as natural antioxidants have some regulatory effects including nutrient uptake, immune defense, and antioxidant properties [10]. Several studies have been performed on the antioxidant capacity of protein hydrolysates or peptides extracted from natural sources such as egg-yolk protein [11], milk kefir and soymilk kefir [12], casein [13], algae protein waste [14], and buckwheat protein [15]. Camel milk is a rich source of proteins, which is suggested to have biological activity including antibacterial, antiviral, and antioxidant activities (PMID: 1319434). A previous finding has shown that due to it antioxidant activity, camel milk can be considered as a potential therapeutic approach for the treatment of autism spectrum disorder (PMID: 24069051, 20175528). The aim of this study was to investigate the antioxidant properties of the three peptides derived from camel milk proteins and also to evaluate the expression of SOD and CAT genes in HepG2 cells treated with the selected peptide YY-11.

2. Materials and methods

2.1. Materials

Enzymes and chemical compounds used in this study including pepsin, pancreatin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), glutathione, butylated hydroxyanisole, potassium persulfate, trichloroacetic acid, 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), EDTA, and trifluoroacetic acid were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). All cell cultured components were purchased from Gibco, Grand Island, New York, USA. RNA isolation and complementary DNA (cDNA) synthesis kits were purchased from Roche (Mannheim, Germany) and Thermo Fisher Scientific Company (168 Third Avenue Waltham, MA 02451, USA), respectively.

2.2. Preparation of hydrolysate from camel milk using pepsin–pancreatin enzymes

Camel milk was hydrolyzed using a mixture of pepsin–pancreatin enzymes according to Quirós et al [16] with some modifications. For this purpose, first the mixture of pepsin and glycine–HCl buffer (pH 2) was prepared and stirred in special conditions (at 37°C for 2 hours). Then using $NaHCO_3$ (0.9M) and NaOH (1.0M), pH values were adjusted to 5.3 and 7.5, respectively. After reaching the desired pH, pancreatin was added and the mixture was kept at 37°C for 4 hours. In order to inhibit the enzyme activity, the hydrolysate were placed in boiling water for 10 minutes, and then incubated at room temperature (25°C) for 15 minutes and finally centrifuged (at 7000*g*, 4°C, for 15 minutes). The resulting hydrolysate (supernatant) was lyophilized and maintained at −20°C for later analysis. The resulting hydrolysate was dissolved in distilled water and passed through Millipore ultrafiltration membrane of 3 kDa (Billerica, MA, USA). The filtered solution was collected, lyophilized, and stored at −20°C.

2.3. High-performance liquid chromatography and amino acid sequencing

High-performance liquid chromatography (Knauer, Berlin, Germany) was used for the purification of camel milk protein hydrolysate. A flow rate of 2.0 mL/min and a linear gradient of 5–65% acetonitrile containing 0.1% trifluoroacetic acid were used for fractionation over 60 minutes. Five hundred microliters of the resulting hydrolysate (at 20 mg/mL) were prepared and filtered through a 0.45-μm filter, and then loaded on a C_{18} column (Macherey-Nagel, Düren, Germany). Elution of peaks was monitored at 220 nm, and fractions were collected and lyophilized. The collected fractions were further purified using the same gradient of acetonitrile as mentioned above at a flow rate of 1 mL/min. The peptides of interest were analyzed through *de novo* sequencing using matrix-assisted laser desorption/ionization time-of-flight (at Proteomics International Pty Ltd (Nedlands, Western Australia).

2.4. Evaluation of DPPH free radical scavenging

DPPH assay was used to evaluate the scavenging ability of the peptides according to Binsan et al [17] with some modifications. Briefly, a mixture containing 0.5 mL of sample (0–1 mg/mL, final concentration) and 0.5 mL of DPPH (0.15mM in 95% ethanol) was prepared. The mixture was incubated for 30 minutes in the dark at room temperature (25°C).

Sample absorbance (A_{Sample}) was measured at 570 nm using a spectrophotometer (Epoch, Winooski, Vermont, USA). Free radical scavenging ability of the peptides was assessed by the following formula [17]:

$$\text{DPPH radical scavenging (\%)} = (A_{Control} - A_{Sample}/A_{Control}) \times 100$$

A mixture containing 0.5 mL water and 0.5 mL DPPH in ethanol was used as a control.

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