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Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides

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Abstract

Yak (*Bos grunniens*) milk casein derived from Qula, a kind of acid curd cheese from northwestern China, was hydrolysed with alcalase. The hydrolysates collected at different hydrolysis times (0 min, 60 min, 120 min, 180 min, 240 min, 300 min, 360 min) were assayed for the inhibitory activity of angiotensin-I-converting enzyme (ACE), and the one obtained at 240 min hydrolysis showed the highest ACE inhibitory activity. The active hydrolysate was further consecutively separated by ultrafiltration with 10 kDa and then with 6 kDa molecular weight cut-off membranes into different parts, and the 6 kDa permeate showed the highest ACE-inhibiting activity. This active fraction was further purified to yield two novel ACE-inhibiting peptides, whose amino acid sequences were Pro–Pro–Glu–Ile–Asn (PPEIN)(κ-CN; f156–160) and Pro–Leu–Pro–Leu–Leu (PLPLL) (β-CN; f136–140), respectively. The molecular weight and IC_{50} value of the peptides were 550 Da and 566.4 Da, and 0.29 ± 0.01 mg/ml and 0.25 ± 0.01 mg/ml, respectively.
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Keywords: Qula; Yak (*Bos grunniens*) milk casein; ACE inhibitory peptide; Alcalase

1. Introduction

The currently used synthetic drugs for the treatment of hypertension, e.g., captopril and enalapril have certain side effects, such as coughing, skin discomfort, and, in particular, excessively low blood pressure (Je, Park, Jung, Park, & Kim, 2005). Natural ACE inhibitors such as bioactive peptides, as alternatives to synthetic drugs, have awoken the attention of both food and medical researchers.

Bioactive peptides possess many activities, such as immunoregulatory, opioid, antioxidant, or antihypertensive (Mao, Nan, Li, & Ni, 2005a, 2005b; Martin, Gwenaele, & Said, 1999; Pihlanto, 2001; Sandre et al., 2001). In recent years, many ACE inhibitory peptides have been isolated from various food proteins, such as fish protein (Curtis, Dennis, Waddell, MacGillivray, & Ewart, 2002), cheese whey (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998; Hernández, Recio, Ramos, & Amigo, 2002; Sandrine, Pascal, Céline, & Adèle, 2003), casein (Maeno, Yamamoto, & Takano, 1996; Mizuno, Nishimura, Matsuura, Gotou, & Yamamoto, 2004; Yamamoto, Maeno, & Takano, 1999), fermented milk products (Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000; Yamamoto et al., 1999), corn gluten (Suh & Whang, 1999), bovine plasma (Janitha et al., 2002) and fermented soybean (Gibbs, Zougman, Masse, & Mulligan, 2004). Bioactive peptides can be generated not only during the manufacture of cheese, yogurt and other dairy products, but also during protein hydrolysis by digestive enzymes, such as pepsin, trypsin, or chymotrypsin. They may also be generated by controlled protein hydrolysis. Using commercially available microbially-derived food grade proteases to hydrolyse casein is advantageous because these enzymes are cheap and safe.

Yak milk is a common product in northwestern China, such as Gansu, Xinjiang and Tibet. In these areas, people

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only use it for the production of butter, and the by-product (Qula, a kind of crude cheese, whose main component is casein) is not fully used. Therefore, improving the value of Qula has received great attention from the Chinese government. Fortunately, the casein content of Qula is greater than 80% on a dry weight basis.

This work was carried out to determine the proper enzymatic conditions to convert Qula into a good source of antihypertensive peptides, and to isolate and characterise ACE inhibitory peptides from yak milk casein hydrolysate. The results would determine the potential of yak milk casein as an ingredient for the production of antihypertensive functional foods.

2. Materials and methods

2.1. Materials and chemicals

Qula (casein) was provided by Tongjian Co. (Gansu province, China); Hippuryl-L-histidyl-L-leucine (HHL), angiotensin-I-converting enzyme (ACE), hippuric acid (HA), gastric porcine pepsin (EC.3.4.23.1, 1:10000) and trypsin (EC.3.4.21.4, 1:250, Gibco-BRL, activity 2–4 U/mg) were purchased from Sigma–Aldrich Company (St. Louis, MO). Alcalase was purchased from Huaqiang Biochem. Inc. (activity, 5000 U/g, Beijing, China).

2.2. Preparation of yak milk casein

Casein was precipitated from Qula through pI (isoelectric point) precipitation. Qula was heated at 85 °C in 0.05 M NaOH until dissolved completely. The solution was cooled to 25 °C and acidified to pH 4.6 with 0.5 M HCl, to induce casein precipitation. The acid curd was lyophilised to get yak milk casein.

2.3. Hydrolysis of yak milk casein

Casein was dissolved in demineralised preheated water and brought to the appropriate pH using 1 N NaOH. Casein was hydrolysed by alcalase from *Bacillus licheniformis*. The hydrolysis conditions were as follows: substrate concentration was 6% (w/w), enzyme dose was 2.5% (w/w, defined as enzyme mass/substrate mass × 100%), pH value was 8.0, temperature was 55 °C. At different hydrolysis times (0 min, 60 min, 120 min, 180 min, 240 min, 300 min, 360 min) during hydrolysis, samples were collected and immediately heated in a boiling-water bath for 10 min to inactivate the enzyme. The samples were defined as A0, A1, A2, A3, A4, A5 and A6, respectively. The unhydrolysed casein was removed by pI precipitation. The hydrolysates were collected and lyophilised for further analysis.

2.4. Degree of hydrolysis

The degree of hydrolysis (DH%), derived from the α-amino nitrogen and the total nitrogen, was calculated as follows (Mahmoud, Malone, & Cordle, 1992):

$$\mathrm{DH}\,\% = \frac{\mathrm{AN_h} - \mathrm{AN_c}}{\mathrm{TN} \times P_f} \times 100$$

$\mathrm{AN_h}$ and $\mathrm{AN_c}$ are the percent amino nitrogen of hydrolysate and intact casein, respectively. No significant difference was found between total nitrogen of the intact casein and that of the hydrolysates. Thus, TN refers to the mean percent total nitrogen of the intact casein solution and all hydrolysate samples, and P_f is a correction factor for side chain nitrogen, which cannot be converted to amino nitrogen by hydrolysis of peptide bonds. The P_f factor was calculated from the amino acid profile of casein.

2.5. Measurement of ACE inhibitory activity

The determination of ACE inhibitory activity was performed by a HPLC method, with a modification of the method of (Cushman & Cheung, 1971). HHL (5.0 mM) was dissolved in 90 mM/L Na-borate buffer (pH 8.3) containing 0.4 M NaCl. ACE was dissolved in the same buffer, at a concentration of 60 mU/ml. A mixture containing 150 μl sample and 75 μl ACE solution was incubated at 37 °C for 5 min, 150 μl HHL solution was then added and incubated for 30 min. The reaction was stopped with 50 μl of 0.1% trifluoroacetic acid (TFA). Hippuric acid liberated by ACE was determined by RP-HPLC on a Zorbax Eclipse (2.5 × 150 mm, Agilent, Palo Alto, CA) column. The mobile phase was acetonitrile and Milli-Q water, and the flow rate was 0.8 ml/min. The effluent was monitored with an ultraviolet detector (Agilent) at 228 nm. A series of standard hippuric acid solutions were prepared to construct a calibration curve of peak area versus hippuric acid concentration. The ACE inhibiting activity of the tested substances was calculated as follows:

$$\text{ACE inhibiting activity}\ (\%) = \frac{[\text{hippuric acid}]_{\text{control}} - [\text{hippuric acid}]_{\text{sample}}}{[\text{hippuric acid}]_{\text{control}}} \times 100$$

2.6. Purification of ACE inhibitory peptides

2.6.1. Ultrafiltration

For purification of ACE inhibitory peptides, the active casein hydrolysate was passed through a polyether–sulfone ultrafiltration membrane with 10 kDa and then with 6 kDa molecular weight cut-off membranes (Millipore Co., Billerica, MA) successively. It was fractionated into three fractions (>10 kDa, 6–10 kDa, and below 6 kDa).

The antihypertensive activities of these fractions were tested by HPLC method.

2.6.2. Gel filtration

The most active fraction was loaded onto a DE-52 column (1.6 cm × 30 cm), previously equilibrated with PBS (5 mM, pH 8.0). After washing with PBS (5 mM, pH 8.0) until the UV absorbance returned to baseline, the absorbed peptides were eluted with a linear gradient of NaCl

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