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Separation and Purification of Angiotensin Converting Enzyme Inhibitory Peptides Derived From Bovine Casein

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Angiotensin-converting enzyme plays an important physiological role in the blood pressure regulation of people, and antihypertensive peptides from natural food have been seen as the hotspots in the physiological bioactive peptides. In the presented work the hydrolysate of bovine casein with high inhibitory activity to angiotensin-converting enzyme (IC50=0.76mg/mL) was produced by alcalase, and its fraction (3-2) with a inhibitory ratio of 93.56% was obtained by the consecutive separation method including ultrafiltration and preparation reverse-phase high performance liquid chromatography. Amino acid sequence analysis by mass spectrography showed the molecular weight of antihypertensive fraction (3-2) was 956 Da and the most probable structure unit is Gly-Ala.

Key words: Angiotensin-converting enzyme (ACE), Inhibitory peptide, Bovine casein.

Hypertension, which carries a high risk of cerebrovascular, cardiac, and renal complication, has become the most common serious chronic health problem over the world in recent years. The number of people who are considered to be suffering from hypertension depends on the levels of "normality" for blood pressure given by different health organizations. Generally, 20-45% of a population and nearly 50–60% of elderly people have elevated blood pressure[1, 2].

Angiotensin converting enzyme (ACE) plays an important physiological role in the regulation of blood pressure by virtue of two different reactions which it catalyzes: conversion of the inactive decapeptide angiotensin I (DRVYIHPFHL) into a powerful vasoconstrictor and salt-retaining octapeptide, angiotensin II (DRVYIHPF), and inactivation of the vasodilator nonapeptide, bradykinin, which is conducive to lower blood pressure[3]. Synthetical ACE inhibitors contain captopril, enalapril, lisinopril, and others which are effective and have been developed for lowering blood pressure. However, they inevitably cause adverse side effects[4]. Therefore, it is necessary to find innovative, safer, and more economical ACE inhibitors for the prevention and remedy of hypertension.

To date, more than 200 kinds of ACE inhibitory peptides have been reported in hydrolysates from diverse food proteins digested with different proteases[5]. Most of these peptides were derived from plants[6, 7], casein[8], fish muscle proteins[9], fermented foods[10-12] and so on. To further understand the relationship between structure and function of ACE-inhibitory peptide, in the presented work ACE inhibitory peptide was produced from casein by alcalase, purified through consecutive separation method and analyzed with mass spectrography (MS).

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MATERIALS AND METHODS

Material and reagents

Casein with a total protein content of 90.5% (w/w) was purchased from Beijing Aoboxing Biotech Co., Ltd. (Beijing, China). ACE(from rabbit lung) and the substrate (hippuryl-histidyl-leucine, HHL) of ACE were purchased from Sigma Chemical Co. (St. Louis, MO). Papain and alcalase were provided by Nanning Pangbo Biological Engineering Co., Ltd. (Nanning, China). All other reagents were analytical reagents.

Enzymatic hydrolysis of bovine casein

To produce bioactive peptides, enzymatic hydrolysis was performed by papain or alcalase under each optimal condition (Table1) respectively. The concentration of substrate casein was 15% (w/v) and the ratio of enzyme to substrate is 0.15% (w/w). The mixture was adjusted to the desired pH by adding 2 mol/L HCl or NaOH and incubated at each optimal temperature with stirring for 6 h, while the standard NaOH solution was added to maintain the enzyme's optimum pH range. After reaction the mixture was heated in a boiling water bath for 15 min and cooled to room temperature. Then the hydrolysate was centrifuged at 4000 r/min for 15min and supernatants were collected.

In vitro ACE inhibitory activity of hydrolysate

ACE inhibitory activities were analyzed by HPLC, ACE(0.1U/mL, 15μL) was added to 0.15 mL buffer solution pH8.3 (consisting of 0.05mol/L $Na_2B_4O_7.H_2O$, 0.2 mol/L H_3BO_3, 0.3 mol/L NaCl, and 5mmol/L Hip-His-Leu) containing 20μL hydrolysate (1mg/mL). The reaction was incubated at 37°C for 30 min and then terminated by the addition of 0.15mL 1mol/L HCl. Then reaction mixture was applied to a JASCO C_{18} column (5μm, 4.6× 250mm, JASCO International Co. Ltd., Japan), and eluted with 30% methanol and 70% purified water containing 0.1% (w/w) trifluoroacetic acid (TFA) at a flow rate of 1 mL/min. The absorbance was detected at 228 nm.

The inhibitory activity of hydrolysate was calculated as [(Ac–As)/Ac]×100, where As is the peak area of hippuric acid with a hydrolysate added to the reaction mixture, and Ac is the peak area of hippuric acid without a hydrolysate added to the reaction mixture.

Purification of ACE inhibitory peptide

The hydrolysate with the highest ACE inhibitory activity was successively passed through an ultrafiltration (UF) membrane with 10 kDa and 3 kDa molecular weight cutoff (MWCO). The permeate (<3kDa) obtained was lyophilized, then redissolved in distilled water and subjected (20μL) to HPLC purification on an ODS C_{18} column (10μm,10 ×300 mm, SHIMADZU, Japan) with a linear gradient elution of HPLC grade methanol (10%-80% in 0-35min, 80%-95% in 35-38min, 95%-10% in 38-40min, 10% in 40-45min) and purified water containing 0.1% TFA at a flow rate of 4.0 mL/min. The elution peaks were detected at 280 nm. The activity peak was concentrated using a rotary evaporator and subjected to further purification as the above procedure again and then lyophilized.

Amino acid sequence of ACE inhibitory peptide

The accurate molecular mass was determined by mass spectrography (MS) (MALDI-TOF-TOF-MS, Bruker, GER) using N_2 as laser source and detected at 337 nm. Detection method is reflection mode (flight tube length 2.7m, accelerating voltage 20KV, and reflected voltage 23KV). The amino acid sequence of the purified peptide was determined by using N-terminal protein sequence instrument (ABI491). To measure the amino acid sequence of the purified peptide by making use of N-terminal protein sequence instrument (ABI491).

RESULTS

Production of ACE inhibitory peptides

To produce ACE inhibitory peptides, bovine casein was hydrolyzed with various enzymes such as alcalase, papain and the combination of these two enzymes. The hydrolysis time of each enzyme was 6 h; for dual enzyme hydrolysis, alcalase's hydrolysis time was 3h and then papain's hydrolysis time was 3h. In this study, these commercial enzymes were selected to evaluate their effectiveness in producing ACE inhibitory peptides from bovine casein. The extent of protein degradation by proteolytic enzymes was

Table 1. The Optimum Reaction Condition of Enzyme

Enzyme	pH	Temperature (°C)
Alcalase	9.5	50
Papain	6.5	45

Table 2. The DH and ACE inhibition ratio of bovine casein treated by different enzymes

Treated enzymes	ACE inhibition(%)	Degree of hydrolysis(%)
Alcalase	64.36	22.71
Papain	48.47	15.55
The combination of two enzymes	59.81	22.41

estimated by assessing the DH, and results were shown in Table 2. Alcalase and its combination with papain both could effectively hydrolyzed casein, and the DH of their hydrolysates are higher than 20%. Due to low-MW peptides being more potent as bioactive peptides than high-MW peptides[13], hydrolysates were further separated successively by using the UF membrane with 10 kDa and 3 kDa MWCO, and permeate was

Table 3. ACE inhibitory activity of fractions isolated by RP-HPLC

Fractions	1	2	3	4	5	6	7
ACE inhibition[a](%)	61.18	63.24	85.63	89.16	68.38	27.05	37.16

a: ACE inhibition was determined with 20 µL of each fraction (1mg/mL).

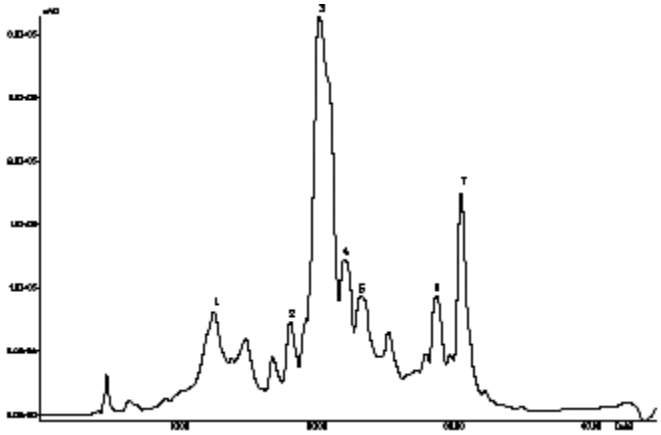

Fig. 1. Separation of ACE-inhibitory peptide from the active fraction by RP-HPLC

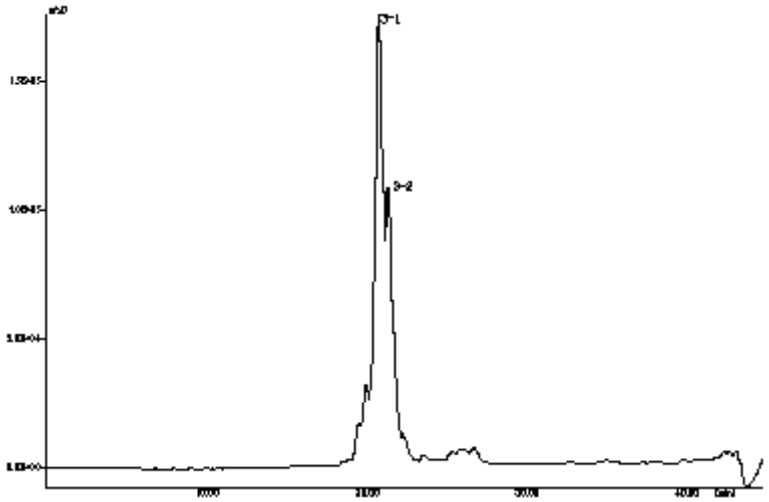

Fig. 2. HPLC Chromatogram of fraction 3 eluted under the same conditions in Fig. 1

lyophilized and assayed for ACE inhibitory activity. As shown in Table 2, the hydrolysate produced by alcalase possessed the highest ACE inhibitory activity, and its IC_{50} is 0.76mg/mL. To identify ACE inhibitory peptides, the hydrolysate produced by alcalase was selected for further analysis.

Identification of ACE Inhibitory Peptide

ACE inhibitory peptides were obtained from the active fraction after UF by RP-HPLC chromatography. The seven main fractions were isolated as shown in Figure 1. The main fraction with higher ACE inhibitory activity was isolated and designated as fraction 3 (Table 3). To identifying the active peptides, fraction 3 was separated and further purified through the same RP-HPLC column with the same gradient elution. After the second purification, the fraction 3 was separated into two components and designated as fraction 3-1 and 3-2, as shown in Figure 2. The ACE inhibition of fraction 3-2 is 93.56%, higher than that of fraction 3-1. So fraction 3-2 was lyophilized and subjected to MS identification, and its fragmentation spectrum contained three major ions at m/z 956 as shown in Figure 3. ACE inhibitory activity of peptide is highly dependent on its structure, most of the strong and competitive ACE inhibitory peptides have an aromatic amino acid (Trp, Tyr or Pro) or Arg at the C-terminal and a hydrophobic or basic amino acid at the N-terminal. The sequence of this peptide was identified as Gly-Ala using N-terminal protein sequence instrument.

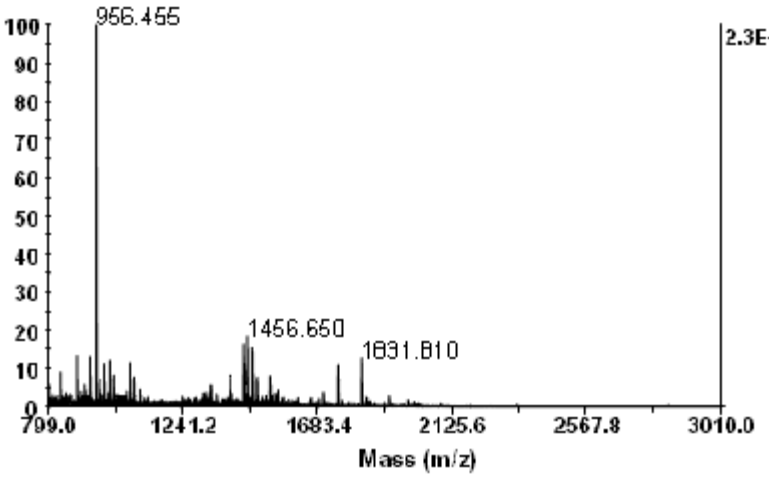

Fig. 3. MALDI-MS spectrum of fraction 3-2

CONCLUSION

The ACE inhibitory peptide with a inhibitory ratio of 93.56% was obtained from bovine casein by alcalase in this study. It contains at least a Gly-Ala dipeptide at the N-terminal and has a high ACE inhibitory activity in vitro. But there is no animal experiments in vivo and needs for further research to determine its activity in vivo.

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