Separation and Purification of Angiotensin Converting Enzyme Inhibitory Peptides Derived from Goat's Milk Casein Hydrolysates

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ABSTRACT : To investigate the basic information and the possibility of ACE-inhibitory peptides for antihypertension materials, goat's caisin (CN) was hydrolyzed by various proteolytic enzymes and ACE-inhibitory peptides were separated and purified. ACE-inhibition ratios of enzymatic hydrolysates of goat's CN and various characteristics of ACE-inhibitory peptides were determined. ACE-inhibition ratios of goat's CN hydrolysates were shown the highest with 87.84% by pepsin for 48 h. By Sephadex G-25 gel chromatograms, Fraction 3 from goat's CN hydrolysates by pepsin for 48 h was confirmed the highest ACE-inhibition activity. Fraction 3 g and Fraction 3 gh from peptic hydrolysates by RP-HPLC to first and second purification were the highest in ACE-inhibition activity, respectively. The most abundant amino acid was leucine (18.83%) in Fraction 3 gh of ACE-inhibitory peptides after second purification. Amino acid sequence analysis of Fraction 3 gh of ACE-inhibitory peptides was shown that the Ala-Tyr-Phe-Tyr, Pro-Tyr-Tyr and Tyr-Leu. IC_{50} calibrated in peptic hydrolysates at 48 h, Fraction 3, Fraction 3 g and Fraction 3 gh from goat's CN hydrolysates by pepsin for 48 h were 29.89, 3.07, 1.85 and 0.87 g/ml, respectively. Based on the results of this experiment, goat's CN hydrolysates by pepsin were shown to have ACE-inhibitory activity. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 5 : 741-746)***

Key Words : ACE-inhibitory Peptide, Goat's Milk CN, Proteolytic Enzyme

INTRODUCTION

Recently, high blood pressure (hypertension) is a major risk factor in cardiovascular disease. Angiotensin converting enzyme (ACE) inhibitors, in particular, is a subject of much study, as it is a material that could directly inhibit hypertension, which is at once the cause of circulatory system diseases and shows an extremely high fatality rate if complicated with cerebral hemorrhage, heart disease or nephropathy (Miyoshi et al., 1989; Brown and Vaughan, 1998).

The factor that inhibits ACE activity in food is low molecular material. This is stable in heat and is easily absorbed in the body (Maruyama et al., 1985). Although, its inhibitory effect is weaker than that of existing hypertensive agents, its promise is gathering hope in that it commands a large intake since it is provided in a dietary form (Shimizu, 1994). Such dietary ACE-inhibitory material is known to exist in the proteolytic hydrolysates of diverse animals, plants and fish (Hara et al., 1987; Kohama, 1988; Miyoshi et al., 1989; Kohmura et al., 1990; Miyoshi et al., 1991; Seki et al., 1993).

Milk proteins are commonly known as precursors of a range of biologically active peptides (Meisel, 1997; FitzGerland and Meisel, 2000; Oukhatar et al., 2000; Kim and Lim, 2004; Kim et al., 2004). Several ACE-inhibitory peptides derived from milk protein have been studied (Mullally et al., 1997; Pihlanto-Leppälä et al., 1998, 2002). Protein from goat's milk shows a stronger digestive ability than that from regular bovine milk protein, while at the same time does not accompany side effects such as allergies or diarrhea; such potential promises a high dietary value (Haenlein, 1995). Hernandez-Ledesma et al. (2002), examined the ACE-inhibitory effects of peptide fragments from β-lactoglobulin of caprine whey. However, little study can be found on ACE-inhibition from proteolytic hydrolysates derived from casein (CN) in goat's milk.

The objective of this study was to separate ACE-inhibitory peptide from goat's milk casein hydrolysates and identify its characteristics.

MATERIALS AND METHODS

Enzymes and reagents

Fresh goat's milk (Saanen; *Ovis aegagrus*) was purchased from Korean Medi-R Co. and then defatted by ultrafiltration (Supra 25 K, Hanil Sci., Korea). The separation of goat's milk CN was performed according to the methods of Sofia and Malcate (2000).

Trypsin (Bovine pancreas, activity 3.3 Anson units g^{-1} protein) and Neutrase 0.8 (*Bacillus subtilis*, activity 0.8 Anson units g^{-1} protein) were from Novo Nordisk A/S (Bagsvaerd, Denmark), Protease S (*Bacillus stearothermophilis*, activity 10,000 units g^{-1} protein) and Papain W-40 (*Carica*

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papara L., activity 400,000 units g^{-1} protein) were from Amano Enzymes (Japan). Pepsin (Porcine gastric mucosa, activity 0.8-2.5 units g^{-1} protein) was purchased from Sigma Chemical Co. (USA).

BSA, TNBS (trinitrobenzensulfonic acid) and Angiotensin Converting Enzyme (rabbit lung) were purchased from Sigma Chemical Co. (USA). All other reagents were of an analytical grade.

Preparation of goat's CN hydrolysates

Hydrolysis of goat's CN was determined from the method of Adamson and Reynolds (1996). Goat's CN was adjusted to pH 8.0 and pH 2.0 by the addition of 0.5 N NaOH and 0.5 N HCl, respectively. Commercial food-grade enzymes preparations, dissolved in distilled water, were added to the reaction mixture at the ratio of 1:100 (enzyme:substrate, w/w, protein basis). The pH of the reaction mixture was maintained at a constant through the continuous addition of 0.5 N NaOH using a pH-stat (Metrohm Ltd., Herisan, Switzerland). During hydrolysis, samples were withdrawn after 0.25, 0.5, 1, 2, 4, 8, 16, 24, 36, 48, 60 and 72 h, and the enzyme was inactivated by heating for 10 min at 90°C. The hydrolysates were centrifuged (12,000×g) and the precipitate discarded. The supernatant was freeze-dried and stored at -20°C.

Determination of degree of hydrolysis (DH) and protein concentration

The DH for all enzymatic hydrolysates was determined according to the method of Adler-Nissen (1986). Protein concentrations in hydrolysates and fractions were determined by the dye-binding method of Lowry et al. (1951). BSA was used as the standard.

Measurement of ACE-inhibitory activity

The ACE-inhibitory activity was measured by the method of Cushman and Cheung (1971). The method is based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL) catalyzed by ACE. The assay mixture contained the following components: 5 mM HHL, 200 mM potassium phosphate buffer (pH 8.3), 200 L of ACE from rabbit lung. After 30 min of incubation at 37°C the reaction was stopped by addition of 250 L 1 N HCl. The hippuric acid formed by action of ACE was extracted with ethyl acetate, and after removal of ethyl acetate by heat evaporation the amount of hippuric acid was measured spectrophotometrically at 228 nm. The amount of hippuric acid liberated from HHL under test conditions-but in the absence of an inhibitor-is defined as 100% ACE activity.

The inhibitory activity of hydrolysate/peptide fraction was expressed as percentage of ACE inhibition at a given protein concentration or as the concentration needed to inhibit 50% of the original ACE activity (IC_{50}).

Separation and purification of ACE-inhibitory peptides

Gel filtration chromatography : Separation of ACE-inhibitory peptides in peptic hydrolysates adopted the method of Maruyama et al. (1985). Gel filtration chromatography was performed on Sephadex G-25 (Sigma Co., USA). The column (2.5×50 cm) previously equilibrated with the distilled water, operated at a flow rate of 0.2 ml min^{-1} and fraction of 2 ml were collected and analyzed by UV-absorbance (Perkin Elmer Lambda EZ 201, USA) at 280 nm. The samples were filtered through 0.5 m syringe filters prior to application to the column. The fraction with the highest ACE-inhibitory activity was further analyzed by reverse phase high performance liquid chromatography (RP-HPLC).

Reverse phase-HPLC : The first and the second purification of ACE-inhibitory peptides in goat's CN hydrolysates were analyzed by RP-HPLC on a Nucleosil (Nucleosil C-18 5 Micron, Alltech Associates, Inc., USA) C-18 column (4.6×250 mm), equilibrated with solvent A [0.1% trifluoroacetic acid (TFA) in H_2O] and eluted with a linear gradient to solvent B (0.1% TFA in acetonitrile) for 40 min. Runs were conducted at a room temperature using a Dionex HPLC system (ASI 100, Dionex Co., USA) at a flow rate of 1.0 ml min^{-1}, and the absorbance of the column elute was monitored at 214 nm. The injection volume was generally 10 L, and the concentration of peptide material applied was approximately equivalent to 0.5 mg ml^{-1} protein. The samples were filtered through a 0.2 m syringe filters prior to application to the C-18 column.

Amino acid analysis and amino acid sequence

The amino acid analysis was performed by the method of Moore et al. (1958). The purified ACE-inhibitory peptides (exactly 1 mg of protein) were dialyzed exhaustively against distilled water and lyophilized, and then hydrolyzed in 1 ml of 6 N HCl in evacuated tubes at 110°C for 24 h. After being concentrated by speed vacuum concentration (MAXI-DRY PLUS, Heto-Holten A/S., Denmark), the sample was dissolved in 0.2 M sodium citrate loading buffer (pH 2.2), and then filted through a 0.2 m syringe filters. The analysis of amino acid was carried out on an amino acid analyzer (Biochem 20, Pharmacia, Sweden). The N-terminal sequence of the purified ACE-inhibitory peptides was identified by sequence analysis with a protein sequencer (962592A, Perkin-Elmer, USA).

RESULTS AND DISCUSSION

Degree of hydrolysis

DH of goat's milk CN from commercial enzymes is shown in Figure 1. The CN hydrolysates from goat's milk exhibited various DH from 18.1% to 60.8% depending on the characteristics of each enzyme. The hydrolysis of all

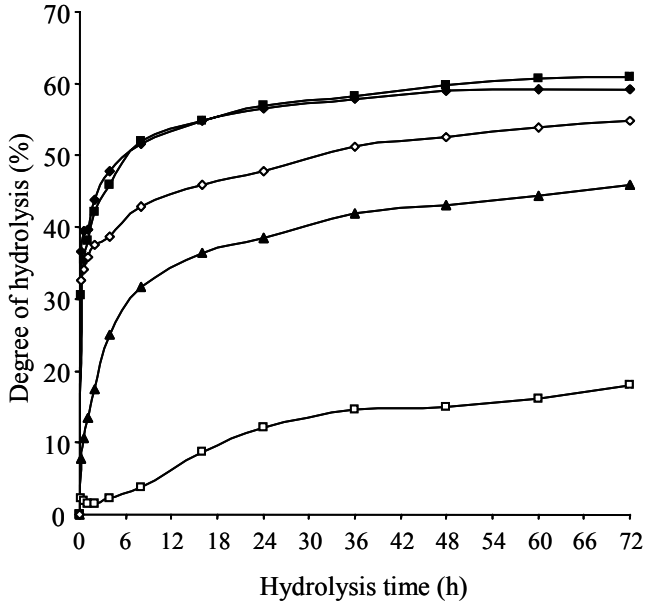

Figure 1. DH (Degree of hydrolysis) of goat's CN by commercial proteases. Legend: Trypsin (♦), Papain W-40 (◊), Protease S (■), Neutrase 1.5 (□) and Pepsin (▲).

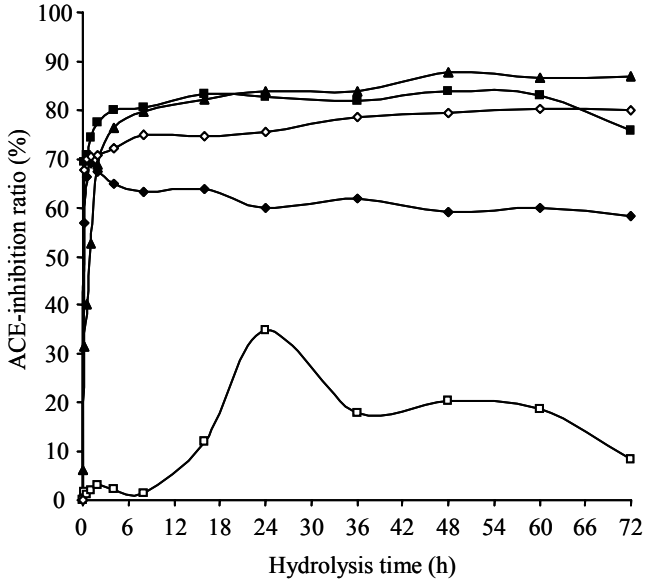

Figure 2. ACE-inhibition ratios of goat's CN hydrolysates by commercial proteases. Legend: Trypsin (♦), Papain W-40 (◇), Protease S (■), Neutrase 1.5 (□) and Pepsin (▲).

enzymes showed a precipitous climb in the first 8 h but a gradual growth thereafter. Of all the hydrolyzed enzymatic hydrolysates, protease S showed the highest DH (60.8%), by 72 h. The result of this study shows that protease S, which is an enzyme derived from a microorganism, had a higher hydrolysis ratio than that of trypsin which is an animal enzyme. This can be explained by the fact that protease S has the characteristic of decomposing in random whereas trypsin has a limited cutting section.

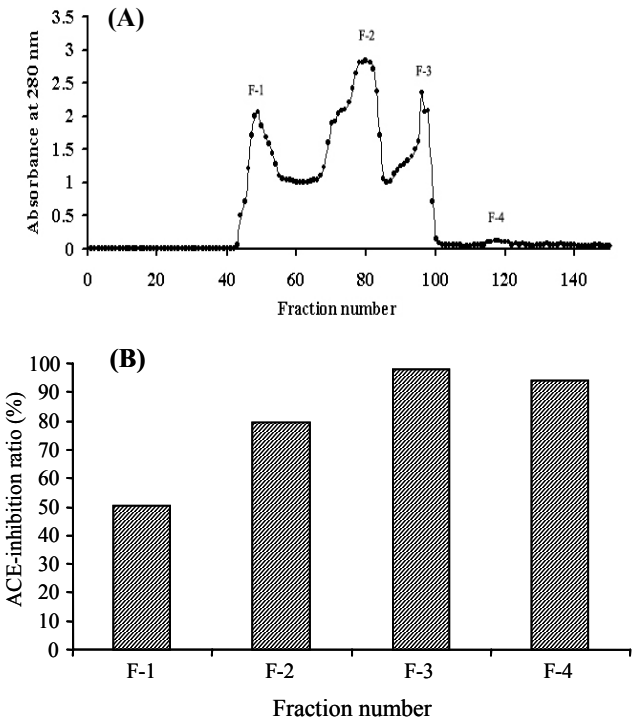

Figure 3. Sephadex G-25 gel chromatograms of the goat's CN hydrolysates at 48 h by pepsin (A) and ACE-inhibition ratios of the collected fractions from the Sephadex G-25 gel chromatograms (B). Legend: F-1 (tube no. 41-52), F-2 (tube no. 63-87), F-3 (tube no. 88-107) and F-4 (tube no. 108-182).

ACE-inhibitory activity

Figure 2 shows the ACE-inhibitory activity of goat's milk CN hydrolysates after enzyme hydrolysis. The hydrolysate by pepsin at 48 h sowed the highest activity (87.8%), followed by protease S (83.9%) at 48 h, papain W-40 (80.3%) at 60 h, trypsin (69.0%) at 1 h and neutrase 0.8 (34.9%) at 24 h. This result was in line with that from the study of Ukeda et al. (1991), which concluded that hydrolysis by pepsin and chymotrypsin was the most effective after various enzyme hydrolysis of sardine meat and an ensuing examination of ACE-inhibitory activity. Goat's milk CN before enzyme treatments showed a zero hydrolysis ratio. In general, the ACE-inhibitory ratio showed a rapid growth in the initial stage of hydrolysis. This result is similar to that of Yeum et al. (1993), in which bovine CN hydrolysis by trypsin or chymotrypsin increased the ACE-inhibitory activity dramatically for the first 8 h but the activity was quickly decreased. This suggested that an increase in DH might not always lead to an increase in ACE-inhibitory activity and that hydrolysis beyond a certain level proved to be ineffective. Therefore, ACE-inhibition requires an ACE-inhibitory peptide that has a special sequence for ACE-inhibitory activity.

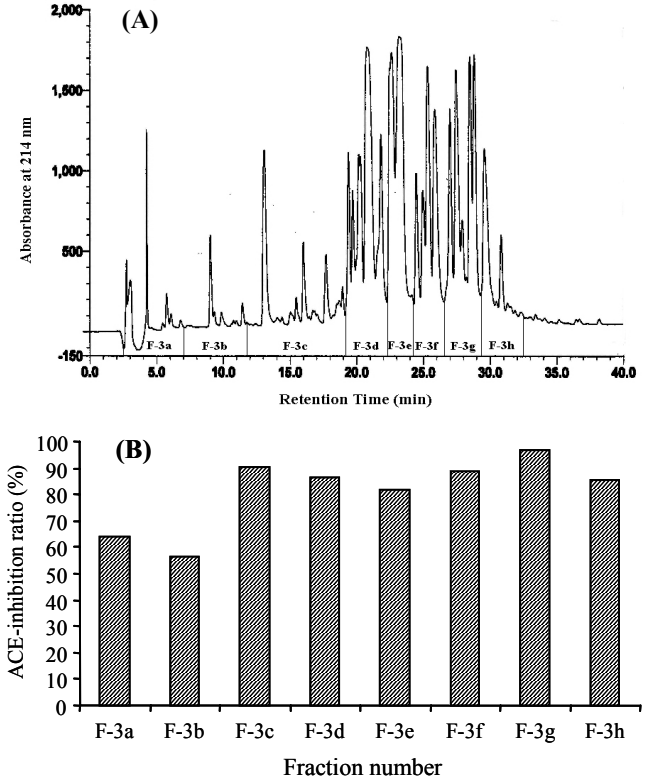

Figure 4. The first fractionation by RP-HPLC of the most active fraction (F-3) obtained by Sephadex G-25 gel chromatography from goat's CN hydrolysates by pepsin at 48 h (A) and ACE-inhibition ratios of the collected fractions by the first fractionation (B). Fractions were termed with F-3a to F-3h followed by a number, respectively.

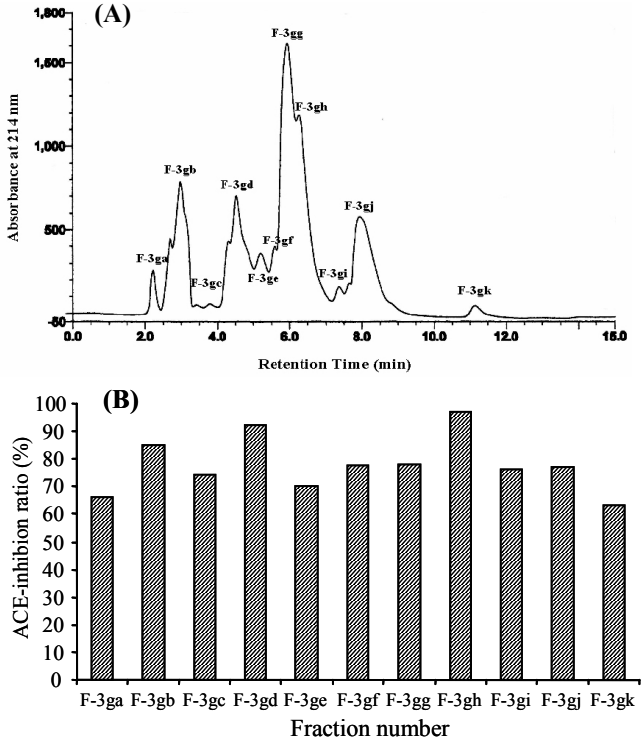

Figure 5. The second fractionation by RP-HPLC of the most active fraction (F-3g) obtained by the first RP-HPLC from goat's CN hydrolysates by pepsin at 48 h (A) and ACE-inhibition ratios of the collected fractions by the second fractionation (B). Fractions were termed with F-3ga to F-3gk followed by a number, respectively.

Separation and purification of ACE-inhibitory peptides

Pepsin hydrolysates (48 h) were divided into 4 fractions by gel filtration. Fraction 3 (F-3) and F-4 showed a high inhibition of 90% or above. In particular, F-3 showed the highest ACE-inhibition of 97.8% (Figure 3). Figure 4 is the result of separating ACE-inhibitory peptide from F-3 that was divided by gel filtration and purifying with linear gradient using RP-HPLC that is equipped with a C-18 column. Divided into a total of 8 fractions, the ACE-inhibitory activity was measured for each fraction. F-3 g (96.8%) exhibited the highest inhibition ratio. Next, to increase the level of purity in F-3 g, RP-HPLC was used again under the same C-18 column. The result is shown in the chromatogram of Figure 5. A measurement of ACE-inhibitory activity of each fraction showed that F-3 gh had the highest inhibitory activity of 97.0%.

Amino acid analysis

Table 1 shows the amino acid composition of F-3 gh, which showed an excellent ACE-inhibitory activity derived from pepsin hydrolysates of goat's milk CN. The concentration of leucine is higher than that of the others. It is known that specific amino acid residues (Leu, Ile, Tyr, Pro and Glu) are involved in ACE-inhibitory activities (Cheung et al., 1980; Miyoshi et al., 1991; Matsumura et al., 1993). In the present study, the concentration of Leu, Ile, Tyr, Pro and Glu in F-3 gh account for 82% of total amino acids, suggesting that this fraction has a high potential as inhibitor of ACE activities.

Amino acid sequence

Peptides Ala-Tyr-Phe-Tyr, Pro-Tyr-Tyr and Tyr-Leu, which were found in F-3 gh, seem to have been derived from α_{s1}-CN, κ-CN, and α_{s1}-CN, α_{s2}-CN, respectively. Ala-Tyr-Phe-Tyr that was found in this experiment is similar to an ACE-inhibitory peptide (Ala-Tyr-Phe-Tyr-Pro-Glu) derived from the hydrolysis of bovine α_{s1}-CN. On the other hand, Cheung et al. (1980), reported that peptides with branched-chain aliphatic amino acids, such as Trp, Phe, Tyr and Pro for C-terminal amino acid residues and Val and Ile for N-terminal amino acid residues, displayed ACE-inhibitory activity. In addition, Maruyama et al. (1985) said that a peptide in bovine β-CN which shows an Ala-Val-Pro-Tyr-Pro-Gln-Arg base sequence also has the capability of inhibiting ACE. Compared with the above papers, peptides separated in this experiment, namely, Ala and Leu of the N-terminal kind and Tyr, Leu and Phe of the C-terminal kind

Table 1. Amino acid composition of ACE-inhibitory peptides derived from goat's CN hydrolysates by pepsin

Amino acids	ACE-inhibitory peptides
Asp	0.86
Thr	0.47
Ser	0.43
Glu	15.31
Pro	15.94
Gly	0.49
Ala	4.20
Cys	6.46
Val	0.73
Met	0.79
Ile	17.47
Leu	18.83
Tyr	14.27
Phe	0.22
His	0.08
Lys	2.98
Arg	0.47
Total Mol. ratio	100.00

show a consistency with the previous reports.

IC_{50} value

IC_{50} value at each stage of the separation and purification of ACE-inhibitory peptide from goat's milk CN hydrolysates was determined. The IC_{50} value of peptic hydrolysate at 48 h, F-3, F-3 g and F-3 gh were 29.89, 3.07, 1.85 and 0.87 g/ml, respectively. This experiment indicated that pepsin IC_{50} value of F-3 was quite higher than that of hydrolysates. The reason is thought to be that hydrolysates can produce a high level of ACE inhibitants only with gel filtration without a complex purification process.

Our results show that ACE-inhibitory peptides are applicable in therapeutic formula concerning hypertension. A future study shall be carried out on the commercialization of the peptides with ACE-inhibitory activity by compounding a large amount of such peptides and conducting an *in vivo* test.

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