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Storage Stability of the Synthetic Angiotensin Converting Enzyme (ACE) Inhibitory Peptides Separated from Beef Sarcoplasmic Protein Extracts at Different pH, Temperature, and Gastric Digestion

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Abstract The angiontensin converting enzyme (ACE) inhibitory peptides were separated from beef sarcoplasmic protein extract and their amino acid sequences were identified as GFHI, DFHINQ, FHG, and GLSDGEWQ. The 4 peptides were synthesized in a laboratory and the ACE inhibitory activities of peptides was measured after 2 months of storage at 4°C under different pH conditions (6.0, 6.5, 7.0, 7.5, and 8.0) and the exposure of different temperatures (70, 80, 90, and 100°C) for 20 min to evaluate industrial use. No significant difference was detected by pH and temperature abuse for 20 min during storage. When the synthetic peptides were digested by pepsin, trypsin, and chymotrypsin, the ACE inhibitory activity was not changed. These results indicated that the 4 synthetic peptides with ACE inhibitory activity were pH-stable, heat-stable, and resistant to proteinases in gastro-intestinal tracts. Therefore, those 4 peptides can be used as a source for functional food product with various applications.

Keywords : angiontensin converting enzyme (ACE), synthetic peptide, stability, gastric digestion, sarcoplasmic protein

Introduction

Bioactive peptides can be released by enzyme proteolysis of food proteins and may act as potential physiological modulators of metabolism during intestinal digestion. The bioactive peptides usually contain 3-20 amino acid residues and their activities are based on their amino acid composition and sequence (1-3). These peptides containing only a few amino acid residues are able to cross the digestive epithelial barrier and reach the blood vessels, which allow them to reach peripheral organs and have beneficial effects for the organism (4-6). Of the bioactivities of these peptides, antihypertensive peptides have been studied extensively (5-9). The mechanism of activity involves inhibition of ACE, the key enzyme responsible for the regulation of blood pressure by the rennin-angiotensin system (9). Therefore, commercial interest in the production of bioactive peptides with the purpose of using them as active ingredients in functional food has been increased. However, pH and temperature can affect functional properties of the peptides during processing. When bioactive peptides encounter gastric enzymes it also may affect their activities during digestion in the body.

There are enormous studies of enzymes that can be used for the cleavage of specific peptide bonds and the bioactive production (10, 11). Specific cleavage sites of enzyme pepsin, trypsin, α-chymotrypsin, and thermolysin were C-terminus of Phe, Leu, Tyr, and Trp, C-terminus of Arg and Lys, C-terminus of Tyr, Trp, Phe, and Leu, and N-terminus of Leu and Phe, respectively (12, 13). Arihara *et al.* (14) reported that digestive enzymes (pepsin, trypsin, and α-chymotryspn) in gastrointestinal tracts generated ACE inhibitory activity from muscle proteins, myosin as 61.4, 60.2, and 70.1%, respectively. Also, several authors have reported an increase in ACE-inhibitory activity by the action of digestive enzymes on fermented casein solutions (1, 15). For an industrial application of those antihypertensive peptides as functional food material, a main consideration would be not only the purification of active peptides but also the stability of the active peptide under different environments including various pH, temperature, and gastric digestion. Because, when the bioactive peptides are applied for certain food additives, the different environments of different food systems may differ, it is important to investigate the stability of the peptides in different environments (16).

In this respect, those synthetic peptides previously isolated and identified from beef sarcoplasmic protein in our previous study were synthesized and stored with different pH and temperature conditions for 2 months to evaluate their storage stability. In addition, the peptides were digested with such commercial gastrointestinal enzymes as pepsin, trypsin, and α-chymotrypsin and their activities were evaluated.

Materials and Methods

Enzymes Pepsin (porcine stomach mucosa), trypsin (bovine pancreas), α-chymotrypsin (bovine pancreas), ACE from rabbit lung, hippuryl-histydyl leucine (HHL) were obtained from Sigma, Co. (St. Louis, MO, USA). All the other reagents where used as analytical grade.

Peptides synthesis Peptides GFHI, DFHINQ, FHG, and GLSDGEWQ were isolated from beef sarcoplasmic protein hydrolysates and identified as ACE inhibitory peptides (data not shown). These peptides were

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synthesized by PeptrEX™ using Fmoc-Solid phase system (Peptron Co., Daejeon, Korea).

Effect of pH and temperature on the stability of peptides Each peptide (0.1 mg/mL) was resuspended in distilled water and adjusted to pH 6.0, 6.5, 7.0, 7.5, and 8.0 (pH meter, MD 230; Mettler-Toledo, Manchester, UK) using 0.5 M HCl solution. For measuring heat stability of peptides, a test tube containing 1 mL of distilled water was preheated in water bath (EX-B; Taitec, Tokyo, Japan) and when temperature was reached to the designated temperature (70, 80, 90, and 100°C), the peptides were added and heating was stopped after 20 min. Those pH and temperature adjusted peptides were kept at 4°C for 2 months to examine the change of its ACE inhibition activity.

***In vitro* gastric digestion of synthetic peptides** The synthetic peptides were dissolved in distilled water (0.1 mg/mL), adjusted to pH 2.0 using 0.5 M HCl solution and digested by pepsin at 37°C for 5 hr. The peptic digests were successively adjusted to pH 7.6 using 0.5 M NaOH and digested by trypsin for 2.5 hr at 37°C. Then pH of the digest was adjusted to 7.8 using the 0.5 M NaOH and further digestion was carried out by chymotrypsin for another 2.5 hr at 37°C. The reaction was inactivated by boiling at 100°C for 5 min. The ratio of enzyme and substrate was 1:100 (w/w).

ACE inhibitory activity assay ACE inhibitory activities of the peptides were determined for the stability of their function. The determination of ACE inhibitory activity was performed by the spectrophotometric method described by Cushman and Cheung (17). For each assay, 100 μL of protein hydrolysates and 100 μL of hippuryl-L-histidyl-L-leucine (HHL, 12.5 mM in 50 mM sodium borate buffer) were incubated at 37°C for 5 min. After incubation, 150 μL of ACE (peptidyldipeptide hydrolase, from rabbit lung acetone extract) was added and the mixture was incubated for another hour. The enzymatic reaction was stopped by adding 250 μL of 0.5 N HCl. The hippuric acid forced by the action of the angiotensin-converting enzyme on HHL was extracted from the acidified solution into 1.5 mL ethyl acetate by vortexing for 15 sec. This was centrifuged at 3,290×g for 10 min at 4°C, and a 0.5 mL aliquot of each ethyl acetate layer was transferred to clean tubes and evaporated by heating at 120°C for 20 min on a heating plate. The hippuric acid was redissolved in 3 mL of 1 M NaCl, and the amount formed was determined by its absorbance at 228 nm. The IC_{50} value, defined as the concentration of a peptide that inhibits 50% of the ACE activity, was determined by measuring the ACE inhibitory activity and protein levels of each sample. The protein content was measured by the methods of Lowry *et al.* (18).

Reversed-phase HPLC system High performance liquid chromatography on a reversed phase column (RP-HPLC) is widely utilized to generate a peptide map from digested protein (19). The peptide mixture was separated by RP-HPLC on a symmetry C18 column (Zorbax 25× 0.46 cm; Agillent, San Francisco, CA, USA) in an aseptic condition with flow rate at 0.8 mL/min. The mobile phase was delivered by a HP1100 pump (Agillent). Separation was made under linear gradient elution conditions and acetonitrile was used as the organic modifier and trifluoroacetic acid (TFA) as the volatile buffer. Eluent A consisted of 0.1% TFA in milli-Q water (v/v); eluent B of 0.07% TFA in acetonitrile. The chromatographic column was conditioned with 100% of eluent A, after which 20 μL of the peptide solution was applied on the C18 column and eluted by the remaining eluent A for 10 min and with the following increasing eluent B concentrations: 0-10 min, 0%; 10-30 min, 0-65%; and 10 min, 100%. The UV absorbance of the eluent was monitored at 214 nm.

Statistical analysis Statistical analysis was performed with the SAS program (2000) (20). One-way ANOVA was performed and the mean and standard error were reported. When the significance was formed, Duncan's multiple range test was carried out to analyze the significant differences among mean values at $p<0.05$.

Results and Discussion

Effect of pH changes on ACE inhibitory activity of synthetic peptides ACE inhibitory activity of peptides was measured after 2 months of storage at 4°C under conditions at pH 6.0, 6.5, 7.0, 7.5, and 8.0 to evaluate the storage stability for industrial use (Table 1). The activity of the 4 peptides was not affected by different pH with storage for 2 months. This indicates that weak acidic, neutral, and weak alkali conditions during storage did not affect on ACE inhibitory activities of those peptides.

In the conventional industrial manufacture of various foodstuffs, the proteins contained in raw materials are readily subjected to alterations with regard to their functional or biological properties (21). Changes of pH and certain chemical treatments affect functional properties by modifying specifically one or more amino acids. For example, acidic treatments destroy glutamine and asparagine, whereas alkaline treatments destroy cystine, serine, and threonine, and produce lysinoalanine and D-amino acids (21, 22). From the result, however, the synthetic ACE inhibitory peptides identified from beef sarcoplasmic protein hydrolysates were stable on pH.

Effect of temperature on ACE inhibitory activity of synthetic peptides Heating is one of the oldest, most common, and most widely used methods of modifying

Table 1. ACE inhibitory activity (IC_{50}, μg/mL) of synthetic peptides on different pH after 2 months storage at 4°C

pH	Peptides			
	DFHINQ	GFHI	GLSDGEWQ	FHG
6.0	50.6±0.4	64.2±0.4	117.5±0.8	52.6±0.7
6.5	51.0±0.6	64.3±0.6	117.9±0.5	53.0±0.5
7.0	50.9±0.3	64.5±0.5	117.1±0.4	52.6±0.5
7.5	50. 6±0.8	64.9±0.5	117.2±0.6	52.7±0.6
8.0	50.4±0.4	64.0±0.6	117.6±0.5	52.3±0.4

proteins. For example, different heat treatment is applied to make food proteins more edible (21). Heat denaturation temperatures of different dietary proteins vary from 60 to 90°C (21, 23). However, heat treatment often causes undesirable secondary effects, frequently called processing damage including interactions between the major components such as proteins, lipids, and carbohydrates (24). Accordingly, the activity of bioactive proteins may be reduced by different heat treatments.

The temperature changes of the peptides were conducted by heating at 70, 80, 90, and 100°C for 20 min and stored for 2 months at 4°C (Table 2). In all temperatures, the activity of peptide DFHINQ and GFHI were shown 50.4-50.6 and 64.2-64.5 μg/mL, respectively, and had no significant difference. Those synthesized peptides DFHINQ and GFHI had ACE inhibitory activity (IC_{50}) as 50.5 and 64.3 μg/mL, respectively in initial stage (pre-enzyme in Table 3). Peptide GLSDGEWQ, identified from thermolysin + proteinase A hydrolysates of beef sarcoplasmic protein, showed almost same inhibitory activity in all temperature treatment.

Figure 1 shows that HPLC chromatogram of the 4 synthetic peptides without enzyme hydrolysis. The retention time of peptide DFHINQ, GLSDGEWQ, FHG, and GFHI was 19.4, 20.6, 15.5, and 23.5 min, respectively. After heating the synthetic peptides, there was no change of retention time of original in all temperature treatment.

There is commercial interest in the production of bioactive peptides with the purpose of using them as active ingredients in functional foods. The development of technology for industrial-scale production of such peptides is in progress (25). Three major kinds of synthetic ACE inhibitors captopril, lisinopril, and enalapril are known to have severe side effects. In contrast, the ACE inhibitory peptides derived from food proteins have not shown these side effects yet (13). Previously, our study revealed that ACE inhibitory peptides, VLAQYK, separated from beef sarcoplasmic protein showed no abnormal effect on spontaneously hypertensive rat (SHR) after oral administration for 2 months (7).

Korhonen *et al.* (21) suggested that although the

Table 2. ACE inhibitory activity (IC_{50}, μg/mL) of synthetic peptides stored for 2 months at 4°C after the exposure of different temperatures

Temperature (°C)	Peptides			
	DFHINQ	GFHI	GLSDGEWQ	FHG
70	50.4±0.3	64.4±0.3	117.1±0.1	51.9±0.7
80	50.4±0.5	64.5±1.0	117.6±0.7	52.1±0.6
90	50.6±0.2	64.2±1.4	117.3±0.5	52.0±0.4
100	50.6±0.8	64.2±0.5	117.6±1.4	52.0±0.7

Table 3. Effect of pepsin, trypsin, and chymotrypsin on the ACE inhibitory activity (IC_{50}, μg/mL) of synthetic peptides

Enzyme	Peptides			
	DFHINQ	GFHI	GLSDGEWQ	FHG
Pre-enzyme	50.5±1.4	64.3±0.6	117.3±0.1	52.0±0.1
Pepsin	50.5±1.0	64.4±0.5	117.6±0.1	52.5±0.4
Trypsin	50.6±0.4	64.4±0.8	117.7±0.4	52.3±0.8
Chymotrypsin	50.6±0.2	64.5±0.4	117.4±0.2	52.1±0.6

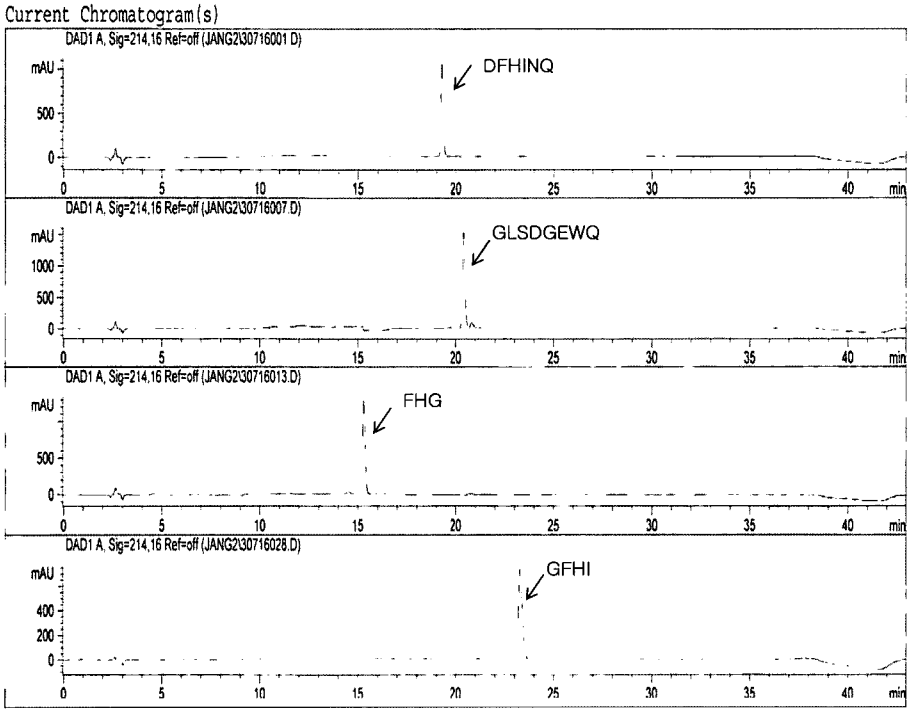

Fig. 1. HPLC chromatogram of original 4 synthetic peptides with ACE inhibitory activity.

bioactive proteins or peptides, described in their study, are not known to possess specific allergic or toxic effects, their addition to any different food system warrants careful consideration about potential health risks. Specifically, their interactions during processing or storage with other proteins, sugars, and lipids need to be researched with a view to possible formation of toxic, allergenic, or carcinogenic substances. In present study, we determined the stability of ACE peptides against different pH and temperature but a further study is needed to clarify the safety of the peptides *in vivo*.

Effect of gastric enzymes on ACE inhibitory activity of synthetic peptides When the 4 synthetic ACE inhibitory peptides were consecutively treated with pepsin, trypsin, and α-chymotrypsin. However, the ACE inhibitory activity of the peptides was not significantly affected (Table 3). In the present study, we did not determine if the pepsin, trypsin, and chymotrypsin cleaves the exact residue, amino acid residue for specific those enzymes. However, there was no change in ACE inhibitory activities of the synthetic peptides after digestion. These results agree well with Yoshii *et al.* (26). There is no guarantee that the enzymes will cleave the expected bond in all peptides, since sometimes the structure of the peptide prevents this happening. Gómez Ruiz *et al.* (27) reported that the presence of proline makes amino acid sequences less susceptible to proteolytic enzymes. Although the proline was not contained any of the synthetic peptide identified from beef sarcoplasmic protein, the synthetic peptides were survived through gastric digestion. These results indicated that if the ACE inhibitory peptides were orally administered, it would be stable in the stomach.

In conclusion, results indicated that the synthesized bioactive peptides were stable by different pH conditions during 2 months of storage at 4°C. Temperature and gastric enzymes also did not affect on the stability. Therefore, the ACE inhibitory peptides including GFHI, FHG, DFHINQ, and GLSDGEWQ may be stable in different food processing environment when it is applied as a functional ingredient in industry.

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