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Inhibitory Profile of Nonapeptide Derived from Porcine Troponin C against Angiotensin I–Converting Enzyme

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A novel angiotensin I-converting enzyme (ACE) inhibitory peptide (RMLGQTPTK; 9mer) from porcine skeletal troponin C was investigated for its inhibitory profile. This peptide was noncompetitive and as hydrophobic as the known ACE inhibitory peptides. Aminopeptidase M quickly hydrolyzed 9mer, resulting in production of MLGQTPTK and LGQTPTK with inhibitory activities similar to those of 9mer. The main hydrolysis product of 9mer with carboxypeptidase A and B was RMLGQTPT showing very weak activity. Most products derived from 9mer hydrolysis by ACE, aminopeptidase, or carboxypeptidase showed weak but definite ACE inhibitory activities. Thus, 9mer was estimated to be a wholly efficient inhibitor with these fragment peptides.

KEYWORDS: Angiotensin I-converting enzyme inhibitory peptide; inhibitory profile; kinetics; peptic digestion; porcine skeletal troponin C

INTRODUCTION

Recently, many angiotensin I-converting enzyme (ACE) inhibitory peptides from foods have been reported (*1*, *2*) but rarely have the inhibitory mechanisms of these peptides been examined. To know the effectiveness of a newly identified peptide after oral administration, the inhibitory mechanism against ACE, the target enzyme, and the action of its derivative peptides need to be proved. In addition, it is important to clarify the resistance of these peptides against digestive proteases.

Yokoyama et al. (*3*) studied the penta-, hexa-, and nonapeptides showing ACE inhibitory activity from the thermolysin digest of dried bonito. Among various fragments of these peptides derived by chemical synthesis or enzymatic hydrolysis, the peptides obtained by further hydrolysis with thermolysin or chymotrypsin had higher levels of activity than did the original peptides, and were suggested to be absorbed across the intestinal epithelium when they were administered orally. Saito et al. (*4*) investigated the digestive-enzyme resistance of ACE inhibitory tetra- or penta-peptides from sake or sake lees. They obtained some correlations between the structure and ACE inhibitory activity. Fujita et al. (*5*) reported the ACE resistance of ACE inhibitory peptides from the enzymatic digests of chicken breast muscle and ovalbumin, as well as those from dried bonito, which had been reported by Yokoyama et al. (*3*). They tentatively classified these peptides into three categories: inhibitor type, substrate type, and prodrug type. In all of the reports described above, it was emphasized that the resistance of the ACE inhibitory peptide against ACE itself, or against digestive enzymes, was a prerequisite for their action in vivo.

A novel ACE inhibitory nonapeptide, Arg-Met-Leu-Gly-Gln-Thr-Pro-Thr-Lys (RMLGQTPTK: 9mer), was isolated from peptic hydrolysate of porcine troponin C (*6*). The 50% inhibitory concentration (IC_{50}) of 9mer was 34 μM, and it was the strongest peptide among those from porcine protein previously reported (IC_{50} = 549 and 945.5 μM) (*2*). This peptide was slowly hydrolyzed by ACE and was estimated to be a substrate-type inhibitor to ACE according to the classification by Fujita et al. (*5*). However, 9mer showed relatively high resistance against gastrointestinal endoproteases: pepsin, chymotrypsin, and trypsin (*6*). Therefore, it may stay in the intestine for a long time, which would increase the possibility of paracellular absorbance of the peptide across the intestinal epithelium (*7*). However, exopeptidases, another type of enzyme, are secreted into the alimentary canal or localized in the intestinal mucosa. Thus, it is crucial to understand the activity of the resulting peptides after exopeptidase digestion.

In the present study, the ACE inhibitory action of 9mer and its fragment peptides were kinetically studied to categorize their inhibitory types. The hydrophobicity of the peptides was also

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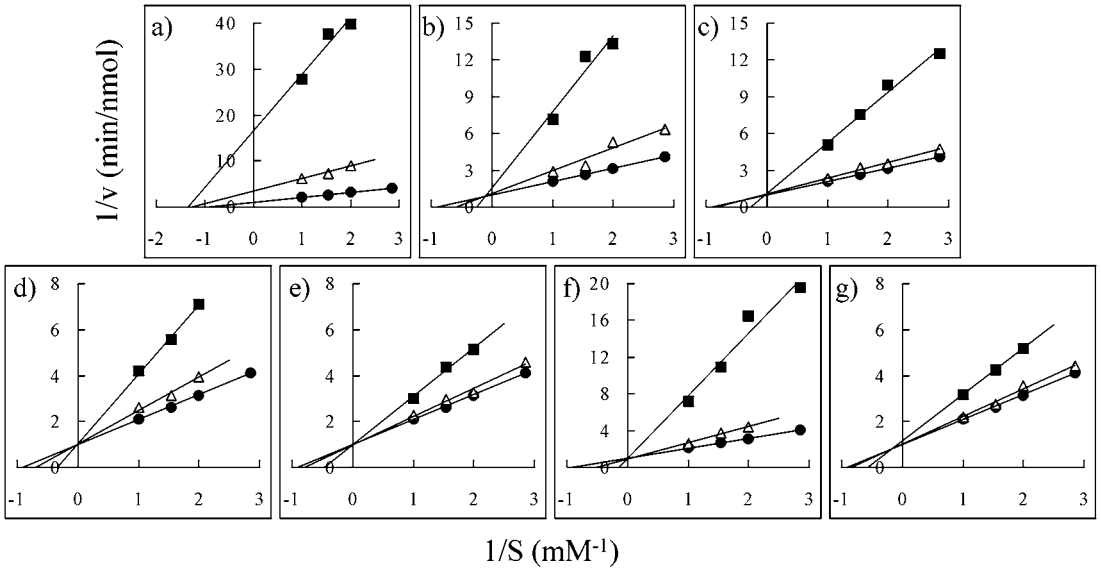

Figure 1. Kinetic evaluation of ACE inhibition of synthetic peptides. RMLGQTPTK (9mer) and its fragment peptides were kinetically analyzed with their inhibitory types at the concentrations of 50 (△) and 500 μM (■). ● = without peptides. (a) 9mer, (b) RMLGQTP, (c) RMLGQ, (d) RML, (e) GQ, (f) TP, and (g) TK.

estimated as a measure of affinity to ACE. Finally, the resistance of the peptides against exopeptidases was investigated.

MATERIALS AND METHODS

Synthetic Peptides. RMLGQTPTK (9mer), RMLGQTPT (8mer), RMLGQTP (7mer), and RMLGQ (5mer) were purchased from Sigma Genosys Japan Co. (Ishikari, Hokkaido, Japan); RML and TK from Sawady Technology Co., Ltd. (Tokyo, Japan); ML and TP from Bachem AG (Bubendorf, Switzerland); and GQ from Sigma Chemical Co. (St. Louis, Mo.).

VW (*8*) and PTHIKWGD (*9*), which are ACE inhibitory peptides, were obtained from Bachem AG, and Pyr-GLPPRPKIPP (bradykinin potentiator B) (*10*) was obtained from Peptide Institute Inc. (Mino, Osaka, Japan).

ACE Inhibitory Assay. The assay for ACE inhibitory activity was performed following the method of Cushman and Cheung (*11*) with slight modifications (*12*). ACE, a dipeptidyl carboxypeptidase [EC 3.4.15.1], extracted from bovine lung, was obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Hippuryl-L-histidyl-L-leucine (HHL, Nacalai Tesque Inc., Kyoto, Japan) was used as a synthetic substrate. Sample was added to 0.1 M borate buffer (pH 8.5) including HHL and 0.608 M NaCl. The reaction was started by the addition of ACE in 0.25 M borate buffer (pH 8.5). To terminate the enzyme reaction after 30 min, hydrochloric acid was added. Inhibitory activity was calculated using the absorbance at 228 nm of hippuric acid liberated from HHL by ACE. The ACE inhibitory activity of a peptide was expressed as IC_{50} of the peptide in the assay, or as the inhibition ratio (%) at a constant concentration of inhibitors.

Kinetic Evaluation of ACE Inhibitory Peptides. A Lineweaver−Burk plot (*13*) was drawn to estimate the ACE inhibitory types of the peptides. Briefly, the ACE inhibitory activities of the peptides (50 and 500 μM) were measured in various concentrations of substrate (HHL). These activities were transformed into the velocity of ACE (nmol/min). The resulting data were reciprocally plotted (substrate concentration for horizontal axis and velocity for vertical axis). The ACE inhibitory types of peptides were estimated by comparing the curves of these data with those in the absence of the inhibitor. The inhibition constant (K_i) of a peptide inhibitor was obtained from the secondary plot of Lineweaver−Burk plot, the slope for vertical axis, and inhibitor concentration for horizontal axis. The intercept on the horizontal axis, (I), is the absolute value of the K_i.

Estimation of Hydrophobicity of Peptides. The hydrophobicity of the peptides was analyzed by reverse-phase (RP) high-performance liquid chromatography (HPLC) using a Cosmosil $5C_{18}$ AR−II (4.5- × 150-mm, Nacalai Tesque Inc.), according to the method of Browne et al. (*14*). Samples were fractionated by a gradient elution of 1−30% of acetonitrile in 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 mL/min. Under this condition, VY, a hydrophilic peptide, was eluted at 13.31 min, and VW, a hydrophobic peptide, was at 23.50 min. The peptide eluted at an earlier retention time (RT) was judged to be more hydrophilic, and that with longer RT was judged to be more hydrophobic.

Digestion of Synthetic Peptides with Aminopeptidase M or Carboxypeptidase A and B. A 1-mg/mL sample of 9mer (in 10 mM Na-phosphate, pH 8.0) was used as a substrate. Aminopeptidase M (APase, type IV−S, EC 3.4.11.2, from porcine kidney microsomes) was obtained from Sigma Chemical Co.; phenymethylsulfonyl fluoride-treated carboxypeptidase A (EC 3.4.17.1, from bovine pancreas) and carboxypeptidase B (EC 3.4.17.2, from porcine pancreas) were from Worthington Biochemical Co. (Lakewood, NJ). Each exopeptidase was used at the concentration of 50 mU/mL. Two kinds of carboxypeptidases (CPases) were used at the same time. In every case, the enzyme was reacted at 37 °C and inactivated by heating at 95 °C for 20 min. After that, the reaction mixture was applied to RP-HPLC using a Cosmosil $5C_{18}$ AR−II (4.5- × 150-mm) (Nacalai Tesque Inc.) and fractionated by a gradient elution of 1−30% of acetonitrile in 0.1% TFA at a flow rate of 1 mL/min. The concentration of 9mer was calculated from the peak area of HPLC. The major fractions among the detected peaks were collected, concentrated by a centrifugal vaporizer (CEV-100D; Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and dissolved with H_2O. Their ACE inhibitory activities were then measured.

Determination of Peptide Concentration. The concentrations of peptides were calculated from their weights at the time when their solutions were prepared. Those of fragment peptides from the isolation by RP-HPLC were measured by the UV method (*15*) using wavelengths of 215 and 225 nm.

RESULTS AND DISCUSSION

ACE Inhibitory Activities and Inhibitory Types of 9mer and the Related Peptides. The ACE inhibitory activities of 9mer, as well as those of its fragment peptides synthesized from a theoretical calculation based on the substrate specificity of the ACE, were measured (**Table 1**). The activities were then kinetically analyzed with their inhibitory types (**Figure 1** and **Table 2**). It was observed that 9mer was the strongest inhibitory peptide among them. The activities of intermediary peptides, 7mer and 5mer, were about 10 times weaker than that of 9mer

Table 1. ACE Inhibitory Activity of Synthetic Peptides

peptide	MW	IC_{50} (μM)
RMLGQTPTK	1031.2	34
RMLGQTP	802.0	503
RMLGQ	603.8	358
RML	419.6	1019
GQ	203.2	5630
TP	216.2	2071
TK	248.1	1634

Table 2. ACE Inhibitory Types of Synthetic Peptides

peptide	K_i (μM)[a]	inhibition type[b]
RMLGQTPTK	60.6	N
RMLGQTP	121.5	mixed
RMLGQ	167.2	C
RML	317.6	C
GQ	554.7	C
TP	100.4	C
TK	616.4	Mixed

[a] K_i, Inhibition constant. [b] N, noncompetitive inhibition; mixed, mixed type of competitive and noncompetitive inhibitions; C, competitive inhibition.

but still stronger than those of the final products, the di- and tri-peptides. This means that the level of 9mer activity weakens as hydrolysis by ACE proceeds. Unlike the fragment peptides, the ACE inhibitory type of 9mer had a nature of noncompetitive inhibition. However, it was previously reported that 9mer was slowly hydrolyzed by ACE and was suggested a competitive inhibitor (*6*). From these results, it was considered that 9mer bound ACE and inhibited a binding of HHL to ACE for a long time, because 9mer was relatively highly resistant against ACE. Therefore, we thought that 9mer apparently acted as a noncompetitive inhibitor in this condition of 30 min incubation. The ACE inhibitory types of 7mer and TK were similar to competitive inhibitions but with slight differences, suggesting they were the mixed types of competitive and noncompetitive inhibitors. The other peptides showed typically competitive inhibitions. The K_i value of 9mer was the smallest among all peptides, and 9mer showed the strongest affinity to ACE. TP and 7mer, which have Pro at their carboxyl terminals, showed relatively small K_i values.

Because 9mer was hydrolyzed with ACE (*6*), it was important to evaluate the ACE inhibitory activities of the fragment peptides from 9mer by ACE hydrolysis. All fragment peptides from 9mer in this study showed weak but definite ACE inhibitory activity. This suggested a little participation of these small and weak peptides in the ACE inhibition by 9mer. The weak activities of these small peptides suggested that the orally administered 9mer temporarily inhibited ACE in vivo and was then hydrolyzed by ACE, and consequently the blood pressure regulatory system concerned with ACE was restored to almost the pre-administration state of 9mer.

Hydrophobicity of ACE Inhibitory Peptides. Hydrophobic interaction between the substrate peptide and ACE is known to play a role in the activity. Although the hydrophobicity of a peptide can be defined to a certain extent by that of the amino acid in the peptide (*16*), we evaluated hydrophobicity by RP-HPLC analysis (*14*). The hydrophobicity of 9mer was relatively high but a little lower than that of 7mer (**Table 3**). This difference changed with the presence or absence of the TK dipeptide at the carboxyl terminal of 9mer, and the role of TK was investigated further for comparison with the other parts of 9mer. The hydrophobicity of 9mer and that of the related peptides in **Table 1** were analyzed (**Table 3**). The hydrophobicity of TK and that of GQ were very low, while that of TP was relatively high. RML showed the longest retention time among the related peptides, and ML, a partial peptide of RML, largely participated in it. Thus, RML in 9mer was estimated to enhance the affinity of 9mer to ACE by hydrophobic binding.

Table 3. Retention Time of ACE Inhibitory Peptides at RP-HPLC Analysis

peptide	RP-HPLC RT (min)
RMLGQTPTK	20.61
RMLGQTP	22.62
RMLGQ	20.37
RML	22.47
ML	21.59
GQ	2.14
TP	7.16
TK	2.36

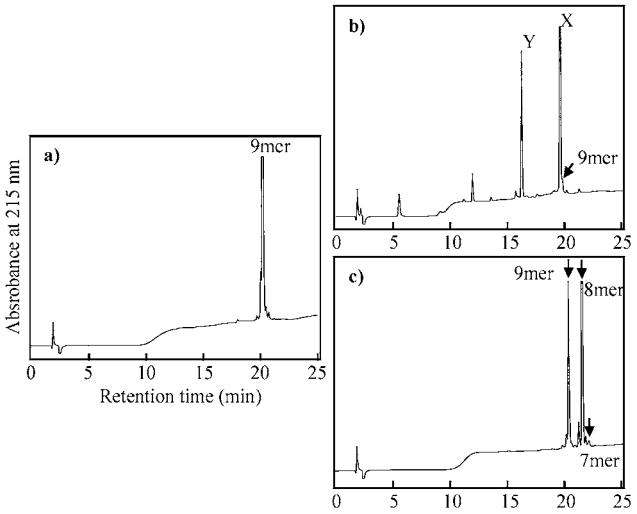

Figure 2. RP-HPLC chromatograms of RMLGQTPTK and its hydrolysates by aminopeptidase or carboxypeptidases. a) Original RMLGQTPTK (9mer), b) 9mer hydrolyzed with aminopeptidase M for 30 min, and c) 9mer hydrolyzed with the mixture of carboxpeptidase A and B for 30min. 8mer, RMLGQTPT; 7mer, RMLGQTP. Fraction X and Y showed ACE inhibitory activity.

When a peptide expresses ACE inhibitory activity, the affinity of the peptide to ACE is one of the important factors. The actions of many ACE inhibitory peptides previously reported were discussed mainly from the viewpoints of their amino acid sequences; an aromatic amino acid (Pro, Phe, or Tyr) at the carboxyl terminus was fundamental for the ACE inhibitory activity (*4*, *17*). On the other hand, there were some cases (*3*−*5*) that the relatively highly hydrophobic peptides expressed high levels of activity. The already-known ACE inhibitory peptides, VW (RT = 23.50 min), PTHIKWGD (RT = 23.78 min), and Pyr-GLPPRPKIPP (RT = 26.50 min), exhibited high hydrophobicity. From these facts, we can consider the estimation of peptide hydrophobicity to be useful in explaining the affinity of peptide to ACE.

Digestive Profile of ACE Inhibitory Peptide with APase or CPases. The chromatograms of digestion products of 9mer and APase or CPases for 30 min are shown in **Figure 2**. APase gradually hydrolyzed most of 9mer, and two major peaks (X and Y) were found after 30 min incubation. The major product of 9mer with CPase hydrolysis was RMLGQTPT (8mer), which was detected by comparing its retention time with authentic peptide, and 7mer was scarcely generated. This showed that the liberation rate of Thr at the carboxyl terminal side of Pro

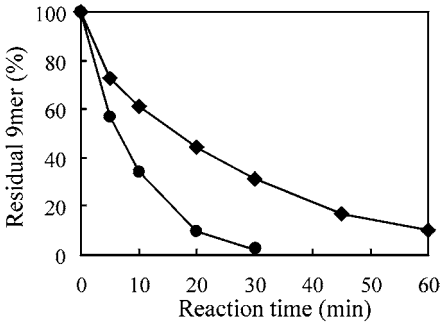

Figure 3. Reduction of RMLGQTPTK as substrate for aminopeptidase or carboxypeptidases. RMLGQTPTK (9mer) was hydrolyzed with aminopeptidase M (●) or the mixture of carboxypeptidase A and B (◆) for various durations indicated in figure. The hydrolysis ratio of each reaction mixture was analyzed by RP-HPLC, and the amount of residual 9mer was calculated from peak area.

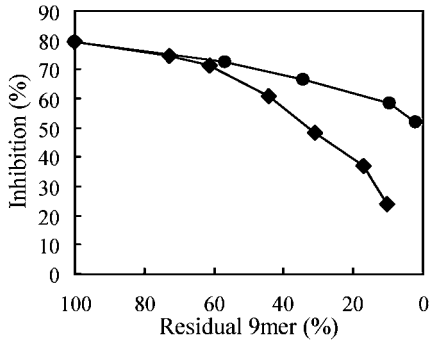

Figure 4. ACE inhibitory activity of RMLGQTPTK and its hydrolysates by aminopeptidase or carboxypeptidases. The ACE inhibitory activity of RMLGQTPTK (9mer) hydrolyzed with aminopeptidase M (●) or the mixture of carboxypeptidase A and B (◆) was measured. The hydrolysis ratio (the amount of residual 9mer) was measured as in **Figure 3**.

was very slow because CPases scarcely hydrolyzed the peptide (8mer) with Pro at the second position from the carboxyl terminal. The decreasing profile of 9mer with increasing time is shown in **Figure 3**. After 30 min of incubation, APase hydrolyzed most of 9mer, and the residual 9mer was 2%. On the other hand, CPases hydrolyzed 9mer more slowly than did APase, and the residual 9mer was 31 or 10% after 30 or 60 min incubation, respectively.

The ACE inhibitory activities of the hydrolysates (1 mg/mL of the original 9mer) were measured and expressed as inhibition ratios (%) (**Figure 4**). Although the original 9mer showed a high inhibition ratio (80%), the activities of all hydrolysates decreased as the remaining 9mer decreased by APase or CPase hydrolysis. The decreasing velocity of inhibition in comparison with that of the residual 9mer was higher by CPases than that by APase hydrolysis. The reaction mixture at 20 min incubation with APase had 9% of the residual 9mer and showed 58% inhibition. In contrast, the reaction mixture at 60 min incubation with CPases showed only 23% inhibition, although it had 10% of the residual 9mer, which was similar to the case with 20 min incubation with APase. This result suggests that the amino acid sequence at the carboxyl terminal is more important to the activity of 9mer than is the amino acid sequence at the amino terminal. This consideration is also supported by the fact that the inhibitory activity of 9mer is more than 10 times stronger than that of 7mer, which lacks two amino acid residues at the carboxyl terminal of 9mer.

From these results, and because the reaction mixture of 9mer and APase was thought to include some active peptides derived from 9mer, two major fractions (fractions X and Y in **Figure 2**) were collected, dried, and dissolved with H_2O, and their ACE inhibitory activities were measured. They showed relatively strong levels of activity, and their IC_{50}s were 34 μg/mL (fraction X) and 45 μg/mL (fraction Y). These values were very close to that of 9mer (35 μg/mL) (*6*). The amino acid sequence of X or Y fraction was estimated as MLGQTPTK or LGQTPTK, which lacked Arg or Arg-Met from 9mer, respectively, according to the analysis of the prosperity and decay of the RP-HPLC peak area. This indicated that neither of these amino acid residues at the amino terminal of 9mer was very important for the activity of 9mer, although RML was important for the hydrophobicity of 9mer. Thus, RML in 9mer was considered to work for the affinity of 9mer to the nonactive site of ACE. The IC_{50} of 8mer, the major product in the reaction mixture of 9mer and CPases, was 1038 μM, and its activity level was fairly lower than that of 9mer. This, too, supported the importance of the amino acid sequence at the carboxyl terminal of 9mer.

Although 9mer showed relatively high resistance against gastrointestinal endoproteases (*6*), it was easily hydrolyzed with exopeptidases. However, most products derived from 9mer hydrolysis by ACE, aminopeptidase, or carboxypeptidase showed weak but definite ACE inhibitory activities. Thus, 9mer was estimated to be a wholly efficient inhibitor with these fragment peptides.

An ACE inhibitory peptide, 9mer, was a noncompetitive inhibitor and was as hydrophobic as the known ACE inhibitory peptides. The amino acid residues at the amino terminal participated in the affinity of 9mer to ACE; that at the carboxyl terminal was important for the activity of 9mer. Most products derived from 9mer hydrolysis by ACE, aminopeptidase, or carboxypeptidase showed weak but definite ACE inhibitory activities. Thus, 9mer was estimated to be a wholly efficient inhibitor with these fragment peptides.

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