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Cyclic and Linear Peptides Derived from α-Amylase Inhibitory Protein Tendamistat[1]

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Tendamistat is a strong inhibitory protein of porcine pancreatic α-amylase (PPA) with a K_i value of 0.2 nM. To develop potent α-amylase inhibitors, we synthesized six odd-length cyclic peptides (5–15 residues) and four even-length cyclic peptides (10 and 12 residues) having the inhibitory sequence of tendamistat. Their PPA inhibitory activities were evaluated, and, among them, the 11-residue cyclic peptide Ten(15-23) (K_i = 0.27 μM) exhibited the strongest inhibitory activity (K_i = 0.27–1.41 μM). To examine the effect of cyclic structure on PPA inhibition, ten linear peptides corresponding to the cyclic peptides were also synthesized, and their PPA inhibitory activities were evaluated (K_i = 0.28–1.00 μM). Interestingly, the 11-residue linear peptide Ten(15-23) exhibited almost the same inhibitory activity (K_i = 0.28 μM) as that of cyclic Ten(15-23). The results of a circular dichroism study indicated that stabilization of the β-hairpin structure occurred only for cyclic Ten(15-23). Also, the results of proteolytic digestion experiments of the cyclic and linear Ten(15-23) peptides by trypsin and chymotrypsin suggested no differences in protease resistance between the cyclic and linear structures. Therefore, we demonstrated that both cyclic and linear peptides containing the inhibitory sequence of tendamistat exhibit potent PPA inhibitory activity.

Key words: α-amylase inhibitory activity, circular dichroism, cyclic and linear peptides, protease resistance, tendamistat.

Tendamistat, an α-amylase inhibitory protein of 74 residues, is produced by *Streptmyces tendae*. This protein potently inhibits porcine pancreatic α-amylase (PPA) with an inhibitory constant K_i of 0.2 nM (*1*). Structural analyses of tendamistat itself and its complex with PPA, accomplished by X-ray crystallographic and NMR spectroscopic techniques (*2–6*), have shown that tendamistat consists of a small and a large loop (Cys^{11}–Cys^{27} and Cys^{45}–Cys^{73}, respectively) bridged by disulfide bonds, and that the small loop functions as the inhibitory site for PPA. The small loop forms a β-hairpin structure composed of a type I β-turn connecting two β-strands containing the inhibitory sequence Trp^{18}-Arg^{19}-Tyr^{20} (Fig. 1).

Recently, attention has focused on the small loop β-turn, and many conformationally constrained small β-turn models of linear and cyclic peptides have been synthesized (*7–12*). Etzkorn *et al.* synthesized 12 kinds of β-turn mimic cyclic and linear peptides on the basis of the small loop of tendamistat. Among them, two cyclic hexapeptides containing the inhibitory sequence Trp^{18}-Arg^{19}-Tyr^{20} adopted β-turn conformations and exhibited more potent inhibition (K_i 14–32 μM) than the linear peptides (*9*). Matter *et al.* also designed 15 kinds of cyclic hexapeptides and reported the relationship between the β-turn structure and PPA inhibitory activity (*10*). The data suggested the existence of factors other than the inhibitory sequence in the β-turn. Therefore, the design of small and potent α-amylase inhibitors has been unsuccessful so far.

In our latest study, we designed and synthesized six small cyclic peptides having the inhibitory sequence of tendamistat, Ten(18-24), Ten(17-21), Ten(16-22), Ten(15-23), Ten(14-24), and Ten(13-25) (see Figs. 1 and 2A), in which the two N- and C- terminal cysteine residues were used to form the disulfide bridged cyclic structure (*13*). Among these cyclic peptides consisting of different odd-numbered peptide lengths (5, 7, 9, 11, 13, and 15 residues), the 11-residue cyclic peptide Ten(15-23) exhibited potent inhibitory activity (K_i = 0.29 μM) toward PPA. Interestingly, a circular dichroism (CD) study of these six cyclic peptides showed that only cyclic Ten(15-23) forms a predominant β-structural conformation. These results suggested that there is a relationship between α-amylase inhibitory activity and the structural properties of these peptides.

In this study, to improve the inhibitory activity of cyclic peptides derived from tendamistat, four cyclic peptides with even numbers of peptides (10 and 12 residues, Fig.

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Abbreviations: CD, circular dichroism; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PPA, porcine pancreatic α-amylase; TMSBr, trimethylsilyl bromide.

2B) were newly designed on the assumption that such even peptide lengths should be more effective in forming stable β-hairpins than the odd-numbered (11 residues) cyclic Ten(15-23). These cyclic peptides were synthesized, and their inhibitory activities toward PPA and CD spectra were compared to those of the six odd-length cyclic peptides. Moreover, we synthesized ten corresponding carbamoylmethyl linear peptides in which the N- and C-terminal Cys side chains were modified by iodoacetamide, and evaluated their PPA inhibitory activities to examine the effect of cyclization. In addition, trypsin and chymotrypsin digestion of the cyclic and linear carbamoylmethyl Ten(15-23) peptides was investigated to compare the protease resistances of the cyclic and linear peptides.

MATERIALS AND METHODS

Materials—Porcine pancreatic α-amylase (PPA) [EC 3.2.1.1] was purchased from Merck (Darmstadt, Germany). TPCK-treated trypsin [EC 3.4.21.4] and TLCK-treated chymotrypsin [EC 3.4.21.1] were purchased from Sigma Chemicals (St. Louis, MO, USA). Other chemicals were of analytical grade.

Analytical Instruments—HPLC analyses were performed using a Jasco high-pressure gradient system consisting of two PU 980 pumps (Tokyo) and a Shimadzu SPD-6A UV detector (Tokyo). All of the peptides were analyzed and purified on ODS-80Ts (4.6 × 250 mm) from Tosoh (Tokyo) and $5C_{18}$-AR (8.0 × 250 mm) from Nacalai Tesque (Kyoto) columns, respectively, using the acetonitrile-trifluoroacetic acid (TFA) system as the mobile phase. Molecular ion masses of the peptides were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on a PerSeptive Biosystems Voyager-RP STR (Framingham, MA, USA) using α-cyano-4-hydroxycinnamic acid as the matrix. Enzyme reactions were monitored on an Iwaki Glass microtiterplate reader Wellreader SME 3400 (Tokyo) using a 405-nm filter. CD spectra were obtained at 25°C with a 1-mm pathlength cell using a Jasco J-720W spectropolarimeter.

Peptide Synthesis—The synthesis of the cyclic and linear carbamoylmethyl Ten(14-23) peptides is briefly described in the legend to Fig. 3 as a representative example. All of the peptides were synthesized by the solid phase method with super acid-labile (SAL) resin from Watanabe Chemical (Hiroshima) using Fmoc chemistry. After deprotection and cleavage from the resin by Reagent K [trifluoroacetic acid (82.5%), H_2O (5%), thioanisole (5%), phenol (5%), and ethandithiol (2.5%)] followed by trimethylsilyl bromide (TMSBr) treatment, the obtained crude peptides were purified by reversed-phase HPLC and lyophilized to give the corresponding linear SH peptides in reduced form. These products were characterized by MALDI-TOF-MS.

Cyclization of the linear SH peptides was performed by air oxidation in ammonium acetate solution (pH 8.0) and monitored by analytical reversed-phase HPLC. After disappearance of the linear SH peptide peaks, the cyclic peptides were obtained by lyophilization without further purification and characterized by MALDI-TOF-MS.

To prepare the linear carbamoylmethyl peptides, the linear SH peptides were treated with iodoacetamide. First, 0.5 mg each of the linear SH peptides was dissolved in 500 μl of an aqueous solution containing 5% pyridine and 60% acetonitrile, and completely reduced in the presence of 1 mg of dithiothreitol in a micro test tube. An excess amount of iodoacetamide (2.5 mg) was added to the reduced peptide solution, and the pH was adjusted to 8.5 by the addition of NH_4OH. The atmosphere was replaced with N_2, and the solution was shielded from light for the 1-h reaction at room temperature. The crude product was purified by re-

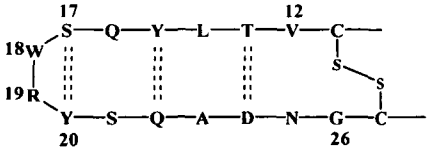

Fig. 1. **Schematic representation of the small loop region of tendamistat.** Broken lines represent hydrogen bonds.

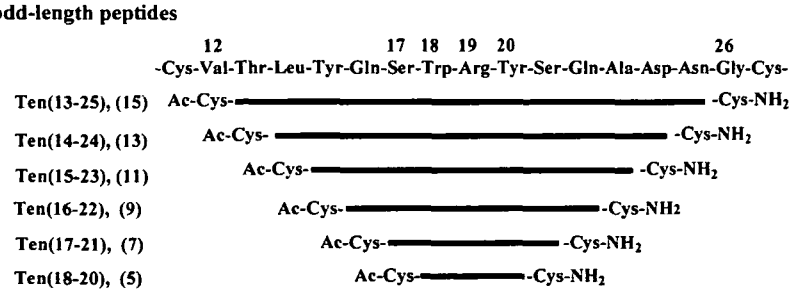

Fig. 2. **Comparison of amino acid sequences of odd-length peptides (A) and even-length peptides (B).** Peptide lengths are shown in parentheses.

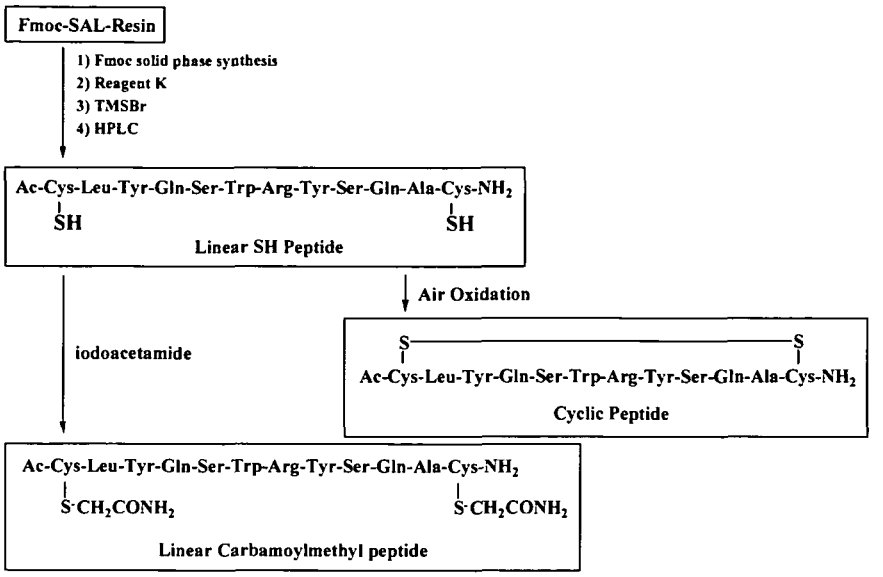

Fig. 3. **Synthetic procedure for cyclic and linear carbamoylmethyl Ten(14-23) peptides.** Details are described in "MATERIALS AND METHODS."

versed-phase HPLC and lyophilized. The final linear carbamoylmethyl peptide was characterized by MALDI-TOF-MS.

Inhibitory Activity—Inhibitory activities of all of the peptides toward PPA were measured using an amylase assay kit Diacolor Liquid AMY from Toyobo (Osaka). This kit is composed of a substrate solution (2-chloro-4-nitrophenyl 4^4-*O*-β-D-galactopyranosyl-β-maltotetraoside) and a reaction enzyme solution containing α-glucosidase and β-glucosidase (*14*). In this assay, 20 mM Tris-HCl buffer (pH 7.0) was used, and the substrate solution was adequately diluted by the buffer for our modification of the α-amylase assay method described by Majima *et al.* (*14*). The concentrations of peptides (ε_{280} = 5,570 and 1,420 M^{-1} cm^{-1} for Trp and Tyr, respectively), substrate (ε_{405} = 17,600 M^{-1} cm^{-1}), and PPA ($A^{1\%}$ = 25 at 280 nm) were determined from the UV absorption spectra. PPA (30 μl, 0.12 μM) and the sample peptide solutions (83 μl, 2.5–10 μM) were mixed and incubated in a micro test tube at 37°C for 10 min. An aliquot (23 μl) of the mixture was transferred to a 96-well plate, and a reaction enzyme solution (96 μl) containing α-glucosidase and β-glucosidase was added. After 2-min incubation at 37°C, 48 μl of adequately diluted substrate solution was added to initiate the reaction. The final substrate concentrations were in the range 0.14–0.47 mM. The initial rates were obtained by monitoring the absorbance change at 405 nm for 7 min using a microtiterplate reader Wellreader SME3400. Under these conditions, the increases in the absorbance at 405 nm showed good linearity, as shown in Fig. 4A. K_i values for the cyclic and linear carbamoylmethyl peptides were determined from Lineweaver-Burk plots using the initial velocities obtained during a period of 5 min (from 2 to 7 min during a 7-min period of monitoring). The inhibitory activities were evaluated from the results of 4–6 independent experiments. In this method, reproducibility of the inhibitory activity was improved compared to that in the previous method (*13*) without the use of the microtiterplate reader, because several independent experiments could be performed simultaneously under the same conditions, *e.g.*, a decrease in K_i values for the cyclic peptide Ten(15-23) from 0.29 ± 0.09 μM (*13*) to 0.27 ± 0.03 μM (Table I).

CD Measurement—The CD spectra of all peptides in 20 mM Tris-HCl (pH 7.0) were measured at 25°C in a 1-mm pathlength cell at a peptide concentration of 2.5–10 μM. For evaluation of the conformational change in linear carbamoylmethyl Ten(15-23) in the presence of PPA, 5 μM each of linear Ten(15-23) and PPA in solution were mixed and incubated at 37°C for 10 min before CD measurement. To obtain the CD spectrum of linear Ten(15-23) induced by PPA, the CD spectrum of PPA was subtracted from that of the mixture of linear Ten(15-23) and PPA.

Proteolytic Digestion—Proteolytic cleavage rates for the cyclic and linear carbamoylmethyl Ten(15-23) peptides were compared. The peptides were incubated with TPCK-treated trypsin or TLCK-treated chymotrypsin, and the reaction products were analyzed by reversed-phase HPLC. Internal standard peptides, Leu-enkephalin and $(Pro\text{-}Pro\text{-}Gly)_5$, were included for the purpose of quantification. The final concentrations of the reaction components for the linear carbamoylmethyl Ten(15-23) were 144 μM peptide, 34 μM internal standard peptide, and 200 nM TPCK-treated trypsin or 200 nM TLCK-treated chymotrypsin in 20 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$. For cyclic Ten(15-23), the final concentrations of the reaction components were 155 μM cyclic peptide and 7% DMSO, and the other conditions were the same as for the linear peptide. After incubation at 37°C for the desired reaction time, trypsin and chymotrypsin were removed from the reaction mixture by ultrafiltration through a 10,000 MW cutoff filter, and the filtrates were frozen at −20°C until chromatographic analysis and MALDI-TOF-MS.

RESULTS

Inhibitory Activities of the Synthetic Peptides toward PPA—The inhibition constants of the cyclic and linear carbamoylmethyl peptides for PPA were determined from the Lineweaver-Burk plots. Figure 4, B and C, shows that both the cyclic and linear Ten(15-23) peptides competitively in-

hibit PPA. Kinetic data for the other peptides also indicated that all of the synthetic peptides act as competitive inhibitors, similar to the other mimics previously synthesized (*9–13*) (data not shown).

The even-length cyclic peptides, cyclic Ten(15-22), Ten(16-23), Ten(15-24), and Ten(14-23), exhibit potent PPA inhibitory activities with K_i values of 0.40 –0.67 μM (Table I). The K_i values for the 12-15-residue cyclic peptides increase with increasing peptide length, while the K_i values for 5-10-residue cyclic peptides increase with decreasing peptide length. As a result, cyclic Ten(15-23) with 11 residues has the strongest inhibitory activity (K_i = 0.27 μM) among the cyclic peptides synthesized.

To examine the effect of the peptide cyclic structure on the PPA inhibitory activity, the inhibition constants of the linear carbamoylmethyl peptides were also evaluated (Table I). These linear carbamoylmethyl peptides were found to have potent PPA inhibitory activities (K_i = 0.28–1.00 μM), and the effect of peptide structure was variable.

TABLE I. **Inhibition constants of cyclic and linear carbamoylmethyl peptides derived from tendamistat.**

Sequence	Peptide length[a]	Inhibition constants K_i (μM)[b]	
		Cyclic peptide	Linear peptide
Ten(13-25)	15	1.04 ± 0.01	0.77 ± 0.18
Ten(14-24)	13	0.80 ± 0.03	1.00 ± 0.28
Ten(14-23)	12	0.50 ± 0.01	0.92 ± 0.12
Ten(15-24)	12	0.67 ± 0.07	0.85 ± 0.03
Ten(15-23)	11	0.27 ± 0.03	0.28 ± 0.02
Ten(15-22)	10	0.40 ± 0.04	0.36 ± 0.01
Ten(16-23)	10	0.41 ± 0.01	0.59 ± 0.08
Ten(16-22)	9	0.48 ± 0.06	0.48 ± 0.04
Ten(17-21)	7	1.04 ± 0.11	0.53 ± 0.08
Ten(18-20)	5	1.41 ± 0.01	0.55 ± 0.10

[a]Peptide lengths including N- and C-terminal Cys residues were expressed. [b]K_i values are means ± SD of 4–6 experiments. The inhibition constants were evaluated in 20 mM Tris-HCl buffer (pH 7.0) at 37°C using Lineweaver-Burk plots. Final concentrations of PPA, peptides, and substrate were 3 nM, 0.25 μM, and 0.14–0.47 mM, respectively.

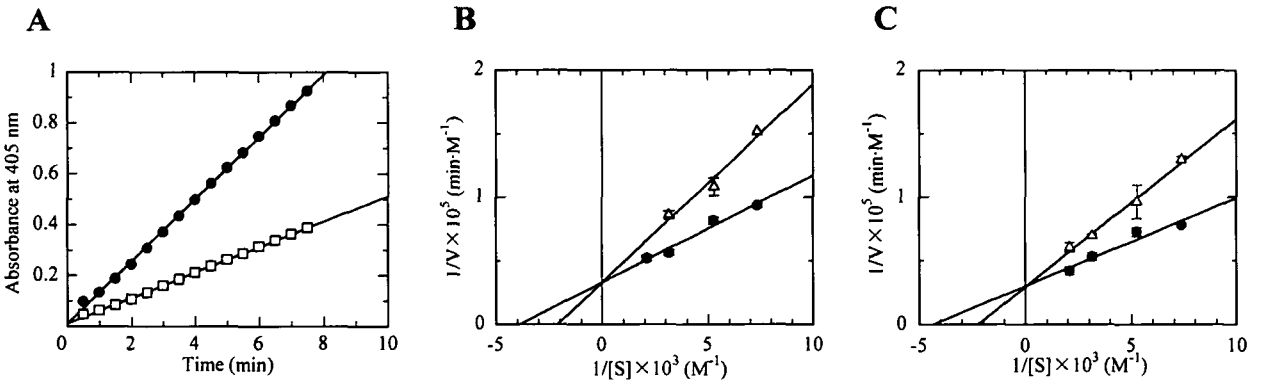

Fig. 4. **PPA inhibition by the cyclic and linear carbamoylmethyl Ten(15-23) peptides.** (A) Time courses of the release of 2-chloro-4-nitrophenol by PPA in 20 mM Tris-HCl buffer (pH 7.0) at 37°C in the absence (●) and presence (□) of cyclic Ten(15-23) (0.25 μM). (B)(C) Lineweaver-Burk plots for PPA inhibition by cyclic Ten(15-23) (B) and linear Ten(15-23) (C). Peptide concentrations were 0.25 (●) and 0 μM (△). The measurements were conducted in 20 mM Tris-HCl buffer (pH 7.0) at 37°C. Details are described in "MATERIALS AND METHODS."

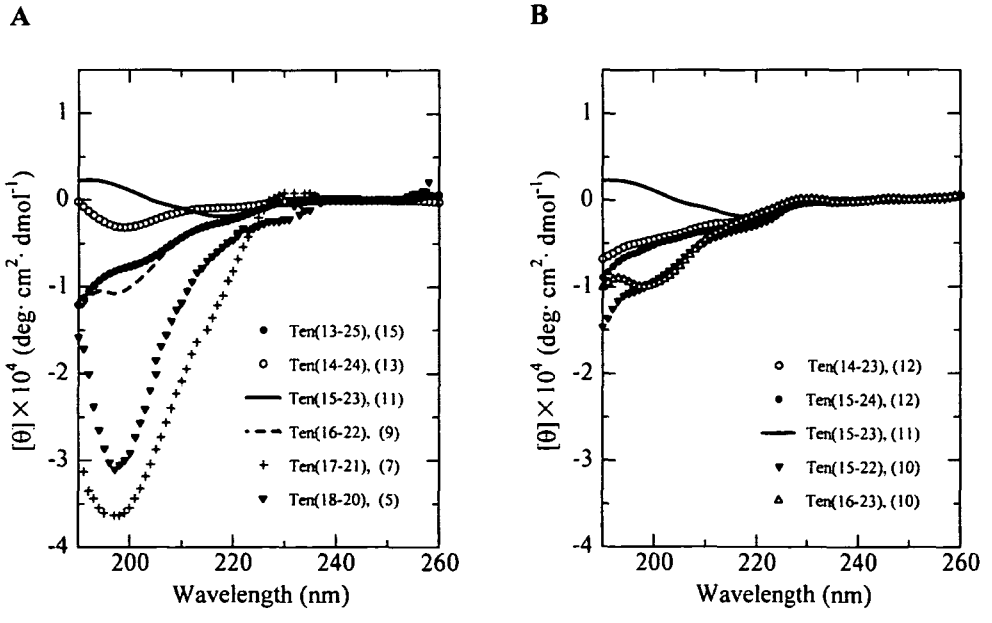

Fig. 5. **CD spectra of cyclic peptides with odd (A) and even lengths (B).** Peptide concentrations were 2.5–10 μM. The measurements were conducted in 20 mM Tris-HCl buffer (pH 7.0) at 25°C.

A change in the peptide structure from cyclic to linear produced a decrease in the PPA inhibitory activities of the peptides Ten(14-24), Ten(14-23), Ten(15-24), and Ten(16-23), while such a change led to an increase in the PPA inhibitory activities of the peptides Ten(13-25), Ten(17-21), and Ten(18-20). In the case of the 10- and 11-residue peptides Ten(15-23), Ten(15-22), and Ten(16-22), no significant difference in PPA inhibition was observed between the cyclic and linear forms. It is particularly noteworthy that the linear Ten(15-23) with 11 residues is still the strongest PPA inhibitor among the linear peptides tested, and its inhibitory activity is almost the same as that of cyclic Ten(15-23). These results indicate that a cyclic structure to force the peptide into take a β-hairpin structure is not necessarily required for PPA inhibition.

CD Studies—The structure-inhibitory activity relationship was examined by comparing the CD spectra of the cyclic and linear carbamoylmethyl peptides in 20 mM Tris-HCl buffer (pH 7.0). For the cyclic peptides, a peptide length-dependent CD spectral change was observed (Fig. 5). The cyclic 11-residue peptide Ten(15-23) took a predominant β-structural conformation characterized by a negative minimum at 217 nm. Other cyclic peptides with odd and even numbers of residues showed CD spectra similar to each other without any characteristic CD bands other than a slight trough at around 217 nm. These CD patterns suggest the presence of several structures, including a β-structure. The conversion of the cyclic structure to a linear one by Cys side chain modification was found to result in a disintegration of the β-structural conformation, especially for cyclic Ten(15-23), because a disappearance of the CD band around 217 nm or a decrease in intensity was observed (Fig. 6). In addition, linear Ten(14-23) exceptionally showed a characteristic CD spectral pattern with a negative minimum at around 217 nm. However, this structure is thought to have been formed intermolecularly because a peptide concentration-dependence was observed for the CD spectral pattern (data not shown). These data for cyclic peptides suggest that there is a relationship between β-structure formation and PPA inhibitory activity, while it seems that there is no such relationship for linear peptides.

To examine whether a peptide structural change is induced by the interaction between a linear peptide and PPA, the CD spectra for linear Ten(15-23) at 5 μM in the absence and presence of PPA (5 μM) were measured (Fig. 7). For this experiment, we assumed that no noticeable secondary structural change in PPA would occur when it bound to linear Ten(15-23), and, consequently, evaluated the CD spectrum for linear Ten(15-23) bound to PPA. Compared to the CD spectrum for linear Ten(15-23) alone, a trough at 217 nm clearly appeared, and the positive maximum (226 nm) shifted to a longer wavelength. This CD change implies

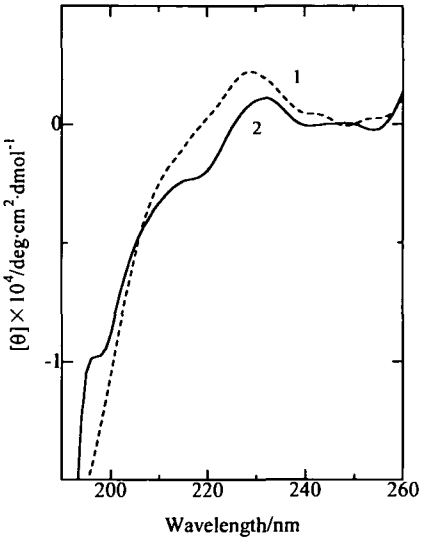

Fig. 7. **Comparison of the CD spectra of linear carbamoylmethyl Ten(15-23) in the absence (1) and presence (2) of PPA.** Concentrations of PPA and linear Ten(15-23) were identical (5 μM). Mixtures were incubated at 37°C for 10 min before the CD measurement [20 mM Tris-HCl buffer (pH 7.0) at 25°C].

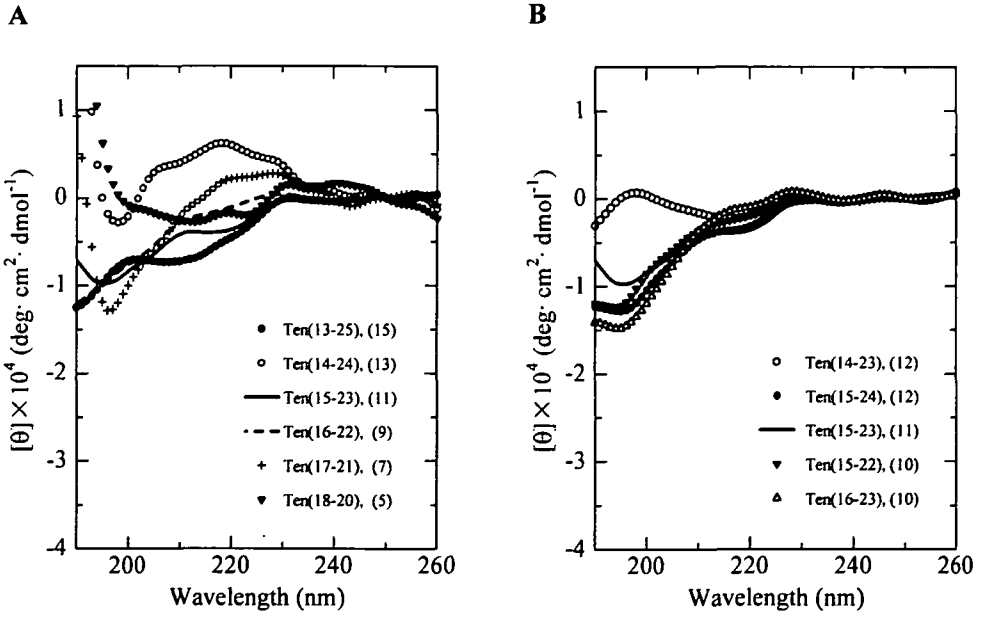

Fig. 6. **CD spectra of linear carbamoylmethyl peptides with odd (A) and even lengths (B).** Peptide concentrations were 2.5–10 μM. The measurements were conducted in 20 mM Tris-HCl buffer (pH 7.0) at 25°C.

that an ordered structure such as a β-structure may be induced when linear Ten(15-23) interacts with PPA.

Protelytic Hydrolysis of Cyclic and Linear Carbamoylmethyl Ten(15-23)—Since a cyclic peptide structure often shows an enhanced protease resistance compared to a linear peptide structure, in order to compare the protease resistances of cyclic and linear peptides, the cyclic and linear carbamoylmethyl Ten(15-23) peptides were treated with trypsin and chymotrypsin as a model assay. We chose trypsin as a specific protease because our designed peptides contain only one trypsin cleavage site, Arg^{19}-Tyr^{20}, located in the inhibitory site of tendamistat, and tryptic cleavage must cause a disappearance in PPA inhibition; chymotrypsin was chosen as a non-specific protease. Leucine-enkephalin and $(Pro\text{-}Pro\text{-}Gly)_5$ peptide were used as the internal standards for tryptic and chymotryptic digestion, respectively, and aliquots of cyclic and linear carbamoylmethyl Ten(15-23) peptides were analyzed by reversed-phase

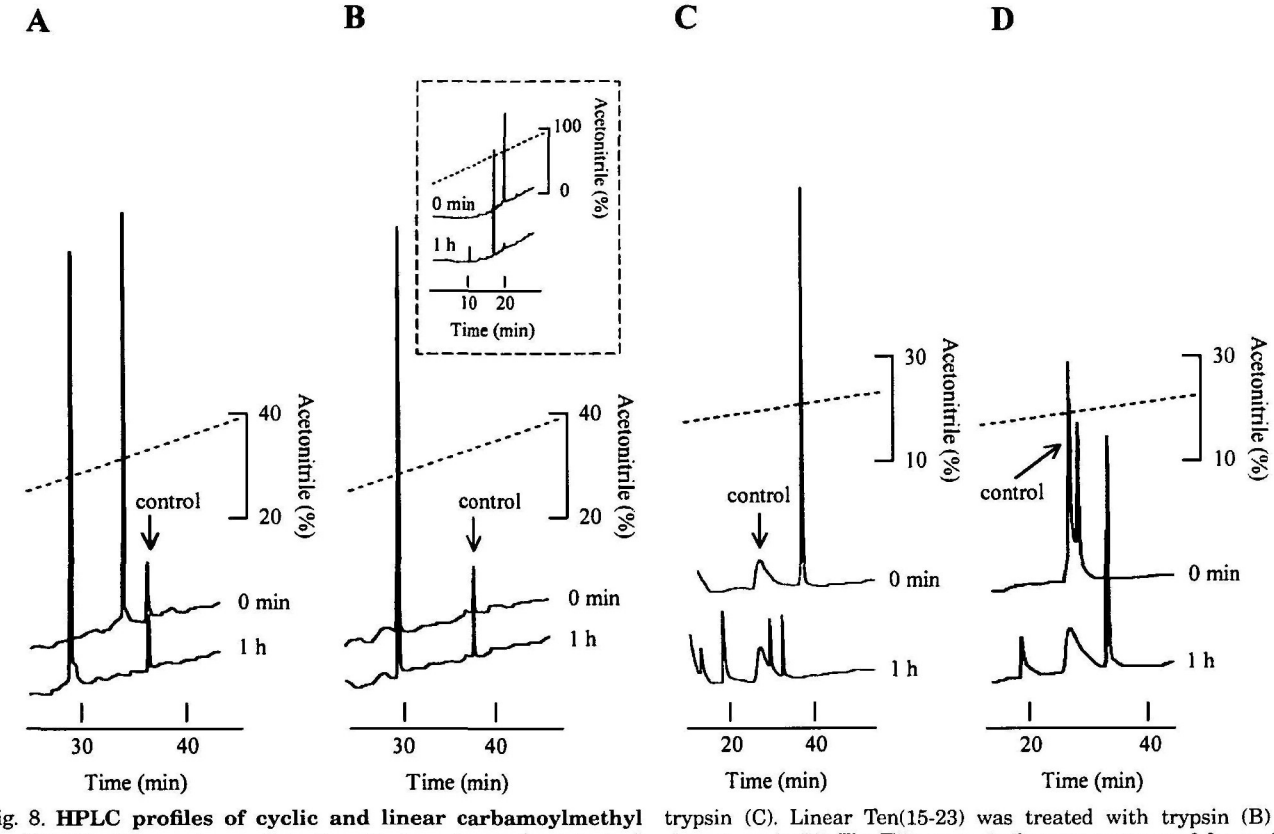

Fig. 8. **HPLC profiles of cyclic and linear carbamoylmethyl Ten(15-23) after protease digestion.** Each peak was characterized by MALDI-TOF-MS. Leu-enkephalin and $(Pro\text{-}Pro\text{-}Gly)_5$ peptide were used as internal standards for tryptic and chymotryptic digestion, respectively. Cyclic Ten(15-23) was treated with trypsin (A) and chymotrypsin (C). Linear Ten(15-23) was treated with trypsin (B) and chymotrypsin (D). The TFA-acetonitrile system was used for analyses A, B, C, and D, while 10 mM triethylammonium acetate-acetonitrile system was used for the analysis shown in the inset in B.

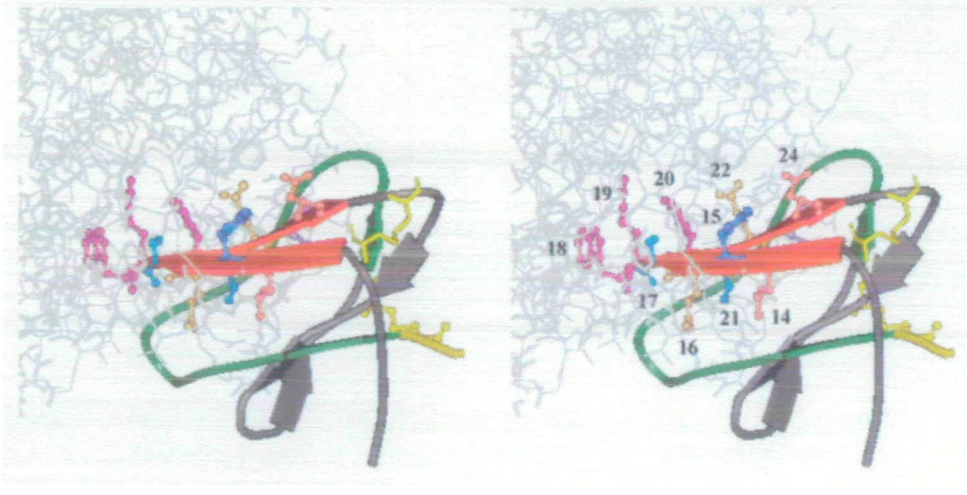

Fig. 9. **Stereoview of tendamistat bound to PPA based on the X-ray crystal structure (PDB entry 1BVN) by Wiegand *et al.* (5).** The active small loop (red), large loop (green), and other regions (dark gray) in tendamistat are shown in solid ribbon format. Several amino acid side chains on the small loop of tendamistat are presented in ball and stick format. The side chains of disulfide bridged Cys11–Cys27 and Cys45–Cys73 are also shown (yellow). Partial structure of PPA around the active site is presented in stick format (silver). The figure was generated by Weblab Viewer (MSI, San Diego, USA).

HPLC and MALDI-TOF-MS (Fig. 8).

No significant difference in trypsin resistance was observed between the cyclic and linear Ten(15-23) peptides. Cyclic Ten(15-23) was completely hydrolyzed by trypsin within 1 h under the conditions used, and a new peak corresponding to the linear product (MS 1451) appeared (Fig. 8A). Linear carbamoylmethyl Ten(15-23) was also hydrolized under the same conditions (Fig. 8B). In this case, since the linear peptide and the degradation product could not be separated sufficiently using the TFA-acetonitrile system (Fig. 8B), we used another triethylammonium acetate-acetonitrile system (inset in Fig. 8B) for the analysis. In the case of chymotryptic digestion, there was also no difference in chymotrypsin resistance between the cyclic and linear Ten(15-23) peptides (Fig. 8, C and D). These results suggest that both the cyclic and linear Ten(15-23) peptides are degraded to the same extent by proteases and lose their inhibitory activities in biological systems.

DISCUSSION

To search for lead compounds in the development of α-amylase inhibitors, we have designed 10 kinds of disulfide-bridged cyclic peptides of various lengths on the basis of the inhibitory sequence of tendamistat. The most potent inhibitor among the peptides synthesized was the 11-residue cyclic peptide Ten(15-23). The results of our CD study indicated that only cyclic Ten(15-23) takes a predominant β-structural conformation. A more detailed structural study of cyclic Ten(15-23) using an NMR technique was conducted by Kawano *et al.*, and their data suggest that the Trp-Arg-Tyr sequence of the peptide forms a turn structure similar to that in tendamistat (*15*). Even-length cyclic peptides with 10 and 12 residues, Ten(14-23), Ten(15-24), Ten(15-22), and Ten(16-23), exhibited potent but slightly less inhibitory activity compared to cyclic Ten(15-23). On the other hand, no noticeable characteristics of a β-structural conformation were observed in the CD spectra of the even-length cyclic peptides, indicating that even peptide lengths of 10 and 12 residues have no stabilizing effect of a β-hairpin on peptide structure. It was, therefore, demonstrated that the 11-residue peptide length is suitable for the formation of a tendamistat-like β-hairpin structure and, consequently, cyclic Ten(15-23) shows potent PPA inhibitory activity among our designed cyclic peptides based on the inhibitory sequence of tendamistat.

It was surprising that the linear carbamoylmethyl peptides have potent PPA inhibitory activities (K_i = 0.28–1.00 μM) comparable to those of the cyclic peptides (Table. I). On the other hand, the CD data for the linear peptides other than Ten(14-23) in buffer do not show the existence of a predominant β-structure. It is particularly noteworthy that linear Ten(15-23) exhibited almost the same PPA inhibitory activity as that of cyclic Ten(15-23), even though it does not form a predominant β-structure. These results indicate that the constrained structure of cyclic peptides does not contribute effectively to the enhancement of PPA inhibitory activity.

The potent PPA inhibitory activity observed for the linear carbamoylmethyl peptides can be explained by several factors. First, the flexibility of the linear peptide backbone contributes to the maintenance of potent PPA inhibitory activity. The CD data for linear Ten(15-23) in the presence of PPA suggest the induction of a β-structural conformation upon binding to PPA. Additionally, in the case of the shorter linear peptides with 7 and 5 residues, Ten(17-21) and Ten(18-20), PPA inhibitory activities increase when the cyclic peptide structure is lost, indicating an unfavorable effect of the constrained structures. These results support the speculation that linear peptides form a tendamistat-like β-hairpin structure favorable to PPA binding. Second, the introduction of the two carbamoylmethylated Cys residues must influence the PPA inhibition. This is supported by the fact that a linear tripeptide, Ac-Trp-Arg-Tyr-OCH_3, shows a much weaker PPA inhibitory activity, with a K_i value of 100 μM, than linear Ten(18-20), with a K_i value of 0.55 μM (*9*). In addition, since linear Ten(15-23) shows the most potent PPA inhibitory activity among the linear peptides tested, the contribution of some favorable amino acid residues to binding to the PPA active site is also suggested.

The inhibitory site in the small loop of tendamistat forms a β-hairpin structure, and the side chains of Trp^{18}, Arg^{19}, and Tyr^{20} in the β-turn region emerge to interact with the PPA active site (Fig. 9), as previously described (*5*). The Arg^{19} side chain is sandwiched between the aromatic rings of Trp^{18} and Tyr^{20} by cation-π interaction in the native tendamistat. Therefore, it is thought that the construction of a tendamistat-like turn structure is important for PPA inhibition. Besides the β-turn region, the side chains of Thr^{13}, Tyr^{15}, Gln^{22}, and Asp^{24} face the PPA molecule, while the side chains of Leu^{14}, Gln^{16}, Ala^{23}, and Asn^{25} oppositely face the large loop of tendamistat itself. In addition, the Thr^{13} and Asp^{24} side chains are not in close contact with the PPA molecule because these residues are located outside the PPA active site when binding to PPA. These previous findings suggest that the Tyr^{15} and Gln^{22} residues are important for PPA binding, while the Thr^{13}, Leu^{14}, Gln^{16}, Ser^{21}, Ala^{23}, and Asp^{25} residues are less important. The peptide length-dependent PPA inhibition observed not only for the cyclic peptides but also for the linear peptides can be explained by these suggestions. Therefore, both our designed cyclic and linear peptides should interact with PPA in a manner similar to that of tendamistat. Moreover, these suggestions provide a reason why our cyclic and linear peptides have more potent PPA inhibitory activity than the mimics designed so far, even though these mimics adopt a tendamistat-like β-turn conformation (*9–12*).

In conclusion, we have shown that small cyclic and linear peptides containing the sequence of the tendamistat small loop region have potent PPA inhibitory activities depending on peptide lengths. We also demonstrated that a peptide length of 11 residues is suitable for potent PPA inhibition in the case of both cyclic and linear peptides. The results of our CD study suggest that there is only an additional effect of cyclic structure on PPA inhibition. Since no significant differences between the protease resistances of cyclic and linear peptides were observed, both cyclic and linear peptides containing the inhibitory sequence of tendamistat should be considered as lead compounds in the design of potent α-amylase inhibitors. In addition, *Streptomyces* produces several proteinous α-amylase inhibitors, Paim (*15*), Haim (*16*), Z-2685 (*17*), AI-409 (*18*), and T-76 (*19*), whose active site sequences are homologous to that of tendamistat. Therefore, cyclic and linear peptides designed on the basis of the inhibitory sequence of tendamistat should

also be useful for studies on such proteinous α-amylase inhibitors.

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