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WATER-SOLUBLE CHYMOTRYPSIN SPECIFIC INHIBITORS CONTAINING ARGININE

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Summary: In an attempt to develop water-soluble, potent inhibitors for chymotrypsin, structurally rigid dipeptides containing arginine were designed and synthesized. The dipeptide H-D-Arg-Phe-NHBzl inhibited chymotrypsin very strongly (K_i=5.9 μM). The dipeptide with the inverse sequence, H-D-Phe-Arg-NHBzl, was also a moderate inhibitor for chymotrypsin with K_i of 240 μM. In spite of the presence of arginine in these dipeptides, they inhibited trypsin only weakly, indicating that they are highly specific for chymotrypsin. High resolution ^{1}H-NMR (400-MHz) indicated that these dipeptides can make a strong intramolecular hydrophobic interaction between Arg-β, γ, δ-methylenes and Phe-phenyl, producing a rigid hydrophobic core which interacts with the chymotrypsin S_2 site. Since these dipeptides are easily soluble in water, they are regarded as the sophisticated and effective inhibitors for chymotrypsin.

Several chymotrypsin-like proteases have recently been found in cells present in intractable diseases such as cancer (1), rheumatism (2), and muscular dystrophy (3). Although the roles of these enzymes have not been clarified yet in detail, their specific inhibitors are expected to be useful not only for therapeutics but also for elucidation of their enzymatic mechanisms. Although various types of inhibitors for chymotrypsin or chymotrypsin-like enzymes have been designed and synthesized, the number of effective compounds is not so large (4). Since the interactions between chymotrypsin and inhibitors at the binding site are all hydrophobic, this inevitably imposes a serious problem of water-insolubility upon the development of effective inhibitors.

Recently, we found that dipeptides H-D-Xxx-L-Phe-NHBzl (or OBzl), where Xxx denotes the hydrophobic amino acid such as Leu, Val and Ala, can inhibit chymotrypsin very strongly and that they can take a specific conformation suitable for this inhibition. The conformational feature of these dipeptides is the formation of hydrophobic core due to an

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Abbreviations: Abbreviations used are according to IUPAC-IUB Commissions (1984) *Eur. J. Biochem.* **138**, 9-37. Additional abbreviations: ATEE, Ac-L-Tyr-OEt; BAPA, Bz-L-Arg-*p*NA; DMSO, dimethyl sulfoxide; and NOE, nuclear Overhauser effect.

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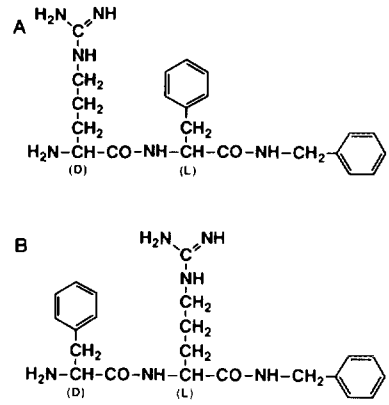

Fig. 1. Structure of arginine-containing dipeptides as chymotrypsin specific inhibitors. (A) H-D-Arg-Phe-NHBzl and (B) H-D-Phe-Arg-NHBzl.

intramolecular side chain-side chain interaction between D-Xxx and Phe residues (5, 6). In this inhibitory conformation, the amide-benzyl group appeared to interact with the S_1 site of the enzyme, while the hydrophobic core with the S_2 site. However, poor water-solubility of this series of inhibitors hampered their effective use. If the substitution of hydrophobic D-Xxx in H-D-Xxx-Phe-NHBzl by hydrophilic amino acids can hold the inhibitory conformation, it appears to become feasible to obtain water-soluble compounds which inhibit chymotrypsin and chymotrypsin-like enzymes.

CPK modeling of dipeptides has suggested that any amino acids having methylene or methyl groups at the β, γ, and/or δ carbons of Xxx can make hydrophobic interaction with adjacent Phe-phenyl group, even if Xxx is hydrophilic amino acid such as Arg. Arg has three methylene groups at the β, γ, and δ positions. In this study, dipeptides containing Arg at the position 1 or 2 (Fig. 1) were synthesized and their inhibitory activities were assayed against chymotrypsin as well as trypsin.

EXPERIMENTAL PROCEDURES

Materials: Arginine-containing peptides were prepared by the conventional solution method of peptide synthesis. For instance, H-D-Arg-Phe-NHBzl was obtained by HF treatment of Boc-D-Arg(Tos)-Phe-NHBzl, which was prepared from Boc-D-Arg(Tos)-OH and H-Phe-NHBzl using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in the presence of 1-hydroxybenzotriazole. Purification was carried out by gel filtration on a Sephadex G-15 column, and the purity was verified by HPLC and HP-TLC. Bovine chymotrypsin was purchased from Worthington Biochemical Co. (Freehold, NJ), and trypsin from Sigma (St. Louis, MO). Ac-L-Tyr-OEt (ATEE) and Bz-L-Arg-*p*NA (BAPA) were purchased from Peptide Institute (Osaka).

Enzyme assays: Assays for inhibition of chymotrypsin were performed in 50 mM phosphate buffer (pH 7.8) using ATEE as a substrate. Assays for trypsin were performed using BAPA in 50 mM Tris-HCl buffer (pH 8.2) containing 10 mM $CaCl_2$. The rate of hydrolysis of ATEE and BAPA was monitored at 237 and 410 nm, respectively, using a Hitachi Model 100-60 spectrophotometer. Kinetic analyses of inhibitions were carried out by Lineweaver-Burk plot (7) and Dixon plot (8).

Solubility test: The extent to which dipeptide inhibitors can dissolve in aqueous alcohol was assessed by inspecting the presence or absence of precipitation after centrifugation of

the solution mixed and stirred. Dipeptides (5-6 mg, the amount to make 50 mM concentration in final) was added to 0, 10, 30, or 50% MeOH (250 µl) and the solutions were vortexed for 10 min at room temperature and centrifuged (10,000 rpm for 2 min). When the precipitation was observed, peptides were first dissolved in neat MeOH (25 µl for 10%, 75 µl for 30%, and 125 µl for 50%) and then diluted with water up to 250 µl in total volume.

^{1}H-NMR measurements: All ^{1}H-NMR spectra were recorded on a Bruker AM-400 spectrometer interfaced to an Aspect 3000 computer. The chemical shifts were determined at 30°C using tetramethylsilane as an internal standard. Samples were dissolved in 0.5 ml DMSO-d_6 and the signal assignments were made using two-dimensional COSY. The H-{H} nuclear Overhauser effect (NOE) data were recorded by a gated irradiation pulse sequence. The difference NOE spectra were obtained by subtracting the control spectrum from an original NOE spectrum produced by presaturation of selected proton signal with a low decoupling power for 3 sec before pulse.

RESULTS AND DISCUSSION

The arginine-containing dipeptides synthesized inhibited chymotrypsin competitively (Fig. 2). The K_i value (5.9 µM) for H-D-Arg-Phe-NHBzl was almost comparable to that (3.6 µM) obtained for H-D-Leu-Phe-NHBzl, which is the strongest inhibitor in a series of dipeptide inhibitors so far tested. Interestingly, the dipeptides with the inverse sequence, namely H-D-Phe-Arg-NHBzl (240 µM) and H-D-Phe-Leu-NHBzl (26 µM), inhibited chymotrypsin much more weakly; 7-40 times less active than the parent dipeptides (Table 1). These results suggested that these two series of dipeptides with Phe at position 1 or 2 are different in conformation.

Dipeptide lacking the amide benzyl group, H-D-Arg-Phe-NH_2, was completely inactive, indicating that the presence of this benzyl group is critical for chymotrypsin inhibition. When inhibitory activities of H-D-Arg-Phe-NHBzl and H-D-Arg-Ala-NHBzl were compared, the latter was about 17 times less active than the former (Table 1). This shows that the Phe-phenyl group is also an important structural element for inhibition of chymotrypsin. Dipeptide H-D-Ala-Phe-NHBzl, which is the D-Arg/D-Ala substituted

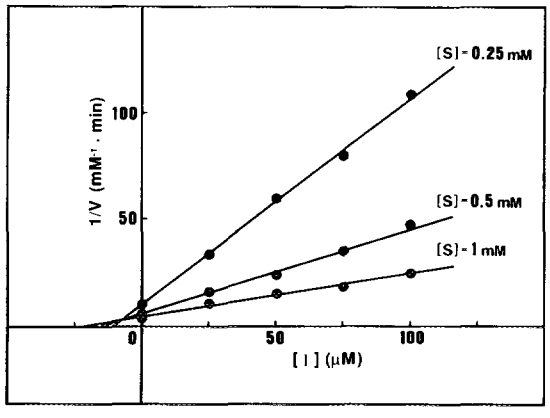

Fig. 2. Dixon plot for hydrolysis of Ac-Tyr-OEt by chymotrypsin in the presence of H-D-Arg-Phe-NHBzl at pH 7.8 (50 mM phosphate buffer) and 25°C. The substrate concentrations used are indicated in figure.

Table 1. Inhibitory activities of dipeptides for chymotrypsin and trypsin

Dipeptide	K_i (μM)		Selectivity Ratio
	Chymotrypsin	Trypsin	K_i (Tsin)/K_i (Csin)[a]
H-D-Arg-Phe-$NHCH_2C_6H_5$	5.9	588	98
H-D-Arg-Phe-NH_2	inactive	inactive	—
H-D-Arg-Ala-$NHCH_2C_6H_5$	100	600	6
H-D-Ala-Phe-$NHCH_2C_6H_5$	34	inactive	—
H-D-Phe-Arg-$NHCH_2C_6H_5$	240	inactive	—

a) The chymotrypsin (Csin)/trypsin (Tsin) selectivity ratio was calculated by dividing the K_i value obtained for trypsin by that obtained for chymotrypsin.

derivative of H-D-Arg-Phe-NHBzl, retained considerably strong inhibitory activity (K_i=34 μM) (Table 1).

The solubility of dipeptides was tested using water and aqueous alcohol (Table 2). To prepare 50 mM stock solution for inhibition assay, peptides were first attempted to dissolve in water directly. Leu-containing dipeptides were sparingly soluble in water. They were unable to dissolve directly even in aqueous methanol (10-30%), unless they were first dissolved in neat MeOH and then diluted to 30% solution with water. In contrast, Arg-containing dipeptide benzyl amides dissolved in water without difficulty.

In order to examine whether these Arg-containing dipeptide benzyl amides adopt the conformation similar to those of Leu-containing dipeptides, we analyzed their ^{1}H-NMR spectra. The NMR spectra characteristic of Leu-containing dipeptides were (i) large upfield shifts of Leu-β and γ methylene protons, which are shielded from the Phe-phenyl group, (ii) non-equivalent splitting of Phe-β methylene protons, and (iii) distinct NOE between Leu-isobutyl and Phe-phenyl groups. These NMR data indicated that dipeptides can construct the strong isobutyl-phenyl hydrophobic interaction between the side chains of Leu and Phe residues. In ^{1}H-NMR spectra of H-D-Arg-Phe-NHBzl, distinct upfield shifts of β ($\Delta\delta$=0.243 ppm), γ ($\Delta\delta$=0.235 ppm), and δ ($\Delta\delta$=0.096 ppm) methylene proton signals of Arg were observed when compared with those of Boc-Arg-OH (Table 3). Similar upfield shifts of proton signals of Arg-β, γ, δ-methylenes were also observed for

Table 2. Solubility of arginine- or leucine-containing dipeptides in aqueous methanol

Dipeptide	Solubility[a]			
	H_2O	10% MeOH	30% MeOH	50% MeOH
H-D-Arg-Phe-$NHCH_2C_6H_5$	+++	+++	+++	+++
H-D-Phe-Arg-$NHCH_2C_6H_5$	+++	+++	+++	+++
H-D-Leu-Phe-$NHCH_2C_6H_5$	−(−)	−(−)	−(+)	++
H-D-Phe-Leu-$NHCH_2C_6H_5$	−(−)	−(−)	−(+)	++

a) Solubility is expressed by the symbols + (soluble) and − (insoluble). The solubility extent is shown by single, double or triple symbols. Symbols in the parentheses indicate the solubility when dipeptides were first dissolved in neat MeOH and then diluted with water.

Table 3. Upfield shifts in ^{1}H-NMR chemical shifts of arginine-methylene protons

Dipeptides	Upfield shifts of arginine side chain methylene protons (ppm)[a]		
	β	γ	δ
H-D-Arg-Phe-$NHCH_2C_6H_5$	0.243	0.235	0.096
H-D-Arg-Phe-NH_2	0.154	0.516	0.147
H-D-Arg-Ala-$NHCH_2C_6H_5$	-0.070	0.053	-0.012
H-D-Phe-Arg-$NHCH_2C_6H_5$	0.014	0.196	0.043

a) For calculation of upfield shifts, the chemical shifts of arginine side chain methylenes of Boc-Arg-OH were utilized as control values.

H-D-Arg-Phe-NH_2 lacking the amide benzyl group. However, no significant shifts were observed for those in H-D-Arg-Ala-NHBzl lacking the Phe-phenyl group (Table 3). These provide the direct evidence that upfield shifts of Arg-β, γ, δ-methylene protons are due to the shielding effect from the Phe-phenyl group but not from the amide benzyl group. Non-equivalent splitting of Phe-β methylene protons was also prominent. Furthermore, positive NOEs were observed for proton signals of Phe-phenyl when signals of β- or γ-methylene of Arg were irradiated. All these results demonstrate that the side chains of Arg and Phe are spatially in close proximity to each other, and thus conformation of H-D-Arg-Phe-NHBzl is similar to those of Leu-containing dipeptides. As proposed for H-D-Leu-Phe-NHBzl, it is highly likely that the amide benzyl group fits the S_1 site of chymotrypsin, while the side chain-side chain complexing hydrophobic core fits the S_2 site (Fig. 3).

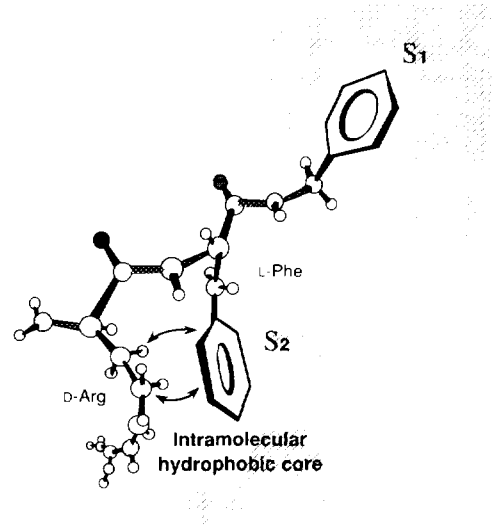

Fig. 3. A proposed model for the interaction between an inhibitor H-D-Arg-Phe-NHBzl and chymotrypsin active center. The dipeptide is in a specific inhibitory conformation produced by the side chain-side chain hydrophobic interaction.

Dipeptide H-D-Phe-Arg-NHBzl with the inverse sequence of highly active H-D-Arg-Phe-NHBzl showed smaller upfield shifts of side chain protons of Arg (Table 3). This might be a cause of its diminished activity (Table 1). Dipeptide H-D-Arg-Ala-NHBzl exhibited a moderate inhibitory activity (Table 1) despite the lack of Phe-phenyl. Hydrophobic D-Arg-β, γ, δ-methylenes and Ala-methyl in this dipeptide seem to interact similarly as seen in D-Arg-β, γ, δ-methylenes and Phe-phenyl of H-D-Arg-Phe-NHBzl and to produce an inhibitory conformation. In this case, the hydrophobic interaction cannot be detected by ^{1}H-NMR measurements because of the lack of magnetic shielding effects between D-Arg-β, γ, δ-methylenes and Ala-methyl.

Since the dipeptides synthesized contain Arg, they might be possible to inhibit trypsin too. When hydrolysis of BAPA by trypsin was carried out in the presence of H-D-Arg-Phe-NHBzl, it in fact inhibited trypsin in a competitive manner but with 100 times less weakly than chymotrypsin (Table 1). Dipeptide H-D-Arg-Ala-NHBzl inhibited trypsin similarly as observed for H-D-Arg-Phe-NHBzl, although its selectivity toward chymotrypsin was greatly lower than that of H-D-Arg-Phe-NHBzl (Table 1). On the other hand, H-D-Phe-Arg-NHBzl exhibited no inhibition against trypsin (Table 1).

Water-solubility of compounds is one of the most important structural requisites for chymotrypsin inhibitors. Incorporation of hydrophilic amino acids as well as construction of an intramolecular hydrophobic core with more proper geometry appear to be essential for creating more effective and selective inhibitors for chymotrypsin and chymotrypsin-like enzymes.

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