[Chem. Pharm. Bull. 34(7)2931—2936(1986)]

Synthesis and Structure of Prolinal-Containing Peptides, and Their Use as Specific Inhibitors of Prolyl Endopeptidases

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(Received January 20, 1986)

Peptide aldehydes are potent inhibitors of serine and cysteine proteases. In the present work, *N*-benzyloxycarbonyl (Z) dipeptides containing prolinal at the carboxyl terminus were synthesized as inhibitors of prolyl endopeptidases. Since no aldehyde proton was detected by proton nuclear magnetic resonance (^{1}H-NMR) spectrometry, a cyclic structure was proposed for these peptides. Compounds with a Z–L-X–L-prolinal structure were strong inhibitors of prolyl endopeptidases from the ascidian, *Halocynthia roretzi*, and *Flavobacterium meningosepticum*. The potency was in the order of Z–L-Val–L-prolinal $\simeq$ Z–L-Ile–L-prolinal > Z–L-Phe–L-prolinal > Z–L-Ala–L-prolinal with IC_{50} values of 10^{-8}—10^{-6} M order for both enzymes. Conversion of the aldehyde into an alcohol or an acid moiety resulted in a considerable decrease in the inhibitory activity. The diastereomers of Z–L-Phe–L-prolinal were much less inhibitory. This result is not compatible with the reported stereospecifity of the *Flavobacterium* enzyme for its substrates [T. Yoshimoto, R. Walter and D. Tsuru, *J. Biol. Chem.*, **255**, 4786 (1980)]. This implies that the open species binds preferentially to the enzyme active site.

Keywords——prolyl endopeptidase; post-proline cleaving enzyme; *Halocynthia roretzi*; *Flavobacterium meningosepticum*; peptide aldehyde; prolinal; protease inhibitor

Introduction

Prolyl endopeptidase [EC 3.4.21.26; post-proline cleaving enzyme] that catalyzes the hydrolysis of peptides at the carboxyl side of L-proline residues has been found in various organs of mammals,[1] several strains of *Flavobacterium*,[2] and ascidian sperm.[3] Involvement in neuropeptide metabolism has been suggested for the mammalian enzymes.[1] Recently, we suggested the possible involvement of the ascidian enzyme in fertilization.[3] This was based on the finding that the fertilization of the ascidian was inhibited by the chloromethyl ketone derivative of benzyloxycarbonyl(Z)–Gly–L-Pro, a specific irreversible inhibitor of prolyl endopeptidase synthesized by Yoshimoto *et al.*[4] Wirk and Orlowski[5] recently prepared Z–L-Pro–L-prolinal and found it to be a specific and potent inhibitor of the enzyme from rabbit brain. We have also prepared L-prolinal-containing peptides, Z–L-X–L-prolinal (X = Ala, Val and Phe),[6] by a route different from that of Wirk and Orlowski. These compounds proved to be strong inhibitors of prolyl endopeptidase from ascidian sperm, Z–L-Val–L-prolinal being the strongest (K_i = 2.4 nM). The findings that reduction of an aldehyde group of the inhibitors with $NaBH_4$ virtually abolished the inhibitory activity and that preincubation of the enzyme with the inhibitors was required for full inhibition suggest that the aldehyde group reacts with the enzyme to form a hemiacetal adduct as a transition-state analogue. In this paper, we describe the details of the synthesis of prolinal-containing peptides, their structures, and their inhibitory activities toward prolyl endopeptidases from the ascidian, *Halocynthia roretzi*, and *Flavobacterium meningosepticum*.

Experimental

Instruments——Absorbance was measured with a Shimadzu UV-240 spectrophotometer. Fluorescence intensity was measured with a Hitachi 203 fluorescence spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-GX 270FT NMR spectrometer with tetramethylsilane as an internal standard. Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected.

Materials——The following materials were obtained from the indicated sources: Z–X *p*-nitrophenyl esters (X = L-Ala, L-Val, L-Phe and D-Phe) and Z–X hydroxysuccinimide esters (X = L-Ile and L-Phe) (Kokusan Chemical Works, Tokyo, Japan); L-prolinol (Sigma Chemical Co., St. Louis, U.S.A.); diisobutylaluminum hydride (Mitsuwa's Pure Chemicals, Osaka, Japan); succinyl–Gly–L-Pro 4-methylcoumaryl-7-amide (Peptide Institute Inc., Osaka, Japan); Z–Gly–L-Pro *p*-nitroanilide and prolyl endopeptidase from *Flavobacterium meningosepticum* (lot 0228A) (Seikagaku Kogyo Co., Tokyo, Japan). Prolyl endopeptidase from sperm of the ascidian, *H. roretzi*, was prepared according to the method of Yokosawa *et al.*[3] Other chemicals were reagent-grade commercial products.

Synthesis of Prolinal Semicarbazone Tosylate——First, Z–L-prolinal semicarbazone was prepared according to the method of Ito *et al.*[7] A 1.76 M diisobutylaluminum hydride solution in hexane (57 ml) was added dropwise to a precooled (−50——−60 °C) solution of Z–L-Pro–OMe (13.2 g, 50 mmol) in dry toluene (250 ml) over a period of 30 min with stirring. After additional stirring for 30 min, 2 M HCl (250 ml) was added dropwise to decompose the reducing agent. The organic layer was washed with water and dried (Na_2SO_4). The oil obtained by evaporation of the solvent was treated with semicarbazide hydrochloride (6 g) and sodium acetate (4.5 g) in 70% ethanol (180 ml) at 80 °C for 7 min. After being concentrated to a small volume, the solution was extracted with ethyl acetate (200 ml). The extract was washed with water, dried (Na_2SO_4) and evaporated to give an oil. The oil was then dissolved in a small amount of benzene and a large amount of petroleum ether was added. The resulting white gum was triturated in petroleum ether and dried *in vacuo* to yield Z–L-prolinal semicarbazone as an amorphous powder (6.5 g, 45%). Next, the benzyloxycarbonyl group was removed by catalytic hydrogenation. Z–L-Prolinal semicarbazone (6.35 g, 20 mmol) was dissolved in methanol (200 ml) and palladium–carbon (10% Pd, 1.2 g) was added. The mixture was hydrogenated for 1 h, then *p*-toluenesulfonic acid (7.6 g, 40 mmol) was added. The whole was filtered and the filtrate was evaporated to an oil. While the oil was being washed with ether, white crystals of L-prolinal semicarbazone tosylate appeared (3.9 g, 59%). mp 175—178 °C. *Anal.* Calcd for $C_{13}H_{20}N_4O_4S$: C, 47.55; H, 6.14; N, 17.06. Found: C, 47.61; H, 6.11; N, 17.17. D-Prolinal semicarbazone tosylate was also prepared by essentially the same procedure as the L-isomer. mp 185—188 °C. *Anal.* Found: C, 47.49; H, 6.19; N, 16.68.

Synthesis of Z–X-prolinal——Generally, Z–X-prolinal was prepared by coupling Z–X *p*-nitrophenyl ester (X = L-Ala, L-Val, L-Phe or D-Phe) or Z–X hydroxysuccinimide ester (X = L-Ile) with L- or D-prolinal semicarbazone tosylate. The synthetic procedure for Z–L-Val–L-prolinal is described below as a typical example. A solution of L-prolinal semicarbazone tosylate (635 mg, 2 mmol) and triethylamine (280 μl, 2 mmol) in dimethylformamide (1 ml) was added to a solution of Z–L-Val *p*-nitrophenyl ester (819 mg, 2.2 mmol) in dimethylformamide (1 ml). The mixture was allowed to stand at room temperature for one day, then the solvent was evaporated off and the resulting oil was washed several times with ether. The oil was dissolved in ethyl acetate (30 ml) and washed successively with 1% citric acid, water, 1 M NH_4OH, and water. The organic layer was dried (Na_2SO_4) and evaporated to a small volume. On addition of petroleum ether, Z–L-Val–L-prolinal semicarbazone precipitated as an amorphous powder (250 mg, 32%). This product (250 mg, 0.64 mmol) was taken up in ethanol (5 ml), then 0.12 M HCl (12.5 ml) and formalin (37% formaldehyde, 2.5 ml) were added. After being stirred at room temperature for 5 h, the solution was concentrated to small volume, neutralized with $NaHCO_3$ and extracted with ethyl acetate (35 ml). The extract was washed with water, dried (Na_2SO_4) and evaporated to dryness. The residual oil of Z–L-Val–L-prolinal was crystallized from chloroform–heptane (91 mg, 43%). ^{1}H-NMR (in $CDCl_3$) δ: 1.1 (d, 6H, $-CH(C\underline{H}_3)_2$ from valine side chain), 1.8 (m, 1H, $-C\underline{H}(CH_3)_2$ from valine side chain), 2.0 (m, 4H, $N-CH_2-C\underline{H}_2-C\underline{H}_2-$ from pyrrolidinyl ring), 3.0 (br, 1H, CH–O$\underline{H}$, exchangeable with D_2O), 3.6 (m, 3H, $C\underline{H}-N-C\underline{H}_2$ from pyrrolidinyl ring), 4.4 (br, 1H, $N-C\underline{H}-CO$, valine α-proton), 5.3 (s, 2H, $Ph-C\underline{H}_2$), 6.1 (br, 1H, $C\underline{H}-OH$), 7.5 (s, 5H, Ph). Other prolinal-containing peptides were similarly synthesized. Analytical data for the synthesized peptides are shown in Table I.

Synthesis of Z–L-Phe–L-prolinol——This compound was prepared by coupling Z–L-Phe hydroxysuccinimide ester with L-prolinol in tetrahydrofuran, and was obtained as an oil. The analytical data are shown in Table I.

Synthesis of Z–L-Phe–L-Pro——This compound was obtained by saponification of the corresponding methyl ester, prepared from Z–L-Phe and L-Pro–OMe by the mixed anhydride method. The analytical data are shown in Table I.

Inhibition Experiment——The *Flavobacterium* enzyme was preincubated with or without various concentrations of inhibitors in 1 ml of 0.1 M phosphate buffer (pH 7.0) at 25 °C for 1 h. To this solution, 50 μl of 1.36 mM Z–Gly–L-Pro *p*-nitroanilide was added, and the release of *p*-nitroaniline was measured at 25 °C at 410 nm. The inhibitor concentration at which the enzyme activity is half of the uninhibited value (IC_{50}) was estimated from the inhibitor concentration *vs.* activity curve.

The incubation of the ascidian enzyme was measured similarly: 30 μl of 5 mM succinyl–Gly–L-Pro 4-methylcoumaryl-7-amide was added to the incubation mixture in 3 ml of 25 mM phosphate buffer (pH 6.8) containing

0.1 M NaCl and 0.5 mM dithiothreitol, and the release of 7-amino-4-methylcoumarin was monitored by measuring its fluorescence intensity (excitation and emission at 380 and 440 nm, respectively). The K_m values for the hydrolysis of Z–Gly–L-Pro *p*-nitroanilide by the *Flavobacterium* enzyme and succinyl–Gly–L-Pro 4-methylcoumaryl-7-amide by the ascidian enzyme were reported to be 1.25[8] and 1.0 mM,[6] respectively. The substrate concentrations employed are well below these values (about 0.05 K_m), so the IC_{50} values should be nearly equal to the inhibition constants, K_i.

The preincubation of the enzymes with prolinal inhibitors seems necessary, as described previously.[6] We chose a preincubation time of 1 h in the present experiment, because essentially the same residual activity was observed after 30 min and 1 h of preincubation for both enzymes when checked with 30 nM Z–L-Val–L-prolinal. No loss of enzyme activity was observed for either enzyme during 1 h preincubation without inhibitors.

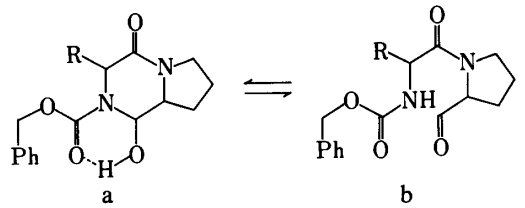
Results

Structure of Prolinal-Containing Peptides

As shown in Table I, the elemental analysis of the prolinal-containing peptides gave quite satisfactory results. However, the ^{1}H-NMR spectra of these peptides did not show any evidence that they possess free aldehyde groups, *i.e.*, no signals corresponding to the aldehyde proton (around δ 9—10) were observed. The assignment of the NMR signals of Z–L-Val–L-prolinal (described in Experimental) and those of other prolinal-containing peptides (not shown) led to the tentative proposal that the synthesized prolinal-containing peptides have a cyclic structure, as shown in Fig. 1a. On the other hand, Wirk and Orlowski[5] detected an aldehyde proton in the NMR spectrum of Z–L-Pro–L-prolinal. This may be explained in terms of the inability of this compound to take a cyclized form. When D_2O was added to the sample solution ($CDCl_3$), only one proton (–OH) disappeared from the NMR spectra of Z–L-Val–L-prolinal, Z–L-Ala–L-prolinal, Z–L-Phe–L-prolinal and Z–L-Ile–L-prolinal, confirming the structure of Fig. 1a. On the other hand, no change in the NMR spectrum was observed for Z–D-Phe–L-prolinal or Z–L-Phe–D-prolinal on addition of D_2O. It is possible that the hydroxyl groups of these two compounds lie between two phenyl rings (those of the benzyloxycarbonyl group and the phenylalanine residue) spatially and, thus, the hydroxyl groups can not freely interact with the solvent. The proposed structure in Fig. 1a may be stabilized by the six-membered ring formed by the reaction of the aldehyde group with the α-

Table I. Analytical Data for the Synthesized Peptides

Peptide	mp (°C)	Formula	Molecular weight	Analysis (%) Calcd (Found)		
				C	H	N
Z–L-Ala–L-prolinal	150.5—152	$C_{16}H_{20}N_2O_4$	304.3	63.15	6.62	9.21
				(63.11	6.54	9.30)
Z–L-Val–L-prolinal	134—135	$C_{18}H_{24}N_2O_4$	332.4	65.04	7.28	8.43
				(65.13	7.23	8.39)
Z–L-Phe–L-prolinal	138—140	$C_{22}H_{24}N_2O_4$	380.4	69.47	6.36	7.36
				(69.33	6.40	7.50)
Z–L-Ile–L-prolinal	118—119	$C_{19}H_{26}N_2O_4$	346.4	65.88	7.57	8.09
				(65.68	7.57	8.05)
Z–D-Phe–L-prolinal	103—104	$C_{22}H_{24}N_2O_4$	380.4	69.47	6.36	7.36
				(69.29	6.38	7.27)
Z–L-Phe–D-prolinal	100—101	$C_{22}H_{24}N_2O_4$	380.4	69.47	6.36	7.36
				(69.34	6.53	7.32)
Z–L-Phe–L-prolinol	Oil	$C_{22}H_{26}N_2O_4$	382.4	69.10	6.85	7.33
				(68.48	6.68	6.99)
Z–L-Phe–L-Pro	108—110	$C_{22}H_{24}N_2O_5$	396.4	66.66	6.10	7.07
				(66.47	6.25	7.08)



a cyclized form b open form

Fig. 1. Structure of Z–X–prolinal

R represents the side chain of amino acid X.
------ is a possible hydrogen bond.

TABLE II. Inhibition of Prolyl Endopeptidases from Ascidian and *Flavobacterium meningosepticum* by Prolinal-Containing and Related Peptides

Peptide	IC_{50} (10^{-8} M)	
	Ascidian	*Flavobacterium*
Z–L-Ala–L-prolinal	200	100
Z–L-Val–L-prolinal	1	3
Z–L-Ile–L-prolinal	2	3
Z–L-Phe–L-prolinal	20	60
Z–D-Phe–L-prolinal	>10000[a]	90000
Z–L-Phe–D-prolinal	5000	80000
Z–L-Phe–L-prolinol	10000	10000
Z–L-Phe–L-Pro	2000	80000

a) 42% inhibition at 1×10^{-4} M.

nitrogen atom of amino acid X, as well as by the possible hydrogen bond between the carbonyl oxygen of the benzyloxycarbonyl group and the hydroxyl hydrogen. The cyclic structure is expected to be stable in aqueous solution because the opening reaction is not a hydrolytic reaction. Further, the NMR spectrum of Z–L-Val–L-prolinal dissolved in D_2O containing 33% $(CD_3)_2SO$ and kept for one week was essentially the same as that of the compound dissolved in CD_3Cl except that one proton (–OH) was missing.

Inhibition by Prolinal-Containing Peptides

Table II shows the inhibitory activities toward the *H. roretzi* enzyme and the *Flavobacterium* enzyme. Compounds with the general structure Z–L-X–L-prolinal were strong inhibitors of the two enzymes with IC_{50} values of 10^{-8}—10^{-6} M order. Z–L-Val–L-prolinal and Z–L-Ile–L-prolinal were nearly equally potent, and Z–L-Phe–L-prolinal and Z–L-Ala–L-prolinal followed in that order for both enzymes. The IC_{50} values of each inhibitor for the two enzymes were not greatly different. Z–L-Phe–D-prolinal and Z–D-Phe–L-prolinal, the diastereomers of Z–L-Phe–L-prolinal, inhibited both enzymes much more weakly. This observation is consistent with the fact that the subsites S_1 and S_2 of the *Flavobacterium* enzyme are highly stereospecific for L-configuration.[9] The inhibitory power of Z–L-Phe–L-prolinal was greatly reduced by reduction (Z–L-Phe–L-prolinol) or oxidation (Z–L-Phe–L-Pro) of the aldehyde group. It is apparent that the aldehyde group is essential for the strong inhibition. The inhibitory effect of Z–L-Val–L-prolinal (1 mM) on bovine trypsin, porcine elastase and bovine chymotrypsin was examined at pH 8.0 using specific substrates, benzoyl–L-Arg *p*-nitroanilide, succinyl–(L-Ala)$_3$ *p*-nitroanilide and glutaryl–L-Phe *p*-nitroanilide, respectively, at concentrations well below the K_m values (about 0.05 K_m). No inhibition was observed with trypsin or elastase, and only 25% inhibition was observed with chymotrypsin.

Discussion

Peptide aldehydes are known to be strong inhibitors of serine and cysteine proteases.

Their specificities are determined by the C-terminal amino aldehydes. Since prolyl endopeptidase is known to be a serine protease and hydrolyzes peptides at the carboxyl side of L-proline, it was expected that the enzyme would be specifically inhibited by peptide aldehydes containing L-prolinal. In fact, Wirk and Orlowski,[5] Yoshimoto *et al.*[10] and we[6] reported that L-prolinal-containing peptides are strong inhibitors of the enzyme. The synthetic procedures of Wirk and Orlowski,[5] and Yoshimoto *et al.*[10] involved preparation of L-prolinol-containing peptides followed by oxidation of the hydroxyl group. Our procedure was different from theirs; prolinal semicarbazone-containing peptides were first synthesized and the semicarbazone group was removed by acid treatment. Although the two procedures seem to be equally useful in preparing simple dipeptides, the use of L-prolinol may be inappropriate in some cases if the hydroxyl group is not blocked suitably. The stable crystalline compound, L- or D-prolinal semicarbazone, that we employed as a key intermediate has been blocked at the aldehyde group, but the aldehyde group can be easily reproduced by acid treatment. This compound should also be useful in the preparation of an affinity adsorbent containing L-prolinal as a specific ligand for prolyl endopeptidase.

Although Z–L-Phe–L-prolinal is a strong inhibitor of the *Flavobacterium* enzyme as well as of the ascidian enzyme, its diastereomers are much less inhibitory. The same stereospecificity has been observed for the hydrolysis of peptide substrates by the *Flavobacterium* enzyme.[9] Therefore, the mode of binding of our prolinal inhibitors to the enzyme is thought to be very similar to that of the substrates, *i.e.*, the L-prolinal and the adjacent L-amino acid residues of the inhibitors interact with the S_1 and S_2 subsites of the enzyme, respectively. Although the major proportion of each prolinal inhibitor seems to be cyclized in aqueous media as judged from the NMR spectra, it is assumed that a trace amount of the open species (Fig. 1b) exists in equilibrium with the cyclized species, and the former binds to the enzyme preferentially. In the previous[6] and present studies, it was observed that prolonged preincubation of the prolinal inhibitors with the ascidian and the *Flavobacterium* enzymes is essential for strong inhibition. Such a phenomenon would occur if the conversion from the cyclized form to the open form is slow.

If the prolinal inhibitors interact with the enzymes to form a hemiacetal adduct as a transition-state analogue, and if this process is rate-limiting as seen for the leupeptin–trypsin system,[11] this might also account for the above phenomenon. Z–L-Pro–L-prolinal, which we did not prepare in the present study, may clarify which is the main reason, because this compound is unable to cyclize. Yoshimoto *et al.*[10] observed that Z–L-Pro–L-prolinal inhibited the *Flavobacterium* enzyme 200 times more strongly than Z–L-Val–L-prolinal, which we prepared. Though they did not determine the time course of inhibition, their observation may be a result of inability of Z–L-Pro–L-prolinal to cyclize. It is interesting that the IC_{50} values of the prolinal inhibitors for the ascidian and the *Flavobacterium* enzymes were not greatly different. The nature of the active site, especially the S_1 and S_2 subsites, of these two enzymes seems to be quite similar in spite of the difference between their molecular properties.

Wirk and Orlowski,[5] and Yoshimoto *et al.*[10] observed that the inhibition of prolyl endopeptidase by prolinal inhibitors was noncompetitive. This type of inhibition may also be experimentally observed if the enzyme-inhibitor complex binds so strongly that equilibrium among the enzyme, the inhibitor and the substrate is not attained within the period of the kinetic experiment even if the actual type of inhibition is competitive. We feel that the inhibition by prolinal peptides is likely to be competitive because these peptides seem to bind to the subsite-binding region of the enzyme, as described above.

In the present experiment, Z–L-Val–L-prolinal and Z–L-Ile–L-prolinal were the strongest inhibitors. At 30 nM, these compounds could inhibit more than half of the activity of the *Flavobacterium* and the ascidian enzymes within 1 h. On the other hand, another type of specific inhibitors of prolyl endopeptidase, chloromethyl ketone derivatives of L-proline,

seems to be less effective. As reported by Yoshimoto *et al.*,[9] these compounds act as irreversible inhibitors, and the second-order rate constant of, for example, Z–Gly–L-Pro chloromethyl ketone for the inhibition of *Flavobacterium* prolyl endopeptidase has been estimated to be $21.0\,M^{-1}\cdot s^{-1}$. This means that at the above concentration (30 nM) of Z–Gly–L-Pro chloromethyl ketone, as long a time as 300 h may be required to inhibit half of the *Flavobacterium* enzyme. The strong inhibition by the prolinal derivatives as compared with chloromethyl ketone derivatives seems to be especially valuable in experiments where organic solvents should be avoided, because the latter inhibitors are difficult to dissolve in aqueous solution directly and must be dissolved in organic solvents first in most cases. Recently, we described the purification of prolyl endopeptidase from the ascidian sperm and its possible involvement in fertilization.[3] More recently, we also observed the presence of this enzyme in the ascidian egg (unpublished). Using prolinal derivatives, we are now examining in more detail the physiological role of the enzyme in fertilization and the subsequent cleavage processes, which do not proceed normally in the presence of organic solvents.

Acknowledgment We thank Dr. Eisuke Sato of the Faculty of Pharmaceutical Sciences, Hokkaido University for valuable advice on the analysis of NMR spectra. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 59780159) from the Ministry of Education, Science and Culture of Japan to M. N.

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