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Amino Acid Sequence of a Carboxypeptidase Inhibitor from Tomato Fruit†

G. Michael Hass* and Mark A. Hermodson

ABSTRACT: The amino acid sequence of a 37 residue carboxypeptidase inhibitor from tomato fruit has been determined. The amino terminus was shown to be 2-oxopyrrolidine-5-carboxylic acid by digestion of reduced and S-carboxymethylated inhibitor with pyroglutamate aminopeptidase. The remainder of the sequence was assigned by analysis of peptides which had been generated by specific cleavage at the Asp_4–Pro_5 bond under acid conditions and by treatment with trypsin. The amino acid sequence of this inhibitor is identical with that of an analogous inhibitor from potatoes in 26 positions, and two of the replacements are highly conservative. The identification of the nonconservative replacements has been used to better define regions of the inhibitor which are not believed to contribute significantly to the free energy of association of the enzyme–inhibitor complex.

Although proteinaceous inhibitors of the serine proteinases are widely distributed in nature, polypeptides which specifically inhibit the pancreatic carboxypeptidases have only been found in roundworms (Homandberg & Peanasky, 1976), potatoes (Ryan et al., 1974), and, quite recently, in tomato fruit (Hass & Ryan, 1980). Since the tomato and potato plants are close phylogenetic relatives, it is not unexpected that the carboxypeptidase inhibitors from these sources exhibit similar physical and chemical properties. That the free energies of association of these two inhibitors with their target enzymes are virtually indistinguishable is, however, somewhat surprising (Hass & Ryan, 1980).

This report presents the amino acid sequence of the carboxypeptidase inhibitor from tomatoes to provide a means of comparison with the sequence of the inhibitor from potatoes (Hass et al., 1975). These data should be of particular utility both in assessing the extent of homology between these proteins and in identifying unusually important regions of these molecules.

Experimental Section

Materials. The carboxypeptidase inhibitor was prepared from ripened tomato fruit as described by Hass & Ryan

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(1980). The *S*-Cm[1] and *S*-Pe derivatives of the inhibitor were prepared by reduction with 0.1 M 2-mercaptoethanol in 6 M guanidine-HCl and 0.1 M Tris-HCl (pH 8.0) followed by treatment with a 1.5-fold molar excess of bromo[1-^{14}C]acetate (Crestfield et al., 1963) or vacuum-distilled 4-vinylpyridine (Cavins & Friedman, 1970), respectively.

Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone and thermolysin were purchased from Sigma Chemical Co., pyroglutamate aminopeptidase was from Boehringer-Mannheim, and Sephadex G-25 SF was a product of Pharmacia Fine Chemicals. All chemicals were reagent grade or better and were used without further purification.

Analytical Procedures. Amino acid analyses were performed by the method of Spackman et al. (1958) on peptides which had been hydrolyzed for 18–24 h in 6 N HCl at 110 °C (Moore & Stein, 1963).

High-voltage paper electrophoresis was performed on peptides at either pH 3.6 or pH 6.5 at 2 kV for 1 h (Bennett, 1963). Peptides were routinely detected by fluorescence after treatment of dried chromatograms with fluorescamine (Mendez & Lai, 1975). In certain instances, the Cl_2–starch iodide reagent described by Pan & Dutcher (1956) was used as well. Tryptophan was detected in peptides by staining electrophoretograms with *p*-(dimethylamino)benzaldehyde (Smith, 1953).

Automatic Edman Degradation. The amino acid sequences were determined with a Beckman 890C sequencer according to the procedures of Hermodson et al. (1972) with the exception that Polybrene (3 mg) was added to the sequencer cup with the peptides (100–500 nmol). The program used was identical with that used by Hermodson et al. (1977) except that a 10-min extraction with ethyl acetate was performed immediately following the benzene extraction step. Pth-amino acids were identified by high-pressure liquid chromatography, employing a slight modification of the procedures of Zimmerman et al. (1977).

Digestion with Pyroglutamate Aminopeptidase. The *S*-Cm derivative of the inhibitor (0.4 μmol) was dissolved in 1 mL of 0.1 M ammonium bicarbonate and 0.05 M 2-mercaptoethanol (pH 8.0). Pyroglutamate aminopeptidase (50 μL of a solution containing 0.025 unit/mL) was added, and the reaction vessel was flushed with N_2. After 9 h of incubation at 4 °C, the sample was incubated for an additional 14 h at 25 °C (Podell & Abraham, 1978). An aliquot (50 μL) of the digest was electrophoresed at pH 4.1 (Doolittle, 1970) and stained with the Cl_2–starch iodide reagent (Pan & Dutcher, 1956). 2-Oxopyrrolidine-5-carboxylic acid was used as a control. The remainder of the digest was subjected to automatic Edman degradation. Controls containing either inhibitor alone or enzyme alone were also subjected to electrophoresis and Edman degradation.

Cleavage of the Asp–Pro Bond and Characterization of the Resulting Peptides. The *S*-Cm and *S*-Pe derivatives of the inhibitor were incubated for 4 days at 40 °C in 10% acetic acid adjusted to pH 2.5 with pyridine (Fraser et al., 1972). The resulting peptide mixtures were chromatographed on a column (1.5 × 80 cm) of Sephadex G-25 SF by using 1% acetic acid as eluent. Fractions of approximately 1.3 mL were collected, and peptide elution was monitored by the absorbance at 280 nm.

Two pools containing peptide were taken, lyophilized, and treated as follows. Peptide AP-1 (residues 5–37) was subjected to automatic Edman degradation. Peptide AP-2 (residues 1–4) (160 nmol) was dissolved in 0.16 mL of 1 mM $CaCl_2$ and 50 mM Tris-HCl (pH 8.0), and 20 μL of a 1 mg/mL solution of thermolysin was added to initiate digestion. After a 20-h reaction at 37 °C, the sample was applied in a zone 3 cm wide to Whatman 3MM chromatography paper, and electrophoresis was performed at pH 3.6 and 2 kV for 1 h. The entire chromatogram was stained with fluorescamine, and then a guide strip was stained with the Cl_2–starch iodide reagent (see above). Peptide-containing zones were eluted with 10% acetic acid, and the eluted peptides were subjected to acid hydrolysis and amino acid analysis.

Digestion with Trypsin and Characterization of the Resulting Peptides. *S*-Cm inhibitor (0.75 μmol) was dissolved in 2 mL of 0.2 M ammonium bicarbonate, and 0.125 mL of an 0.8 mg/mL solution of trypsin was added. After 6 h at 40 °C, the digest was lyophilized. The peptide mixture was dissolved in 3 mL of 1% acetic acid and chromatographed on a column (1.5 × 80 cm) of Sephadex G-25 SF. The eluent was 1% acetic acid, and fractions of 1.3 mL were collected. Two pools were taken based on the absorbance at 280 nm. Peptide TP-1 (residues 1–28) was analyzed for amino acid content after acid hydrolysis and for tryptophan by reaction with *p*-aminobenzaldehyde (Smith, 1953) and by absorbance (Edelhoch, 1967). Peptide TP-2 (residues 29–37) was analyzed for amino acid content and was also subjected to automatic Edman degradation.

Results

Identification of the N Terminus. Automatic Edman degradation of the *S*-Cm derivative of the inhibitor revealed no free-amino terminus. The blocking group was identified as 2-oxopyrrolidine-5-carboxylic acid by digestion of the *S*-Cm inhibitor with pyroglutamate aminopeptidase. A fluorescamine negative, Cl_2–starch iodide positive zone was detected which migrated 17.7 cm when electrophoresis was performed at pH 4.1 for 1 h at 2 kV (Doolittle, 1970). Commercial L-2-oxopyrrolidine-5-carboxylic acid migrated 17.5 cm under these conditions. A similar zone was not observed in either enzyme or inhibitor blanks. Unfortunately, the preparation of pyrogultamate aminopeptidase possessed a significant endoproteolytic activity which precluded sequence analysis of unblocked inhibitor.

Cleavage of the Asp–Pro Bond. Cleavage of the Asp_4–Pro_5 bond of the *S*-Cm and *S*-Pe derivatives was effected by incubation at pH 2.5 (Fraser et al., 1972). When only the large fragment (residues 5–37) was required, 6 M guanidine-HCl was included during cleavage. Under these conditions, cleavage was approximately 80% complete as judged by amino acid analysis (see below); however, separating the small peptide (residues 1–4) from the salt proved to be difficult. Thus, for characterization of both peptides, guanidine-HCl was omitted from the reaction even though the cleavage was somewhat less effective (ca. 70%) under these conditions.

Peptide AP-1 (residues 5–37) containing some uncleaved inhibitor was cleanly separated from peptide AP-2 (residues 1–4) by chromatography on Sephadex G-25 (Figure 1). The presence of uncleaved inhibitor in fractions 51–61 was indicated by the presence of some tyrosine and slightly greater values for aspartic acid and glutamic acid than would be expected of pure AP-1 (Table I). Fractions 73–85 did not contain appreciable amounts of peptide, and fractions 87–98 contained peptide AP-2 in reasonable yield.

Amino Acid Sequence of AP-2 (Residues 1–4). Peptide AP-2 had the amino acid composition Asx, Glx_2, and Tyr and was fluorescamine negative. On the basis of the specificity

[1] Abbreviations used: *S*-Cm, *S*-carboxymethyl; *S*-Pe, *S*-pyridylethyl; Pth, phenylthiohydantoin.

Table I: Amino Acid Compositions of Peptides Derived from the Carboxypeptidase Inhibitor from Tomatoes

amino acid	inhibitor composition[a]	sequence	AP-1	AP-2	TP-1	TP-2
Asp	3	3	2.7 (2)	1.0 (1)	2.8 (3)	
Thr	3	3	2.6 (3)		1.8 (2)	1.0 (1)
Ser	2	2	1.8 (2)		1.7 (2)	
Glu	4	4	3.0 (3)	2.0 (2)	3.4 (3-4)	
Pro	3	3	3.0 (3)		1.8 (2)	1.1 (1)
Gly	4	4	4.3 (4)		2.0 (2)	2.1 (2)
Ala	2	2	1.8 (2)		1.0 (1)	1.1 (1)
Val	2	2	2.0 (2)		1.0 (1)	1.1 (1)
Tyr	2	2	0.81 (1)	0.8 (1)	0.9 (1)	0.9 (1)
Phe	2	2	2.0 (2)		1.0 (1)	1.0 (1)
His	1	1	1.0 (1)		0.9 (1)	
Lys	1	1	1.1 (1)		1.1 (1)	
Arg	1	1	1.0 (1)		1.0 (1)	
Cm-Cys	6	6	5.0 (6)		3.8 (5)	0.9 (1)
Trp	2[b]	1	+[c] (1)	-[c]	0.9[d] (1)	0.15[d] (0)
total	38	37	33	4	28	9
yield (%)			95	65	81	80

[a] From Hass & Ryan (1980). [b] Numbers in parentheses are those expected from the sequence (Figure 2). [c] Determined by reaction with *p*-aminobenzaldehyde (Smith, 1953). [d] Determined spectrophotometrically (Edelhoch, 1967).

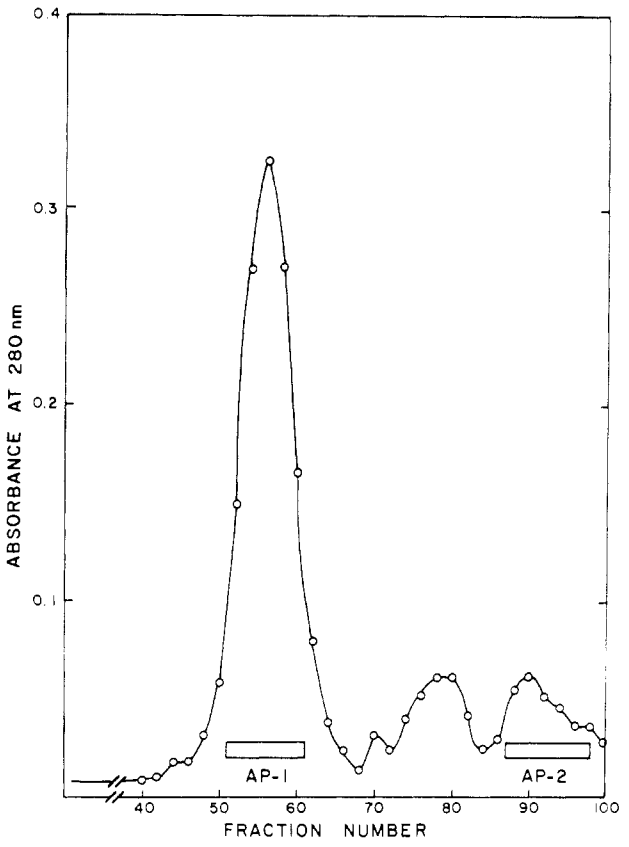

FIGURE 1: Separation of the peptides generated by cleavage of the *S*-Cm derivative of the inhibitor at the aspartyl–proline bond. Gel filtration was performed on a 1.5 × 80 cm column of Sephadex G-25 in 1% acetic acid. Fractions of 1.3 mL were collected, and peptide elution was monitored by the absorbance at 280 nm.

Table II: Edman Degradation of AP-1 and TP-2[a]

cycle no.	AP-1	TP-2
1	Pro (211)	Phe (160)
2	Val (222)	Ala (160)
3	Cys (119)	Gly (344)
4	His (90)	Thr (76)
5	Lys (293)	Cys (153)
6	Pro (147)	Gly (355)
7	Cys (104)	Pro (297)
8	Ser (58)	Tyr (250)
9	Thr (35)	Val (238)
10	Gln (70)	
11	Asp (89)	
12	Asp (118)	
13	Cys (35)	
14	Ser (34)	
15	Gly (111)	
16	Gly (147)	
17	Thr (18)	
18	Phe (58)	
19	Cys (25)	
20	Gln (23)	
21	Ala (62)	
22	Cys (22)	
23	Trp (11)	
24	Arg (ND)	
25	Phe (32)	
26	Ala (43)	

[a] AP-1 (∿300 nmol) and TP-2 (∿400 nmol) were degraded in the presence of Polybrene as the *S*-pyridylethyl and *S*-carboxymethyl derivatives, respectively. Approximate yields in nanomoles of each residue are shown in parentheses. No corrections were made for the substantial overlaps observed in the AP-1 degradations. Arg-24 in AP-1 was detected by its reaction with phenanthrenequinone and was not quantitated.

of the method of generating this peptide (Fraser et al., 1972) and the presence of 2-oxopyrrolidine-5-carboxylic acid (<Glu) at the amino terminus of the inhibitor (see above), the partial amino acid sequence of AP-2 was deduced to be <Glu-(Glx,Tyr)Asp. AP-2 exhibited a mobility of 0.77 relative to aspartic acid when electrophoresis was performed at pH 6.5. Thus, a charge of −2 at this pH was indicated for the peptide (Offord, 1977), and the Glx residue was determined to be glutamine.

Exhaustive digestion of AP-2 with thermolysin at pH 8.0 produced two distinct zones upon electrophoresis at pH 3.6. One of these migrated 3 cm toward the anode, was fluorescamine negative and Cl_2–starch iodide positive, and exhibited an amino acid composition identical with that of AP-2. The second zone was fluorescamine positive, migrated approximately 2 cm toward the cathode, and contained equimolar amounts of aspartic acid and tyrosine. From the data, the amino acid sequence of AP-2 was assigned as <Glu-Gln-Tyr-Asp. The failure to detect the dipeptide <Glu-Gln after electrophoresis of thermolytic digest of AP-2 was not surprising, since hydrolysis of the Gln–Tyr bond was only about 10–25% complete and since the Cl_2–starch iodide reagent, which would have detected <Glu-Gln, was much less sensitive than fluorescamine.

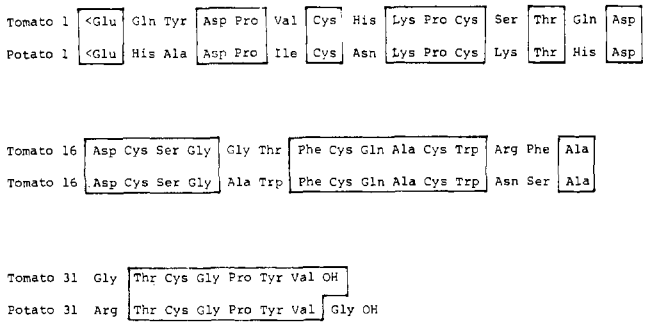

FIGURE 2: Comparison of the amino acid sequences of the carboxypeptidase inhibitor from tomato and potato. The residues enclosed in brackets are identical in both sequences. Data on the inhibitor from potatoes are from Hass et al. (1975).

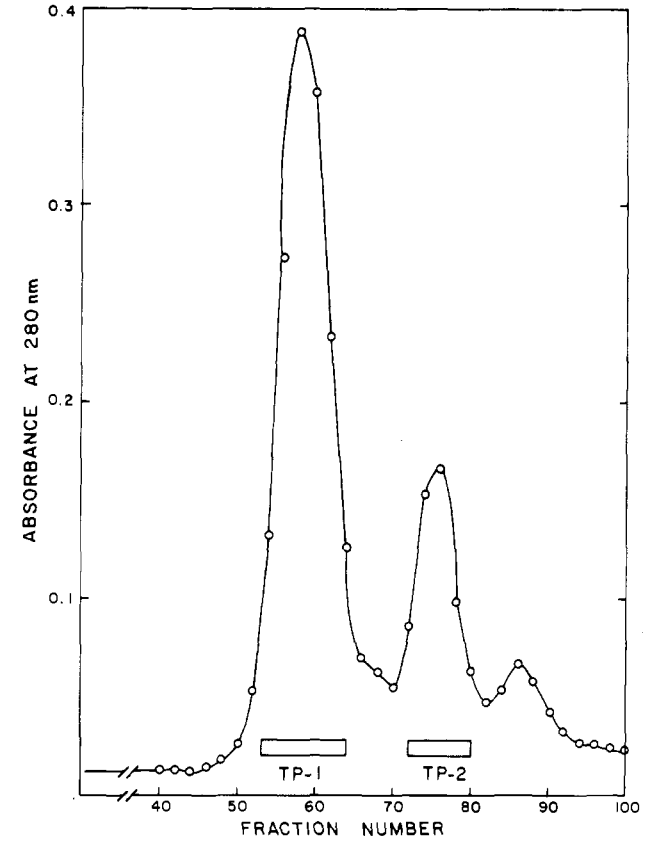

FIGURE 3: Separation of the tryptic peptides derived from *S*-Cm carboxypeptidase inhibitor. Gel filtration was performed on a 1.5 × 80 cm column of Sephadex G-25 in 1% acetic acid. Fractions of 1.3 mL were collected, and peptide elution was monitored by the absorbance at 280 nm.

Edman Degradation of AP-1. Degradation of AP-1 yielded a sequence for the first 26 residues of the peptide (Table II; residues 5–30 of the inhibitor, Figure 2). This sequence ended with -Arg-Phe-Ala-, the only site where trypsin could cleave the inhibitor.

Digestion with Trypsin and Sequence Analysis of TP-2 (Residues 29–37). Digestion of the *S*-Cm derivative of the inhibitor with trypsin produced the two tryptic peptides TP-1 and TP-2 which were readily separated by gel filtration on Sephadex G-25 (Figure 3). Peptide TP-1 exhibited an amino acid composition identical with that expected from sequence analysis of peptides AP-1 and AP-2 except for a slightly low value for Cm-cysteine (Table I). This discrepancy was most likely due to the high susceptibility of Cm-Cys to oxidation.

With the exception of the value of tryptophan, which will be discussed later, the composition of TP-2 accurately reflected the difference between that of the inhibitor and that of residues 8–27. Automatic Edman degradation of this peptide provided the sequence of the carboxyl-terminal nine residues of the inhibitor, beginning with the Phe_{29}-Ala_{30}- sequence at the end of the AP-1 degradation (Table II).

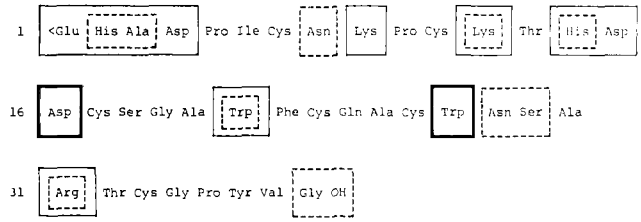

FIGURE 4: Amino acid residues of the carboxypeptidase inhibitor which are not believed to be in contact with enzyme in the complex. Evidence based upon a comparison with the sequence of the inhibitor from tomatoes (- - -) and upon chemical modification (—) from Hass et al. (1976).

Discussion

The carboxypeptidase inhibitor from tomatoes was shown to possess amino-terminal 2-oxopyrrolidine-5-carboxylic acid by digestion with pyroglutamate aminopeptidase. The amino acid sequence was determined from the two tryptic peptides and the two Asp-Pro cleavage fragments. Analysis of fragment AP-2 provided the sequence of residues 1–4, and automatic Edman degradation of AP-1 (residues 5–37) yielded the sequence of residues 5–30. Peptide TP-1 (residues 1–28) formally provided the overlap of AP-1 and AP-2, whereas the remainder of the sequence (residues 29–37) was determined by automatic Edman degradation of TP-2. Except for the presence of one rather than two residues of tryptophan per molecule, the amino acid sequence (Figure 2) was consistent with the amino acid composition published earlier (Hass & Ryan, 1980). Recent tryptophan determinations on intact molecules as well as on the tryptic peptides (Table I) have supported the presence of one residue of tryptophan per molecule.

The carboxypeptidase inhibitor from tomatoes and from potatoes are clearly homologous proteins (Figure 2). Of the 37 residues of these inhibitors, 26 are identical, and 7 of the 11 replacements could have arisen by single base changes. Moreover, all six residues of half-cystine, which occur as three disulfide bonds (Hass & Ryan, 1980), are aligned without the need to introduce gaps in either sequence.

Because of the extensive homology between the inhibitors from the two plants, their structural differences rather than similarities provide the greatest insight into regions of the inhibitor which are critical for binding to target enzymes. The virtually indistinguishable K_i values of the two inhibitors for their target enzymes (carboxypeptidase A, carboxypeptidase B, and *Streptomyces griseus* carboxypeptidase) (Hass & Ryan, 1980) suggest that they bind through the same interactions. If this is so, nonconservative amino acid replacements cannot occur at sites on the inhibitor in contact with the enzyme.

Figure 4 presents the amino acid sequence of the carboxypeptidase inhibitor from potatoes with nonconservative replacements (Figure 2) enclosed in broken lines. Residues of the inhibitor which may be chemically modified or removed without appreciable effect on inhibitory activity are indicated in Figure 4 by solid lines (Hass et al., 1976). On the basis of these data, the region from Cys_{17} to Cys_{26} and the carboxyl-terminal section of the inhibitor should be of particular importance in dictating conformation or in direct interaction with enzymes.

The interaction of the carboxyl-terminal region of the inhibitor from potatoes with carboxypeptidases has been clearly indicated by X-ray crystallographic analysis of the enzyme–inhibitor complex at 2.5-Å resolution (Rees & Lipscomb, 1980). That report demonstrates that the inhibitor binds like an extended substrate. The most unusual features of the inhibitor–enzyme interaction are that the Val_{37}–Gly_{38} peptide bond is cleaved in the complex and that Gly_{38} appears to be trapped in the binding pocket of the enzyme (Rees & Lipscomb, 1980). The comparison of the amino acid sequences of the inhibitors from tomatoes and potatoes (Figure 2) is of particular interest in this respect. The amino acid residue which would correspond to Gly_{38} of the inhibitor from potatoes is not found in the inhibitor from tomatoes. This observation has led to recent studies which demonstrate that carboxypeptidase A rapidly removes Gly_{38} from the potato inhibitor and that this residue is not required for enzyme–inhibitor interaction.[2]

The X-ray diffraction analysis of the enzyme–inhibitor complex (Rees & Lipscomb, 1980) indicates that residues 36–38 of the inhibitor interact extensively with the enzyme active site, an observation consistent with the complete homology in this region between the inhibitors from potatoes and from tomatoes (Figure 2). Additional enzyme–inhibitor contacts involve the rings of Trp_{27}, Phe_{21}, and, to a lesser extent, His_{14}. Of these residues, the only replacement observed in $His_{14} \rightarrow Gln_{14}$ (Figure 2). The final enzyme–inhibitor contact involves the backbone of Trp_{27}-Ser_{29} of the inhibitor and Ile_{247}, Tyr_{248}, and Lys_{239} of the enzyme. Although Trp_{27} is conserved, nonconservative replacements are found at residues 28 and 29. Apparently, these replacements do not significantly affect the conformation of the polypeptide backbone in this region.

In addition to residues in contact with enzyme, other highly conserved regions (e.g., residues 9–11, 15–19, and 23–26) are observed. Ascribing any unusual importance to these segments should await the purification and amino acid sequence analysis of other homologous carboxypeptidase inhibitors.

[2] G. M. Hass and C. A. Ryan, manuscript in preparation.

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