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CARBOXYPEPTIDASE INHIBITOR FROM RIPENED TOMATOES; PURIFICATION AND PROPERTIES

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Key Word Index—*Lycopersicum esculentum*; Solanaceae; tomato; fruit; carboxypeptidase inhibitor; carboxypeptidase; exopeptidase inhibitor.

Abstract—A polypeptide which inhibits pancreatic carboxypeptidases has been purified from extracts of tomato fruit (*Lycopersicum esculentum*). The purification procedure involves chromatography on immobilized carboxypeptidase A and gel filtration, and yields homogeneous inhibitor as judged by disc gel electrophoresis and amino acid analysis. The inhibitor from tomatoes contains 38 amino acid residues and is related to one from potato tubers as determined by a comparison of their amino acid compositions and by cross-reactivity against anti-potato inhibitor antibodies. Inhibition studies indicate that bovine carboxypeptidase A, porcine carboxypeptidase B, and a carboxypeptidase from *Streptomyces griseus* bind to the inhibitors from tomato and potato with approximately the same free energy of association.

INTRODUCTION

Inhibitors of the pancreatic CPDases (carboxypeptidases) have been found in yeast [1], roundworms [2], and the fungus *Aspergillus ochraceus* [3]. Among higher plants there seem to be at least two distinct classes of CPDase inhibitor, the inhibitor from potato tubers representing one of these classes [4]. This peptide binds to target enzymes through complementary interactions in a manner similar to the association between serine endopeptidases and most of their naturally occurring inhibitors. In contrast, the inhibitor from legumes affects metalloenzymes by chelating metal ions [5]. A given CPDase inhibitor would be assigned to the latter group if inhibitory activity were abolished by preincubation with excess metal ion. In this report we describe the purification of tomato fruit CPDase inhibitor that is not affected by metal ion, and we compare its physical and kinetic properties with those of the CPDase inhibitor from potatoes.

RESULTS AND DISCUSSION

Purification

The purification of the CPDase inhibitor from 4.6 kg of tomatoes is summarized in Table 1. Amounts of inhibitor found in extracts from several lots of tomatoes varied between *ca* 0.1 and 0.7 μmol/kg tomatoes (fr. wt). Thus, it was of advantage to estimate the amount of inhibitor in extracts from a representative sample of each large batch of material prior to processing. Although much activity was lost during the 0–90% ammonium sulfate fractionation step, the loss was tolerable since a large amount of unwanted material was eliminated.

The most effective single step in the purification was affinity chromatography on CPDase A–Sepharose 4B (Fig. 1). No trace of inhibitory activity was detected in the breakthrough (fractions 3–25) and the inhibitor was released by the high pH buffer. The efficiency of this procedure has also allowed its use recently to quantitatively remove the carboxypeptidase inhibitor from extracts of several tissues of the potato plant [6]. However, this procedure does not yield pure inhibitor since several non-inhibitor proteins also adsorb to the affinity column (Fig. 2).

The final step in the purification of the inhibitor was chromatography on a column of BioGel P-10 (Fig. 2). The late elution of the inhibitor from this resin (fractions 64–68), which excludes proteins over MW 10000, suggested that it had a relatively low MW. The final yield of inhibitor from 1 kg tomatoes was 1 mg.

Inhibitor which has been prepared by this procedure appeared to be homogeneous as judged by polyacrylamide gel electrophoresis at pH 3.2 [7] and by disc gel electrophoresis at pH 8.3 [8]. In addition, a single

Table 1. Purification of carboxypeptidase inhibitor from 4.6 kg tomatoes

Step	Vol. (ml)	A_{280}*	Inhibitor (μmol)†	Yield (%)
Extract	3320	14.3	8.0	100
Ammonium sulfate	625	8.7	3.3	41
CPDase–Sepharose	12	5.8	1.5	19
BioGel P-10	23	0.48	1.3	15

* 1 cm light path.

† Estimated by titration against carboxypeptidase A.

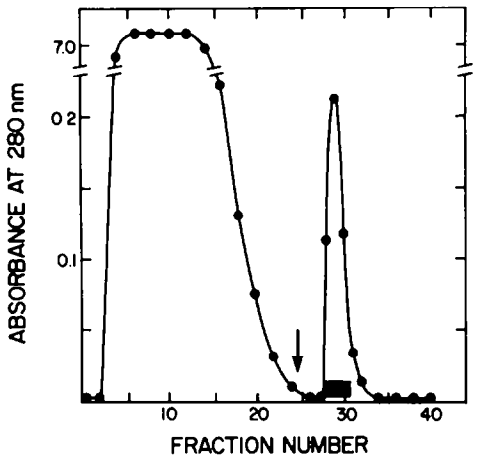

Fig. 1. Chromatography of partially purified carboxypeptidase inhibitor from tomatoes on a column of CPDase A-Sepharose. A 30 ml aliquot of the ammonium sulfate fraction in 1 M NaCl, 10 mM Mes (pH 6) was applied at 4° to a 1.5 × 6.5 cm solumn of resin. The column was washed with buffer and the inhibitor was eluted with 10 mM L-phenylalanine, 0.2 M Na_2CO_3 (pH 11.4) beginning at fraction 24. Fractions of 4.6 ml were collected and monitored for A_{280} and inhibitory activity. Fractions indicated by the bar were pooled.

zone of inhibitor activity (pI = 3.8) was detected in isoelectric focusing. These data both attested to the purity of the inhibitor and indicated that there probably were not multiple isoinhibitor species present. In contrast, at least 3 major CPDase isoinhibitors have been purified from potato tubers [9].

Structural characterization

Gel filtration of the inhibitor from tomatoes on BioGel P-10 yielded a V_e/V_o value of *ca* 1.9. The V_e/V_o ratio of the inhibitor from potatoes determined

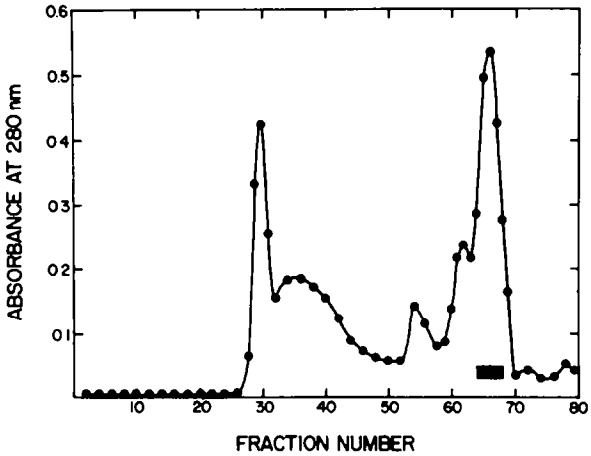

Fig. 2. Chromatography of the carboxypeptidase inhibitor from tomatoes on a 1.5 × 80 cm column of BioGel P-10. Lyophilized material from the affinity chromatography step derived from 2.3 kg tomatoes (fr. wt) was dissolved in 3 ml of 20 mM ammonium bicarbonate and chromatographed at 25°. Fractions of 1.1 ml were collected and monitored by A_{280} and for inhibitory activity. Fractions indicated by the bar were pooled.

under identical conditions was 2.0–2.1, suggesting that these two peptides were very similar in size.

The amino acid composition presented in Table 2 indicated the inhibitor from tomatoes contained 38 amino acid residues and, thus, had a minimum MW of *ca* 4200. The virtual absence of methionine, leucine, and isoleucine provided further support for the purity of our preparations. Titration of the tomato inhibitor with Ellman's reagent, 5,5-dithiobis(2-nitrobenzoate), in 6 M guanidine HCl revealed less than 0.05 residue of free sulfhydryl group per mol. Thus, the 6 residues of half-cystine per mol (Table 2) were present as 3 disulfide bonds.

Ouchterlony double diffusion assays were employed to determine if the CPDase inhibitor from tomato fruit cross-reacted immunologically with rabbit antibodies prepared against potato inhibitor. The tomato inhibitor strongly cross-reacted with anti-potato inhibitor antiserum giving one clean precipitin line. This line intersected the precipitin line from potato inhibitor with a definite spur present and indicated that, even though a considerable number of antigenic determinants on tomato inhibitor were identical to those of potato inhibitor, some potato inhibitor determinants were missing from the tomato inhibitor. These results were consistent with the composition of the two proteins (Table 2) which indicated that the inhibitors possessed an overall similarity in amino acid composition with a few differences present among the individual amino acid residues.

Table 2. Amino acid compositions of carboxypeptidase inhibitors

	Tomato		Potato†		
Amino acid	Average*	Integral	I	II	III
Aspartic acid	3.0	3	5	5	5
Threonine	2.7‖	3	2	2	2
Serine	2.1‖	2	1	2	2
Glutamic acid	3.7	4	2–3	2–3	1
Proline	2.9	3	3	3	3
Glycine	4.0	4	4	3	3
Alanine	2.1	2	5	4	4
Valine	1.9‖	2	1	1	1
Isoleucine	< 0.1	0	1	1	1
Tyrosine	1.9	2	1	1	1
Phenylalanine	2.0	2	1	1	1
Lysine	0.8	1	2	2	2
Histidine	0.8	1	2	2	2
Arginine	1.0	1	0	1	1
Trytophan	1.9‡	2	2	2	2
Half-cystine	5.5§	6	6	6	6
Total		38	38–39	38–39	37

* Values given were obtained on 24 hr acid hydrolysates performed in triplicate unless indicated otherwise.

† Data for the three carboxypeptidase isoinhibitors were from ref. [9].

‡ Estimated spectrophotometrically [16].

§ Determined as cysteic acid after treatment with performic acid [17]. An 85% conversion to cysteic acid was assumed [17].

‖ Data for these amino acids are expected to be slightly low when based upon one time of hydrolysis.

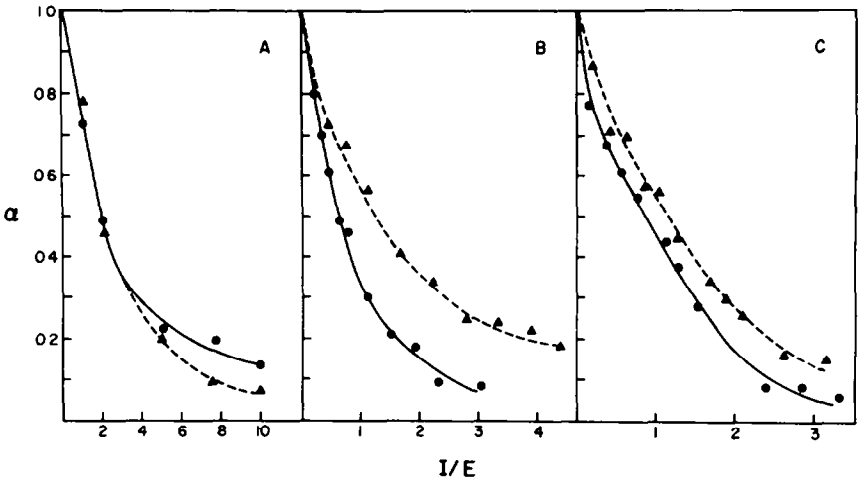

Fig. 3. Titration of carboxypeptidase activity with inhibitors from tomato and potato. Enzyme and varying amounts of tomato (▲- - -▲) or potato (●——●) carboxypeptidase inhibitor were incubated, and the fraction of free enzyme, α, was measured as a function of enzyme/inhibitor ratio, I/E. A: Bovine carboxypeptidase A (4.0×10^{-9} M) assayed using hippuryl-DL-phenyllactate (1 mM), B: porcine carboxypeptidase B (7.9×10^{-8} M) assayed with hippuryl-L-lysine (1 mM), and C: *S. griseus* carboxypeptidase (5.9×10^{-8} M) assayed with hippuryl-DL-phenyllactate (1 mM).

Inhibition studies

Two distinct classes of naturally occurring inhibitors of the CPDases have been identified. Inhibitors from potato [4], round worm [2], and yeast [1] formed complexes with their enzymes via complementary surface interactions and masked or blocked the enzyme active site. Members of the second class of inhibitor including that from red kidney beans were strong chelating agents and inhibited not only pancreatic carboxypeptidases, but also other enzymes which require Zn^{2+} as a cofactor [5]. The similarity in amino acid compositions between the inhibitors from potato and tomato and their immunological cross-reactivity suggested that the inhibitor from tomatoes belonged to the first class. Two studies were performed which supported this assignment. First, the inhibitor from tomatoes had no effect on alkaline phosphatase, an enzyme requiring Zn^{2+} and shown to be inhibited by the inhibitor from red kidney beans [5]. In addition, preincubation of the tomato inhibitor with 50 μM $ZnCl_2$ did not diminish its capacity to inhibit CPDase A. Under these conditions, the red kidney bean inhibitor was completely inactivated [5].

The strength of binding of the inhibitors from tomatoes and potatoes to target enzymes was compared using the method of Green and Work [10]. The association between CPDase A and these inhibitors was quite strong. Plots of α, the fraction of free enzyme, against the inhibitor to enzyme ratio, I/E, were linear when hippuryl-L-phenylalanine was used as substrate, from α values of 1 to 0.2 or less. When inhibitor concentrations were determined by amino acid analysis, extrapolation of the linear region to an α of 0 yielded an I/E value of *ca* 1.1. Thus, the inhibitor from tomatoes binds enzymes with a 1:1 stoichiometry.

Although titration of CPDase A using hippuryl-L-phenylalanine as substrate provided a convenient means of estimating amounts of inhibitor, the small amount of free enzyme present at equivalence precluded an accurate comparison of binding energies.

Table 3. Apparent K_i values for the carboxypeptidase inhibitors

Enzyme	K_i, app. (M) Tomato	Potato
Bovine CPDase A	$4.8–5.0\times10^{-9}$	$5.4–6.0\times10^{-9}$
Porcine CPDase B	$5.7–6.0\times10^{-8}$	$0.7–1.1\times10^{-8}$
S. griseus CPDase	$2.8–4.0\times10^{-8}$	$1.4–1.9\times10^{-8}$

Suitable titrations of this enzyme by the inhibitors were achieved using lower concentrations and a substrate, hippuryl-DL-phenyllactate, which had a lower K_m than hippuryl-L-phenylalanine (Fig. 3A). Clearly, there was little difference in the ability of the tomato and potato inhibitors to bind this enzyme.

Titration curves for porcine CPDase B (Fig. 3B) and *S. griseus* CPDase (Fig. 3C) demonstrated that these enzymes bind the inhibitor from tomatoes slightly less well than from potatoes. However, the apparent K_i values (Table 3), that is, K_i values which are not corrected for the binding of substrate, revealed that none of the enzymes was very specific in binding one inhibitor in preference to the other.

These inhibition studies suggested two major conclusions. Firstly, inhibitors from tomato and from potato bound to carboxypeptides A and B and to the *S. griseus* carboxypeptidase through the same types of interactions. In addition, the major structural differences between the two inhibitors probably did not involve residues which were in contact with enzyme in the complex. Amino acid sequence determination of the carboxypeptidase inhibitor from tomatoes might, thus, better define the residues involved in binding to target enzymes.

EXPERIMENTAL

Materials. The polypeptide CPDase inhibitor from potato tubers was purified as described previously [4]; porcine CPDase B was purified from trypsin-activated Me_2CO powder extracts by affinity chromatography [11]; *S. griseus*

CPDase of MW *ca* 40000 was purified by chromatography on immobilized CPDase inhibitor from potatoes [11, 12]; and bovine CPDase A was purchased from Worthington Biochemical Co. Alkaline phosphatase, hippuryl-L-arginine, hippuryl-L-phenylalanine, and hippuryl-DL-phenyllactic acid were purchased from Sigma, and hippuryl-L-lysine was a product of Vega Biochemicals. BioGel P-10 (200–400 mesh) was purchased from BioRad and Sepharose 4B was a product of Pharmacia.

Purification of inhibitor. Ripe tomatoes were obtained locally, chilled to 4° and homogenized in a large Waring blender for 1 min at low speed. The homogenate was centrifuged at 4° for 30 min at 10000 *g* and the supernatant decanted and adjusted to 90% satn with solid $(NH_4)_2SO_4$ (622 g/l.). After standing 18 hr at 4°, the suspension was centrifuged for 30 min at 10000 *g*. The ppt. was dissolved in H_2O (*ca* 200 ml/500 g tomatoes original) and adjusted to 1 M NaCl, 10 mM MES (pH 6). The small amount of ppt. which formed was removed by centrifugation. An aliquot of the crude $(NH_4)_2SO_4$ fraction which contained 0.25 μmol of inhibitor by titration against CPDase A (see below) was applied to a 1.5×6.5 cm column of CPDase A–Sepharose 4B [6] at 4°. After exhaustively washing the column with 1 M NaCl, 10 mM MES (pH 6), the inhibitor was eluted with 0.2 M Na_2CO_3 (pH 11.4) containing 10 mM L-phenylalanine. Fractions of 4.6 ml were collected and monitored by A_{280} and inhibitory activity against CPDase A (see below). Active fractions were pooled, dialyzed exhaustively against H_2O and lyophilized. The affinity chromatography fraction derived from 2.3 kg of tomatoes (fr. wt) was dissolved in 3 ml of 50 mM ammonium bicarbonate and chromatographed on a $1.5 \times$ 80 cm column of BioGel P-10 which was eluted with this buffer. Fractions of 1.1 ml were collected and monitored by *A* at 280 nm and for inhibitory activity. Fractions containing the inhibitor were pooled and this material was used in all subsequent studies.

Electrophoresis. Purified carboxypeptidase inhibitor was subjected to PAGE (15% gels containing 6 M urea) at pH 3.2 for 3.5 hr at 3 mA/gel [7]. Electrophoresis was also performed for 2.5 hr at 3 mA/gel in 15% gels using the pH 8.3 system of ref. [8]. Gels were stained for protein with 1% amido black in 7% HOAc saturated with $HgCl_2$ and destained by diffusion using this solvent.

Isoelectric focusing was performed in 7.5% polyacrylamide gels containing 2% (v/v) Bio-Lyte ampholytes (pH 3–10) (BioRad). Focusing was at 4° for 24 hr at a potential gradient of 20 V/cm. The gels were cut into 2.5 mm sections and soaked 18 hr in deaerated H_2O. The pH value and inhibitory activity of each eluate was then monitored.

MW estimation. Elution vols (V_e) for the carboxypeptidase inhibitors from tomatoes and from potatoes were obtained using a column (1.5×80 cm) of BioGel P-10 (200–400 mesh) with 50 mM ammonium bicarbonate as buffer. Potato chymotrypsin inhibitor I (MW 40000) was used to measure the void vol., V_o [13]. Vols were estimated from sample application to the inflection point of the ascending limb of the elution profile.

Amino acid analysis. Acid hydrolysates were prepared by heating the inhibitor in 6 M HCl for 24 hr *in vacuo* [14] at 110°. Amino acid analyses were performed using a Beckman Amino Acid Analyzer with a single column buffer system [15]. Trytophan was estimated spectrophotometrically by the method of ref. [16]. Half-cystine was estimated as cysteic acid on acid hydrolyzates of inhibitor which had been treated with performic acid [17]. Cysteine was determined by reaction of inhibitor (3×10^{-5} M) in 6 M guanidine–HCl with 5,5′-dithiobis (2-nitrobenzoate) as described in ref. [18]. A molar extinction coefficient at 412 nm of 13600 was assumed for the thionitrobenzoate ion.

Immunological assays. Ouchterlony double diffusion assays [19] were carried out in 2% Noble Agar prepared in 0.1 M Na barbital, pH 8.2, 0.9% NaCl and 1×10^{-4}% Thimerosal. The precipitation lines of tomato inhibitor were compared with those of the potato inhibitor (both at 50 μg/ml) when challenged with a 1:1 dilution of rabbit anti-potato CPDase inhibitor serum [20].

Inhibition studies. Association of inhibitors with target enzymes was measured by the method of ref. [10]. In this procedure, enzymatic activity was determined in the presence of varying amounts of inhibitor. For bovine CPDase A and the *S. griseus* CPDase, the hydrolysis of 1 mM hippuryl-DL-phenyllactic acid was monitored spectrophotometrically at 25° using 0.5 M NaCl, 0.05 M Tris–HCl (pH 7.5) as buffer [21]. The concns of stock CPDase A solns were estimated from the $A_{280}^{0.1\%}$ value of 1.88 [22] and by assay with hippuryl-L-phenylalanine as described below. Concns of the *S. griseus* enzyme were estimated based on $A_{280}^{0.1\%} = 1.77$ and a reported turnover number of 9400 min^{-1} for the hydrolysis of 1 mM hippuryl-DL-phenyllactate [12]. In all cases, protein concns based on *A* agreed with those estimated by enzymatic activity to within 5–10%. The inhibition of porcine CPDase B was determined at 25° using 1 mM hippuryl-L-lysine as substrate in 0.5 M NaCl, 0.05 M Tris–HCl (pH 7.5). The protein concn of stock solns was estimated by their activity toward 1 mM hippuryl-L-arginine [23]. The activity of alkaline phosphatase was determined spectrophotometrically at pH 8 and 25° using *p*-nitrophenylphosphate as substrate [24]. The concn of inhibitor solns was routinely estimated by titration against a known amount of CPDase A using 1 mM hippuryl-L-phenylalanine as substrate [25]. Because of the tight binding of enzyme and inhibitor, the relatively high K_m value of carboxypeptidase A for this substrate, and the high enzyme levels ($0.5–1 \times 10^{-7}$ M) used in this assay, enzyme was inhibited with a 1:1 stoichiometry over the range 0–80% inhibition. Inhibitor concns determined by amino acid analysis of appropriate aliquots of stock solns agreed with values obtained by titration to within 10%.

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