

Pectinesterase

The primary structure of the tomato enzyme

Oskar MARKOVIČ² and Hans JÖRNVALL¹

¹ Department of Chemistry I, Karolinska Institutet, Stockholm

² Institute of Chemistry, Slovak Academy of Sciences, Bratislava

(Received February 10/April 22, 1986) — EJB 86 0143

The first primary structure of a pectinesterase has been determined by analysis of the main form of this plant enzyme from tomatoes. The analysis was untraditional in the sense that few data were obtained by frequently used methods. Thus, digestion with trypsin, Glu-specific protease and several other enzymes gave limited cleavages and highly insoluble products. Instead, the determination was to a large extent based on peptides derived from chemical cleavages with CNBr at Met, *N*-chlorosuccinimide at Trp and hydroxylamine at Asn-Gly, plus an enzymatic cleavage at modified cysteine residues after chemical derivatizations.

The structure shows the protein chain to be 305 residues long, with four half-cystine residues and five tryptophan residues. The N-terminus has a free α -amino group (from isoleucine). Two types of residue, tyrosine and histidine, have been functionally implied in the catalytic activity of the enzyme. Tyrosine is common in the protein (at 18 positions). However, only two histidine residues are found, and both are close to tyrosine residues in the primary structure (His-128 separated by one intervening residue from Tyr-126, and His-269 adjacent to Tyr-268), defining segment(s) of possible interest in relation to the active site.

Pectinesterases have been found in all species of higher plants tested and are also produced by a number of plant pathogenic fungi and bacteria. Plant pectinesterases participate in the conversion of protopectin to soluble pectin and pectate, and are involved in plant maturation processes as well as in mechanisms that protect the plant from infection [1]. The enzyme catalyzes the de-esterification of pectin and is highly specific for the D-galacturonan structure of the substrate.

Much attention has been devoted to tomato pectinesterase because of the high content of this enzyme in the ripe fruit. Highly purified products have been prepared and partly characterized [2–5]. However, very little is known about the enzyme structure. The relative molecular mass has been reported to be 22 000–35 500 from estimates based on exclusion chromatography [3–5] and 26 300–27 800 from those based on sedimentation equilibrium [4, 6]. Multiple molecular forms have been described [3–6] and the dominant form has been isolated by chromatography on CM-Sephadex C-50 [6]. The presence of a single N-terminal group, as well as the total composition related to a tryptic peptide map of this form of the enzyme, further suggest that it is pure and composed of one type of protein chain [7].

Functionally, the enzyme can attack the pectin substrate at different positions. About half of the observed hydrolysis catalyzed by tomato pectin esterase affects the region near the reducing end of highly esterified pectin, while the remaining cleavages affect secondary loci near free carboxyl groups [8].

The tomato enzyme de-esterifies pectin with the formation of blocks of free carboxyl groups, whereas with fungal pectinesterase from the fungi *Aspergillus foetidus* and *Aspergillus niger*, de-esterification proceeds randomly [9]. For tomato pectinesterase, the partially esterified penta(D-galactosiduronic acid) was found to be the shortest unit that served as substrate, whereas the *Aspergillus* pectinesterase was able to catalyze attacks on the dimer subunit [10]. Organophosphates, carbamates and Ellman's reagent do not inhibit the tomato pectinesterase, but strong inhibition is observed with iodine [11]. Selective acetylation of tyrosine residues with *N*-acetylimidazole reduces the activity of tomato pectinesterase, and restoration of the enzyme activity has been achieved by de-acetylation with hydroxylamine [12]. Diethylpyrocarbonate inhibits the enzyme. An activity decrease to 40% appears to be associated with the modification of one histidine residue, as determined by absorbance changes at 240 nm [13].

Apart from this scattered information, nothing is known about the enzymatic mechanism or structure-function relationships. In order to provide a sound basis for further assessment of the structure and function of this enzyme, the primary structure has now been determined.

MATERIALS AND METHODS

Protein

Pectinesterase was prepared from ripe tomatoes (*Lycopersicon esculentum*; Immuna variety) and carboxymethylated with ¹⁴C-labelled iodoacetate, as given in the Supplement. This material was utilized for three different types of chemical cleavage and five different types of enzymatic cleavage.

Correspondence to H. Jöرنvall, Kemiska Institutionen I, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden

Enzymes. Pectinesterase, pectin pectyl-hydrolase (EC 3.1.1.11); chymotrypsin (EC 3.4.21.1); trypsin (EC 3.4.21.4); staphylococcal extracellular Glu-specific protease (EC 3.4.21.19); pepsin (EC 3.4.23.1); *Achromobacter lyticus* lysyl endopeptidase (EC 3.4.21.50).

Chemical cleavages

CNBr. The carboxymethylated protein (10 mg/ml in 70% formic acid) was treated with CNBr (15-fold weight excess over protein) for 24 h at room temperature. Reagents were removed by rotary evaporation and the peptide mixture was fractionated by chromatography on Sephadex G-50. Final purification of individual CNBr fragments was achieved by reverse-phase high-performance liquid chromatography on Ultropac TSK ODS-120, 5 μ m (LKB, Sweden) [14].

Tryptophan cleavage. Selective cleavage of tryptophanyl peptide bonds [15, 16] of 100 nmol carboxymethylated protein was achieved by incubation at room temperature for 1 h with 2 mg *N*-chlorosuccinimide (added in two portions) in 30% acetic acid/5 M urea. Excess reagent was removed by addition of 4 mg *N*-acetyl-L-methionine. After dialysis against 30% acetic acid, the sample was subjected to chromatography on Sephadex G-50.

Hydroxylamine cleavage. Cleavage of Asn-Gly peptide bonds [17] was performed with 2 M hydroxylamine, 0.2 M Tris, pH 9.36 (adjusted with 2 M NaOH) at 50°C for 5 h with 50 nmol of the carboxymethylated protein. The reaction was stopped by acidification to pH 4 with 50% trifluoroacetic acid. The sample was then subjected to chromatography on Sephadex G-50.

Enzymatic cleavages

Trypsin, chymotrypsin, staphylococcal Glu-specific protease. Digestions with these enzymes were incomplete and difficult to control, since the carboxymethylated protein is quite insoluble at basic pH. Digestions with trypsin after initial solubilization in 6 M guanidine $\cdot$ HCl, and with two protease additions were therefore carried out as detailed in the Supplement. Digests were separated by high-performance liquid chromatography as also detailed in the Supplement.

Pepsin cleavage. Conditions for this treatment are given in the Supplement.

Cleavages at cysteine residues. For selected cleavage after original cysteine/half-cysteine residues, a chemically modified form of the protein was digested with an endopeptidase ordinarily directed against lysine residues (*Achromobacter lyticus* protease I, obtained as a kind gift from Dr Sumusawa, Osaka University, Japan). To restrict cleavages to original Cys residues, lysine residues were blocked by succinylation before digestion, and Cys residues were converted by aminoethylation after reduction. The native protein (100 nmol) was dissolved in 6 M guanidine $\cdot$ HCl, 0.1 M sodium pyrophosphate, pH 8.4, succinic anhydride (50-fold molar excess over total Lys) was added in portions, pH was kept at pH 8.5–8.9 with 1 M NaOH, and reagents were removed by dialysis against 0.1 M ammonium bicarbonate, as previously reported for maleylation [18]. Cysteine residues were then converted to the lysine analogs by treatment with ethyleneimine to produce the *S*-(2-aminoethyl)-cysteine derivatives. For this, the lyophilized product was dissolved in 6 M guanidine $\cdot$ HCl, 0.4 M Tris/HCl, 2 mM EDTA, pH 8.1, reduced with dithiothreitol (50% molar excess over protein Cys residues) and aminoethylated by addition of ethyleneimine (80% molar excess over total SH) in three portions [19]. Finally, the succinylated, reduced, and aminoethylated protein was dialyzed against 0.1 M ammonium bicarbonate, lyophilized, and digested with the lysine endopeptidase (200 μ g added in two portions) for 8 h at 37°C in 0.1 M ammonium bicarbonate, pH 8.1.

Structural analysis

Total compositions were determined on a Beckman 121M amino acid analyzer after hydrolysis for 24 h at 110°C with 6 M HCl/0.5% phenol. Amino acid sequences were determined by manual degradations with the dimethylaminoazobenzene isothiocyanate method and by liquid-phase sequencer degradations in a Beckman 890D sequencer with a 0.1 M Quadrol peptide program in the presence of pre-cycled Polybrene, as described [14].

Nomenclature

Peptides are given by a letter/figure combination indicating the digest from which they were obtained and the peptide order in the amino acid sequence. T indicates peptide generation by digestion with trypsin, T* tryptic digestion in the presence of 1.5 M guanidine hydrochloride, E cleavage with the Glu-specific protease, P with pepsin, C with chymotrypsin, CB with CNBr, W with *N*-chlorosuccinimide, N with hydroxylamine, and CC cleavage after an original cysteine residue by treatment with the lysyl protease subsequent to succinylation of lysine, and reduction plus aminoethylation of cysteine. All peptide numbers are consecutive along the sequence, including only those peptides identified in each digest; in the case of the different trypsin treatments, T and T* peptides are numbered together.

RESULTS

The primary structure

The primary structure was determined by analysis of peptides derived from essentially eight types of proteolytic treatment and by direct analysis of the intact protein. Three types of chemical cleavage had to be utilized since ordinary enzymatic digestions gave only limited data because of low solubility of the protein, incomplete digestions, and low peptide yields. With the exception of the batch for cleavage at original half-cysteine residues, all protein samples were first carboxymethylated by reduction and subsequent reaction with ^{14} C-labelled iodoacetate.

Peptides analyzed, and the final structure deduced, are summarized in Fig. 1. The total composition of the whole protein is given in Table 1, showing good agreement between the values derived from acid hydrolysis and those from the sum of the sequence determined. Data for the peptides analyzed are given in the Supplement (compositions in Supplement Table 1, peptide purifications in Supplement Figs 1–4).

Direct sequencer degradations of the intact, carboxymethylated protein revealed the first 40-odd residues (Fig. 1). Since ordinary enzymatic digestions gave only limited data of value, much of the remaining parts of the structure were established by two approaches: different chemical cleavages and extensive use of pepsin digestions.

Eight CNBr fragments were characterized (Fig. 1). Several cleavages were obtained in low yield (especially at Met-210, presumably because of unintentional methionine oxidations [20]), although the Met-110–Ser-111 bond, of low expected susceptibility to cleavage [21] was cleaved (Fig. 1). Treatment with *N*-chlorosuccinimide yielded fragments with six different N-terminal structures, corresponding to cleavages at all five tryptophan residues, partly non-stoichiometrically but with high specificity since no other structures were found cleaved.

Table 1. *Total composition of carboxymethylated tomato pectinesterase*. The hydrolytic values show the molar ratios, calculated for an M_r of 33239 as deduced from the sequence analysis. Values were obtained from acid hydrolysis for 24 h, 48 h and 72 h (extrapolation to zero time for Ser and Thr; and correction for slow release of Val and Ile by use of the 72-h values; Trp was not determined). For comparison, values from an earlier report [7] of uncorrected values from 20-h hydrolysis are shown within parentheses, after recalculation for M_r 33239. In that analysis, Cys was determined as Cya and Trp was determined after hydrolysis with thioglycolic acid

Amino acid	Acid hydrolysis		Sequence
	present	[7]	
Cys(Cm)	4.3	(4.8)	4
Asp			31 {
	30.8	(31.4)	
Asn			25 {
Thr	24.7	(21.5)	
Ser	15.1	(17.7)	13 {
Glu			
	26.3	(25.6)	26 {
Gln			
Pro	12.2	(14.3)	11
Gly	29.6	(28.8)	30
Ala	31.9	(28.8)	33
Val	27.3	(20.4)	28
Met	6.5	(6.1)	7
Ile	15.7	(13.2)	16
Leu	17.5	(17.0)	17
Tyr	17.1	(14.6)	18
Phe	9.6	(11.0)	9
Trp	+	(4.8)	5
Lys	16.3	(15.5)	16
His	2.3	(2.4)	2
Arg	13.7	(13.2)	14
Sum			305
			M_r 33239

Similarly, hydroxylamine gave the expected cleavage at Asn-103 – Gly-104 and only one additional cleavage, at Asp-208 – Gly-209 (Fig. 1). Remaining overall controls of overlapping segments were ascertained by recovery of all fragments from cleavage at original half-cystine residues by use of the lysine-specific endopeptidase after lysine protection by succinylation, and cystine 2-aminoethylation by reduction and ethylenimine treatment. High specificity of the lysine endopeptidase is obvious from lack of recovery of false cleavage products (Fig. 1), in spite of extensive digestion conditions (cf. Methods).

Residues not unequivocally defined from the sequencer analyses of the large peptides were obtained by manual degradations of 42 peptic peptides as shown in Fig. 1. All peptic peptides were not completely resolved by high-performance liquid chromatography, but results could be interpreted in mixtures since the general outline of the entire amino acid sequence was known from the other digests.

DISCUSSION

Analytical results and structural properties

Nearly all regions of the structure were recovered in more than one type of digest and analyzed in multiple degradations (cf. Fig. 1). Results obtained agree with the hydrolytically

derived total compositions for both the entire protein (Table 1) and individual peptides (Supplement). Consequently, the structure deduced is considered reliable in establishing the first primary structure of a pectinesterase protein chain.

In relation to previous studies, it may be noticed that an early report on preliminary characterizations of pectinesterase [7] was largely correct. Thus, total compositions then reported agree with present data (Table 1). Also, the N-terminus of the entire protein chain then reported (isoleucine), is in agreement with the present result (Fig. 1), but previous attempts at C-terminal determinations are not in equal agreement. Finally, a previous mapping of tryptic peptides gave largely correct estimates. It may be concluded that the preparations purified at different occasions are identical and homogeneous.

The relative molecular mass of the protein chain is now found to be 33239 (Table 1). This value is within the spread of earlier estimates, 22000 – 35000 [3 – 7], even if slightly larger than the estimates previously considered most representative from structural properties [7].

Distributions of most residues appear fairly even, with similar amounts of acidic and basic residues, and with approximately half of all Asx and Glx residues amidated (Table 1), but with a small excess (32 versus 29) of basic residues, compatible with the basic character of intact pectinesterase. However, several regions are quite hydrophobic, over one quarter (81/305) of all strictly hydrophobic residues are adjacent to another such residue and long segments are highly hydrophobic, especially in the N-terminal region (15 strictly hydrophobic residues among the first 26 residues) and in some other segments (14 such residues at positions 270 – 298). These segments probably explain the solubility problems encountered during the structural analysis of the protein (below), although they also have charged residues (Fig. 1) and therefore somewhat mixed properties.

Special problems and methodological aspects

Essentially, only strong formic acid or guanidine · HCl gave acceptable solubilization of the carboxymethylated pectinesterase. One of the tryptic digestions was therefore performed after solubilization of the protein in 6 M guanidine · HCl, and dilution to 1.5 M guanidine · HCl immediately before trypsin addition, as detailed in the Supplement. Multiple enzyme additions and prolonged digestion times finally gave a soluble peptide mixture. However, some untraditional cleavages were then noticed (after Asn-4, Tyr-74, Asn-170, Phe-190, Thr-240, Trp-265, Pro-266; all giving T* peptides). A few of these cleavages are similar to those expected for chymotryptic-like activity and may be explained by the extensive digestions. However, remaining unexpected cleavages (at Pro, Thr and Asn) appear to suggest a different substrate specificity, possibly because of altered specificities from the presence of guanidine · HCl during the enzyme action.

The solubility problems motivated the use of chemical cleavages. In this regard, the cleavages at tryptophan with *N*-chlorosuccinimide should be noted. Thus, although this protein has no less than five tryptophan residues (Table 1) and although both the specificity and stoichiometry for tryptophan cleavage with *N*-chlorosuccinimide is incomplete [15], cleavages at all five tryptophan residues were now encountered, without substantial cleavages at other residues (Fig. 1). Similarly, the Asn-Gly cleavage worked very well with this protein.

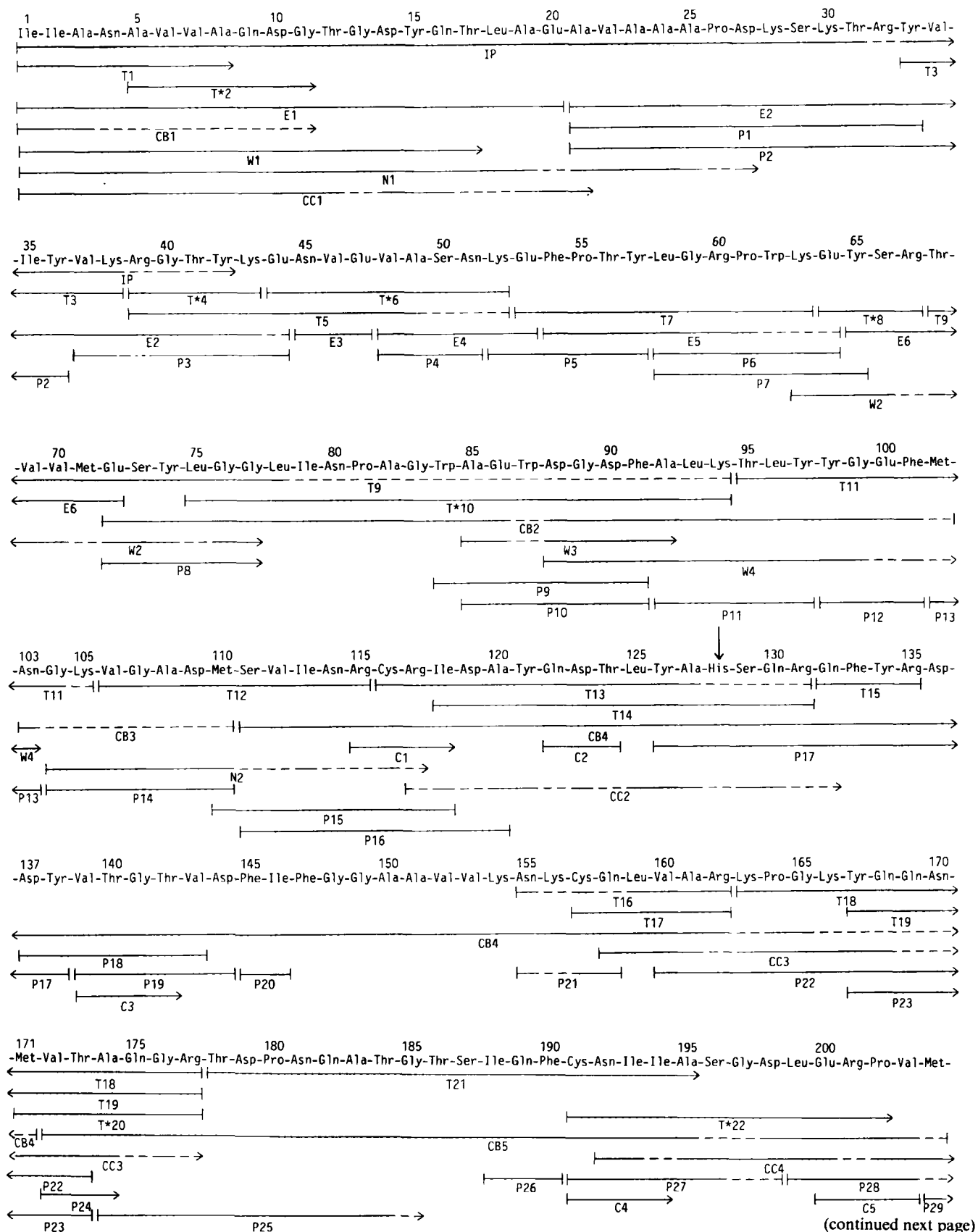


Fig. 1. The primary structure of tomato pectinesterase and a summary of all peptides analyzed to determine the structure. Peptides are indicated as given under Nomenclature in Materials and Methods. Compositions, sequence analyses, and purifications of fragments relevant to deduce the structure are detailed in the Supplement (Supplement Table 1 and Supplement Figs 1–4). Parts of peptides analyzed by sequence degradations are indicated by solid lines; remaining parts, analyzed by total compositions only, are dashed. Histidine and tyrosine are the only two residue types that have been ascribed functional importance in the enzyme activity. The positions of the two histidine residues in the entire structure (at positions 128 and 269, close to adjacent tyrosine residues) are indicated by arrows

16. Jörnvall, H., Carlquist, M., Kwauk, S., Otte, S. C., McIntosh, C. H. S., Brown, J. C. & Mutt, V. (1981) *FEBS Lett.* **123**, 205–210.
17. Bornstein, P. & Balian, G. (1977) *Methods Enzymol.* **47**, 132–145.
18. Butler, P. J. G. & Hartley, B. S. (1972) *Methods Enzymol.* **25**, 191–199.
19. Raftery, M. A. & Cole, R. D. (1966) *J. Biol. Chem.* **241**, 3453–3461.
20. Ozols, J. & Gerard, C. (1977) *J. Biol. Chem.* **252**, 5986–5989.
21. Schroeder, W. A., Shelton, J. B. & Shelton, J. R. (1969) *Arch. Biochem. Biophys.* **130**, 551–555.

Supplement to

Pectinesterase

The primary structure of the tomato enzyme

Oskar MARKOVIČ and Hans JÖRNVALL

Department of Chemistry I, Karolinska Institutet, Stockholm

MATERIALS AND METHODS

Pectinesterase was prepared from ripe tomatoes (*Lycopersicon esculentum*; Immuna variety) by extraction with 0.5 M NaCl, pH 7.8, salt fractionation with ammonium sulphate, separations on DEAE-Sephadex and Sephadex G-75, and final chromatography on CM-Sephadex C-50 [3]. The final product is the major component (also one of the most cathodic forms) in tomatoes; its homogeneity was checked by ultracentrifugation, gel electrophoresis [3, 6] and N-terminal analysis [7].

Carboxymethylation was carried out by dissolving the protein (10 mg/ml) in 0.1 M Tris/HCl, 6 M guanidine · HCl, 2 mM EDTA, pH 8.1, before reduction for 2 h at 37°C with dithiothreitol (40% molar excess over protein cysteine/half-cysteine residues) and subsequent addition (25% molar excess over total SH groups) of neutralized iodo[2-¹⁴C]acetic acid (Amersham) [14].

Enzymatic cleavages

Ordinary treatments with trypsin, chymotrypsin and staphylococcal glu-specific protease were insufficient for digestion because of a largely insoluble pectinesterase substrate. Therefore, the chymotryptic and Glu-specific protease treatments were carried out mainly as secondary digestions on the largest CNBr fragments. In the case of trypsin, a second type of digest was performed after solubilization of the protein in 6 M guanidine · HCl, dilution with 0.1 M ammonium bicarbonate to 1.5 M guanidine · HCl, pH adjustment to 8.1, two additions of trypsin (50 µg each to 100 nmol carboxymethylated pectinesterase) and incubation each time for 5 h at 37°C. With this treatment, the tryptic digest was eventually soluble and peptides from all regions of the molecule were recovered.

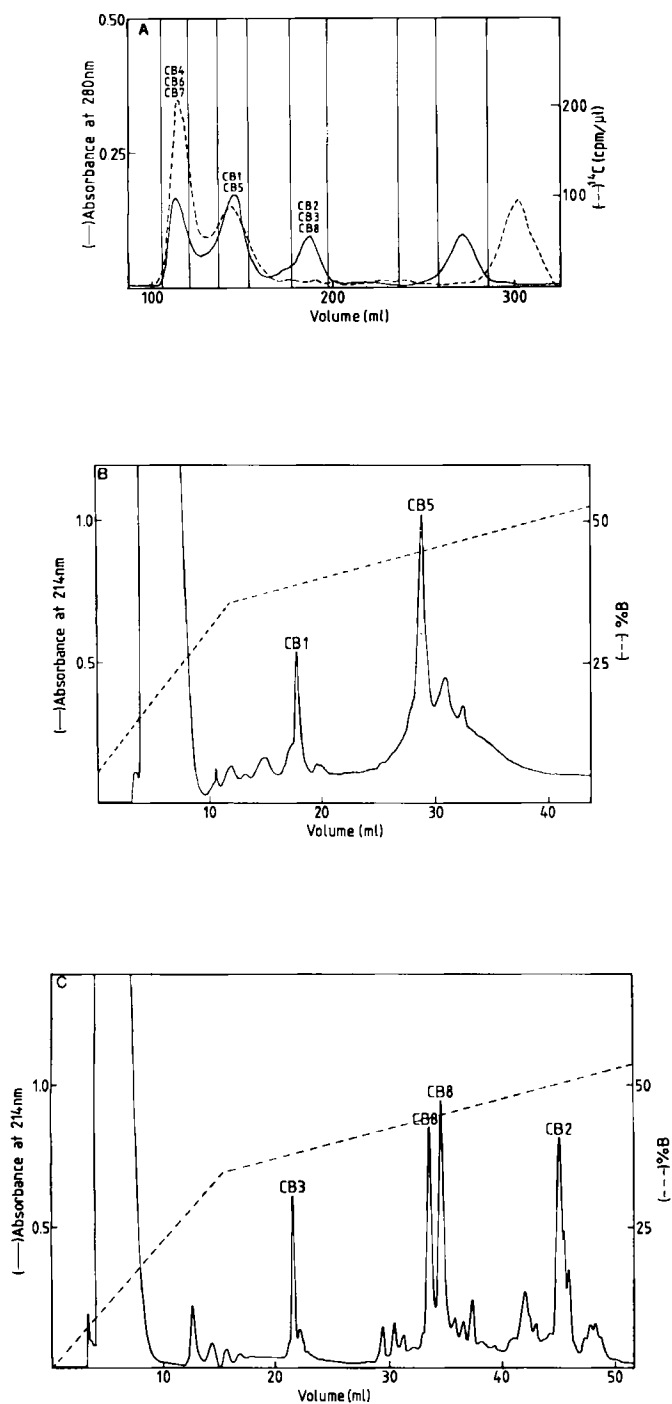
The two types of tryptic digest were prefractionated by chromatography on Sephadex G-50 (1.5 × 200 cm) in 30% acetic acid, before final purification by reverse-phase high-performance liquid chromatography on Ultropac TSK ODS-120, 5 µm (LKB, Sweden), utilizing a gradient of acetonitrile in 0.1% trifluoroacetic acid [14]. The digests with chymotrypsin and the Glu-specific protease were directly separated by high-performance liquid chromatography.

Pepsin cleavage. The carboxymethylated pectinesterase (100 nmol) in 5% formic acid was incubated for 4 h at 37°C with 60 µg pepsin. After lyophilization, the sample was dissolved in 30% formic acid and directly submitted to high-performance liquid chromatography as detailed above.

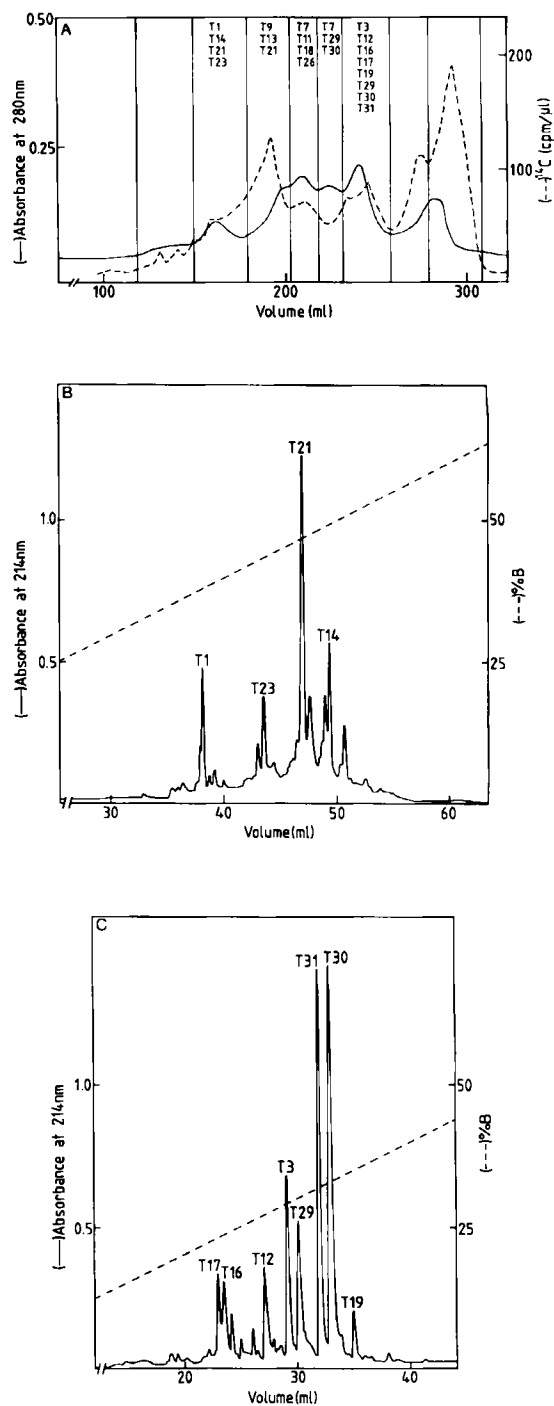
Supplement Table 1. Amino acid compositions of relevant tryptic peptides.

The peptides include five methionine-overlapping fragments. All peptides were purified by initial Sephadex G-50 fractionation and final reverse phase high performance liquid chromatography as shown in Supplement Figs. 2 and 3. Values shown are molar ratios obtained after acid hydrolysis for 24 h; values from the sequence analysis are within parentheses. + indicates the presence of Trp as shown by a high absorbance at 280 nm of the peptide material upon high performance liquid chromatography.

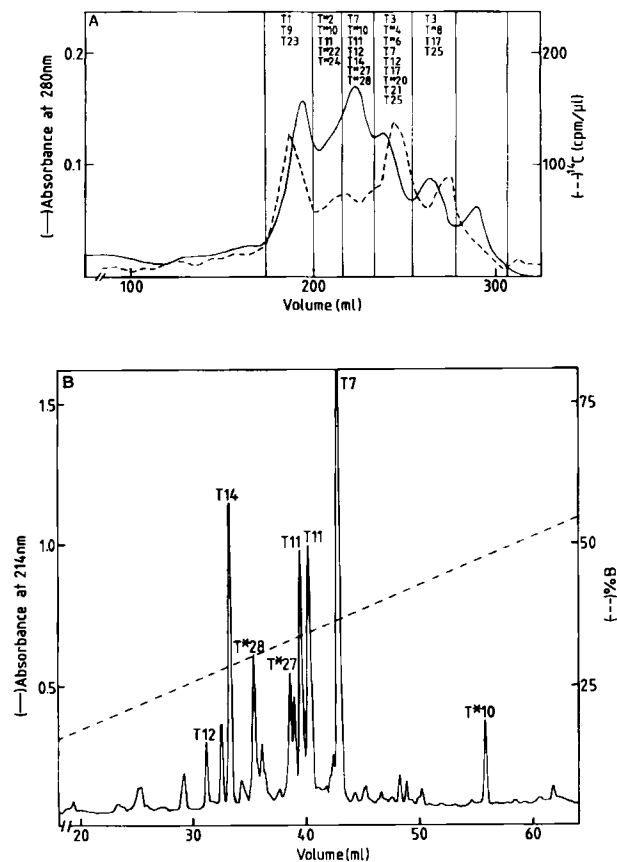
Peptide	T3	T*6	T7	T9	T*10	T11	T12	T13	T14	T18	T26	T29	T30	T31
Cys(Cm)	-	-	-	-	-	-	-	0.7 (1)	-	-	-	-	-	-
Asx	-	1.9 (2)	-	2.8 (3)	2.8 (3)	1.1 (1)	2.0 (2)	2.1 (2)	2.1 (2)	1.2 (1)	1.1 (1)	-	-	1.7 (2)
Thr	-	-	0.8 (1)	1.3 (1)	-	0.9 (1)	-	0.9 (1)	1.1 (1)	1.1 (1)	0.9 (1)	0.7 (1)	-	0.6 (1)
Ser	-	1.2 (1)	-	1.4 (1)	-	-	1.1 (1)	1.2 (1)	1.3 (1)	-	-	-	1.2 (1)	1.2 (1)
Glx	-	2.0 (2)	1.1 (1)	2.3 (2)	1.3 (1)	0.7 (1)	-	1.8 (2)	2.1 (2)	2.9 (3)	-	-	1.3 (1)	-
Pro	-	-	1.9 (2)	1.3 (1)	1.4 (1)	-	-	-	-	0.9 (1)	2.4 (2)	0.9 (1)	-	-
Gly	-	-	1.3 (1)	4.4 (4)	3.9 (4)	1.8 (2)	1.2 (1)	-	-	2.2 (2)	1.1 (1)	-	2.3 (2)	2.2 (2)
Ala	-	1.1 (1)	-	2.8 (3)	2.7 (3)	-	1.0 (1)	2.3 (2)	1.7 (2)	1.2 (1)	0.8 (1)	1.7 (2)	-	0.9 (1)
Val	2.2 (2)	1.7 (2)	-	1.6 (2)	-	-	1.7 (2)	-	-	1.0 (1)	1.9 (2)	1.1 (1)	-	2.2 (2)
Met	-	-	-	0.9 (1)	-	0.9 (1)	0.7 (1)	-	-	0.8 (1)	-	0.9 (1)	-	-
Ile	0.7 (1)	-	-	1.1 (1)	0.8 (1)	-	0.8 (1)	0.6 (1)	0.7 (1)	-	0.9 (1)	-	0.7 (1)	-
Leu	-	-	0.9 (1)	2.6 (3)	2.7 (3)	0.7 (1)	-	1.2 (1)	1.1 (1)	-	-	-	2.2 (2)	1.0 (1)
Tyr	1.7 (2)	-	0.8 (1)	0.9 (1)	-	1.8 (2)	-	1.6 (2)	1.7 (2)	0.8 (1)	0.7 (1)	-	-	1.6 (2)
Phe	-	-	0.8 (1)	1.0 (1)	1.1 (1)	0.7 (1)	-	-	-	-	-	0.7 (1)	-	-
Trp	-	-	+ (1)	+ (2)	+ (2)	-	-	-	-	-	+ (1)	-	+ (1)	-
Lys	0.9 (1)	1.1 (1)	0.9 (1)	1.3 (1)	1.0 (1)	0.8 (1)	-	-	-	1.6 (2)	1.7 (2)	0.8 (1)	-	-
His	-	-	-	-	-	-	-	0.9 (1)	0.8 (1)	-	0.8 (1)	-	-	-
Arg	-	-	0.8 (1)	-	-	-	1.1 (1)	1.9 (2)	1.0 (1)	0.9 (1)	-	-	1.1 (1)	-
Sum	6	9	11	27	20	11	10	16	14	15	14	8	9	12
N-terminus	Tyr	Glu	Glu	Thr	Leu	Thr	Val	Cys	Ile	Lys	Val	Ala	Leu	Ser



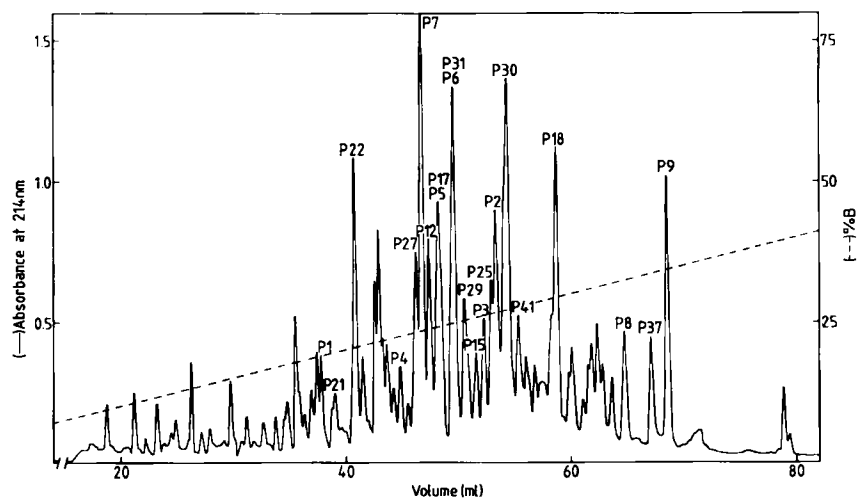
Supplement Fig. 1. Chromatographic separation of CNBr fragments of $[^{14}\text{C}]$ carboxymethylated tomato pectinesterase. (A) Elution profile from Sephadex G-50 Fine (1.5 $\times$ 190 cm) of the whole CNBr digest in 30% acetic acid. Flow 9 ml/h, fraction size 3 ml; pools are indicated by the thin vertical lines. Elution positions of CNBr fragments are given above the peaks. (B) Purification of the pool of CB1 and CB5 from A by reverse-phase high-performance liquid chromatography on Ultropac TSK ODS-120, 5 μm (LKB, Sweden), utilizing a gradient (%B) of acetonitrile in 0.1% trifluoroacetic acid, as shown by the dashed line. (C) Purification of the pool of CB2, CB3 and CB8 from A by high-performance liquid chromatography as in B.



Supplement Fig. 2. Chromatographic separation of the tryptic digest of $[^{14}\text{C}]$ carboxymethylated pectinesterase. (A) Elution profile from Sephadex G-50 Fine of the whole tryptic digest. Column dimensions and conditions as in Supplement Fig. 1. Fractions were pooled as indicated by the vertical lines, and the constituent peptides identified in each pool are given at the top. (B) Purification of T1, T14, T21 and T23 from the first peptide pool in A, by reverse-phase high-performance liquid chromatography as in Supplement Fig. 1. (C) Purification of T3, T12, T16, T17, T19, T29-T31 from the last peptide pool in A, by high-performance liquid chromatography as in B.



Supplement Fig. 3. Chromatographic separation of the second tryptic digest of $[^{14}C]$ carboxymethylated pectinesterase (performed in 1.5 M guanidine $\cdot$ HCl, pH 8.1). (A) Elution profile from Sephadex G-50 Fine (conditions as in Supplement Fig. 1). Pools and peptides identified are indicated as in Supplement Fig. 2. (B) Purification of the peptide-rich third pool from A, by reverse-phase high-performance liquid chromatography (conditions as in Supplement Fig. 1)



Supplement Fig. 4. Separation of the peptic digest of $[^{14}C]$ carboxymethylated tomato pectinesterase. Fractionation was by direct reverse-phase high-performance liquid chromatography (conditions as in Supplement Fig. 1)