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Disulfide bridges in tomato pectinesterase: Variations from pectinesterases of other species; conservation of possible active site segments

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Abstract

Analysis of tomato pectinesterase by carboxymethylation, with and without reduction, shows that the enzyme has two intrachain disulfide bridges. Analysis of fragments obtained from the native enzyme after digestion with pepsin identified bridges connecting Cys-98 with Cys-125, and Cys-166 with Cys-200. The locations of disulfide bridges in tomato pectinesterase are not identical to those in three distantly related pectinesterases (18–33% residue identities) from microorganisms. However, one half-Cys (i.e., Cys-166) position is conserved in all four enzymes. Sequence comparisons of the overall structures suggest a special importance for three short segments of the entire protein. One segment is at the N-terminal part of the tomato pectinesterase, another in the C-terminal portion near the distal end of the second disulfide loop, and the third segment is located in the central part between the two disulfide bridges. The latter segment, encompassing only 40 residues of the entire protein, appears to highlight a functional site in a midchain segment.

Keywords: active site segments; disulfides; pectinesterase; structural variation

Pectic enzymes are degradative enzymes, involving both deesterifying hydrolases, deglycosidating hydrolases, and deglycosidating lyases. Together, they control breakdown of the heteropolysaccharide pectin in plants (Rombouts & Pilnik, 1980) and regulate both physiological processes and pathogenic processes such as infection by phytopathogenic microorganisms (Collmer & Keen, 1986). To date, four different types of pectinesterase have been determined in primary structure, the pectinesterase from tomato (Ray et al., 1988; Markovič & Jörnvall, 1990), *Aspergillus niger* (Khanh et al., 1990), *Erwinia chrysanthemi* (Plastow, 1988), and *Pseudomonas solanacearum* (Spök et al., 1991). These four enzymes are distantly related, exhibiting a low degree of sequence similarity, with residue identities at the 18–33% level (disregarding an elongated N-terminal overshooting end of the *Pseudomonas* enzyme). Attempts at chemical modifications have shown the presence of reactive tyrosine residues (Markovič, 1983), and overall comparisons suggest relationships within both this family of pectinesterases, and that of polygalacturonases, which are also pectic degradative enzymes (Plastow, 1988; Hinton et al., 1990). Furthermore, pectinesterase homologs exist and help to define elements of structural interest (Albani et al., 1991). However, information on functional mechanisms, active site locations, and overall organization of the proteins is limited.

We have now determined the arrangement of disulfide bridges in the tomato enzyme. Two intrachain bridges were detected. Comparisons of their locations with those of cysteine/half-cystine positions in other pectinesterases reveal fairly large variations. We clearly show regions of conserved primary structure in all of these pectinesterases that suggest a functional role for a mid-chain segment.

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Results

Presence of disulfide bridges

The native enzyme, with a specific activity of 750 U/mg (1 U hydrolyzes 1 μmol ester/min), was analyzed for the presence of disulfide bridges by carboxymethylation un-

der different conditions. Reaction with ^{14}C-labeled iodoacetate in 6 M guanidine-HCl to ensure accessibility of all SH groups was tested directly and after reduction with dithiothreitol. Excess reagents were removed by exclusion chromatography, and incorporation of carboxymethyl groups was evaluated by amino acid analysis after hydrolysis and by radioactivity measurements. No carboxymethylcysteine was obtained in the absence of reduction, whereas after reduction a carboxymethylcysteine content of 4.1 was detectable. In both cases, the total composition is in agreement with that from previous cDNA and protein data (Markovič & Jörnvall, 1986, 1990; Ray et al., 1988). Similarly, regardless of the amount of alkylating agent (10-fold or 25-fold molar excess), the incorporation of radioactivity increased by about 4 nmol carboxymethyl groups per nmol protein after pretreatment by reduction.

Combined, these results suggest that all four Cys residues in the primary structure of tomato pectinesterase are present in the form of disulfide-linked half-Cys residues. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis suggested a molecular weight in the range of 35,000 for the protein subunit, both with and without reduction, establishing that disulfide bridges are intramolecular. Finally, the presence of enzyme activity in the preparations suggests that disulfide bridges are native constituents of the active enzyme and not just derived by oxidation of the enzyme preparations after isolation. Consequently, it is concluded that native tomato pectinesterase appears to contain two intrachain disulfide bridges interconnecting four half-cystine residues. The presence of native disulfide bridges is consistent with the fact that pectinesterase is an extracellular enzyme, secreted from the cells and present in the extracellular fluid during native growth (Markovič & Slezárik, 1969; Rombouts & Pilnik, 1980; Collmer & Keen, 1986; Plastow, 1988).

Localization of disulfide bridges

The native protein was cleaved with pepsin in 5% formic acid, i.e., under conditions minimizing disulfide rearrangements, and the resulting peptide mixture was separated by reverse-phase high-performance liquid chromatography. All major fractions were submitted to analysis for total composition after acid hydrolysis in order to detect all peptides containing half-Cys. As shown in Figure 1, two fractions were found to contain half-Cys residues, and both were submitted to sequence analysis by degradation in a MilliGen ProSequencer 6600 to identify the constituent peptides. The first-eluting, Cys-containing fraction (fraction 1 in Fig. 1) contained two Cys residues, Cys-98 in the peptide segment at positions 97–112, and Cys-125 in the peptide segment at positions 120–128. These two sequences were recovered in about equal yield, and the result was reproducible in two different preparations of the enzyme. In addition, these fractions also contained segments 56–63 and 311–317 in yields that varied between the two preparations. The other Cys-containing fraction also contained two Cys residues, Cys-166 in the segment 165–168, and Cys-200 in the segment 200–208. Again, both were obtained in approximately equimolar amounts, together with two other fragments in variable yield and not containing carboxymethylcysteine.

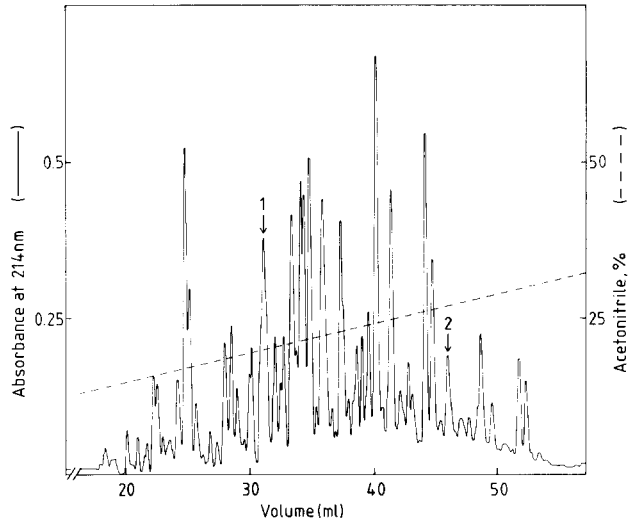

Fig. 1. Separation by high-performance liquid chromatography on Vydac C4 of the peptic peptides from tomato pectinesterase. The two fractions corresponding to the numbered arrows were the only ones found to contain half-cystine by acid hydrolysis; sequence analysis showed each to contain two half-cystine residues.

Combined, these results suggest that disulfide bridges connect Cys-98 with Cys-125, and Cys-166 with Cys-200. This would mean a linear arrangement along the polypeptide chain, with two consecutive disulfide bridges, the first in a loop with 26 other residues, the second in a loop with 33 other residues and a connecting segment of 40 residues (positions 126–165).

Discussion

The present study quantitates Cys residues in tomato pectinesterase and suggests that all four residues are involved in disulfide bridges in the native enzyme. The disulfide pattern shows two disulfide bridges near the middle of the molecule, separated linearly by a segment of 40 residues. The disulfide-loop structures are of a size typical for proteins in general, around 25 residues (Thornton, 1981). It is concluded that tomato pectinesterase is a disulfide-stabilized enzyme containing two separate loops.

Determination of the disulfide bridges in tomato pectinesterase, and the fact that primary structures of four highly divergent pectinesterases are now known, permits comparisons between pectinesterases in general. The four structures exhibit distant similarities (Hinton et al., 1990; Markovič & Jörnvall, 1990; Spök et al., 1991; Fig. 2); however, the Cys residues differ in number and are not

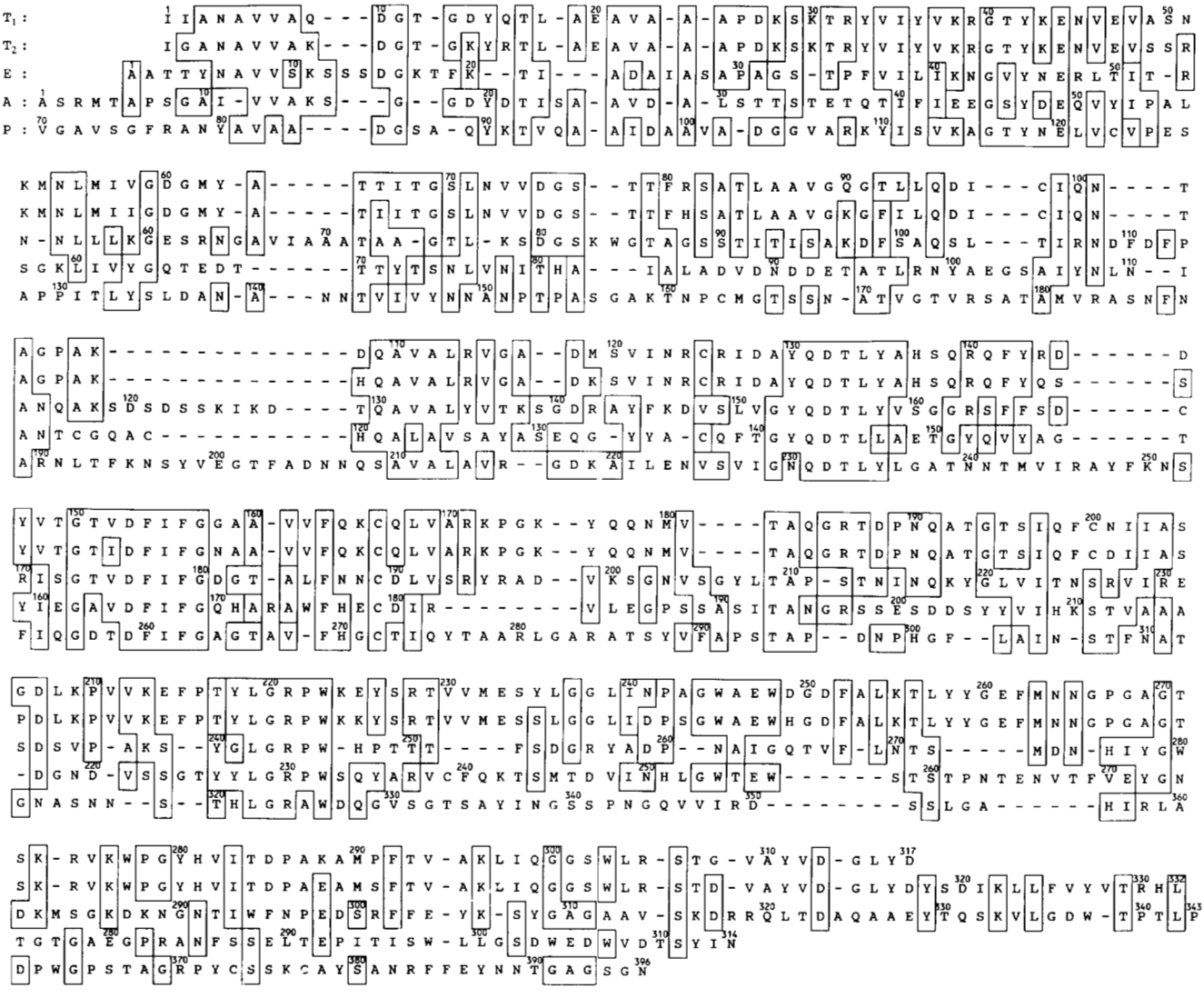

Fig. 2. Sequence alignment of characterized pectinesterases. The top two lines show the two isoforms deduced from protein (T1) and cDNA (T2) data of tomato pectinesterase (*Lycopersicon esculentum*), the third line shows the enzyme analyzed from the nucleotide sequence of the *Erwinia chrysanthemi* pectinesterase gene (E), the fourth line the cDNA-deduced and peptide-confirmed structure of the enzyme from *Aspergillus niger* RH 5344 (A), and the bottom line the nucleotide sequence from the cloned pectinesterase gene of *Pseudomonas solanacearum* (P). Data from Plastow (1988), Ray et al. (1988), Hinton et al. (1990), Khanh et al. (1990), Markovič and Jörnvall (1990), and Spök et al. (1991). Boxes indicate residue identities; dashes gaps. All gap ends and starts are not well defined, and a few may be slightly moved without affecting alignments at large. However, gap positions are largely locked by the segments of extensive similarities, and single gap movements have no decisive influence on residues strictly conserved (Table 1) or other major aspects of the alignments.

coaligned at corresponding residue positions. However, all four pectinesterases have the common properties of being extracellular, secreted, and derived from proproteins with leader sequences. Furthermore, one Cys position (corresponding to Cys-166 in the tomato enzyme) is conserved in all four forms, and another Cys (Cys-125 in the tomato enzyme) is common to the tomato and *Aspergillus* forms (Fig. 3). Therefore, except for the highly dissimilar *Pseudomonas* enzyme, homology alignments (Fig. 2) appear compatible with a similar protein-folding pattern.

Overall similarities are also compatible with the fact that glycine is a well-conserved residue in these enzymes (Table 1), which is a pattern typical for proteins distantly related but with conserved functional properties (Smith & Margoliash, 1964; Jörnvall, 1973). Only 32 of the total 314–396 residues are strictly conserved between each species, corresponding to 8–10% of all residues. Few polar residues are conserved, essentially only single Tyr, Glu, Asp, Thr, and Arg residues (Table 1) that could be of interest for providing reactive groups in enzyme catalysis and may relate to previous labeling experiments

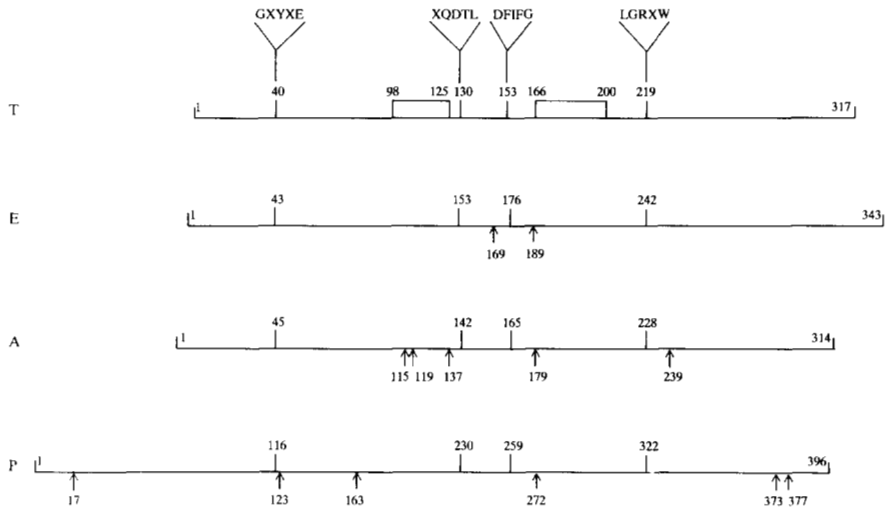

Fig. 3. Schematic representation of the four highly divergent pectinesterases characterized. Positions of disulfide bridges (⌐¬) in the tomato pectinesterase (T) and positions of Cys residues (↑) in *Erwinia* (E), *Aspergillus* (A), and *Pseudomonas* (P) pectinesterases are shown to scale, with positional numbers referring to the positions in Figure 2. The scale also follows that alignment, with the gaps included. The only 5-residue segment strictly conserved (top, segment without X), as well as the other segments with many consecutive conserved residues but also minimally one nonconserved residue (top, segments with X) include or are close to Tyr residues and most of the conserved acidic residues although only three short segments of the whole protein are involved (around positions 40, 130–153, and 219).

(Markovič, 1983). Most of the conserved residues are in only three segments of the molecule; the two Tyr residues, conserved in all but the *Pseudomonas* enzyme, and two of the three strictly conserved acidic residues are confined to essentially four separate five-residue segments, of which one is the only conserved five-residue segment in the whole alignment (Fig. 2). This pattern is summarized in Figure 3. Other regions of similarity have been observed in previous comparisons of three of these structures (Spök et al., 1991). Notably, a central portion of the molecule, involving the 40-residue segment between the two disulfide bridges of the tomato enzyme, contains two of these conserved segments and two of the polar residues (2 Asp, cf. Fig. 3). Interestingly, a gene highly expressed in developing pollen of *Brassica napus*, clone Bp 19 (Albani et al., 1991) codes for a 584-residue polypeptide, the carboxy-terminal half of which, starting at position 269, has sequence similarity to tomato and microbial pectinesterases compared here. This part of the Bp 19 protein contains 30 residues strictly conserved in the four pectinesterases and has all four of the five-residue segments outlined in Figure 3 located at corresponding positions. This conservation in another protein supports our hypothesis that these segments in pectinesterases may play an important structural or functional role.

Table 1. *Residues strictly conserved in pectinesterases of four highly divergent species, corresponding to the tomato, Erwinia, Aspergillus, and Pseudomonas forms, as shown in Figure 2*

Cys	1
Asp	2
Asn	–
Thr	4
Ser	–
Glu	1
Gln	1
Pro	–
Gly	5
Ala	6
Val	1
Ile	3
Leu	2
Tyr	1
Phe	3
Trp	1
Arg	1
Sum	32

In summary, the present results may suggest that the four pectinesterases have one to three disulfide loops and a conserved midchain segment. Such a segment could be a candidate for an active site. Interestingly, the only five-residue segment strictly conserved is localized in this segment (DFIFG, at position 153 of the tomato enzyme, Fig. 3), and the conserved parts of the five-residue segments indicated in Figure 3 include two acidic residues (Fig. 3) and adjacent Tyr residues. This corresponds to no less than half of the total hydrophilic residues strictly conserved (Table 2). Therefore, provided these enzymes have common enzymatic mechanisms and utilize common catalytic residues, the conserved structures in the midchain segment are undoubtedly functionally or structurally essential to the active site.

Materials and methods

Pectinesterase was prepared from ripe tomato (*Lycopersicon esculentum*, Immuna variety), by extraction with 0.5 M NaCl, pH 7.8, fractionation with ammonium sulfate, separations on DEAE-Sephadex A-50 and Sephadex G-75, and final chromatography on CM-Sephadex C-50 (Markovič & Slezárik, 1969).

Carboxymethylation was carried out by dissolving the protein (0.5 mg) in 0.1 M Tris-HCl, 6 M guanidine-HCl, 2 mM EDTA, pH 8.1 (200 μL), with or without reduction

for 2 h at 37 °C with dithiothreitol (17-fold molar excess over protein half-Cys) and addition of neutralized iodo[2-^{14}C]acetic acid at a two-fold molar excess over total Cys and reagent thiols (Markovič & Jörnvall, 1986). Carboxymethylated samples were desalted on an Ultropac TSK G 2000 SW column, 7.5 × 600 mm (LKB, Sweden) in 30% acetic acid at a flow of 0.5 mL/min.

Total compositions were determined with a Beckman 121M amino acid analyzer, after hydrolysis for 24 h at 110 °C with 6 M HCl/0.5% phenol. Enzymatic cleavage with pepsin was performed with native pectinesterase (20 nmol) in 5% formic acid for 4 h at 37 °C with a protease:substrate ratio of 1:20–40. After lyophilization, the samples were dissolved in 30% formic acid and submitted to reverse-phase high-performance liquid chromatography on Vydac C4 (4.6 × 250 mm) in 0.1% aqueous trifluoroacetic acid with a gradient of acetonitrile. All fractions were checked for the presence of half-cystine by amino acid analysis after acid hydrolysis, and peptides containing half-cystine were subjected to sequence analysis on a solid-phase MilliGen ProSequencer 6600 utilizing both DPITC membranes and arylamine membranes for covalent attachment.

Structures for the comparisons were obtained from known amino acid sequences for tomato, *Aspergillus*, *Erwinia*, and *Pseudomonas* pectinesterases (Markovič & Jörnvall, 1986, 1990; Plastow, 1988; Ray et al., 1988; Khanh et al., 1990; Spök et al., 1991). In the case of tomato pectinesterase, initial reports differed, both because of analytical problems and because of the presence of two different isoforms now known to reflect a true heterogeneity (Markovič & Jörnvall, 1990 and W. Schuch, pers. comm.) apparently affecting 27 positions (Fig. 2).

Acknowledgments

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