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Research Article

Characterization of carrot pectin methylesterase

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Abstract. The most alkaline form of pectin methylesterase was purified from ripe carrot roots and used for structural analysis. Determination of an N-terminal blocking group and of the primary structure allowed comparisons with other forms, and facilitated crystallographic determination of the three-dimensional structure. The mature enzyme has 319 residues and the N-terminal blocking group was shown to be a pyroglutamyl residue derived from a glutaminyl cyclization. Few other methylesterases have been isolated and assigned to exact mature forms, and together with the present enzyme, only two have been analyzed in three-dimensional structure. However, comparison of 39 forms, mainly from GenBank data, reveals clear relationships and identifies subgroups of this enzyme type, deviating in structure but centering around two functionally important and conserved Asp residues at positions 136 and 157 in the carrot enzyme.

Key words. Pectic enzyme; methylesterase; amino acid sequence; aspartyl esterase; pyroglutamyl N terminus; phylogenetic relationship.

Pectin methylesterase (EC 3.1.1.11) is a pectic enzyme (table 1) which catalyzes the deesterification of methylesterified D-galactosiduronic acid units in pectic compounds, yielding substrates for depolymerizing enzymes. Pectin methylesterase participates in modulating cell wall structural features during fruit ripening [1], cell wall extension [2] during pollen germination [3, 4], and in defense mechanisms against pathogens [5]. The enzyme has been found in all higher plants examined and is also produced by phytopathogenic bacteria and fungi**.** The enzyme generally occurs in multiple forms**.** It was identified in carrots more then 140 years ago [6] and has also been described more recently [7–9]. The most alkaline form of carrot pectin methylesterase was prepared in a homogeneous state and found to have an N terminus resistant to Edman degradation [10]. A blocked N terminus was also found in two alkaline isoforms of the enzyme

Table 1. Pectic enzymes and their EC numbers.

Esterases	
Pectin methylesterase	EC 3.1.1.11
Pectin acetylesterase	EC 3.1.1.6
Rhamnogalacturonan acetylesterase	EC 3.1.1.-
Glycosyl hydrolases	
Polygalacturonase (endo)	EC 3.2.1.15
Exopolygalacturonase	EC 3.2.1.67
Exo-poly-α-D-galactosiduronidase	EC 3.2.1.82
Oligo-D-galactosiduronate hydrolase	EC 3.2.1.-
Rhamnogalacturonase	EC 3.2.1.-
Endo-xylogalacturonan hydrolase	EC 3.2.1.-
Lyases	
Pectate lyase	EC 4.2.2.2
Oligogalacturonid lyase	EC 4.2.2.6
Exopolygalacturonate lyase	EC 4.2.2.9
Pectin lyase	EC 4.2.2.10
Phamnogalacturonan lyase	EC 4.2.2.-

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from *Vigna radiata* (PE_α and PE_β [11]), in one *Erwinia chrysanthemi* enzyme (3937 PemB [12]), in the enzyme purified from transgenic tobacco expressing the tomato plant gene *pmeu1* [13], and in the enzyme from orange fruit peels [14]. In the latter enzyme, the blocked N terminus was found to be acetylated [14], while a few forms have been shown to have a free N terminus [11, 15–23].
The aim of the present work was to prepare for the complete structural characterization of carrot root plant methylesterase through X-ray crystallography [24] by determination of the primary structure, establishment of the relationships with other plant methylesterases, and characterization of the nature of the blocked N terminus.

Materials and methods

Enzyme preparation

Pectin methylesterase was prepared from ripe carrot roots (*Daucus carota* L., cv. Tiptop) by homogenization, extraction with 1 M NaCl and filtration through a fruit press as described elsewhere [10]. The first extract was used for exopolygalacturonase purification and for identification of pectin acetylesterase and rhamnogalacturonase. The press residue was extracted with 2 M NaCl at pH 7.8, and after ammonium sulfate fractionation (30–75%), the extract was purified by ion exchange chromatography on DEAE Sephadex A-50 and CM Sephadex C-50, and by exclusion chromatography on Sephadex G-75 and Superose 12 (FPLC) [10]. The final product was obtained by desalting and lyophilization.

Enzyme characterization

The most alkaline form of carrot pectin methylesterase gave one distinct band on isoelectric focusing with a pI of 9.8 and one band on SDS/PAGE with an M_r close to 33,000. This form had a specific activity of 32 mol s^{-1} kg^{-1}, with a K_m of 1.5×10^{-6} mol^{-1} for methyl D-galactopyranosyl residues, and a pH optimum at 7.8.

Primary-structure determination

The carrot pectin methylesterase was carboxymethylated and cleaved in separate batches with Lys-protease (from *Achromobacter lyticus*; Wako Chemicals, Neuss, Germany) and with Asp-protease (from *Pseudomonas fragi;* Boehringer Mannheim, Germany). Peptides obtained were separated by reverse-phase HPLC on Vydac C4 and C8. Sequence analysis of the peptides isolated was performed on Applied Biosystems 494 Protein Sequencers. The native enzyme was also analyzed by C-terminal degradation [25] in an Applied Biosystems 494C Sequencer. Mass spectrometry of blocked peptides was performed on a Q-TOF tandem mass spectrometer [26] equipped with a Z-spray nano-ES interface (Micromass, Manchester, UK). Samples were sprayed from gold-coated borosilicate capillaries (Protan a A/S, Odense, Denmark).

Sequence alignments

Alignments were performed using the program CLUSTAL W [27] , while an evolutionary tree was constructed with the neighbor-joining method [28] and drawn with the program TreeView [29].

Results and discussion

Homogeneity of enzyme preparation

The most alkaline form of carrot pectin methylesterase was purified and established to lack a free N terminus detectable by N-terminal sequence analysis, but to have a single C-terminal sequence, determined by C-terminal degradation [25] to be -Phe-Ser-Leu-Gly-Leu. Attempts at N-terminal de-blocking [30, 31] were unsuccessful, suggesting that the blocked N terminus is not acetylated. The lack of N-terminal contaminating sequences and the presence of only one C-terminal sequence establish the homogeneity of the enzyme preparation.

N-terminal blocking group

For isolation of the N-terminal structure in a peptide fragment, the carboxymethylated enzyme was digested with Lys-protease, the peptides were separated by reverse-phase HPLC and analyzed. The N-terminal structures of these peptides identified them, versus homologous pectin esterases from other sources, as internal fragments and established much of the protein primary structure. However, one of the peptides was resistant to Edman degradation and corresponded to the blocked N-terminal part of the original preparation. This structure was determined with tandem mass spectrometry [26] and was found to start with pyroglutamate, derived from cyclization of an N-terminal glutaminyl residue, thus explaining the nature of the blocked N terminus. The 20-residue amino acid sequence of this fragment was fully determined by tandem mass spectrometry (fig. 1).

Primary structure

In addition to the blocked N-terminal peptide mentioned above, all the remaining 12 peptides from the Lys-protease digest of carboxymethylated pectin methylesterase were isolated and analyzed. All were fully determined by sequencer degradations, except for the longest peptide K7 which was analyzed for 30 cycles, to His141 (cf. fig. 1). The remaining part of this peptide, as well as the relative positions of all K-peptides were ascertained by sequence analysis of D-peptides separately obtained by digestion of carboxymethylated pectin methylesterase with Asp-protease and subsequent peptide separation by HPLC. Combined with additional digestions utilizing

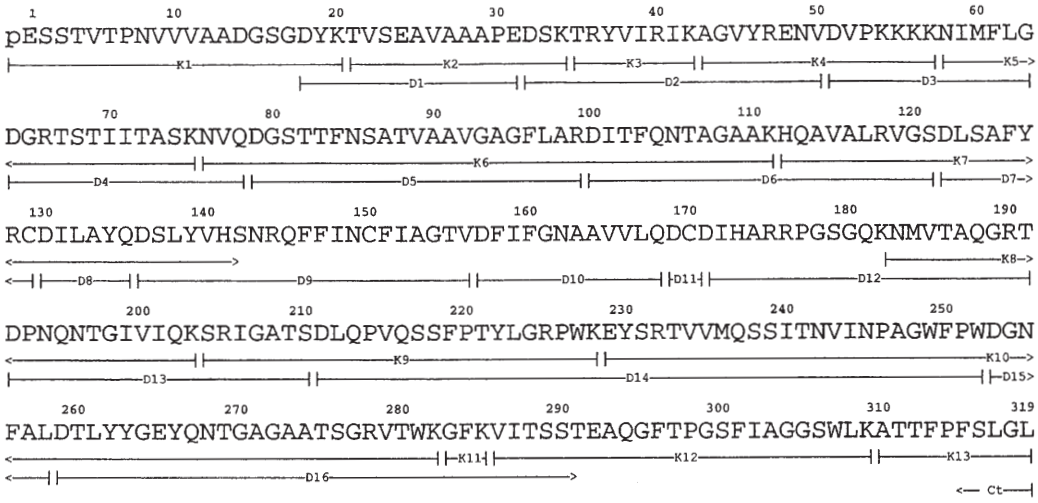

Figure 1. Amino acid sequence of the presently analyzed most alkaline form of carrot pectin methylesterase. Peptides D and K denote those peptides derived from cleavages with Asp- and Lys-proteases, respectively. Continuous lines indicate identifications by sequencer analysis, continuities then not covered were overlapped by determination of total masses and by separate digests with chymotrypsin and trypsin.

trypsin, all overlaps were established to give the complete amino acid sequence of carrot pectin methylesterase shown in figure 1, with all K- and D-peptides marked. The carrot enzyme polypeptide consists of 319 residues with a mass of 34,237.4, as deposited in the Swiss-Prot data bank (accession number P83218), and does not contain potential glycosylation sites.

Structural relationships and functional residues.

With the present enzyme available for X-ray crystallography [24], a three-dimenional structure of a plant pectin esterase will be ascertained. Only one other three-dimensional structure of this type of enzyme is known, but for a bacterial form [32]. Similarly, although the corresponding cDNAs have been cloned from many plants, the mature enzyme with N-terminal modifications and start points has only been directly established in a few cases. Therefore, further comparisons are required to establish common properties and deviating characteristics. All forms analyzed at any level are summarized in table 2. As shown, an N-terminal blocking group has been proven in three cases (the present carrot form, an orange form of the enzyme, and the bacterial *Erwinia* form), with the pyroglutamic acid identified only in the carrot enzyme, and with clearly different blocking groups in the two plant forms where blocking groups have been established. This shows that the N-terminal blocking is multifactorial and not enzyme unique throughout pectin esterases. Similarly, open reading frames in the cDNAs and start points of the mature enzymes appear to differ, where characterized (table 2).

We aligned the amino acid sequences of all the table 2 enzymes. Specific details of this show the relative importance of individual residues and are outlined in relation to the presentation of the crystallographically determined three-dimensional structure [24]. Three general principles however, can be seen to emerge from the overall comparisons of the 39 known forms in table 2, as summarized below.

The first is that the many enzymes, although strictly homologous, with overall residue identity from 24 to 85% versus the carrot form, are quite divergent. As ordered in table 2, there is a continuous drop in extent of similarities, and the only other form with a known tertiary structure (the bacterial *Erwinia* enzyme [32]) is only distantly related. This is visible directly at the active site segment, where the segments around the two catalytically important Asp residues are shown aligned in figure 2. Some of the distantly related forms differ in intervening chain lengths (fig. 2) and few residues are strictly conserved. This makes analysis of the three-dimensional structures important, motivating comparisons of the carrot and *Erwinia* enzyme structures [24]. In fact, among all the 39 forms (table 2, and shown in part in fig. 2), only 13 amino acid residues are strictly conserved: Gly44, Tyr46, Asn105, Ala114, Ala116, Asp136, Gly154, Asp157, Gly161, Leu223, Gly224, Arg225, and Trp227. Significantly, this includes the two active-site Asp residues (Asp136 and Asp157), proving the nature of pectin esterases as aspartyl enzymes (see below), and the conservation of many Gly residues (no less than four Gly residues, at positions 44, 154, 161, and 224). This conservation means that one-third of all residues actually conserved are Gly residues. Such a conservation of many Gly residues at reverse-turn positions [26] indicates a largely conserved fold, and a functionally homogeneous family, as in other well-defined protein families [cf. ref. 33].

Table 2. Structurally analyzed pectin methyl esterases. The enzymes are listed with the present carrot enzyme (from *Daucus carota*) at the top, and with all subsequent forms according to structural relationships in an apparent phylogenetic tree after alignment of determined sequences. Five groups are then apparent as subdivided by the group numbering. Groups 1 and 2 appear to represent separate branches of one line, group 3 a separate line, and groups 4 (with pollen and flower pectin methylesterases) and 5 separate branches of a third line. In this listing, the extent of amino acid residue identity between the carrot enzyme and all other forms (identity, % gradually falls. SwissProt and GenBank/EMBL columns give the corresponding databank annotation entries, ORF indicates the open reading frame for the enzymes deduced from DNA data (or the mature enzyme for the present carrot protein) and the column N-t indicates the nature (position in the ORF and actual N terminus when analyzed; 1pGlu meaning pyroglutamic residue in position 1, 45AcSer meaning acetyl-Ser at ORF position 45, and ordinary three-letter codes for the other N termini determined). nd, not determined; na, not available.

Group	Enzyme source	SwissProt	GenBank or EMBL	ORF	N-t	Identity (%)
1	*Daucus carota* (var. Tip-Top, roots)	P83218	na	319	1pGlu	100
	Nicotiana tabacum (cell wall protein fraction)	Q9LEB0	AJ2401158	579	nd	85
	Lycopersicon esculentum (leaves, GN: *pmeu.1*)	Q43143	U49330	583	nd	85
	Solanum tuberosum (potato GN: PEST2)	Q9SEE6	AF152172	576	nd	87
	Populus tremula (poplar, GN: pme1)	na	AJ277547	579	nd	82
	Citrus sinensis (Valencia orange PECS-1.1)	O04886	U82972	584	nd	81
	Citrus sinensis (*Navelina* orange pOPME1b)	na	E1260478	362	45AcSer	80
	Linum usitatissimum (GN: 'pme3')	na	AAG17110	555	nd	78
	Arabidopsis thaliana (GN: AtPM2)	Q42534	U25649	582	nd	74
	Arabidopsis thaliana (GN: PME3)	Q49006	AF033204	582	nd	77
2	*Solanum tuberosum* (potato sprouts, GN: BPE2)	Q9SEE7	AF152171	530	nd	60
	Lycopersicon esculentum (fruit, clone B8)	P14280	X74638	546	230Ile	61
	Lycopersicon esculentum (fruit, clone B16)	S46528	X74639	550	234Ile	61
	Nicotiana plumbaginifolia (mesophyl protoplast)	Q42936	Z71753	315	nd	63
	Vigna radiata (mung bean hypocothyl, GN: PEγ)	Q43234	S78456	321	3Asp	62
	Pisum sativum (GN: pmeA)	O24298	X67425	554	nd	58
	Oryza sativa (genomic, chromosome '1')	na	BAA84618	611	nd	50
	Prunus persica (peach mature fruit, clone PPE83)	Q43062	X95991	522	nd	55
	Carica papaya (GN: spe1)	Q96548	Y07899	222	1Ser	48
	Citrus sinensis (Valencia orange PECS-2.1)	O04887	U82975	510	nd	61
	Arabidopsis thaliana (GN: F4L23.27)	O22149	AC002387	510	nd	62
3	*Phaseolus vulgaris* (green beans pods, GN: MPE3)	Q43111	X85216	581	nd	51
	Arabidopsis thaliana (GN: AtPM1)	Q43867	X81585	586	nd	48
4	*Salix gilgiana* (male flowers, clone pSgPME1)	na	AB029461	581	nd	52
	Oryza sativa (GN: HO423H10.13)	na	AL442112	717	nd	52
	Zea mays (pollen specific, GN: ZmC5)	O24596	Y13285	563	nd	52
	Arabidopsis thaliana (flower specific GN:PME4)	na	AAC27719	586	nd	43
	Brassica napus (pollen specific, GN: Bp19)	P41510	X56195	584	269Ile	44
	Brassica campestris (anther specific)	Q42608	L48178	571	256Ile	42
	Petunia inflata (pollen expressed)	Q43043	L27101	374	nd	50
	Medicago sativa (pollen specific)	na	U28148	447	nd	46
5	*Melandrium album (Silene latifolia)* (flower buds)	Q96497	Y08155	379	nd	44
	Vitis riparia (flower buds, GN: PME)	na	AAD51853	336	nd	34
	Aspergillus aculeatus (strain KSM 510)	Q12535	U49378	331	18Ala	35
	Aspergillus tubingensis (A. niger, strain RH 5344)	P17872	X52902	331	18Ala	33
	Aspergillus oryzae (strain KBN 616)	O94162	AB011211	331	18Ala	35
	Erwinia chrysanthemi (PemA)	P07863	Y00549	366	25Ala	31
	Erwinia chrysanthemi (PemB)	Q47474	X84665	433	Blocked	24
	Burkholderia solanacearum (strain DSM 50905)	P24791	M49747	396	nd	30

The second principal point evident from the overall comparisons is the fact that the two Asp residues implied at the active site in the two crystallographically investigated forms are indeed conserved, but no Ser residues. Hence, all forms are aspartyl esterases, and not serine esterases as initially suspected (SwissProt releases until 1999).

Finally, the overall alignments derived from the structures in table 2 allow construction of phylogenetic relationships. Some of the sub-branching then obtained has low significance (low bootstrap numbers) and the phylogenetic trees are therefore not considered final, but some sub-grouping is significant, as summarized in table 2. Thus, the top ten forms constitute one clear clade (identity values 74% and above; table 2), and in this sub-group, many additional residues are conserved, including three cysteine residues (at positions 129, 150, and 170), proving further similarities in potentially important residues within each sub-group. Similarly, two of the plant enzymes (corresponding to the enzymes from flower buds of *Melandrium album* and *Vitis riparia*, group 5 in table 2)

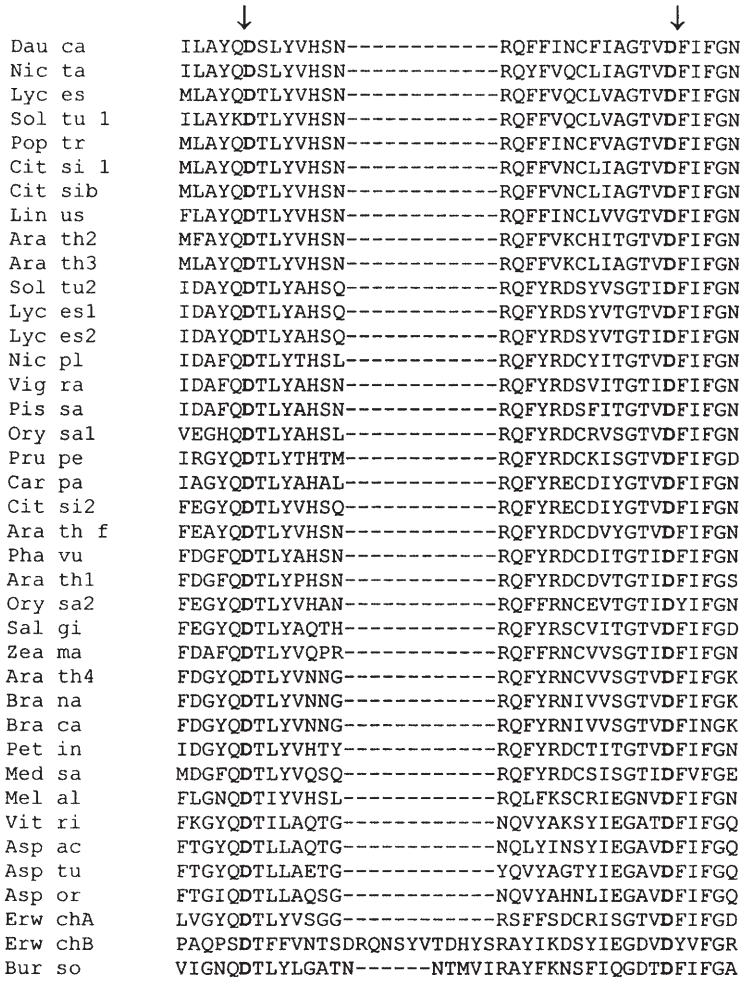

Figure 2. Amino acid sequences around the two active site Asp residues (Asp 136 and Asp 157 in the carrot enzyme) in the 39 homologous enzymes with determined structures that have now been compared. The two Asp residues are marked in bold and indicated by arrows (top). Enzyme order is the same as the phylogenetically derived order of table 2, (abbreviated species names are shown on the left). As shown, inter-Asp-distances are different and residue conservation minimal in the most distantly related forms at the bottom.

form a separate branch in the corresponding phylogenetic tree, and group with the microbial enzyme forms rather than with other plant forms. Two other forms (from *Phaseolus vulgaris* and *Arabidopsis thaliana*, group 3 in table 2) also form a deep-rooted separate branch in the phylogenetic tree, showing that distinct, separate enzyme-folding solutions were formed early on. Most of the intervening plant enzymes (group 2, table 2) are more poorly distinguished, with insignificant branch separations in the present phylogenetic trees. In conclusion, the tree relationships establish at least four fairly well separated enzyme groups within this large protein family. The successive decrease in identity levels in table 2 represents both an overall similarity in a clear enzyme family, and distinct branch differences in particular cases.

The 13 strictly conserved residues outlined above, including the functionally important catalytic Asp residues, are inter-related in the three-dimensional structure interpretations [24] but were noticeable at an early stage from sequence alignments of the multiple species in table 2. Structural characterization of species variability is clearly useful, and comparisons of all forms can identify residues with structural or functional importance.

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