Role of lysine, tryptophan and calcium in the β -elimination activity of a low-molecular-mass pectate lyase from *Fusarium moniliformae*

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An extracellular pectate lyase from *Fusarium moniliformae* was purified to homogeneity by affinity chromatography followed by gel filtration, with a yield of 76.5%. Laser desorption MS of the enzyme gave a molecular mass of $12\,133.5 \pm 2.5$ Da. The pectate lyase was a glycoprotein with a 5% carbohydrate content and had a pI value of 9.1. Atomic-emission spectrometry showed that Ca^{2+} was a part of the holoenzyme held by carboxy groups of the protein. These results support the hypothesis of a putative Ca^{2+} site suggested by Yodder, Keen and Jurnak [(1993) *Science* **260**, 1503–1507] in the crystal structure of pectate lyase C of *Erwinia chrysanthemi*. Loss of Ca^{2+} was observed by treatment with EGTA or carboxy-modifying Woodward's reagent K, with subsequent loss of enzyme activity. Tryptophan fluorescence quenching showed that Ca^{2+} does not affect binding of substrate

to enzyme. Chemical-modification and substrate-protection studies showed the presence of lysine and tryptophan at or near the active site of the pectate lyase. Chemically modified enzyme showed no major structural changes as determined by CD. Amino acid analyses of native, trinitrobenzenesulphonate (TNBS)-treated and substrate-protected TNBS-treated enzyme showed that a single essential residue of lysine is present at or near the active-site. Substrate-affinity studies showed that tryptophan could be essential for substrate binding, whereas lysine could be involved in the catalysis. Fluorescence quenching further confirmed the involvement of tryptophan in substrate binding. The reaction mechanism involving β -elimination by this enzyme is discussed.

INTRODUCTION

Pectin-degrading enzymes are produced by many plant-associated micro-organisms and several saprophytes. Pectic enzymes have been reported from many molds such as species of *Aspergillus* [2], *Cladosporium* [3], *Fusarium* [4,5], *Geotrichum* [6], *Penicillium* [7] and *Sclerotinia* [8]. Pectic polymers, consisting predominantly of 1,4-linked D-galacturonic acid and methoxylated derivatives, are major constituents of the middle lamellae and primary cell walls of higher plants [9]. Although a lot of work has been done on the purification and characterization of pectate lyase of various micro-organisms [1,3,5,10–17], nothing is known about the identity of the functional groups involved in the catalysis. Pectate lyase exhibits a β -elimination mechanism [18] in the cleavage of D-(1-4) glycosidic bonds of polygalacturonic acid. Calcium was found to be essential for the activity of all pectate lyases reported, but it is not clear whether it is part of the enzyme or involved in substrate binding. Crawford et al. [16] had suggested that a dimer of polygalacturonic acid with Ca^{2+} was the substrate for the pectate lyase, whereas Yodder et al. [18a] had identified a putative calcium site in the crystal structure of pectate lyase from *Erwinia chrysanthemi*.

We report here the characterization of a pectate lyase from *Fusarium moniliformae*, with respect to the active site and the role of calcium in the activity of this enzyme.

METHODS

A strain of *Fusarium* was isolated from a tropical mangrove community and was identified as *Fusarium moniliformae*. It was subcultured on Czapek–Dox agar, modified with the addition of 0.5% pectin. The organism has been deposited in the National Collection of Industrial Micro-organisms as *Fusarium moniliformae* N.C.I.M. 1276.

Enzyme assay

Pectate lyase activity was measured in 60 mM Tris/HCl buffer, pH 9.6, with 0.6 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, by the increase in absorbance at 232 nm of 0.24% polygalacturonic acid. One unit of lyase activity was defined as the amount of enzyme which produced 1 μmol of unsaturated galacturonide (ϵ 4600 $\text{M}^{-1} \cdot \text{cm}^{-1}$)/min [9].

Purification and preliminary characterization of pectate lyase

The organism was grown in medium containing 1% pectin, 0.2% glucose, 0.5% $(\text{NH}_4)_2\text{SO}_4$, 0.2% K_2HPO_4 , 0.2% KH_2PO_4 and 0.15% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The pectate lyase was purified by the method of Ward and DeBoer [19], except that calcium was replaced by barium. The enzyme was then loaded on to a Bio-Gel P-30 gel-filtration column equilibrated with 0.1 M Tris/HCl (pH 8.0)/0.1 M NaCl. Homogeneity was checked by SDS/PAGE.

The molecular mass of the pectate lyase was determined by gel filtration using standard molecular-mass markers and was rechecked by equilibrium sedimentation on a Beckman model E analytical ultracentrifuge with schlieren optics. A laser-desorption time-of-flight analysis of the protein to determine the molecular mass was performed on a Finnigan LaserMat mass analyser with an accuracy of 0.1%. An internal standard of β -lactoglobulin B, with a molecular mass of 18278.2 Da, was used. The pI of the enzyme was determined by the modified straight-tube method [20].

Active-site studies

Chemical-modification studies were performed by incubating 0.73 μM enzyme with various modifiers in appropriate buffers for a period of 30 min. Control tubes without the inhibitor were

Abbreviations used: DEP, diethyl pyrocarbonate; HNBB, hydroxynitrobenzyl bromide; TNBS, trinitrobenzenesulphonate; DTNB, dithiobis-(nitrobenzoic acid); PHMB, *p*-hydroxymercuribenzoate; WRK, Woodward's reagent K (2-ethyl-5-phenylisoxazolium-3'-sulphonate); CM, carboxymethyl.

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incubated under identical conditions. *N*-Acetylimidazole, *p*-hydroxymercuribenzoic acid (PHMB), hydroxynitrobenzyl bromide (HNBB) and PMSF were used at a concentration of 10 mM in 50 mM phosphate buffer, pH 7.0. Dithiobis(nitrobenzoic acid) (DTNB), succinic anhydride, diethyl pyrocarbonate (DEP) and trinitrobenzenesulphonate (TNBS) were used at a concentration of 10 mM in 50 mM phosphate buffer, pH 8.0. Phenylglyoxal at a concentration of 10 mM was used in 50 mM carbonate buffer, pH 8.0. Photoreaction of 0.73 μ M enzyme with 0.0005% Methylene Blue in 50 mM phosphate buffer, pH 7.0, was allowed to take place with irradiation with a 250 W visible light placed 20 cm away from the reaction mixture.

The effect of pH on the enzyme was studied by incubating the enzyme and the inhibitor in buffers at various pH values. Sodium phosphate buffer was used for pH 5.5, 6.5 and 7.5, and carbonate buffer for pH 8.5, 9.5 and 10.5.

Amino acid analyses of native enzyme, TNBS-treated enzyme and substrate-protected TNBS-treated enzyme were done. The TNBS-treated pectate lyase was obtained by incubating 20 nmol of enzyme with 5 mM TNBS for 30 min followed by extensive dialysis in deionized water. For the substrate-protected enzyme, polygalacturonic acid at a concentration of 2 mg/ml was added prior to addition of the inhibitor. Amino acid analysis was done on a Pharmacia LKB Alpha Plus amino acid analyser by hydrolysing the purified enzyme in 6 M HCl at 110 °C for 22 h. Cysteine, methionine and tyrosine were protected. Tryptophan was measured by titration with *N*-bromosuccinimide [21].

The kinetics of inactivation of pectate lyase by TNBS and HNBB were carried out by incubating the enzyme at various concentrations of the inhibitors. Aliquots were removed at regular intervals and assayed for residual activity. The TNBS incubations were carried out in the dark because of the sensitivity of the TNP-derivatives.

CD spectra over the range of 190–350 nm were obtained for the native pectate lyase and TNBS-, HNBB- and EGTA-treated enzyme using a JASCO J-500 spectropolarimeter.

Fluorescence scans of the pectate lyase and the enzyme/substrate mixtures were obtained using a Kentron SFM-25 spectrofluorimeter. Tryptophan fluorescence was excited at 297 nm by using a 150 W xenon high-pressure lamp and a monochromator with concave holographic gratings. Emission scans over the range 310–390 nm were obtained using a similar monochromator and a R212 photomultiplier.

Pectate lyase was modified with Woodward's reagent K (WRK) by adding aliquots of a 1 M stock of the inhibitor in 1 mM HCl. WRK has been known to react with acidic side groups of aspartate or glutamate residues in proteins [22,23].

The calcium content of 50 μ M native pectate lyase, EGTA-treated enzyme and WRK-modified enzyme was determined by inductively coupled plasma/optical-emission spectrometry on a Perkin-Elmer Plasma 1000 emission spectrometer at an emission wavelength of 393.366 nm. Enzyme samples were dialysed in deionized water in polycarbonate bottles which were previously washed in 1 M HCl, 1 M HNO₃ and deionized water

RESULTS and DISCUSSION

Enzyme purification

The pectate lyase was purified by affinity chromatography on the basis of the method of Ward and DeBoer [19], the only change being the use of a different bivalent metal ion for precipitation of polygalacturonic acid. We obtained a greater yield with Ba²⁺ than with Ca²⁺ (results not shown). Further purification to obtain a pure enzyme preparation was achieved by gel filtration

Figure 1 Gel filtration of pectate lyase on Bio-Gel P-30

The affinity eluate was concentrated and loaded on a P-30 column equilibrated with 0.1 M glycine/NaOH buffer, pH 9.6. ○, A_{280} ; ▲, pectate lyase activity [units (U)/ml].

Table 1 Purification of pectate lyase

	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Broth	1000	5200	582	8.9	—	—
Affinity elution	118	4420	11.2	395	85	44.4
Bio-Gel P-30	15	3978	7.5	530.1	76.5	59.5

Figure 2 Analytical ultracentrifugation of pectate lyase

(a) Sedimentation coefficient determination on a Beckman model E analytical centrifuge with Schlieren optics in a 4–12 mm cell in an AnHTi rotor at 60 000 rev./min. (b) Archibald sedimentation equilibrium run at 20 000 rev./min. (c) Synthetic boundary run at 10 000 rev./min.

on Bio-Gel P-30 (Figure 1) with an high yield of 76.5% and a purification of 59.5-fold (Table 1). The molecular mass of this pectate lyase as determined by gel filtration was 11 000 and by equilibrium-sedimentation ultracentrifugation was 10 600 (Figure 2). However, SDS/PAGE indicated a molecular mass of about 17 500. Glycoproteins are known to behave differently on SDS/PAGE compared with non-glycosylated proteins and give a higher molecular mass. Using time-of-flight (TOF) laser-desorption MS, the molecular mass of the pectate lyase was determined to be 12133.5 ± 2.5 Da, compared with an internal standard of β -lactoglobulin B having a molecular mass of 18 278.2 Da. We therefore conclude that the protein has a molecular mass of 12133.5 ± 2.5 . Pectate lyase from *Fusarium solani* has been reported to have a molecular mass of 26 kDa [16],

Figure 3 Loss of calcium of pectate lyase with WRK inhibition

The calcium content of 50 μ M native pectate lyase and 28%, 43%, 67%, 88% and 100% WRK-inhibited pectate lyase was determined.

Figure 4 CD spectra of native and EGTA-treated pectate lyase

Trace a, native enzyme; trace b, EGTA-treated pectate lyase.

while the enzyme for *F. oxysporum* f.sp. *ciceri* has a molecular mass of 25 kDa [5]. The molecular masses of pectate lyases from other species vary from 20 kDa to 74 kDa [3, 10–13, 15, 17]. Thus the present enzyme has the lowest molecular mass reported so far.

The pI of the pectate lyase was 9.1. The enzyme was a glycoprotein with a carbohydrate content of 5%. The K_m of the purified enzyme was 1.2 mg/ml and the V_{max} was 655 units/mg with polygalacturonic acid. The enzyme had an absolute requirement for Ca^{2+} . No other bivalent ion could replace Ca^{2+} for activity, With the exception of Ba^{2+} , which gave an activity 8% of that obtained with Ca^{2+} .

The presence of calcium in the enzyme and its role

Atomic-emission spectroscopy gave a molar ratio (metal/protein) of 0.82, showing that a single Ca^{2+} ion must be bound to the pectate lyase. When the enzyme was treated with 2 mM EGTA or 10 mM WRK, the enzyme lost total activity along with the loss of metal ion. Partial blocking by WRK (Figure 3) modified the enzyme at the carboxy sites and showed loss of activity in proportion to the loss of calcium. Thus the Ca^{2+} ion must be held by carboxy groups, probably those of aspartate or glutamate residues in the enzyme. CD of native and EGTA-treated enzyme (Figure 4) showed that loss of Ca^{2+} did not alter the overall secondary structure of the protein. Thus the metal ion does not

Figure 5 Fluorescence quenching of pectate lyase in presence of substrate

Emission scans of pectate lyase when excited at 297 nm were recorded: trace a, 0.25 mg/ml enzyme; b, 0.25 mg/ml enzyme + 0.08 mg/ml polygalacturonic acid with 1 mM Ca^{2+} ; c, 0.25 mg/ml enzyme + 0.08 mg/ml polygalacturonic acid with 1 mM EGTA.

contribute to the conformation of the protein. Our studies support the theory of Yodder et al. [18a], who identified a putative Ca^{2+} -binding site in the crystal structure of the pectate lyase C of *E. chrysanthemi*. In their studies, Ca^{2+} could not be included in the $(NH_4)_2SO_4$ crystallization solution, but they could use heavy atoms like lutetium, uranium and lead. These atoms were located at a common site, 0.21–0.29 nm (2.1–2.9 Å) from three acidic residues, Asp-131, Glu-166 and Asp-170, suggesting that the common heavy-atom site is the putative Ca^{2+} -binding site on the protein. In the crystal structure of pectate lyase of *Bacillus subtilis* Pickersgill et al. [24] identified aspartic and glutamic residues involved in calcium binding. By using biochemical techniques and atomic-emission spectroscopy, the pectate lyase of *F. moniliforme* in solution form is shown to be a calcium-containing metalloprotein and carboxy groups are shown to be involved in Ca^{2+} binding.

Spectrofluorimeter scans of the present enzyme showed that quenching of tryptophan fluorescence, in the presence of polygalacturonic acid, occurred irrespective of the presence or absence of Ca^{2+} (Figure 5). Since the loss of Ca^{2+} ion is proportional to the loss of activity and the absence of Ca^{2+} does not affect substrate binding, it may be necessary for catalysis. These results argue against the suggestions made by Crawford et al. [16] that Ca^{2+} is required to complex the substrate and is not directly involved in catalysis.

Involvement of lysine and tryptophan in the active centre

Chemical modification of pectate lyase with various inhibitors specific for certain protein side-chain groups was done in order to identify amino acids essential for the activity of the enzyme. There was complete inhibition of pectate lyase by succinic anhydride and TNBS, indicating that an essential lysine residue

Table 2 Amino acid analyses for determination of number of essential lysine residues

Residue	Composition (mol/mol)		
	Native PL	TNBS-treated	Substrate-protected
Asx	10.8	11.1	10.4
Thr	7.8	7.8	8.3
Ser	6.7	6.6	7.4
Glx	7.7	8.1	8.3
Gly	12.1	12.1	12.5
Ala	6.6	6.9	7.2
Val	6.0	5.8	6.1
Cys	0.8	—	—
Ile	3.2	3.0	3.6
Leu	3.6	3.7	4.1
Met	0.7	—	—
Tyr	4.7	4.9	5.2
Phe	2.7	2.7	3.3
His	1.6	2.0	2.4
Lys	6.6	2.1	3.4
Arg	2.2	2.3	2.1
Trp	3.1	—	—

was modified. The enzyme was inactivated by DEP, but there was no reversal of inhibition after addition of hydroxylamine. Furthermore, there was no photoinactivation with Methylene Blue, suggesting that histidine is not involved in the activity of the enzyme [25]. The inhibition by DEP could therefore be due to modification of an essential lysine residue of the enzyme. The pH-dependence of reaction by DEP showed maximum inhibition between pH 9.5 and 10.5, giving a pK of 7.0–7.5 for the probable lysine residue. The lowered pK may be due to the micro-environment of the residue in the protein. There was no inhibition with DTNB or PHMB, so a role for cysteine in enzymic activity could be ruled out. Complete inhibition by HNBB indicated an essential tryptophan residue. Absence of inhibition by *N*-acetyl-imidazole, PMSF and phenylglyoxal indicated that tyrosine, serine and arginine were not essential for enzyme activity. A 2 mg/ml concentration of polygalacturonic acid, added prior to the addition of the inhibitors (succinic anhydride, TNBS, DEP and HNBB), gave complete protection against inhibition. This indicates the presence of essential lysine and tryptophan residues at or near the active site of the enzyme.

The kinetics of inhibition of pectate lyase by TNBS and HNBB were followed by estimating the residual activity at different time periods with different inhibitor concentrations. The inhibition followed pseudo-first-order rate kinetics, with the rate constants varying linearly with inhibitor concentrations [26]. The order of the reaction with TNBS was 0.94 and that with HNBB was 0.96. Thus the inactivation of the enzyme with both the inhibitors followed a single exponential.

The amino acid analyses of the native, TNBS-treated and substrate-protected TNBS-treated pectate lyase (Table 2) showed that a single residue of lysine is present at or near the active site, which is protected by the substrate against reaction with the inhibitor. Thus the inhibition kinetics with TNBS, which follows a single exponential, is due to the inactivation of a single essential lysine residue of the enzyme.

The CD spectra of native and chemically modified pectate lyase were found to be similar, indicating that there were no major structural changes in the protein induced by the chemical reagents.

Figure 6 Fluorescence quenching of pectate lyase at different substrate concentrations

Emission scans of pectate lyase when excited at 297 nm were recorded. Traces: a, 0.25 mg/ml enzyme; b, 0.25 mg/ml enzyme + 0.5 mg/ml polygalacturonic acid; c, 0.25 mg/ml enzyme + 0.1 mg/ml polygalacturonic acid; d, 0.25 mg/ml enzyme + 0.15 mg/ml polygalacturonic acid; e, 0.25 mg/ml enzyme + 0.2 mg/ml polygalacturonic acid.

Affinity studies of pectate lyase were performed by adding 1 ml of 0.3 mg/ml solution of polygalacturonic acid to 1 ml of an 8 μ g/ml solution of the enzyme, followed by precipitation of the substrate with 20 μ l of 1 M CaCl_2 . The supernatant was analysed for activity and protein. Lysine-blocked enzyme co-precipitated with polygalacturonic acid on addition of calcium, whereas the tryptophan-blocked enzyme was retained in the supernatant. This would be the case if tryptophan rather than lysine was involved in substrate binding. Secondly, although the native protein binds irreversibly to carboxymethyl (CM)-Sephadex, the presence of substrate inhibits such binding, probably because it takes place through the active site. Tryptophan-blocked enzyme did not bind to CM-Sephadex, whereas the lysine-blocked enzyme did. Thirdly, fluorescence of the enzyme, when excited at 297 nm, was quenched in the presence of polygalacturonic acid (Figure 6). These results argue strongly that tryptophan is involved in substrate binding. Tryptophan is reported to be present in the active site of various carbohydrases such as the endo-1,4- β -glucanase of *Bacillus* sp.KSM-330 [27], an exo-(1-3)- β -D-glucanase from *Rhizoctonia solani* [28], an enzyme (exhibiting both isomaltase and dextranase activity) from *Arthrobacter globiformis* T6 [29], a xylanase from *Streptomyces* [30] and also in the active site of Abrin-a, a bivalent lectin [31]. Tryptophan appears to have affinity with carbohydrates, and its involvement in the substrate binding of carbohydrate-degrading enzymes is therefore not surprising.

Lysine has been reported to be involved in the catalytic site of arginosuccinase [32], γ -cystathionase [33] and aspartate aminotransferase [34], probably as a proton acceptor. In the present case we suggest that lysine is a good candidate for proton abstraction in the β -elimination cleavage of polygalacturonic acid.

Secondary structure of pectate lyase

The CD spectrum of the native pectate lyase (Figure 4) was investigated to correlate the shape of the spectrum with the tertiary class of the protein as described by Venyaminov [35]. Cluster analysis using pattern-recognition algorithms showed that our pectate lyase falls in the all- β tertiary structure class. Yodder et al. [18a] have shown from its crystal structure that the *Erwinia* pectate lyase folds into a unique motif of parallel β -strands coiled into a large helix. The authors had also suggested that, because of the sequence similarities, all extracellular pectate lyases may have a common β -helix motif, differing only in the size and confirmation of the protruding loops. The crystal structure of pectate lyase of *B. subtilis* also has a similar topology of a parallel β -helix. The dominance of β -folds in the structure of the present pectate lyase suggests that this protein could have a tertiary structure similar to those of other reported bacterial pectate lyases.

Reaction mechanism of pectate lyase

The catalytic mechanism for the degradation of polygalacturonic acid by pectate lyase is a typical β -elimination process. The basic mechanism involves a proton abstraction from C-5 of one galacturonide moiety by the enzyme. The abstraction of a proton, possibly by the ϵ -amino group of lysine in the active site of the enzyme, results in the breaking of the C–O bond and formation of a double bond between C-4 and C-5.

The high rates of enzyme-catalysed abstraction of proton from a carbon adjacent to a carbonyl or carboxy group would not be explained by a simple base-catalysed heterolytic cleavage of C–H bonds. Although the α -proton of a carbon acid is more acidic than an aliphatic proton, the inductive effect of the carboxy group is insufficient to decrease the pK_a difference of the α -proton and the catalytic base. According to Gerlt and Gassman [36], the pK_a of the carbon acid could be sufficiently decreased by an additional acid catalyst acting on the carbonyl or carboxy group. Such concerted general acid–general base catalysis would explain the high rates of the β -elimination reactions. They later proposed that prepositioning of the electrophilic catalyst adjacent to the carboxy group would decrease the intrinsic kinetic component of the activation-energy barrier of the reaction [37], thus favouring the base-catalysed proton abstraction from the α -carbon.

In extending the above theory of a concerted acid–base catalysis to the β -elimination reaction of pectate lyase, the abstraction of the C-5 proton by the base, presumed to be the ϵ -amino group of lysine, would require a general acid catalyst acting on the carboxy group. Therefore it is proposed that the Ca^{2+} of the pectate lyase would satisfy such a requirement by acting as a Lewis acid. The polarization of the carboxy group by Ca^{2+} would acidify the α -proton at C-5 sufficiently for abstraction by the catalytic base of the enzyme. Considering the high pH optimum of the pectate lyase, the requirement of a general acid catalyst would not be satisfied by any side-chain groups in the protein. Although calcium is known predominantly to have structural roles in most calcium-containing metalloenzymes, it has been reported to be catalytically important in some enzymes. Cotton et al. [38] had proposed that calcium facilitates the nucleophilic attack of staphylococcal nuclease on thymidine 3',5'-biphosphate by polarizing the 5'-phosphate group. Similarly Suck and Oefner [39] had proposed that Ca^{2+} of bovine pancreatic deoxyribonuclease I facilitates nucleophilic attack on the substrate through polarization of a phosphate group. Thus pectate lyase appears to catalyse a concerted general acid–general base

β -elimination, using Ca^{2+} as a Lewis acid and ϵ -amino group of lysine as a base.

Conclusion

A low-molecular-mass pectate lyase was purified from the culture filtrate of *F. moniliformae*. We have shown the requirement for lysine, tryptophan and calcium ion in the activity of the pectate lyase. Tryptophan was found to be involved in substrate binding, whereas lysine is suggested to play the role of proton acceptor in the reaction mechanism of the enzyme. The β -elimination cleavage would involve proton abstraction from the galacturonide monomer of the substrate by the enzyme. The splitting of the glycoside bond takes place with the formation of a double bond. Calcium was found to be part of the active enzyme, and we suggest that the calcium ion may be required to polarize the carboxy group of the substrate in order to facilitate proton abstraction.

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