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Purification and characterization of pectate lyase from banana (*Musa acuminata*) fruits

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Abstract

Pectate lyase (PEL) has been purified by hydrophobic, cation exchange and size exclusion column chromatographies from ripe banana fruit. The purified enzyme has specific activity of 680 ± 50 pkat mg protein^{-1}. The molecular mass of the enzyme is 43 kDa by SDS–PAGE. The pI of the enzyme is 8 with optimum activity at pH 8.5. Analysis of the reaction products by paper and anion exchange chromatographies reveal that the enzyme releases several oligomers of unsaturated galacturonane from polygalacturonate. The K_m values of the enzyme for polygalacturonate and citrus pectin (7.2% methylation) are 0.40 ± 0.04 and 0.77 ± 0.08 g l^{-1}, respectively. PEL is sensitive to inhibition by different phenolic compounds, thiols, reducing agents, iodoacetate and *N*-bromosuccinimide. The enzyme has a requirement for Ca^{2+} ions. However, Mg^{2+} and Mn^{2+} can substitute equally well. Additive effect on the enzyme activity was observed when any two metal ions (out of Mg^{2+}, Ca^{2+} and Mn^{2+}) are present together. The banana PEL is a enzyme requiring Mg^{2+}, in addition to Ca^{2+}, for exhibiting maximum activity.

Keywords: *Musa acuminata*; Banana; Enzyme purification; Pectate lyase; Metal ions

1. Introduction

Pectins are present as structural polysaccharide in the middle lamella and primary cells of higher plants, including fruits. They play key physiological and developmental roles in plant cell walls. Its degradation requires the combined action of methylesterases, which remove methoxy groups from pectin, and depolymerases which cleave the bonds between galacturonate units. Pectate lyase [poly (1,4-α-D-galacturonide) lyase; EC 4.2.2.2.] catalyzes the cleavage of $(1 \rightarrow 4)$ galacturonan linkages of pectate by β-elimination reaction, generating 4,5-unsaturated oligogalacturonates. Pectate lyases (PEL) are widely distributed in bacteria (Pèrombelon and Kelman, 1980) and fungi (Benen et al., 2000) and have been extensively studied in plant pathogenic bacteria, *Erwinia chrysanthemi*, which causes maceration of parenchymatous tissues of various dicot plants (Pèrombelon and Kelman, 1980). These enzymes act by depolymerizing cell wall polygalacturonane in the presence of calcium ions, thus destroying the integrity of the plant tissues (Collmer and Keen, 1986). Genes encoding PEL (*pels*) have been reported from several plant sources, e.g., pollens (Wing et al., 1989), style (Budelier et al., 1990), rag weed (Griffith et al., 1991), maize (Turcich et al., 1993), Japanese cedar (Sone et al., 1994), *Zinnia elegans* (Milioni et al., 2001), opium poppy latex (Pilatzke-Wunderlich and Nessler, 2001), strawberry (Medina-Escobar et al., 1997), grape (Nunan et al., 2001). PEL sequences of a recombinant protein have also been reported from banana (Pua et al., 2001; Mańin-Rodŕiguez et al., 2003), but the enzyme activity has only been recently demonstrated (Mańin-Rodŕiguez et al., 2003; Payasi and Sanwal, 2003). PELs have been purified and characterized from several microbial sources (Favey et al., 1992; Bruhlmann, 1995; Liao et al., 1997; Kobayashi et al., 1999; Kluskens

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et al., 2003), but studies from plant sources are lacking (Mañin-Rodŕiguez et al., 2002). To the best of our knowledge no report exists on purification of PEL to homogeneity from plant sources. The following studies were undertaken on purification and characterization of PEL from banana fruits. This is a first report that describes purification and characterization of PEL from plant sources as well as describes Mg^{2+} slightly more efficient than Ca^{2+} in catalyzing the PEL reaction.

2. Results

2.1. Purification of pectate lyase

A partial purification of the enzyme has been achieved by negative adsorption on Q-sepharose and positive adsorption on strong cation exchanger, SP-sepharose. Hydrophobic interaction employing Phenyl sepharose as well as gel filtration through Ultrogel AcA-54 and Sephadex G-75 were most effective. PEL was eluated as a single peak at ~0.57 M $(NH_4)_2SO_4$ from Phenyl sepharose column, resulting in ~3.9 fold purification with 62% recovery of the enzyme (Fig. 1A). The enzyme adsorbed on SP-sepharose column could be eluated in ~0.44 M NaCl with ~1.5 fold purification (Fig. 1B). Ultrogel AcA 54 chromatography resulted in ~3.3 fold purification of the enzyme (Fig. 1C). Gel filtration of the purified enzyme through Sephadex G-75 resulted in further 2 fold purification of the enzyme (Fig. 1D). A typical purification of PEL from banana fruits is summarized in Table 1. These steps led to purification of the enzyme by about 120 fold with about 4% recovery from the initial extract. The average specific activity of the purified enzyme from five trials was 680 ± 50 pkat mg protein^{-1} with 3–5% recovery from the initial extract.

2.2. Biochemical characterization

The purified enzyme gave one single band on PAGE when stained with Coomassie blue as well as by more sensitive Silver nitrate (Fig. 2). The purified enzyme also gave a single peak on elution from protein pak glass 300 SW column employing Waters FPLC system. The molecular weight of the purified enzyme determined by gel filtration on Sephadex G-75 column was 44 kDa. The sub unit molecular weight of the enzyme was found 43 kDa by SDS–PAGE, which indicates that the enzyme is a monomeric protein. The electrophoresis pattern of the purified enzyme in the presence of ampholyte (pH range 3–10) indicated pI of 8 for the enzyme.

2.3. Identification of the reaction products

The enzyme assay with PGA as substrate was run for 3 h and then subjected to descending paper chromatography and visualized by bromophenol blue. Whereas the control did not yield any yellow spot for carboxylic acid, the experimental system yielded several yellow spots on blue background with R_{gal} values (the ratio of product migration to the distance migrated by a D-galacturonic acid standard) of 0.07, 0.18, 0.38, 0.58, 0.68 and 0.79. Thus the reaction products of PEL action consisted of several oligomeric galacturonic acids.

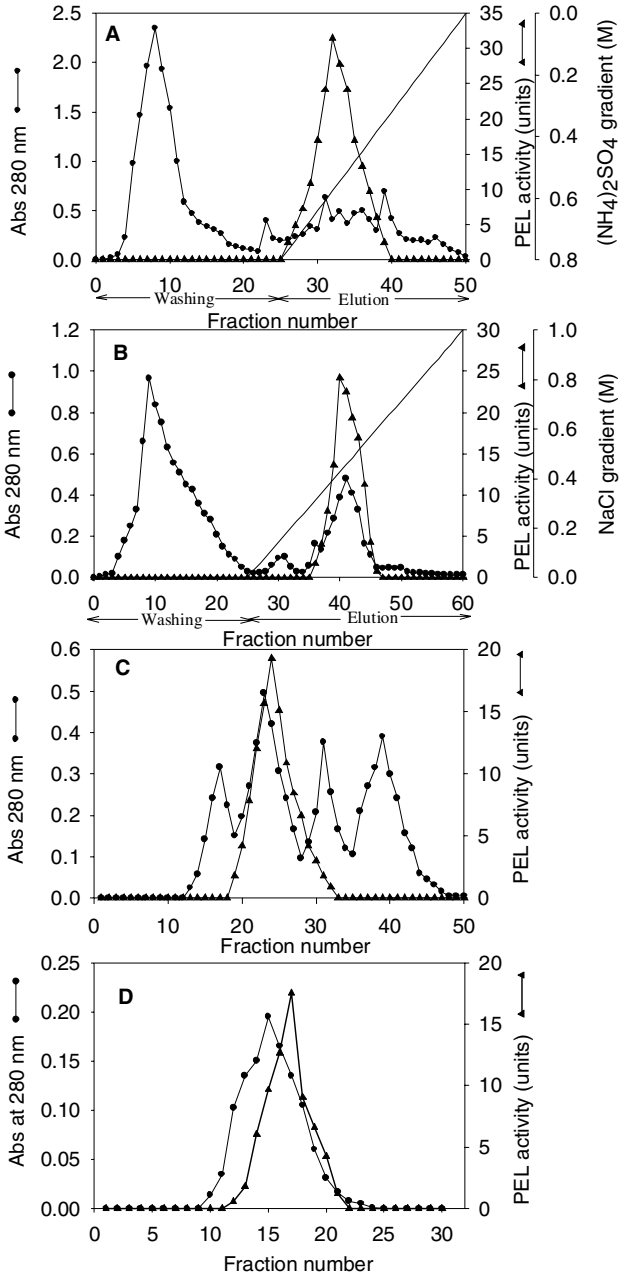

Fig. 1. Purification of PEL from ripe banana fruit employing chromatographies on (A) Phenyl sepharose: 45 ml out of 90 ml of Q-sepharose fraction was loaded. (B) SP-sepharose (C) Ultrogel AcA 54 (D) Sephadex G-75.

The elution of oligomeric galacturonic acid residues from Dowex-1-X-4 column gave six peaks of the reaction product based on absorption at 232 nm. These peaks exhibited maximum absorption between 232 and 236 nm, indicating unsaturation nature of the reaction products.

Table 1
Purification of PEL from ripe banana fruits

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units mg protein^{-1})	Purification (fold)	Recovery (%)
Initial extract[a]	600	631	3660	5.8	1	100
Ammonium sulfate	120	361	3480	9.6	1.7	95
Q-Sepharose	90	135	2340	17	3.0	64
Phenyl sepharose	48	21.6	1440	67	11.6	39
SP-sepharose	32	5.8	580	100	17.2	16
Ultrogel AcA-54	10	0.6	200	333	57	5.5
SephadexG-75	12	0.2	140	700	120	4

[a] Enzyme activity was determined in the extract dialysed against 0.02 M sodium phosphate buffer, pH 7.0.

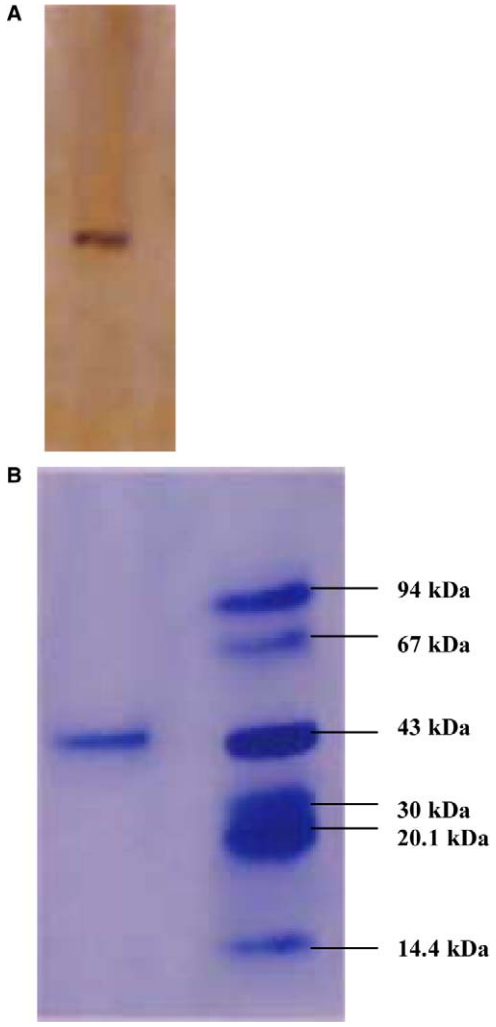

Fig. 2. PAGE and SDS/PAGE of purified PEL. (A) Non-denaturing PAGE of the purified enzyme (5 μg) on a 12% polyacrylamide gel and staining with silver nitrate. (B) SDS–PAGE of the purified Enzyme (15 μg) on a 12% polyacrylamide gel and staining with coomassie blue. The marked line indicates the positions of the molecular mass markers (in kDa).

The product present in the major peak appears to be an unsaturated galacturonic acid polymer as the compound on chromatography in the solvent system: ethylacetate–pyridine–water–acetic acid and spraying with carbazole reagent or bromophenol blue gave a single spot, the compound exhibited maximum absorption at 232 nm and when allowed to react with TBA after periodate oxidation, exhibited absorption with maximum at 548 nm.

2.4. Mode of enzyme action

The rate of reduction in viscosity of polygalacturonic acid (PGA) as well as the liberation of unsaturated digalacturonate by enzymatic degradation were determined every 10 min for 60 min at the room temp (30 °C) . The rate of reduction in viscosity was found higher than the liberation of unsaturated digalacturonate (Fig. 3), indicating *endo*-nature of banana PEL.

2.5. Stability of the purified enzyme

The purified enzyme in sodium phosphate buffer (0.02 M pH 7.0) was stable for at least 15 days when stored at 2–5 °C, while the activity was lost within two days on storage at room temperature (30 °C). The enzyme was unstable under acidic condition and a loss of 30% and 90% activity was observed in 3 and 24 h, respectively, at

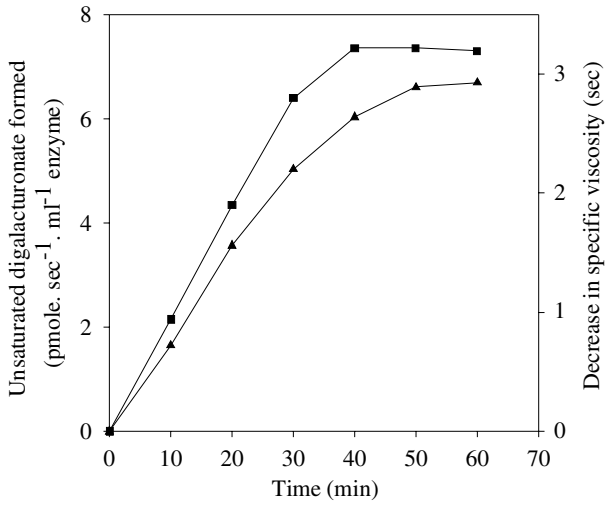

Fig. 3. Changes in viscosity and PEL activity of a PGA solution during incubation with PEL enzyme. Decrease in viscosity, (■); liberation of unsaturated digalacturonate (▲).

pH 5.0. However, the enzyme was stable at neutral (7.0) and alkaline (8.5) pH. The purified enzyme was fairly stable on heating at 40 °C for 5 min, and retained 90–95% of the activity. Heating at higher temperature led to progressive inactivation of the enzyme with a complete loss of activity at 80 °C.

2.6. pH and temperature optima

The purified enzyme showed optimum activity at pH 8.5 in 33 mM Tris/HCl and 15 mM Bicine buffers (Fig. 4).The activity in Tris buffer was slightly low compared to Bicine buffer at all pH values except at pH 8.5, where the activity was almost same. The activity at pH 7.0 was 30 % of the activity at pH 8.5. The enzyme exhibited optimum temperature at 37 °C. Above the optimal temperature, the activity dropped sharply, with only 70% and 55% being retained at 45 and 50 °C, respectively. Arrehenius plot of the data covering the range 25–40 °C indicated Energy of Activation of 43 kJ mol^{-1}.

2.7. Substrate specificity

The purified enzyme was assayed with pectin of various degree of methylation by using the standard assay mixture but substituting polygalacturonic acid (PGA) with methyl esterified pectin. In each case 0.6% (w/v) substrate in Tris buffer (pH 8.5) was used. PGA was better substrate for the enzyme than methyl esterified pectins (Table 2). When PGA degradation rate was taken as 100%, the relative rates towards pectin with degree of esterification of 7.2%, 26% and 65% were 51, 25 and 17, respectively.

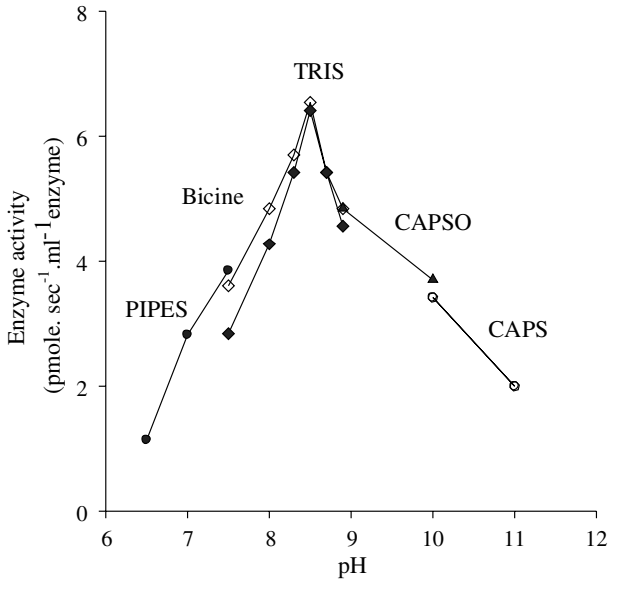

Fig. 4. pH activity profile of PEL enzyme in different buffer. The activity was determined in 0.05 M buffer but, in case of bicine 0.02 M buffer was used. Pipes (●), Bicine (□), CAPSO (▲), CAPS (○), Tris–HCl (■).

Table 2
Substrate specificity of banana PEL

Substrate	Enzyme activity[a] (units)	Relative activity (%)
Polygalacturonate	6.00 ± 0.55	100
Pectin (7.2% methyl esterified)	3.06 ± 0.25	51
Pectin (26% methyl esterified)	1.50 ± 0.16	25
Pectin (65% methyl esterified)	1.02 ± 0.10	17

The assay system consisted of 0.04% of substrate, 0.7 mM $CaCl_2$, 33 mM Tris–HCl buffer, pH 8.5, 0.5 ml of purified enzyme and water in a total volume of 3.0 ml.

[a] The values are mean of three separate experiments.

2.8. Effect of group specific reagents

Treatment of PEL with 5–30 moles NBS per mole of protein at pH 8.5 inhibited progressively the enzyme activity and 50% inhibition was observed at 15 moles NBS, suggesting that tryptophan residues may be involved in the function of PEL. The rate of inactivation of PEL by iodoacetate at pH 7.0 and 37 °C as a function of time was also studied, keeping the concentration of iodoacetate and protein at 6 and 1 g l^{-1}, respectively. The activity was found to be inactivated with time and 50% inactivation occurred in about 1 h, suggesting that glutamic acid residues may be present at the active site of PEL.

2.9. Effect of thiols and reducing agents

PEL activity was progressively inhibited on increasing the concentration of DTT and β-ME (Table 3). Cysteine

Table 3
Effect of thiols and reducing agents on PEL activity

Treatment	Concentration (mM)	Enzyme activity[a] (units)
No		3.3 ± 0.28
Dithiothreitol	1	2.4 ± 0.18
	3	1.8 ± 0.15
	5	1.1 ± 0.10
β-Mercaptoethanol	1	2.0 ± 0.15
	5	1.1 ± 0.10
Cysteine	1	3.3 ± 0.25
	2	3.4 ± 0.30
	5	3.4 ± 0.32
Cystine	1	4.1 ± 0.35
	2	4.8 ± 0.40
	5	5.7 ± 0.45
p-CMB	0.01	1.7 ± 0.10
	0.1	00
$HgCl_2$	0.1	00
Ascorbic acid	0.1	1.7 ± 0.15
	1.0	00
Sodium metabisulphite	0.1	2.0 ± 0.15
	1.0	0.9 ± 0.10

The enzyme was preincubated with thiols and *p*-CMB (*p*-chloromercuric benzoate) for 5 min at 37 °C before starting the reaction with PGA.The reaction system contained 0.3 ml of the purified enzyme.

[a] The values are mean of three separate experiments.

Table 4
Effect of different concentrations of phenolic compounds on PEL activity

Phenolic	Concentration (mM)	Enzyme activity[a] (units)
No		3.3 ± 0.28
Caffeic acid	0.01	1.9 ± 0.16
	0.02	1.5 ± 0.15
	0.05	1.1 ± 0.12
Catechol	0.01	2.5 ± 0.20
	0.02	2.0 ± 0.15
	0.05	1.6 ± 0.15
Chlorogenic acid	0.01	2.4 ± 0.25
	0.02	2.0 ± 0.17
	0.05	1.4 ± 0.12
Cinnamic acid	0.01	2.0 ± 0.15
	0.02	1.6 ± 0.15
	0.05	1.1 ± 0.12
p-Coumaric acid	0.01	2.2 ± 0.20
	0.02	1.7 ± 0.18
	0.05	1.3 ± 0.15
Ferulic acid	0.01	2.8 ± 0.25
	0.02	1.8 ± 0.20
	0.05	1.4 ± 0.15
Salicylic acid	0.01	2.2 ± 0.15
	0.02	1.8 ± 0.13
	0.05	1.3 ± 0.11

The reaction system contained 0.3 ml of the purified enzyme.
[a] The values are mean of three separate experiments.

had, however, no effect on the enzyme activity, but cystine activated the reaction by about 75% in 5 mM concentration. *p*-CMB and $HgCl_2$ were strong inhibitor of PEL activity. Reducing agents, ascorbic acid and sodium metabisulphite also strongly inhibited PEL activity.

2.10. Effect of phenolics

Phenolic acids namely, caffeic, cinnamic, chlorogenic, *p*-coumaric acids and ferulic acid as well as catechol inhibited the enzyme activity. The inhibition increased with the concentration of phenolics (Table 4). Salicylic acids strongly inhibited PEL activity and about 50% inhibition was observed between 0.02 and 0.05 mM concentration of salicylic acid.

2.11. Effect of metal ions

Purified banana PEL exhibited no activity against EDTA-treated PGA. However, in the absence of Ca^{2+} ions the enzyme exhibited 10% of the maximum activity (in the presence of 0.7 mM Ca^{2+}) when tested against untreated PGA. The effect of other divalent cations on PEL activity was determined using the standard reaction system and replacing $CaCl_2$ with chloride of other metals at 0.1 and 1.0 mM concentrations . No activity was observed when Ca^{2+} was replaced with Ba^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, and Zn^{2+}. However Ca^{2+} could be replaced with Mg^{2+} and Mn^{2+}. The effect of various concentrations of Ca^{2+}, Mg^{2+}, and Mn^{2+} on the PEL activity is shown in Fig. 5(A). Mg^{2+} appeared to be slightly better than Ca^{2+}. The optimum activity of the enzyme was found in the presence of 0.7 mM metal ions, irrespective of Mg^{2+}, Mn^{2+} or Ca^{2+} ions. The activity decreased at higher concentrations. The double reciprocal plots of the data (Fig. 5A), indicated K_m values of 0.55, 0.40 and 0.7 mM for Ca^{2+}, Mg^{2+} and Mn^{2+}, respectively, with PGA as a substrate. The activity

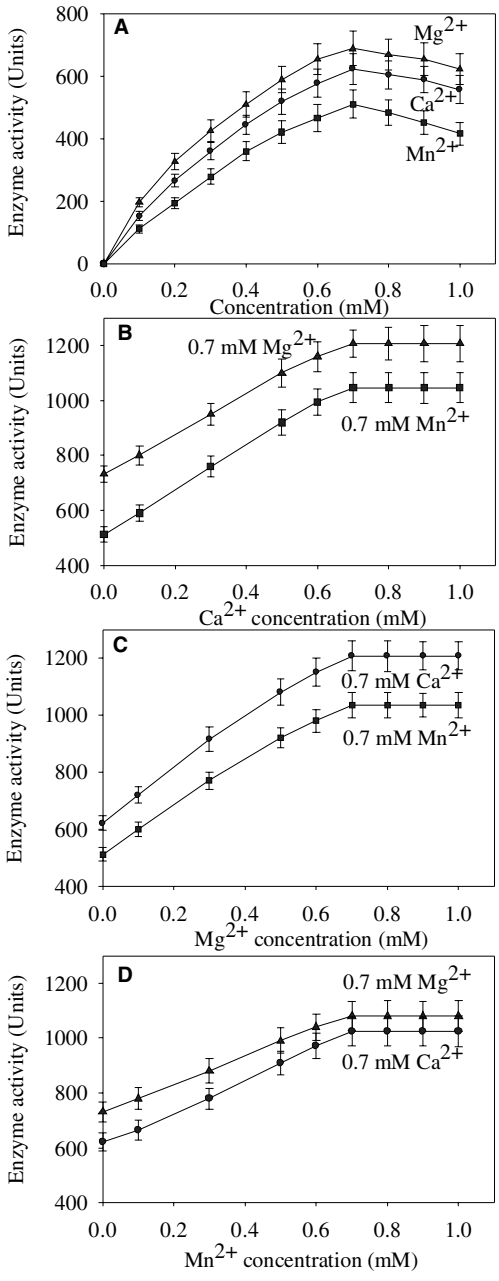

Fig. 5. PEL activity in the presence of various concentrations of metal ions. (A) Ca^{2+} (●); Mg^{2+} (▲); Mn^{2+} (■). (B) PEL activity for a concentration range of Ca^{2+} with a fixed Mg^{2+} and a fixed Mn^{2+} concentration. (C) PEL activity for a concentration range of Mg^{2+} with a fixed Ca^{2+} and a fixed Mn^{2+} concentration. (D) PEL activity for a concentration range of Mn^{2+} with a fixed Mg^{2+} and a fixed Ca^{2+} concentration.

Table 5
Kinetics parameters in the presence of metal ions with PGA as substrate

Metal ions	K_m (g l^{-1})	V_{max} (units mg protein^{-1})
0.7 mM $CaCl_2$	0.40 ± 0.04	1250 ± 75
0.7 mM $MgCl_2$	0.32 ± 0.03	1428 ± 90
0.7 mM $CaCl_2$ + 0.7 mM $MgCl_2$	0.45 ± 0.04	2610 ± 150

The data are mean values of three separate experiments.

in the presence of 0.7 mM $CaCl_2$ + 0.7 mM $MgCl_2$, in the presence of 0.7 mM $CaCl_2$ + 0.7 mM $MnCl_2$ and in the presence of 0.7 mM $MgCl_2$ + 0.7 mM $MnCl_2$ were almost the sum total of the activity in the presence of individual metal ions. The additive effect of these cations can also be seen from curves for a concentration range of Ca^{2+} with a fixed Mg^{2+} or fixed Mn^{2+} concentration (Fig. 5B), a concentration range of Mg^{2+} with a fixed Ca^{2+} or fixed Mn^{2+} concentration (Fig. 5C) and a concentration range of Mn^{2+} with a fixed concentration of Ca^{2+} and fixed concentration of Mg^{2+} (Fig. 5D). These experiments were also conducted by assaying PEL activity by TBA method (Pitt, 1988) and again similar results were obtained. Monovalent metal, Na^+ and K^+ at 1 mM concentration had no effect on the enzyme activity and also could not substitute for Ca^{2+} for exhibiting PEL activity.

2.12. Kinetic studies

The plots of different concentrations of substrate (PGA or 7.2% methyl esterified pectin) were hyperbolic in shape indicating Michaelin kinetics. The double reciprocal plot of the data indicated K_m values of 0.40 ± 0.04 and 0.77 ± 0.08 g l^{-1} for PGA and 7.2% methyl esterified pectin, respectively. The V_{max} values were 1250 ± 75 and 750 ± 50 pkat mg protein^{-1}, respectively, for PGA and pectin. The reaction-rate substrate concentration relationship was also studied with PGA in the range of 0.01–0.04% in the presence of 0.7 mM $CaCl_2$, 0.7 mM $MgCl_2$ and, 0.7 mM $CaCl_2$ + 0.7 mM $MgCl_2$. The substrate saturation curves were hyperbolic in shape. Lineweaver–Burk plots of the data indicated K_m value in the presence of Mg^{2+} lower than in the presence of Ca^{2+}(Table 5). The V_{max} of the enzyme in the presence of combined $Ca^{2+}$and Mg^{2+} ions was almost the sum total of the values obtained individually for Ca^{2+} and Mg^{2+} ions. However, K_m value in the presence of combined Ca^{2+} and Mg^{2+} ions was higher than that of individual K_m values.

3. Discussion

Pectate lyase could be successfully purified from ripe banana fruit pulp employing cation exchange chromatography, hydrophobic chromatography on phenyl sepharose and gel filtration through ultrogel and sephadex G-75. Although the pure enzyme appears homogenous on SDS–PAGE and FPLC, the authors do not claim it as a totally pure preparation, since plant genomes contains several potential pectate lyase genes, which encode homologous proteins, which would be very difficult to separate by biochemical methods. The specific activity of the enzyme was 680 ± 50 pkat mg protein^{-1}, which was low compared to homogenous PEL from microbial sources (Crawford and Koattukuty, 1987; Liao et al., 1997; Benen et al., 2000; Sawada et al., 2000; Kluskens et al., 2003).

The molecular weight of PEL varies from bacterial to fungal sources in the range 17–135 kDa. The subunit molecular weight of banana PEL, as determined by SDS–PAGE was 43 kDa and the enzyme is a monomer. On the basis of complete cDNA sequence, the molecular weight of the translated PEL protein from *Z. elegans* was assumed to be 44 kDa (Domingo et al., 1998). Mańin-Rodŕiguez et al. (2003) carried out yeast transformation with the highly expressed *pel* clone from ripe banana fruit and reported molecular mass of 44 kDa for the recombinant PEL protein.

Banana PEL can be classified as *endo*-pectate lyase on the basis of higher rate of reduction in viscosity of PGA degradation compared to liberation of unsaturated galacturonic acid polymers . This classification is further supported by detection of several oligomeric galacturonic acids as reaction products of the PEL reaction by paper chromatography and anion exchange chromatography. The unsaturation nature of the oligomeric galacturonic acid residues is clear from the fact that the reaction products obtained, after anion exchange chromatography exhibited maximum absorption between 232 and 236 nm.

Banana PEL showed optimum activity at pH 8.5 with only 30% activity at pH 7.0. This may be a regulatory control for the banana enzyme. Domingo et al. (1998) reported optimum pH of 10 for Zinnia PEL.

Inhibition of banana PEL activity by sulfhydryl groups (DTT, and β-ME) and mild activation by cystine suggest no requirement of sulfhydryl groups for the functioning of the enzyme. The enzyme is also inactivated by mild reducing agents indicating the necessity for disulfide bond maintenance. The banana enzyme resembles PEL from *Thermomonospora fusca* (Stutzenberger, 1987) in being inactivated by mild reducing agent.

Salicylic acid, a ubiquitous plant phenolic, as well as other phenolic compounds powerfully inhibited banana fruit PEL. *E. chrysanthemi* PEL is also reported to be inhibited by salicylic acid (Tardy et al., 1997; Pissavin et al., 1998). Tannins are decreased in the ripe banana fruit pulp to about one fifth of their value in the green preclimacteric fruit (Palmer, 1971). The decreased inhibition of PEL activity by plant phenolics in ripe banana is likely to facilitate fruit ripening. PEL plays an important role in banana fruit ripening (Payasi et al., 2004).

Banana PEL exhibited activity with pectin, but the activity decreased with the increase in degree of methylation, thus resembling the enzyme from *E. chrysanthemi* (Tardy et al., 1997) and *Thermotoga maritima* (Kluskens et al., 2003).

Pectin lyase is reported to have no requirement for Ca^{2+} (Pitt, 1988). The abolition of the enzyme activity in the absence of Ca^{2+} ions with pectin as a substrate indicates that the purified banana enzyme is not a pectin lyase, but is a pectate lyase. Similar to the observation of Domingo et al. (1998) for Zinnia PEL and Mańin-Rodŕiguez et al. (2003) for banana PEL, the present authors also observed lack of PEL activity in the presence of EDTA, indicating pectin degradation as primarily calcium-dependent. Of the several divalent ions tested, none were found to substitute for Ca^{2+} except Mn^{2+} and Mg^{2+}. Mn^{2+} is also reported to be a cofactor for PEL from an alkaliphilic *Bacillus* isolate (Kobayashi et al., 1999). Mn^{2+} is reported to be a better cofactor than Ca^{2+} for pectate lyase Pel Z of *E. chrysanthemi* (Pissavin et al., 1998). The banana enzyme can use Mn^{2+} as a cofactor at almost the same levels of Ca^{2+} . The banana PEL is unique in utilizing Mg^{2+} slightly more efficiently than Ca^{2+} as a cofactor. The K_m value of the enzyme for PGA was slightly less in the presence of Mg^{2+} compared to Ca^{2+}. To the best of our knowledge, $Mg^{2+}$is not reported as a cofactor for PEL from plant or microbial sources. The additional effect of Mg^{2+} and Ca^{2+}, Mg^{2+} and Mn^{2+}, Ca^{2+} and Mn^{2+} on banana PEL activity is most likely an effect on the substrate by making more substrate available to the enzyme, a so called swamping effect.

4. Experimental

4.1. Fruit tissue

Mature hands with well developed and rounded fingers were selected from a bunch of Harichhal banana (*Musa acuminata*) fruits purchased from a local supplier. Fruits were washed with water, sterilized by dipping in 0.1% $HgCl_2$ for 5 min and washed again with sterile water. Fruits were ripened in the presence of ethylene. The ripened fruits were again sterilized with 0.01% $HgCl_2$ and washed exhaustly with sterile water.

4.2. Enzyme assays

The PEL activity was routinely assayed by modifying the method of Collmer et al. (1988) by the measurement of absorbancy change at 235 nm in a reaction mixture consisting of 0.4 g l^{-1} PGA (Sigma), 0.7 mM $CaCl_2$, 33 mM Tris buffer (pH 8.5), enzyme and water in a total of 3.0 ml. The reaction was monitored for 30 min at 37 °C. One unit (pkat) of PEL activity is the amount of enzyme which produces 1 pmole unsaturated digalacturonane per sec under the above conditions. The molar extinction coefficient was assumed to be 4600 M^{-1} cm^{-1} for unsaturated digalacturonane.

Experiments on the effect of metal ions on PEL activity were also conducted employing TBA method as described by Payasi et al. (2004) for the enzyme assay.

4.3. Purification of PEL

All procedures were performed at 2–5 °C.

4.3.1. Initial extract

Banana fruit pulp tissue (120 g) was cut into small pieces and homogenized in a Waring blendor with 600 ml of extraction medium consisting of Na–Pi buffer (0.02 M; pH 7.0), freshly prepared cysteine/HCl (0.02 M; pH 7.0), polyvinyl pyrrolidone (1% w/v, mol wt. 360,000) and phenyl methyl sulfonyl fluoride (1 mM). The homogenate was centrifuged at 15,000*g* for 30 min.

4.3.2. Ammonium sulfate fractionation

Solid $(NH_4)_2SO_4$ was added to the initial extract to 0.6% saturation. After 4 h, the precipitate was centrifuged, suspended in Na–Pi buffer (0.02 M; pH 7.0) dialysed overnight against the same buffer and recentrifuged at 15,000*g* for 15 min.

4.3.3. Q-sepharose chromatography

The above fraction was loaded onto a Q-sepharose (Sigma, USA) column (2.5 × 25 cm), previously equilibrated with Na–Pi buffer (0.02 M; pH 7.0) and washed with the buffer. Fractions of 5 ml were collected and active fractions pooled. The PEL activity was mostly recovered in pass through and washing.

4.3.4. Phenyl sepharose chromatography

Q-sepharose fraction (45 out of 90 ml) in neutralized 800 mM $(NH_4)_2SO_4$ was loaded onto a Phenyl sepharose CL 4B (Sigma–Aldrich, USA) column (1.8 × 10 cm), previously equilibrated with buffer A (0.02 M Na–Pi buffer pH 6.8 containing neutralized 0.8 M $(NH_4)_2SO_4$) and washed with buffer A till the protein absorbance at 280 nm became almost zero. The proteins were eluated with 100 ml of a decreasing $(NH_4)_2$ SO_4 gradient (0.8–0.0 M). Fractions of 4 ml were collected at a flow rate of 0.5 ml min^{-1}.The active fractions were pooled, dialysed against acetate buffer (0.02 M; pH 6.5) and concentrated by dialysis against dry sucrose.

4.3.5. SP-sepharose chromatography

The above preparation was applied to a SP-sepharose (Sigma, USA) column (2.5 × 20 cm), previously equilibrated with acetate buffer (0.02 M; pH 6.5) and then the column was washed with acetate buffer till the absorbance at 280 nm was negligible. The adsorbed proteins were eluated with 140 ml of an increasing NaCl gradient (0–1 M). Fractions of 4 ml were collected at a flow rate of 0.4 ml min^{-1}. The active fractions were pooled and concentrated.

4.3.6. Ultrogel AcA 54 chromatography

The above preparation was applied to an Ultrogel AcA 54 (LKB, Sweden) column (1.6 × 80 cm), already equilibrated

with Na–Pi buffer (0.02 M; pH 7.0) containing 0.02 M NaCl. The enzyme was eluated by percolating the same buffer. Fractions of 1 ml were collected at a flow rate of 0.3 ml min^{-1}. The active fractions were collected, pooled and concentrated by dialysis against dry sucrose.

4.3.7. Sephadex G-75 chromatography

The enzyme solution from Ultrogel column was applied to a Sephadex G-75 (Pharmacia, Sweden) column (1.6 × 80 cm), previously equilibrated with Na–Pi buffer (20 mM; pH 7.0) containing 0.02 M NaCl. The elution was carried out with the same buffer at a flow rate of 0.3 ml min^{-1}. Fractions of 1 ml were collected. The active fractions were collected and pooled.

4.3.8. Analytical methods

Protein was estimated according to the method of Lowry et al. (1951) with bovine serum albumin as a standard. Carbohydrate content was determined by Phenol–sulfuric acid method (Dubois et al., 1956), using glucose as a standard. Proteins were analysed by PAGE on 12% polyacrylamide gel as described by Laemmli (1970) and stained with Coomassie blue or Silver nitrate (Blum et al., 1987). The molecular weight of the purified enzyme was estimated by gel filtration chromatography on a Sephadex G-75 column employing Na–Pi buffer (20 mM; pH 7.0) containing 0.02 M NaCl as described by Whitaker (1963). Molecular weight markers used were obtained from Sigma, USA. The sub unit molecular weight was determined from SDS–PAGE on 12% polyacrylamide gel (King and Laemmli, 1971). SDS–PAGE molecular weight standards (Pharmacia, Sweden) were used as the marker proteins. The isoelectric point of the enzyme was determined according to the method described by Robertson et al. (1987), employing a broad range ampholyte (Fluka, Switzerland), ranging in pI from 3 to 10. Viscometry studies were carried out by the method of Hulme (1988).

FPLC of the purified enzyme was carried out employing Waters Advanced Protein Purification System model 650 with gel filtration column, protein pak glass 300 SW. The elution buffer was sodium phosphate buffer (0.02 M; pH 7.0) containing 0.02 M NaCl.

4.3.9. Isolation of reaction products

The reaction mixture for PEL assay, with PGA as substrate was scaled up by 20 times and incubated at 37 °C for 9 h and the reaction halted by heating the mixture in a boiling water bath for 5 min.The mixture was cooled, centrifuged at 15,000*g* for 10 min and then treated with Dowex-50 cation exchange resin (H^+ form). The entire reaction mixture was then filtered into a column of Dowex-1-X-4 anion exchange resin (50–100 mesh) in the acetate form (50 ml of wet resin in a 3 cm diameter glass column). The resin was washed with 100 ml water and elution was carried out with sodium acetate solution adjusted to pH 6.0 with acetic acid. Acetate concentration was increased in stepwise fashion, beginning with 0.2 M and increasing to 1 M by increments of 0.1 M. Each elution buffer was of 50 ml and fractions of 5 ml collected. Absorption of separated fractions was measured at 232 nm in a UV spectrophotometer. Six peaks (one major and five minors) were obtained. Fractions of the major peak were pooled and salts removed by treating the sample with Dowex-50 (H^+ form). Water and acetic acid were removed by heating the sample in an oven at 120 °C till fully dried, cooled and transferred to a desiccator containing calcium chloride.

4.3.10. Paper chromatography

Descending paper chromatography in Whatman No. 1 paper was carried out with ethyl acetate–pyridine–water–acetic acid (5:5:3:1) as a solvent. The chromatogram was run until solvent reached about 1 cm from the paper edge. Carboxylic acids were located by spraying with 0.04% bromophenol blue in 80% ethanol at pH 8.0 (Nagel and Anderson, 1965). Uronic acids were detected by carbazole–sulphuric acid spray reagent (Stahl, 1969).Violet spots for uronic acids were observed on heating for 10 min at 120 °C. Deoxy sugars were detected according to Warren (1960). To determine the components of the reaction products of PEL, the assay system was scaled up by 10 times and incubated at 37 °C for 3 h. The control tube received the substrate, PGA, after termination of the reaction. 1.5 ml EDTA (0.02 M) was added to each tube to chelate calcium and the mixture centrifuged at 1500*g* for 30 min, The supernatant was lyophilized. The dry powder was dissolved in 0.1 ml glass distilled water and an aliquot taken for spotting on a paper chromatogram to identify the reaction products.

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