Partial purification and characterization of a pectin lyase produced by *Penicillium oxalicum* in solid-state fermentation (SSF)

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Received 23 December 2003, revised 21 September 2004, accepted 10 November 2004

An extracellular pectin lyase (PNL) EC. 4.2.2.10, produced by *Penicillium oxalicum* grown on mandarin peel meal in solid-state fermentation was studied. The enzyme was partially purified by ammonium sulphate precipitation. The partially purified preparation showed a pH optima of 8.0 and was stable over a pH range of 4.0-8.0. The optimum temperature of the thermolabile enzyme was 40°C. The enzyme, however, lost its activity quickly at temperatures over 50°C. The *Km* of the enzyme was found to be 2.08 mg/mL of citrus pectin, which was substantially lower than the values already reported. Phenolic compounds except tannic acid and several divalent ions and other inhibitors examined had no significant effect on the enzyme activity.

**Keywords:** ssf, *Penicillium oxalicum*, pectin lyase, pectin transeliminase, pectinases

**IPC code:** Int. Cl. C12N9/88; C12R1:80

**Introduction**

Enzymatic hydrolysis of plant tissues by pectolytic enzymes is called maceration. Factors which produce such tissue maceration are generally random chain splitting pectic enzymes viz. pectate lyase (PL), pectin lyase (PNL) and polygalacturonase (PG). Plant pathogenic fungi produce an array of such extracellular degradative enzymes. A lot of attention has been focused on the enzymes that depolymerize pectin. PNL (E.C 4.2.210) is the only known pectinase capable of lysing α-1,4 bonds of highly esterified pectin without previous action of other pectinases by means of β-eliminative cleavage of glycosidic linkages. On the other hand, PGs and PEs act together to degrade the pectin molecule completely, and liberate methanol as a by-product of PE action. Therefore, the use of PNLs as a major component of commercial preparations is preferred in fruit juices and wine industries as it decreases the viscosity without damaging the volatile ester content responsible for specific aroma of various fruits.

Pectinases are industrially produced by microorganisms either by submerged fermentation (SmF) or solid-state fermentation (SSF). SSF processes present numerous advantages over SmF. The former not only requires a lower volume of liquid for product recovery and a cheap medium for fermentation, it also poses lower risk of contamination on account of unavailability of free flowing substrates. Enzymes produced by SSF have been reported to possess more stable properties and are less affected by catabolic repression than enzymes produced by SmF. Considerable variation in properties of PNLs produced under different conditions has been reported.

Agro-industrial residues and wastes such as wheat bran, rice bran, sugarcane bagasse, corncobs, citrus wastes, apple pomace and a variety of such by-products are potentially good substrates for SSF. Nagpur, a Mandarin orange (*Citrus reticulata* Blanco) growing area, has several orange processing factories in which considerable quantity of fruit peel is used to extract oil. The deoiled peel meal is mostly used as manure. A process was optimized in this laboratory for the production of PNL by SSF, utilizing deoiled peel meal. The present paper describes partial purification and characterization of PNL produced by *Penicillium oxalicum*.

**Materials and Methods**

Chemicals

Mandarin peel meal was provided by Nagpur Orange Growers Association (NOGA) factory. Wheat bran was purchased from the local market. Citrus
pectin was purchased from Sigma Chemical Company, USA and other chemicals used were of analytical grade.

Microorganism and Inoculum
A strain of *P. oxalicum*, isolated in this laboratory was found to produce significant amounts of PNL in the present investigation. The culture was maintained on Czapek-Dox agar slants and subcultured every fortnight. Three-day-old culture was used to feed the SSF medium. Inoculum contained $10^6$ spores/mL of sterile distilled water.

SSF Culture Medium
Each 250 mL conical flask contained: wheat bran, 1.9; deoiled mandarin peel meal, 7.0; ammonium sulphate, 01; and moisture, 71%. Flasks were sterilized at 15 lb in$^2$ for 20 min, cooled and inoculated with *P. oxalicum*. The culture flasks were incubated at 26±2°C for 5 days.

Enzyme Extraction
After fermentation, 35 mL of cold distilled water was added to each culture flask and the mixture was mixed thoroughly, the final volume of it was made to 100 mL. The homogenate was filtered through nylon cloth and the filtrate was centrifuged at 6000 × g for 15 min. The supernatant constituted the crude enzyme and was used for purification.

Enzyme Assay
PNL activity was assayed by the method reported by Albersheim$^9$. For this, 2.7 mL of buffered substrate (0.25% w/v citrus pectin in 0.1 M Tris-HCl buffer, pH 8.0) was mixed with 0.3 mL of enzyme. The mixture was incubated at 40°C. Absorbance (A$_{235}$) was measured at 235 nm at 0 time and after 1 h. The increase in absorbance is a measure of PNL activity. One unit of enzyme activity was defined as that amount of the enzyme, which increased the optical density at 235 nm (A$_{235}$) by 1 in 1 h under assay conditions. Specific activity was defined as units/mg protein. This basic assay procedure was routinely used in all subsequent experiments unless mentioned otherwise.

Protein Estimation
Proteins in enzyme preparations were determined by the method of Lowry *et al*.$^{10}$ with bovine serum albumin as standard.

Results and Discussion

| Table 1—Ammonium sulphate precipitation of PNL produced by *P. oxalicum* by SSF |
|-----------------|----------------|-----------------|----------------------|---------------------|
| Fraction        | Total units   | Total protein   | Specific activity (U/mg protein) | Purification fold | Recovery (% yield) |
| Protocol-A      |               |                 |                                   |                     |                   |
| Crude           | 2250          | 380             | 5.9                                | —                   | 100               |
| 0-30%           | 120           | 7.3             | 16.3                               | 2.7                 | 5.3               |
| 30-60%          | 583           | 18.3            | 31.8                               | 5.3                 | 25.9              |
| 60-90%          | 200           | 15.9            | 13.3                               | 2.2                 | 8.8               |
| Protocol-B      |               |                 |                                   |                     |                   |
| Crude           | 900           | 142             | 5.8                                | —                   | 100               |
| 0-75%           | 465           | 18              | 25.8                               | 4.45                | 51.6              |

Optimum pH
The enzyme activity was assayed over a pH range of 3.0-10.0 using citrate, phosphate and Tris buffers wherever required. As shown in Fig. 1, the enzyme showed a single pH optimum at pH 8.0. Similar pH optima have been reported for PNLs isolated from other organisms such as *Rhizoctonia solani* (pH 7.5) and *Pythium splendens* (pH 8.0) while a PNL from *Penicillium italicum* showed pH optimum between 6.0 and 7.0.$^{11,12}$

pH Stability
The enzyme was incubated for 16 h at 10°C in buffers of pHs ranging from 4.0-10.0 and the residual activity was assayed at pH 8.0 by the usual assay procedure. Fig. 2 shows that the enzyme was reasonably stable over the pH range, highest stability
being recorded at pH 8.0. Similar values of pH stability were found in PNL produced by *P. splendens* while a lower value (7.0) was found in culture filtrates of *Penicillium* sp\textsuperscript{12,13}. It was interesting to note that a PNL produced by *Aspergillus niger*, both in SSF and SmF, was stable between a pH range of 2.5-8.0\textsuperscript{7}. It is believed that pectinases produced by SSF possess a broader range of pH stability than those shown by the enzymes produced in SmF and are also more resistant to denaturation\textsuperscript{7}. Similar observation has also been made with respect to amylases and xylanases\textsuperscript{14,15}.

**Optimum Temperature**

The enzyme activity was assayed at different temperatures (10-60°C). Optimum activity was obtained at 40°C. Temperature optima of 40, 45 and 60°C have been reported for PNLs obtained from *Penicillium janthinellum*, *P. citrinum* and *P. adamatezii*, respectively with citrus pectin as substrate; whereas apple pectin as the substrate, the respective optima were 35, 45 and 55°C\textsuperscript{13}. A maximum PNL activity between 50-55°C was produced by *Aspergillus oryzae*, while a sustained activity over a temperature range of 20-60°C has also been reported earlier\textsuperscript{16,17}.

**Temperature Stability**

The enzyme preparation was allowed to stand at different temperatures (20-70°C) for 15 min and the residual activity was assayed by the usual procedure. As indicated in Fig. 4, the enzyme appeared to retain the activity upto 50°C. The activity, however, rapidly decreased afterwards and was completely lost at 70°C. PNL produced by *Penicillium italicum* has been found to be stable at temperatures upto 50°C, while three different PNLs produced by various *Penicillium* strains have been reported to be stable at 40°C for 60 min\textsuperscript{5,13}. The enzymes, however, readily lost the activity within 15 min at 60°C and above\textsuperscript{13}. A PNL...
produced by A. niger in SSF was found to show sustained activity for 30 min at a temperature range 20-45°C.

**Effect of Substrate Concentration**

The enzyme was assayed at various substrate concentrations (0.08-0.33%). The double reciprocal plot shown in Fig. 5 indicates that the $K_m$ of the enzyme for citrus pectin was 2.08 mg/mL and the $V_{max}$ was 33 U/mg protein. A wide range of $K_m$ values has been reported for PNLs from different sources. PNLs from P. italicum, Erwinia chrysanthemi and A. niger showed $K_m$ values of 3.2, 7.3 and 12.8 mg/mL, respectively for citrus pectin as a substrate. PNL reported by the authors, therefore, appears to be a high affinity enzyme, although a definite picture can be obtained only after extensive purification of the enzyme.

**Effect of Ions**

The enzyme activity was determined in the presence of various ions at 2mM concentration. The ions studied were: Ca$^{2+}$, Cu$^{2+}$, Fe$^{3+}$, Mn$^{2+}$, Mo$^{4+}$, Ba$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, Mg$^{2+}$ and Ag$^{+}$. None of the ions including Ca$^{2+}$ stimulated PNL activity under the present assay conditions. Requirement of Ca$^{2+}$ by PNL has been shown to be dependent on the esterification level of the substrate and the pH of the assay mixture. The assay conditions used by the authors were not apparently conducive for Ca$^{2+}$ stimulation of the enzyme activity. However, it was observed that Mo$^{4+}$ ions were strongly inhibitory as in its presence nearly 62% of PNL activity was denatured (data not given).

**Effect of Phenolic Compounds and Other Protein Inhibitors**

Phenolic compounds are reported to inhibit pectolytic enzymes. Ferulic and caffèic acids at 2 mM concentration had no inhibitory effect on the enzyme activity. Chlorogenic and tannic acids on the other hand were respectively mildly and moderately inhibitory (data not given). It is also reported that tannic acid is a potent inhibitor of a PG produced by *Alternaria alternata*. PMSF (phenyl methyl sulphonyl fluoride) and EDTA (ethylene diamine tetra acetate) at the level of 5 mM also did not inhibit the present enzyme (data not given). A PNL produced by *P. expansum* has been reported to be highly resistant to EDTA and most of the divalent cations over a concentration range of 2-50 mM. However, a bacterial PNL produced by *Bacillus* sp. PN 33 found to be strongly inhibited by PMSF and DEPC (diethylpyrocarbonate) at 5 mM level.

**Acknowledgement**

The authors are grateful to Dr Pratima Jadhav, Head, Department of Biochemistry, University of Nagpur for interest shown in the present work. They are thankful to Dr M B Patil for providing the fungal strain. Financial support received from Nagpur University in the form of a Research Studentship to the first author is gratefully acknowledged. Authors also acknowledge the NOGA for providing mandarin peel meal.
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