Purification and Characterization of Extracellular Polygalacturonase from *Pleurotus Ostreatus* Using Citrus Limonium Waste.

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**Abstract:** Extracellular polygalacturonase (PG) from *Pleurotus ostreatus* NRRL-0366 using Lemon Peel (citrus limonium) as a substrate have been purified and characterized. The enzyme was purified to a homogeneous form with about 27 fold purification and specific activity 158U/mg protein by two chromatographic steps in DEAE cellulose and sephacryl S-300. The extracellular PG was a monomer protein with a molecular weight of 21KDa determined by SDS gel electrophoresis and 20KDa determined by gel filtration techniques. The optimal PH and temperature values were 3.5, 30°C respectively. The enzyme was stable at 30°C, pH (3.0-3.5). PG was able to hydrolyze pectin and partially polygalacturonic acid exhibiting km of 1.33 mg ml⁻¹ and Vmax of 28.6 μmol min⁻¹. The effect of different metal cations at 1mM concentration on the enzyme activity was studied.

**Key words:** polygalacturonase, *Pleurotus ostreatus*, Lemon peel, purification, characterization.

**INTRODUCTION**

The biotechnological potential of pectinolytic enzymes from microorganisms has drawn a great deal of attention worldwide because of their myriad applications. They are of great significance, especially, in fruit processing, textile and paper industries[1-3]. Also, in the treatment of pectic waste waters, oil extraction, juice stabilization…. etc.

Pectinolytic enzymes or pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in higher plants and microorganisms[4].

In relation to their activity, polygalacturonase (PG) cleaving α- (1, 4) glycosidic bonds of non-esterified Gal A residues are classified as endo-PG (E.C.3.2.1.15) and exo-PG (E.C.3.2.1.67). This enzyme is produced by plants[5,6].

Extensive studies have been done for the production of exo-PG from several fungi and bacteria[7-9]. While only few yeast species show this ability[10-11]. Also, few reports were recorded about the production of endo and exo-polygalacturonase from a white rot fungi[12-17]. However, it could be of interest to find new sources of PG in order to display new specificities or mode of action.

In most industrial applications, fungal PGS prove to be the most useful because of higher enzyme activity and optimum activity at a lower pH range, suited to most fruit and vegetable processing applications[18].

Recently there has been increased interest in the production of microbial polygalacturonase from food processing wastes.

For industrial use, polygalacturonase can be produced from several agricultural pectin containing wastes such as apple pomace[18,19] but the main source remains citrus peel[20,21], Lemon peel[22-23], coffee pulp[23], and sugar cane bagasse[24,25]. Since such wastes were derived from food products, they can be considered as food-grade materials to produce enzymes for food processing applications.

Rashad et al.[26] studied the preparation and optimization of *Pleurotus ostreatus* medium using lemon peel as a food processing waste. So, this study will continue for production of PG in a pure form and some kinetic behaviour of the pure enzyme will be studied.

**MATERIALS AND METHODS**

**Microorganism:** Cultures of *Pleurotus ostreatus* NRRL-0366 was obtained from Agricultural Research Service (Peoria, IL). The stock culture was maintained on potato dextrose agar (PDA) slant for 1-2 weeks at 20-30°C and then stored at 4°C[28].

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Agro-industrial Waste: Citrus waste (Lemon peel) was obtained from juice processing shops in the local market at Cairo.

Culture Medium and Conditions: The basal optimized medium described before by [17] consisted of (g%) 2.0 wet lemon waste, 2.0 starch, 2.0 asparagine, extract of 20g of potato and 1.0g glucose. 1cm³ of inoculum mycelium containing 10 mg cells was used to inoculate Erlenmeyer flasks (250ml) containing 25 ml of sterilized basal medium. The flasks were incubated in static incubator at 28-30°C for 4 days.

Preparation of Crude Enzyme: After fermentation, the culture was collected, filtrated on Whatman no.1 filter paper, centrifuged at 13.000 rpm for 30 min at 4°C, dialyzed against 50mM sodium acetate buffer PH 5.0 for 24h at 4°C. The resultant clear liquid was used as crude enzyme solution.

Enzyme Assay: The polygalacturonase (PGase) activity was assayed by determining the liberated reducing end products by [27,28] a method using polygalacturonic acid as a standard. The reaction mixture containing 0.8 ml of 1% (w/v) citrus pectin (Sigma) (dissolved in 0.2 M acetate buffer, PH 5.0) and 0.2 ml of enzyme solution incubated at 55°C for 10 min. One unit of PGase is defined as the amount of enzyme that liberated 1 ìmol of galacturonic acid min⁻¹ ml⁻¹ under the assay conditions.

Protein Determination: Protein was determined either by the method of [29] using bovin serum albumin as a standard or by measuring the absorbance at 280nm [30].

Purification of Extra Cellular P.ostreatus PG: Preparation of Cell- Free Broth: Several batches of the crude enzyme preparation were obtained and designated to purification procedure.

Fractionation by Acetone and Ammonium Sulfate: Fractional precipitation with acetone or ammonium sulfate for crude PGase revealed their unsuitability as precipitating agents due to the poor yield obtained relative to the crude enzyme (date not shown).

Ion –exchange on DEAE cellulose: The crude enzyme solution was loaded directly on a DEAE-cellulose column (25 x 1cm i.d.) equilibrated with 50mM sodium acetate buffer, pH 5.0. The enzyme was eluted with a linear gradient of sodium chloride from 0.0 to 0.5 M prepared in the same buffer. Fractions in 3 ml volume were collected at a flow rate of 30 ml/h. The eluted fractions were monitored at 280nm for protein and assayed for enzyme activity.

Gel filtration on Sephacryl S-300: Fractions with higher activity were pooled, dialyzed, concentrated and loaded on Sephacryl S-300 column (25 x 1cm i.d) previously equilibrated with 50 mM sodium acetate buffer, pH 5.0 and developed at a flow rate of 20 ml/h and 3ml fractions were collected.

Determination of Molecular Weight: The molecular weight of the pure PG was determined either by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration. SDS-PAGE was preformed by the method of [31]. The molecular weight markers for SDS-PAGE (phosphorylase b; 97 kDa, Bovine albumin, 67KDa, Ovalbumin 45KDa, carbonic anhydrase, 30 KDa, Trypsin inhibitor, 20.1 KDa, and a - Lactalbumin, (bovine milk), 14.4 KDa). were obtained from pharmacia Co.

Gel filtration technique was done using sephadex G-200 column (25x 1cm i.d) previously equilibrated and eluted with 50 mM sodium acetate buffer, pH 5.0 [32]. Blue dextran was used for determination the void volume (Vv). A calibration curve was obtained with standard proteins of known molecular weight; cytochrom (12.27 KD), trypsin (24 KD), pepsin (34.7KD), myoglobin (17KD) and bovine serum (66KD).

Characterization of the Pure Enzyme: Optimum pH: The pH optimum was determined using the same assay method with 0.1 M sod. acetate buffer (for the pH range 3.0-5.0) phosphate buffer (for the pH range 6.0-7.0) and 0.1 M tris – HCl buffer (for the pH 8.0-9.0). The pure enzyme was incubated in different PH values at 55°C for 10 min. and then the activity was measured under the standard conditions.

PH Stability: The PH stability was determined using buffer pH ranging from (3.0-8.0) 0.2 ml of pure PG enzyme were incubated at 20°C for 20h., After this period, the activity of PG was measured under the standard assay conditions, then relative activity was calculated.

Optimum Temperature: The maximum activity of the pure enzyme was determined at different incubation temperatures ranged from 20-70°C for 30 min.

Thermostability: Thermal stability was studied by incubating the purified enzyme at various temperatures (20-80°C) for 1h. and the remaining enzyme was then assayed using the standard assay conditions.

Effect of Pectin Concentrations of PG Activity and Michaelis Constant: Apparent Michaelis constant was determined according to [33] using citrus pectin (0.25-3%) as a substrate.
**Determination of Substrate Specificity:** The activity of the purified enzyme on solutions of 1% of several substrates (citrus pectin, Amylopectin, Xylan, Corn starch, dextrin, dextran, glycogen and polygalacturonic acid) were tested under the standard assay conditions.

**Effect of Some Metals and Reagents on PG Activity:** The purified PG enzyme was preincubated with different salts under optimum reaction conditions. Residual activities in the presence of the salts were compared with the controls (without salts).

**RESULTS AND DISCUSSION**

The crude enzyme preparation was obtained by conducting fermentation processes under the optimal culture conditions tested in the previous study[37]. The whole optimum culture media obtained from several batches were collected and the culture filtrate, dialyzed, lyophilized. Partial purification of the crude enzyme was carried out by fractional precipitation using either acetone or ammonium sulfate but the results indicated unsuitability of both for obtaining active enzyme with higher yield (data not shown).

So, it was preferable to load the concentrated culture filtrate directly into anion exchange chromatographic column (DEAE cellulose column) (25 x 1cm i.d.). From the elution profile of this column, a large part of the contaminating proteins was removed and it was found that one peak of higher polygalacturonase (PG) activity was separated (Fig.1) with specific activity of 117.14 units/mg protein which represented 20.1 fold purification over the culture filtrate with 64.23% recovery (Table 1). A Sephacryl S-300 column (Fig. 2) was used to obtain the highest possible specific activity of 158.0 units/mg protein which represented 27.1fold purification over the crude filtrate with 19.43% recovery (Table 1). This results (158 U/mg protein) was nearly at the same range with PG from T.harzianum[9] while it was higher than that obtained for PGS from rhizopus oryzae 45°C[41-43], and 55°C from Streptomyces sp.[44].

The enzyme showed temperature stability retaining 93.6% activity at 40°C and exhibiting 100% stability at 30°C (Fig. 5B). These results are inagreement with that mentioned by[41] for PG from Mucor flavus.

The effect of incubation temperature on the activity of the pure enzyme was tested at pH 3.5 in the range of 10-70°C. As shown in (Fig. 5A), the optimum temperature was found to be 30°C, above 30°C resulted 47% loss of activity at 40°C then gradually loss with increasing the temperature. The relatively high temperature optima were detected for PGS from Sclerotinia sclerotiorum and Rhizopus oryzae 45°C[41-43], while it was higher than that obtained for PG from T.harzianum[9].

**Enzyme Properties:** The pH activity profile of P.ostreatus PG is shown in Fig. (4A). The enzyme showed hydrolase activity from pH 3.0 to 8.0. The results demonstrated a pH optimum at 3.5 which indicated that P.ostreatus PG was an acidic one. The PG activity was found to be stable at pH range from 3.0 to 5.5. This results was very close to those reported for PGS from Sclerotinia sclerotiorum[40], T.harzianum, T.reesei[38] and Lentinus edodes[13]. While it was condradict with P.ostreatus which recorded 7.0[16].

The enzyme showed pH stability retained 97.6%, 98.0% of its activity at pH 3.0 and 4.0 respectively (Fig. 4B). It lost about 60-70% of its activity between pH 6.0-7.0 and 85-90% between pH 8.0 to 9.0. These results were inagreement with that mentioned by[41] for PG from Mucor flavus.

For characterization of P.ostreatus PG with regarded to substrate specificity, non-pectic polysaccharides have been tried as substrates instead of pectins (Table 2). The enzyme activities with non-pectic polysaccharides were compared to the activity with pectin which was also regarded as 100% activity. The results showed that PG had very low activity toward all the examined polysaccharides compared to the purified PG from Penicillium capsulatum which had inactivity against polysaccharides such as xylan, cellulose, and dextran but was active against PGA[60].

The Michaelis constant (Km) value of the pure P.ostreatus PG was 1.33 mg/ml using pectin as a substrate. While its V max was 28.6 μmol/min. It was similar to km's from F.soloni (1.34 mg/ml) [35] and A.niger (1.35 mg/ml) [38].

The effect of different metal cations at the concentration of 1mM on P.ostreatus PG assay system is shown in Table (3). All the examined cations showed different and partial inhibitory effects on the
Table 1: Purification scheme of polygalacturonase from *P. ostreatus* grown on lemon peel medium.

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total units</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg protein)</th>
<th>Fold purification</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude filtrate</td>
<td>248.0</td>
<td>42.40</td>
<td>5.84</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>DEAE cellulose column</td>
<td>159.31</td>
<td>1.36</td>
<td>117.14</td>
<td>20.1</td>
<td>64.23</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>48.20</td>
<td>0.31</td>
<td>158.0</td>
<td>27.1</td>
<td>19.43</td>
</tr>
</tbody>
</table>

Fig. 1: Atypical elution profile of polygalacturonase from *P. ostreatus* on DEAE cellulose: the symbols (●) represent units / fraction, (■) Absorbance at 280nm.

Table 2: Relative activities of *P. ostreatus* PG toward non-pectic polysaccharides.

<table>
<thead>
<tr>
<th>Non-pectic polysaccharides</th>
<th>% Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectin citrus</td>
<td>100.0</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>47.50</td>
</tr>
<tr>
<td>Poly galacturonic acid</td>
<td>39.40</td>
</tr>
<tr>
<td>Xylan</td>
<td>0.0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>1.50</td>
</tr>
<tr>
<td>Potato starch</td>
<td>0.80</td>
</tr>
<tr>
<td>Dextrin</td>
<td>27.70</td>
</tr>
<tr>
<td>Dextran</td>
<td>5.40</td>
</tr>
<tr>
<td>Glycogen</td>
<td>15.30</td>
</tr>
</tbody>
</table>

The activity with pectin was taken as 100% activity. Each value represents the average of two experiments.

Fig. 2: A typical elution profile of polygalacturonase from *P. ostreatus* on Sephacryl S-300: (■) units / fraction (●) Absorbance at 280nm.
Fig. 3: Electrophoretic analysis of the *P. ostreatus* PG (1) standard proteins (2) sephacryl S-300 PG (3) crude extract.

Fig. 4: pH optimum (A) and pH stability (B) of *P. ostreatus* polygalacturonase.
activity of PG. The effectiveness of metal cations as inhibition for PG was in the order of Ba\(^{2+}\) < CO\(^{2-}\) < Zn\(^{2+}\) < Ni\(^{2+}\) < Ca\(^{2+}\) < Cu\(^{2+}\) < Mg\(^{2+}\) < Hg\(^{2+}\) < Fe\(^{3+}\) with 6.5, 15.3, 26.5, 29.4, 33.0, 48.2, 56.3, 90.4 and 95.82% inhibition, respectively.

Kobayashi et al.\(^{[16]}\) reported that Mg\(^{2+}\), Mn\(^{2+}\), Fe\(^{3+}\), Ca\(^{2+}\), Pd\(^{2+}\) and Sr\(^{2+}\) ions stimulated Bacillus PG activity and Cu\(^{2+}\), Ni\(^{2+}\) and Zn\(^{2+}\) ions inhibited the enzyme activity.

REFERENCES


