Characterization of a Polygalacturonase from *Trichoderma harzianum* Grown on Citrus Peel with Application for Apple Juice

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Abstract: A polygalacturonase (PGase) was purified from *Trichoderma harzianum*, grown on citrus peel, by anion exchange and size exclusion chromatographies. The enzyme had molecular mass of 29 kDa. The extent of hydrolysis by PGase was decreased with increasing of pectin esterification degree. \( K_m \) and \( V_{max} \), using polygalacturonic acid as substrate, were 1.42 mg and 0.66 \( \mu \)mol reducing sugar, respectively. PGase had a broad pH optimum ranged from 4.5 to 7.0 and temperature optimum at 55°C. PGase was stable up to 50°C after incubation for 30 min. All the examined metal cations showed partial inhibitory effects, except for Hg which was completely inhibited the enzyme activity. The effect of PGase and wheat \( \alpha \)-amylase on apple juice was studied, where the mixture of the two enzymes increased the quantity (135% fold) and clarity degree of apple juice.

Key words: Polygalacturonase; *Trichoderma harzianum*; Mandarin Citrus Peel; Purification; Properties; Apple Juice.

INTRODUCTION

The enzymes that hydrolyse pectic substances are broadly known as pectinolytic enzymes or pectinases, which include polygalacturonase, pectin esterase, pectin lyase and pectate lyase on the basis of their mode of action (Alkorta *et al.*, 1998). Pectinolytic enzymes of fungal origin attract the most attention since they offer tremendous potential to the industry. Pectinases have widespread applications in retting of flax and vegetable fibres, de-pectinisation and clarification of fruit juices, extraction of oils from vegetables and citrus peels, manufacturing of paper and pulp and pre-treatment of pectic wastewater (Jayani *et al.*, 2005; Moyo *et al.*, 2003; Saito *et al.*, 2004). Almost all of the commercial preparations of pectinases are produced from fungal sources, mainly from *Aspergillus niger*. In fact microbial pectinases account for almost 25% of the global food enzyme sales (Jayani *et al.*, 2005).

The utilization of renewable resources, particularly agricultural residues have focused worldwide attention. The major components of these residues are cellulose, starch, lignin, xylan and pectin. Several microbes are capable of using these substances as carbon and energy sources by producing a vast array of enzymes in different environmental niches (Antranikian, 1992). Pectic substances are abundant in plant biomass, and these constitute 4–30% in citrus fruit peel. Pectins consist of homogalacturonic or “smooth” regions, and rhamnogalacturonic or “hairy” regions carrying neutral sugar side chains. Galacturonic acid (GaLA) residues may be substituted by different compounds such as methanol, acetic acid, and xylose, in the “smooth” as well as in the “hairy” region. The carboxyl groups of pectin are partially esterified with methanol (Pilnik and Voragen, 1970) and the hydroxyl groups at position 2 or/ and 3 of GaLA residues are sometimes acetylated. The degrees of methylation (DM) or acetylation (DAc) are defined as the number of esterified carboxyl or hydroxyl groups per 100 GaLA units.

Generally, polygalacturonase production is induced by galacturonic acid and its polymer (pectin and polygalacturonic acid), glucose, galactose and structural relatives (mucic acid, tartonic acid and dulcitol) (Teixeira *et al.*, 2000; Malvessi and da Silveira, 2004). Pectinases can be also produced from several agricultural pectin-containing wastes such as apple pomac (Hours *et al.*, 1988), but the main source remains citrus peel (Mamma *et al.*, 2008). The present study was carried out on the production of polygalacturonase from *Trichoderma harzianum*, grown on citrus peel as fruit waste, with properties meet the prerequisites needed for efficient application especially in fruit juice processing.
MATERIALS AND METHODS

Organism and Culture Conditions:

*Trichoderma harzianum* was obtained from Plant Pathology Unit, National Research Center, Cairo, Egypt. *T. harzianum* was cultivated and maintained on slants of potato dextrose agar. The extracellular enzyme was produced by fermentation of this fungus using 5% Mandarin *Citrus reticulata* peel as culture medium. Three hundreds ml flasks were incubated for 4 days at 30°C, pH 5.0 and 200 rpm on an orbital shaker before the cell free broth was recovered by filtration.

Purification of Polygalacturonase from *T. harzianum*:

Unless otherwise stated all steps were performed at 4–7°C using 20mM Tris–HCl buffer, pH 7.5. The cell-free broth was concentrated by lyophilization and dialyzed against 20 mM Tris–HCl buffer, pH 7.5 followed by centrifugation at 10,000 xg to obtain clear cell-free broth. The cell-free broth was applied directly to a DEAE–Sepharose column (4x1.6 cm i.d.) equilibrated with 20mM Tris–HCl buffer, pH 7.5. The adsorbed material was eluted with a stepwise gradient ranging from 0 to 0.3M NaCl prepared in the same buffer at a flow rate of 60ml/h and 3ml fractions were collected. Protein fractions exhibiting polygalacturonase activity were eluted with 0.2M NaCl. The enzymatic pooled fractions were concentrated by dialysis against solid sucrose and loaded on Sephacryl S-200 column (90 x 1.6cm i.d.) equilibrated with the same buffer and developed at a flow rate of 20 ml/h and 3 ml fractions were collected.

Preparation of Dinitrosalicylic Acid Reagent (DNS):

Modified DNS reagent containing Rochelle salt (potassium sodium tartrate) was prepared according to Fischer and Kohlts (1951) by dissolving a mixture of 40g dinitrosalicylic acid, 8 g phenol, 2g sodium sulfite and 800 g Rochelle salt in 2 liters of 2 % sodium hydroxide and then diluted to 4 liters with distilled water.

Polygalacturonase Assay:

Polygalacturonase activity was assayed by modified method of Bernfeld (1951). The reaction mixture contained in 0.5ml, 1% polygalacturonic acid, 50 mM sodium acetate buffer, pH 5.0 and 0.2 unit of enzyme. The reaction started by incubation of this mixture at 40°C for one hour, after the incubation the reaction mixture was mixed with 0.5ml DNS reagent, followed by incubation in a boiling water bath for 10 min followed by cooling. The absorbance was recorded at 560nm. The enzymatically liberated reducing sugar was calculated from a standard curve using galacturonic acid. One unit of enzyme activity was defined as the amount of enzyme producing 1μmol reducing sugar as galacturonic acid per hour under the standard assay conditions.

Protein Determination:

Protein was quantified by the method of Bradford (1976) with bovine serum albumin as standard.

Molecular Weight Determination:

Molecular weight was determined by gel filtration technique using Sephacryl S-200. The column (90 x 1.6cm i.d.) was calibrated with molecular weight markers (Sigma). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). SDS-denatured molecular weight markers were used for the calibration curve.

Apple Juice Processing:

Chop one-medium-sized apple into small pieces. Put four equal weight of apple in four beakers. Add 2 ml purified *T. harzianum* polygalacturonase, 5 units (Treatment 1), 2 ml wheat α-amylase, 5 units (previously prepared by Mohamed et al., 2009) (Treatment 2), 1 ml polygalacturonase, 2.5 units and 1 ml α-amylase, 2.5 units (Treatment 3) and 2ml water (Treatment 4) to the four beakers, respectively. Stir the beaker’s contents and incubate the beakers in a water bath at 40°C for 20 minutes. Filter the juice from the apple pieces in funnel placed in measuring cylinders. Record the volume of juice obtained from apple pulp at 5-minute intervals. The turbidity of juice was measured at 540 nm (Chatterjee et al., 2004).

RESULTS AND DISCUSSION

In this study, *T. harzianum* was grown on Mandarin *Citrus reticulata* peel as culture medium and produced an inducible extracellular polygalacturonase. Highest polygalacturonase production was observed on day 4-old culture (10 units/ml). The purification of PGase from *T. harzianum* was summarized in Table 1. The cell-free
broth with specific activity of 293 units/mg protein was applied onto DEAE-Sepharose column (4 x 1.6cm i.d.). From the elution profile, one PGase was separated by 0.2 M NaCl (Fig. 1). The chromatography of DEAE-Sepharose PGase pooled fractions on Sephacryl S-200 column give a purified PGase with specific activity of 1125 units/mg protein and 40% recovery. This specific activity higher than that reported for polygalacturonases from *T. harzianum* (276 units/mg protein) (Mohamed *et al.*, 2006), *T. reesei* (916 and 708 units/mg protein) (Mohamed *et al.*, 2003) and *Aspergillus niger* (418 units/mg protein) (Fahmy *et al.*, 2008). The homogeneity of PGase was detected by SDS-PAGE (Fig. 2).

Table 1: Purification scheme of *T. harzianum* PGase.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total units*</th>
<th>Total protein(mg)</th>
<th>Specific activity(units/mg protein)</th>
<th>Fold purification</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free broth</td>
<td>220</td>
<td>0.75</td>
<td>293</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DEAE- Sepharose</td>
<td>141</td>
<td>0.16</td>
<td>881</td>
<td>3</td>
<td>64</td>
</tr>
<tr>
<td>SEP (PGase)</td>
<td>90</td>
<td>0.08</td>
<td>1125</td>
<td>3.8</td>
<td>40</td>
</tr>
</tbody>
</table>

* One unit of polygalacturonase activity was defined as the amount of enzyme producing 1 mmol reducing sugar per h under standard assay conditions.

Fig. 1: A typical elution profile for the chromatography of *T. harzianum* polygalacturonase on DEAE-Spharose column (4 x 1.6cm i.d.) equilibrated with 20 mM Tris-HCl buffer, pH 7.5 at a flow rate of 60ml/h and 3ml fractions. Absorbance at 280nm (---), polygalacturonase activity (x---x).

Fig. 2: SDS-PAGE for homogeneity and molecular weight determination of *T. harzianum* PGase. 1- Protein markers; 2- Sephacryl S-200 PGase.
The molecular weight of *T. harzianum* PGase was estimated to be 29 kDa by gel filtration. This molecular weight was confirmed by SDS-PAGE (Fig. 2) and estimated to be 29 kDa as single subunit. The majority of purified fungal polygalacturonases have molecular weight in the range from 25 kDa to 82 kDa (Barense *et al.*, 2001; Sakamoto *et al.*, 2002; Fahmy *et al.*, 2008), whereas the polygalacturonase of *Neurodpora sitophila* had a low molecular mass of 13 kDa (Fogarty and Kelly, 1983).

A number of citrus pectins were tested as substrates for *T. harzianum* PGase (Table 2). The enzyme activities with different pectins were compared to the activity with polygalacturonic acid which was regarded as 100% activity. The extent of hydrolysis by enzyme for different pectins was decreased with increasing of degree of esterification (DE), where the degradative rates toward citrus pectins with DE of 8%, 26%, 67% and 89% were 187%, 131%, 120% and 110% respectively. The results showed that all pectins tested had higher affinity toward PGase than polygalacturonic acid, which is the best substrate for the enzyme. Therefore, this enzyme can be considered as potential candidate in fruit processing, where the most fruits contained esterified pectins. These degradative rates were higher than that reported for polygalacturonases from *Bacillus* sp. (the relative rates toward citrus pectins with DE of 31%, 63% and 93% were 42.7%, 8.3% and 0.0% respectively) (Kobayashi *et al.*, 2001) and *M. flavus* (the relative rate toward 64% esterified pectin was 57% and 89% esterified pectin was 22%) (Gadre *et al.*, 2003).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>polygalacturonic acid</td>
<td>100</td>
</tr>
<tr>
<td>Pectin 8% esterification</td>
<td>187</td>
</tr>
<tr>
<td>Pectin 26% esterification</td>
<td>131</td>
</tr>
<tr>
<td>Pectin 67% esterification</td>
<td>120</td>
</tr>
<tr>
<td>Pectin 89% esterification</td>
<td>110</td>
</tr>
</tbody>
</table>

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

By a typical double reciprocal Lineweaver Burk plot, the kinetic constants $K_m$ and $V_{max}$ for *T. harzianum* PGase, using polygalacturonic acid as substrate, were 1.42 mg/0.5 ml and 0.66 μmol reducing sugar, respectively (Fig. 3). This $K_m$ was slightly lower than that reported for polygalacturonase from *Verticillium dahliae* (3.3 mg/ml) (James and Dubery, 2001). Lower Km's were detected for polygalacturonases from *Cryphonectria parasitica* (0.22 mg/ml) (Gao and Shain 1994) and *A. niger* (0.8 mg/ml) (Famy *et al.*, 2008). $V_{max}$ of PGase was higher than that reported for polygalacturonases from *Cryphonectria parasitica* (0.24 μmol) (Gao and Shain, 1994) and *S. thermophile* (0.52 μmol) (Kaur *et al.*, 2004).

![Lineweaver-Burk plot](image)

**Fig. 3:** Lineweaver-Burk plot relating *T. harzianum* PGase reaction velocities to polygalacturonic acid as substrate concentration. The reaction mixture contained in 0.5 ml: 50 mM sodium acetate buffer, pH 5.0, suitable amount of enzyme and concentrations of polygalacturonic acid ranging from 1 to 3 mg.

*T. harzianum* PGase had a broad pH optimum ranged from 4.5 to 7.0 (Fig. 4). Therefore, PGase could be a potential candidate for different applications in the industry requiring broad pH range. The acidic pH
Fig. 4: pH optimum of *T. harzianum* PGase. The reaction mixture contained in 0.5 ml: 1 % polygalacturonic acid, suitable amount of enzyme and 50 mM sodium acetate buffer (pH 3.6 - 6.5), 50 mM Tris-HCl buffer (pH 7.0 - 8.0). Each point represents the average of two experiments.

optimum (pH 4 - 5) was reported for polygalacturonases from *Fusarium moniliforme* (Niture and Pant 2004), *T. reseei* (Mohamed et al., 2003) and *A. niger* (Fahmy et al. 2008). The remarkable property of *A. kawachii* PGI was its high activity in the pH range 2-3 towards soluble and insoluble substrates, while being inactive at pH 5.0 (Contreras-Esquível and Voget, 2004). In contrast, the basic optimal pH of *Bacillus* sp. polygalacturonase was around pH 8.0 in 100 mM Tris-HCl buffer and another optimal pH was observed at pH 9.5 in 100 mM glycine-NaOH buffer (Kobayashi et al., 2001).

*T. harzianum* PGase was found to have temperature optimum at 55°C (Fig. 5). The enzyme retained around 40% of its activity at 65°C and 75°C. The temperature optimum of commercial enzyme Rapidase C80 was determined at the 55°C (Ortega et al., 2004). The same temperature optima were detected for polygalacturonases from *S. thermophil* (Kaur et al., 2004) and *A. sojae* (Tari et al., 2008). Low temperature optima were reported for polygalacturonases from *C. parasitica* (40°C) (Gao and Shain 1994), *T. reesei* (40°C) (Mohamed et al., 2003) and *A. niger* (40°C) (Fahmy et al., 2008).

Fig. 5: Temperature optimum of *T. harzianum* PGase. The enzyme activity was measured at various temperatures using the standard assay method as previously described. Each point represents the average of two experiments.
The thermal stability is defined as the ability of enzyme to resist thermal unfolding in the absence of substrate (Bhatti et al., 2006). *T. harzianum* PGase was stable up to 50°C after incubation for 30 min at temperature ranged from 20 to 70°C (Fig. 6). The thermal stability is an important parameter especially in fruit juice (Tari et al., 2008). Before the addition of pectinases, fruits are first cooked to release more juice. This releases most of the pectin into the juice, resulting in a thick and a cloudy appearance. Apples, stone fruits and berries are normally processed at 30–50°C for about 15 to 90 min (Moyo et al., 2003).

![Fig. 6: Thermal stability of *T. harzianum* PGase. The reaction mixture contained in 0.5 ml: 50 mM sodium acetate buffer, pH 5.0, suitable amount of enzyme and 1 % polygalacturonic acid. The reaction mixture was preincubated at various temperatures for 30 min prior to substrate addition, followed by cooling in an ice bath. The enzyme activity was measured using the standard assay method as previously described. Activity at zero time was taken as 100% activity. Each point represents the average of two experiments.](image)

All the examined metal cations at 1 mM, Zn\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\) and Ca\(^{2+}\) showed different and partial inhibitory effects on the activity of *T. harzianum* PGase except for Hg\(^{2+}\) which was completely inhibited the enzyme activity (Table 3). The similar effect was reported for polygalacturonase from Bacillus, where Cu\(^{2+}\), Ni\(^{2+}\) and Zn\(^{2+}\) inhibited the enzyme activity (Kobayashi et al., 2001). On the contrary, HgCl\(_2\) increased the PG2 activity of *A. niger* 3.4 times but did not effect PG1 (Sakamoto et al., 2002). Devi and AppuRao (1996) found that Hg\(^{2+}\) inhibited PGs activity of *A. carbonarius*, where PGIII was more sensitive than PGII toward Hg\(^{2+}\).

**Table 3:** Effect of metal cations on *T. harzianum* PGase.

<table>
<thead>
<tr>
<th>Metal cations</th>
<th>Relative activity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(^{2+})</td>
<td>87</td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>63</td>
</tr>
<tr>
<td>Ni(^{2+})</td>
<td>61</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>53</td>
</tr>
<tr>
<td>Hg(^{2+})</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Enzyme was preincubated for 15 min at room temperature with 1 mM of listed metal ions as final concentration prior to substrate addition. Activity in absence of metal cations was taken as 100% activity. Each value represents the average of two experiments.

Generally, \(\alpha\)-amylases and polygalacturonases are integral part in fruit juice industry, where the source of these enzyme should be healthy safe. Therefore, we used the purified \(\alpha\)-amylase from wheat as safe source (Mohamed et al., 2009). Concerning the *T. harzianum* PGase, Roco and Pérez (2001) reported that *T. harzianum* is a beneficial microbe that does not cause disease or adverse health effects to humans and is not likely to harm the environment. The effect of the two purified \(\alpha\)-amylase and *T. harzianum* PGase on juice processing was tested through the determination of the quantity and degree of clarity of the apple juice (Table 4). The results showed that the quantity of the juice increased in the order of Treatment 3 (1 ml PGase and 1 ml \(\alpha\)-amylase) > Treatment 2 (1 ml \(\alpha\)-amylase and 1 ml water) > Treatment 1 (1 ml PGase and 1 ml water) > Treatment 4 (2ml water) depending on the measuring of the volume of juice filtrate, where the volume of juice increased 135% in presence of mixture of two enzymes than in presence of water. Concerning the clarity
of the apple juice, the same results were detected where the clarity increased in the order of Treatment 3 > Treatment 2 > Treatment 1 > Treatment 4 depending on the measuring of turbidity at 540nm. The results concluded that the mixture of α-amylase and polygalacturonase give the best resolution. This attributed to the high content of starch and pectin in apple fruit.

Table 4: Effect of T. harzianum PGase and wheat α-amylase on the quantity and clarity of apple juice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume (ml) after 5 min</th>
<th>Volume (ml) after 10 min</th>
<th>Volume (ml) after 15 min</th>
<th>Volume (ml) after 20 min</th>
<th>O.D at 540 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment 1</td>
<td>3.4±0.15</td>
<td>3.8±0.14</td>
<td>4.6±0.17</td>
<td>5.0±0.2</td>
<td>0.6±0.02</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>3.6±0.12</td>
<td>4.0±0.2</td>
<td>4.8±0.18</td>
<td>5.2±0.22</td>
<td>0.5±0.03</td>
</tr>
<tr>
<td>Treatment 3</td>
<td>4.0±0.1</td>
<td>4.4±0.12</td>
<td>5.2±0.16</td>
<td>5.8±0.2</td>
<td>0.35±0.01</td>
</tr>
<tr>
<td>Treatment 4</td>
<td>3.2±0.2</td>
<td>3.6±0.22</td>
<td>4.0±0.19</td>
<td>4.3±0.22</td>
<td>1.0±0.05</td>
</tr>
</tbody>
</table>

Treatment 1, 1 ml polygalacturonase and 1 ml water; Treatment 2, 1ml α-amylase and 1 ml water; Treatment 3, 1 ml polygalacturonase and 1ml α-amylase; Treatment 4, 2 ml water. Each value represents the mean of three experiments ±S.E.

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REFERENCES


