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Characterization of an Exopolygalacturonase from *Aspergillus niger*

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Abstract Polygalacturonase (PGI) from *Aspergillus niger* NRRL3 was purified about 12.0-fold from the cell-free broth using diethylaminoethyl-Sepharose and Sephacryl S-200 columns. The molecular weight of the PGI was 32,000 Da as estimated by gel filtration and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. PGI had an isoelectric point of 7.6 and an optimum pH of 5.0. PGI was active on polygalacturonic acid and esterified pectins, but the activity on pectin decreased with an increase in degree of esterification. PGI had higher affinity (low K_m) and turnover number (V_{max}/K_m and K_{cat}/K_m) toward polygalacturonic acid. PGI was found to have a temperature optimum at 40°C and was approximately stable up to 30 °C. All the examined metal cations had partial inhibitory effects on PGI, while Mn^{+2} at 5 mM caused a complete inhibition for the enzyme. Comparison of viscosity reduction rates with release of reducing sugars indicated that the enzyme from *A. niger* is exoacting. The storage stability study of PGI showed that the enzyme in powder form retained 56% of its activity after 9 months of storage at 4 °C. The above properties of PGI may be suitable for food processing.

Keywords Pectin · Polygalacturonic acid · Polygalacturonase · *Aspergillus niger* · Purification · Characterization · Mode of action

Introduction

Pectinolytic enzymes are classified according to their mode of attack on the galacturonan part of the pectin molecule. They can be distinguished from pectin methylesterase, which de-esterify pectins to low methoxy pectins or pectic acid, and from pectic depolymerases, which split the glycosidic linkages between galacturonosyl (methyl ester) residues. Polygalacturonases (PG) split glycsidic linkages next to free carboxyl groups by hydrolysis,

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while pectate lyases split glycosidic linkages next to free carboxyl groups by β-elimination [1, 2]. PG is produced by plants [3, 4] and by microorganisms such as bacteria and fungi [5–7], but only a few yeast species show this ability [8, 9]. In relation to their activity, PG cleaving α-[1,4]-glycosidic bonds of nonesterified galacturonic acid residues are classified as endoPG (EC 3.2.1.15) and exoPG (EC 3.2.1.67). The prefixes endo and exo denote random or terminal cleavage activity, respectively.

In *Aspergillus niger*, a multigene family encoding endoPGs was identified [10]. Thus far, six out of seven members of the PG-encoding gene family have been sequenced, and the corresponding enzymes have been biochemically characterized [11–13]. Although the extracellular-mature *A. niger* PGs share overall 66–85% of amino acid identity, they exhibit quite different enzymatic properties. The six *A. niger* PGs further differ in their pH optima (ranging from 3.9 to 5.0) and their mode of action on oligogalacturonates of defined chain length. The presence of a PG family suggests that the enzymes have complementary activities and that each one fulfills a unique role during the growth of the fungus on pectic substrates [14].

Generally, each of the *A. niger* PGs has different specific kinetic parameters on polygalacturonic acid and a specific mode of action [11–13]. Furthermore, the enzymes display a different tolerance or preference for partially methylesterified substrates. Therefore, this study focused on the characterization of PG from other strain of *A. niger* NRRL3 especially its effect on the mode of action, the kinetic parameters on polygalacturonic acid and pectins, and the performance on partially methylesterified pectins.

Materials and Methods

Microorganism

A. niger NRRL3 was provided from the culture collection of the Microbial Chemistry Department, National Research Centre, Dokki, Cairo, Egypt.

Cultivation of Organism

The culture of fungus was cultivated and maintained on dextrose agar (DA) 4.3% (w/v) powder from Difco (USA).Conidia was scrapped from mycelia, which was grown on slants (DA) for the fifth day at 28 °C and suspended by hand shaking in sterile distilled water. One-milliliter aliquot of this suspension (5×10^5 spores per milliliter) was used to inoculate under aseptic conditions in 250-ml Erlenmeyer flasks each containing 100 ml of sterile medium of potato dextrose broth with 0.1% citrus pectin. The inoculated flasks were incubated at 30 °C with shaking on a rotatory incubator shaker at 200 rpm for specific time intervals and without shaking before the cell-free broth was recovered by filtration using a clean gauze.

Purification of Polygalacturonase from *A. niger* NRRL3

Unless otherwise stated, all steps of purification were performed at 4 °C using 50 mM sodium acetate buffer, pH 5.0.

Cell-Free Broth

The culture filtrate of *A. niger* was concentrated, using lyophilization, to concentrate large volumes of culture filtrate, then dialyzed against 50 mM sodium acetate buffer, pH 5.0

overnight. The dialyzed filtrate was centrifuged at 13,200×*g* for 30 min at 4–7 °C, and the supernatant was pooled and designated as a cell-free broth. The cell-free broth was frozen at −20 °C for further analysis.

DEAE-Sepharose Chromatography

The dialyzed sample was applied directly on a diethylaminoethyl (DEAE)-Sepharose column (14×1.6 cm inner diameter [i.d.]) previously equilibrated with 50 mM sodium acetate buffer, pH 5.0. The enzyme was eluted with a linear gradient from 0.0 (75 ml) to 0.5 M NaCl (75 ml) in the same buffer at a flow rate of 30 ml/h and 3-ml fractions. Protein fractions exhibiting PG activity were pooled in two peaks (PGI and PGII).

Sephacryl S-200 Chromatography

Pooled PGI fractions were concentrated by dialysis against solid sucrose. The concentrated sample was applied on a Sephacryl S-200 column (95×1.6 cm i.d.) previously equilibrated with sodium acetate buffer, pH 5.0, and developed at a flow rate of 20 ml/h, and 3-ml fractions were collected. The PG enzyme was eluted with the same buffer.

Enzyme Assay

The PG was assayed by determining the liberated reducing end products by the method of Nelson [15] using galacturonic acid as the standard. The reaction mixture contained in 1 ml 0.1% polygalacturonic acid in 50 mM sodium acetate buffer, pH 5.0, and an appropriate amount of enzyme. The reaction started by incubation of this mixture at 40 °C for 1 h, after the incubation the reaction mixture was mixed with colored reagents. The optical density was determined spectrophotometrically at 600 nm. Enzyme and substrate controls were carried out. One unit of enzyme activity was defined as the amount of enzyme that increases the optical density 1.0 per minute per milliliter under standard assay conditions. For kinetic experiments, 1 U of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of galacturonic acid per minute per milliliter under standard assay conditions.

Protein Determination

Protein was determined according to the method of Bradford [16] using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis

Electrophoresis under nondenaturing conditions was performed in 10% (w/v) acrylamide slab gel according to the method of Davis [17] using a Tris–glycine buffer, pH 8.3. Protein bands were located by staining with silver nitrate.

Molecular Weight Determination

Molecular weight was determined by gel filtration technique using Sephacryl S-200. The column (95×1.6 cm i.d.) was calibrated with cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and β-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume (V_o). Subunit

Table 1 Purification scheme for *A. niger* NRRL3 polygalacturonase.

Step	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg protein)	Fold purification	Recovery (%)
Cell-free broth	1.3	45.2	34.7	1	100
DEAE-Sepharose					
PGI	0.2	37.3	186	5.3	82.5
PGII	0.5	3.0	6.0	0.17	6.0
Sephacryl S-200					
PGI	0.043	18	418.6	12.0	39.8

[a] One unit of polygalacturonase activity was defined as the amount of enzyme that increases the OD 1.0 per minute milliliter under standard assay conditions.

molecular weight was estimated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) [18]. SDS-denatured phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and α-lactalbumin (14,200) were used for calibration.

Isoelectric Focusing

The isoelectric point (p*I*) of the enzyme was determined by isoelectrofocusing on polyacrylamide gel as described by Giulian et al. [19]. Isoelectricofocusing markers (amyloglycosidase [p*I* 3.6], soybean trypsin inhibitor [p*I* 4.6], bovine-lactoglobuin [p*I* 5.1], bovine carbonic anhydrase II [p*I* 5.9], human carbonic anhydase I [p*I* 6.6], horse myoglobin [p*I* 6.8, 7.2], lens culinaries lectin [p*I* 8.2, 8.6, 8.8], and bovine trypsinogen [p*I* 9.3]) were used for the calibration curve.

Determination of Change in Specific Viscosity (η)

Viscometric assays were done in an Ostwald viscometer containing of reaction mixture (9 ml of 0.1% polygalacturonic acid [PGA] or esterified pectin in 0.05 M sodium acetate buffer, pH 5.0, and 1 U of enzyme). Measurements were made at room temperature in a glass tube viscometer. Loss in viscosity was determined at 30-min intervals for 180 min.

Results

Purification of *A. niger* Polygalacturonase

The purification scheme of PG from *A. niger* cell-free broth was summarized in Table 1. Purification of PG from *A. niger* was carried out using two steps. The first step was carried out by using a column packed with DEAE-Sepharose, and the anion exchangers have positively charged groups, which will attack negatively charged molecules. By the DEAE-Sepharose column, two isoenzymes of PGs (PGI and PGII) were separated as shown in Fig. 1. Their specific activities were 186, 6.0 U/mg protein, respectively. Completion of the purification steps was carried out for PGI with the highest specific activity. The second purification step was carried out using a Sephacryl S-200 column, which was used to obtain PGI with the highest possible specific activity (418.6 U/mg protein), which represented

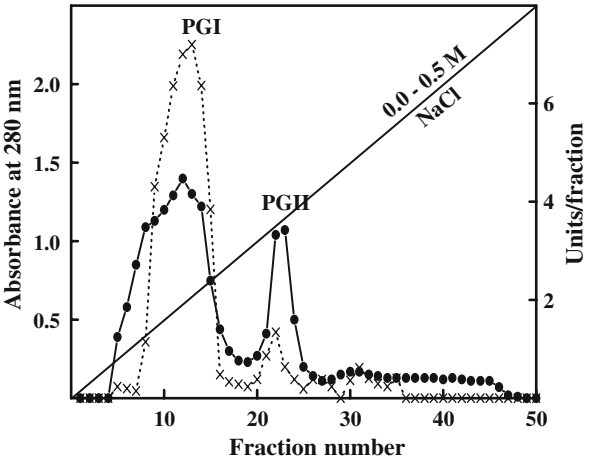

Fig. 1 A typical elution profile for the chromatography of *A. niger* NRRL3 PG cell-free broth on DEAE-Sepharose column (14×1.6 cm i.d.) previously equilibrated with 50 mM sodium acetate buffer, pH 5.0, at a flow rate of 30 ml/h and 3-ml fractions. Absorbance at 280 nm (*solid line with dots*), PG activity (*dashed line with crosses*)

12.0-fold purification over the cell-free broth with 39.8% recovery (Fig. 2). The electrophoretic behavior of the cell-free broth and the final purification step of PGI are shown in Fig. 3a. One band was detected on the gel after gel filtration on Sephacryl S-200, which indicated the homogeneity of the final preparation.

Molecular Weight of *A. niger* PGI

The molecular weight of *A. niger* PGI was calculated from the Sephacryl S-200 calibration curve and estimated to be 32,000 Da. The molecular weight of *A. niger* PGI was confirmed by SDS-PAGE (Fig. 3b) and estimated to be 32,000 as a single subunit from the calibration curve.

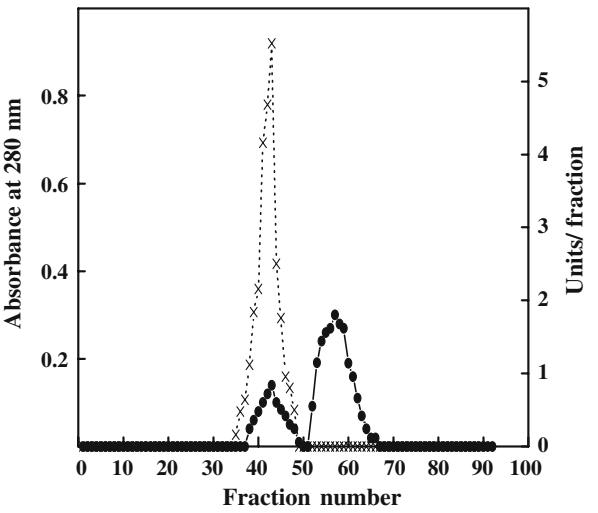

Fig. 2 A typical elution profile for the chromatography of *A. niger* NRRL3 PGI DEAE-Sepharose fraction on Sephacryl S-200 column (95×1.6 cm i.d.) previously equilibrated with 50 mM sodium acetate buffer, pH 5.0, at a flow rate of 20 ml/h and 3-ml fractions. Absorbance at 280 nm (*solid line with dots*), PG activity (*dashed line with crosses*)

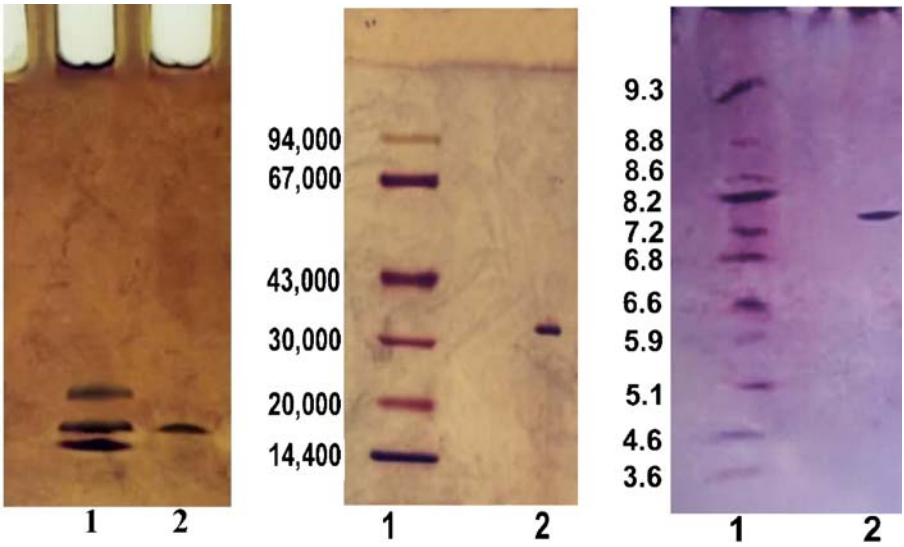

Fig. 3 Electrophoretic patterns for *A. niger* NRRL3 PG. **a** Nondenaturing PAGE: *1* cell-free filtrate, *2* Sephacryl S-200 PGI. **b** SDS-PAGE for molecular weight determination: *1* standard proteins, *2* Sephacryl S-200 PGI. **c** Isoelectric point: *1* standard proteins, *2* Sephacryl S-200 PGI

Isoelectic Focusing

The isoelectric-focusing experiment indicated that *A. niger* PGI was migrated as a single major band with an isoelectric point (p*I*) of 7.6 (Fig. 3c).

Characterization of *A. niger* PGI

All experiments for the characterization of *A. niger* PGI was carried out on the purified enzyme eluted from the Sephacryl S-200 column.

pH Optimum

The pH profile for the purified *A. niger* PGI was performed using PGA as a substrate. The pH range varied from 3.5 to 8.0 using 50 mM sodium acetate buffer for pH values from 3.5 to 5.5, sodium phosphate buffer for pH values from 6.0 to 7.0, and Tris–HCl for pH values from 7.0 to 8.0. PGI had a pH optimum in sodium acetate buffer at pH 5.0, while its activity was gradually decreased at pH levels less or greater than 5.0 indicating that *A. niger* PGI is an acidic PG (Fig. 4).

Kinetic Properties of PGI

Substrate Specificity

A study of substrate specificity for *A. niger* PGI was made using pectin with different degrees of esterification (DE) and methoxyl groups. The enzyme activities with different pectins were compared to the activity with PGA, which was regarded as 100% activity. For

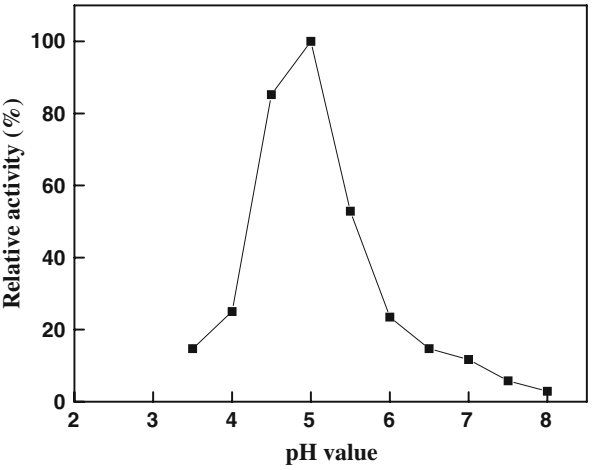

Fig. 4 pH optimum of *A. niger* NRRL3 PGI. The reaction mixture contained in 1.0 ml: 50 mM sodium acetate buffer (pH 3.5–5.5), sodium phosphate buffer (pH 6.0–7.0), and Tris–HCl buffer (pH 7.0–8.0), 0.1% PGA, and a suitable amount of enzyme preparation. The reaction mixtures were incubated for 1 h at different pH and assayed for PG activity. Each *point* represents the average of two experiments

pectins with different DE, the extent of hydrolysis was decreased with increasing of DE % in the order of pectin citrus: DE 26%>DE 67%>DE 89% with 75, 53, and 41% relative activities, respectively, except for pectin citrus with DE 93%, which had 75% relative activity. For pectins with different degrees of methylation, the extent of hydrolysis was increased with increasing of methoxyl content in the order of pectin apple (methyl 6%)> pectin citrus (methyl 7.8%)>pectin citrus (methyl 8.9%) with 73, 78, and 97% relative activities, respectively (Table 2).

K_m, V_{max}, and K_{cat}

Using the Lineweaver–Burk plot approach, kinetic parameters for the PGI acting on PGA and different pectins were determined (Table 3). The K_m value of *A. niger* PGI using PGA as the routinely used substrate was estimated to be 0.8 mg PGA/ml. The enzyme had higher affinity (low K_m), turnover number V_{max}/K_m, and K_{cat}/K_m toward PGA compared to the examined pectins. While PGI had a low affinity (high K_m 1.5 mg/ml) toward pectin (methoxy 12%), it had high turnover number V_{max}/K_m, and K_{cat}/K_m, which was similar to those of PGA, and had also the higher K_{cat} value compared to the other examined pectins.

Table 2 Relative activities of *A. niger* NRRL3 PGI toward PGA and different pectins as substrates.

Pectin	Relative activity (%)
PGA	100
Pectin citrus (DE 26%)	75
Pectin citrus (DE 67%)	53
Pectin citrus (DE 89%)	41
Pectin citrus (DE 93%)	75
Pectin citrus (methyl 6%)	73
Pectin apple (methyl 7.8%)	78
Pectin citrus (methyl 8.9%)	97

The reaction mixture contained in 1.0 ml: 0.1% substrate, 50 mM sodium acetate buffer, pH 5.0, and a suitable amount of enzyme preparation. The activity with PGA was taken as 100% activity. Each value represents the average of two experiments.

Table 3 The kinetic parameters of *A. niger* NRRL3 PGI.

Substrate	K_m (mg/ml)	V_{max} (μmol/min)	V_{max}/K_m	K_{cat}[a] (s^{-1})	K_{cat}/K_m
PGA	0.8	1.6	2.0	85.4	106.7
Pectin (DE 26%)	1.6	2.5	1.5	132.7	82.9
Pectin (methoxy 12%)	1.5	3.0	2.0	160.2	106.8
Pectin (methoxy 8.9%)	1.4	2.1	1.5	53.4	38.1
Pectin (methoxy 7.8%)	1.6	2.3	1.4	122.8	76.7
Pectin (methoxy 6%)	1.7	2.5	1.4	133.5	78.5

[a] K_{cat} nanomoles of product produced per nanomole enzyme protein per second. The nanomole enzyme protein was determined from the calculated molecular weight of *A. niger* NRRL3 PGI (32,000).

Effect of Temperature

Figure 5 show the temperature optimum curve for *A. niger* PGI. The complete enzyme assays were incubated at different temperatures ranging from 20 to 80 °C for 1 h. *A. niger* PGI had a temperature optimum at 40 °C. The enzyme activity was gradually decreased till reached 42.8% at 80 °C. The effect of temperature on the stability of *A. niger* PGI was examined (Fig. 6). The enzyme was incubated for 15 min in 50 mM sodium acetate buffer, pH 5.0 for 15 min at different temperatures ranging from 20 to 80 °C before substrate addition, and the residual activity was determined. The PGI was stable up to 30 °C. Its stability was gradually decreased till 80 °C, where it lost 59 and 80% of its activity at 50 and 80 °C, respectively.

Effect of Metal Ions

The effect of metal ions at the concentrations of 1 and 5 mM on *A. niger* PGI is shown in Table 4. All the examined ions had different inhibitory effects on PGI. At the concentration of 1 mM, the enzyme was inhibited in the order of $MgCl_2$<$NiCl_2$<$HgCl_2$<$CoCl_2$<$CaCl_2$<$CuSO_4$<$MnCl_2$ with 8, 10, 17, 19, 21, 27, and 57% inhibition, respectively. At the concentration of 5 mM, the inhibitory effect was increased in the order of Mg^{2+}<Ca^{2+}<

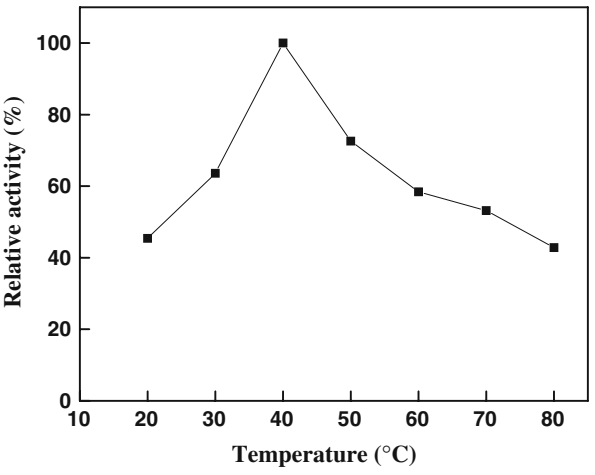

Fig. 5 Optimum temperature of *A. niger* NRRL3 PGI. The enzyme activity was measured at different temperatures ranging from 20 to 80 °C. The reaction mixture contained in 1.0 ml: 0.1% PGA, 50 mM sodium acetate buffer, pH 5.0, and a suitable amount of enzyme preparation. Each *point* represents the average of two experiments

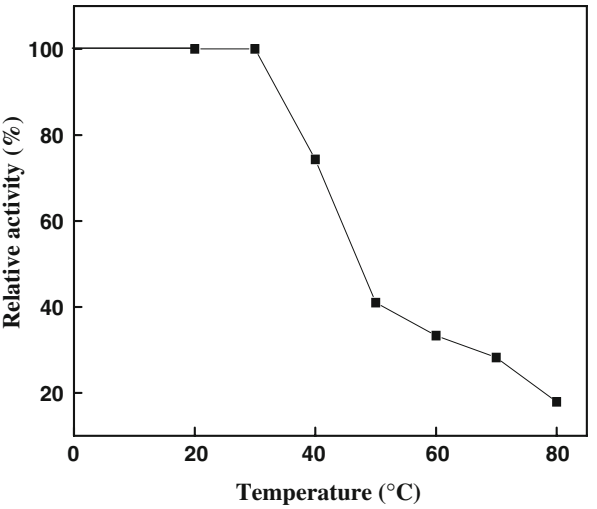

Fig. 6 Effect of temperature on stability of *A. niger* NRRL3 PGI. The reaction mixture contained in 1.0 ml: 50 mM sodium acetate buffer, pH 5.0, and a suitable amount of enzyme preparation. The reaction mixtures were incubated at different temperatures ranging from 20 to 80 °C for 15 min before substrate addition (0.1% PGA) followed by cooling in an ice bath. The residual PG activity was assayed under standard conditions. Activity at zero time was taken as 100%. Each *point* represents the average of two experiments

$Ni^{2+}<Co^{2+}<Cu^{2+}=Hg^{2+}$ with 22, 35, 37, 45, 48, and 48% inhibition, respectively. Mn^{2+} at the concentration of 5 mM caused a complete inhibition for PGI.

Decrease in Specific Viscosity and Reducing Sugars from Esterified Pectin

The action mode of the enzyme (exoPG or endoPG) was determined by using viscometer assays. At the time, PGI affected 7% decrease in the viscosity of pectin solution, while the extent of hydrolysis (release of reducing sugars) was more than 50%. Figure 7 showed that the rate of enzyme that catalyzes the release of reducing sugars of pectin was seven times higher than the rate of reduction in viscosity. This indicated an exo-action of *A. niger* PGI.

Table 4 Effect of metal ions on *A. niger* NRRL3 PGI.

Metal ion	Concentration (mM)	Relative activity (%)
Mg^{2+}	1	92
	5	78
Ni^{2+}	1	90
	5	63
Hg^{2+}	1	83
	5	52
Co^{2+}	1	81
	5	55
Ca^{2+}	1	79
	5	65
Cu^{2+}	1	73
	5	52
Mn^{2+}	1	43
	5	0

Enzyme was preincubated for 15 min at 40 °C with 1 and 5 mM of listed ions as final concentration before substrate addition. The reaction mixture contained in 1.0 ml 0.1% PGA, 50 mM sodium acetate buffer, pH 5.0, and a suitable amount of enzyme preparation. The activity in absence of metal ions was taken as 100% activity. Each value represents the average of two experiments.

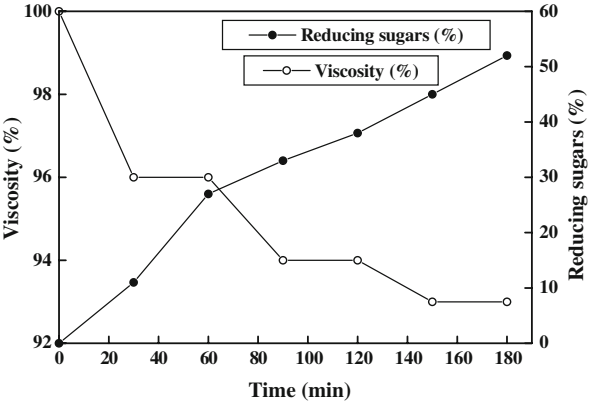

Fig. 7 Changes in viscosity and reducing sugar of citrus pectin (methyl 8.9%) by *A. niger* NRRL3 PGI

Storage Stability

The storage stability of *A. niger* PGI in different forms was studied in different conditions. In liquid form, the enzyme retained 60, 53, and 55% of its activity after 2 weeks at room temperature, 4 and −20 °C, respectively. The enzyme retained 16% of its activity after subjection to freezing and thawing for five times during 4 weeks. In presence of 40% glycerol as a preservative, the enzyme retained 50% of its activity after 3 weeks at refrigerator (4 °C). The enzyme in the powder form retained 56% of its activity after 9 months of storage.

Discussion

Because one of the objectives of this study is the preparation of pure homogenous PG from *A. niger* for further characterization, a simple reproducible method was established. By chromatography on DEAE-Sepharose, two peaks, PGI and PGII, were separated. The purification was restricted for PGI with the highest activity level compared to PGII. The two relatively simply and highly reproducible chromatographic steps were sufficient to obtain homogenous PG from *A. niger*. The specific activity (418.6 U/mg protein) and the recovery (39.8%) of the purified *A. niger* PGI was closely resembled to that obtained by *A. kawachii* PGI (430 U/mg protein and 40% recovery) [20]. However, specific activity of *A. niger* PGI was higher than that of PGs from *Fusarium moniliforme* NCIM 1276 (178.6 U/mg protein) [21] and *F. moniliforme* PGII (18.66 U/mg protein) [22].

The native molecular weight of *A. niger* PGI was determined by the gel filtration technique and was found to be 32,000 Da. This value was confirmed by SDS-PAGE, where the denatured protein migrated as a single band to the position corresponding to molecular weight of 32,000 Da, indicating that the enzyme was a monomeric protein. In comparison with other characterized PGs, the molecular weight value of *A. niger* PGI was close to the values of PGs from *Rhizopus oryzae* NBRC 4707 (31,000 Da) [23], *F. moniliforme* (30,600 Da) [22], and *Postia placenta* (34,000 Da) [24]. In contrast, two exo-PGs 1 and 2 from *A. niger* had higher molecular masses of 82, 000 and 56,000, respectively [25].

With regard to *A. niger* PGI p*I* value (7.6), most PGs reported to be acidic [26, 27]. In the present study, the relatively alkaline p*I* value of *A. niger* PGI resembled PG from Zimbabwean *Armillaria* (p*I* 7.3) [28]. The alkaline p*I* was detected for PGs from *F. moniliforme* (p*I* 8.1) [21] and *M. flavus* (p*I* 8.3) [29].

All the PG forms had the same pH optimum value that lies in the range of 4.0–6.5 reported for most endo- and exoPGs [1]. The *A. niger* PGI showed optimal activity at pH 5.0. Sakamoto et al. [25] reported that the optimum activities occurred at pH 3.4–3.8 for exoPG1 and 3.4–4.2 for exoPG2 from *A. niger*, respectively [25]. In contrast, the PG from *A. kawachii* had an optimum activity at low pH (2.0–3.0), while being inactive at pH 5.0 [20]. The previous low pH value was very useful in the process of enzyme extraction because it minimizes the action of native pectin methylesterase, reduces the risk of microbial contamination, and stabilizes pectin in solution [8, 30]. However, the highest pH optimum (pH 10.0) value was reported for PG from *Bacillus* sp. MG-cp-2 [31].

For characterization of *A. niger* PGI with regard to substrate specificity, a series of pectins with different DE and different methoxy content were tested as substrates. The affinity of *A. niger* PGI was decreased with the increase in degree of esterification and increased with the increase in methoxy content, where the extent of hydrolysis was decreased with the increasing of DE % in the order of pectin citrus: DE 26%>DE 67%>DE 89% with the exception of pectin citrus DE 93%, which had 75% relative activity. These results were resembled to that obtained from *Bacillus* exoPG [32], where the PGA degradation rate was taken as 100%; the relative rates toward citrus pectin with DE of 31, 63, and 93% were 42.7, 8.3, and 0%, respectively.

Using the Lineweaver–Burk plot approach, kinetic parameters for the PGI acting on PGA and different pectins were determined. Using PGA as a substrate, the K_m of *A. niger* PGI was 0.8 mg/ml. The same K_m value was reported for *S. sclerotiorum* using the same substrate (K_m 0.8 mg/ml) [33]. Binding affinities (K_m) of the reported PGs using PGA were ranged from 1.34 to 6.7 mg/ml [34, 35]. Using different pectins as substrates, the K_m of *A. niger* PGI was ranged from 1.4 to 1.7 mg/ml. The varied K_m were also reported for PGs from *Neurospora crassa* (5.0 mg/ml) [36] and *Paenibacillus amylolytics* (4.6 mg/ml) [37]. In the present study, using PGA, the kinetic values as V_{max} (1.6 μmol/min), K_{cat} (85.4/s), V_{max}/K_m (2.0), and K_{cat}/K_m (106.7) were detected for *A. niger* PGI, while, using pectins with different DE, these values ranged from 2.1 to 3.0 μmol/min for V_{max}, 53.4 to 160.2/s for K_{cat}, 1.4 to 2.0 for V_{max}/K_m, and 38.1 to 106.8 for K_{cat}/K_m. The apparent K_m and K_{cat} values for hydrolyzing PGA reported from *Bacillus* were 0.00086 mg/ml and 22.2/s, respectively [32]. Niture and Pant [22] reported that the PGI of *F. moniliforme* had K_m 0.110 mg/ml, V_{max} 111.11 μmol/min, and K_{cat} 4,200/min, while PGII had K_m 0.16610 mg/ml, V_{max} 13.33 μmol/min, and K_{cat} 403/min. For *F. moniliforme* NCIM 1276, K_{cat}/K_m was 3.5×10^4 [21].

The *A. niger* PGI was found to have maximum activity at 40 °C. This value was close to that reported for several other PGs from *A. niger*, *S. sclerotiorum*, and *C. lindemuthianum* (42 °C) [38] and *F. moniliforme* (40 °C) [22] but lower than that for PGs from *S. cerevisiae* (60 °C) [39], *A. niger* (60 °C) [25], and *S. thermophile* Apinis (55 °C) [40]. A thermal stability study indicated that the hydrolytic activity of *A. niger* PGI was stable up to 30 °C and decreased gradually as temperature increased from 40 to 80 °C. In comparison to other PGs from *T. reesei*, PG1 and PG2 were stable up to 40 and 60 °C and lost most of their activities at 60°Cand 80 °C, respectively [41]. For other purified PGs from *A. niger*, *S. sclerotiorum*, and *C. lindemuthianum*, the three enzymes from these fungi were stable below 35 °C but were rapidly inactivated after incubation at temperatures above 35 °C [38].

The effect of various metal ions on *A. niger* PGI was tested at the concentrations of 1 and 5 mM. All the examined cations (Ca^{2+}, Mn^{2+}, Hg^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, and Mg^{2+}) had partial inhibitory effects on the activity of the enzyme, while Mn^{2+} caused a complete inhibition at the concentration of 5 mM. Strong inhibitory effect of Mn^{2+} appeared to be similar to that reported for PG from *Bacillus* sp. MG-cp-2 [31] but different in that Ni^{2+} and

Ca^{2+}stimulated the enzyme activity. *Bacillus* exo-PG was stimulated by Ca^{2+}, Mn^{2+}, and Mg^{2+}, while it was inhibited by Cu^{2+} and Ni^{2+} [32]. Generally, $HgCl_2$ had a complete inhibitory effect toward many enzymes. The testing of a higher concentration of $HgCl_2$ (1 and 5 mM) leads to a partial inhibition effect toward PGI activity. Therefore, this enzyme had good resistance toward $HgCl_2$. However, the addition of 0.01 mM $HgCl_2$ increased the PG2 activity of *A. niger* 3.4 times but did not effect PG1 [25]. *A. niger* exoPGs reported by Mill [42] and Hara et al. [43] were also enhanced 11 and 1.4 times in the presence of 0.001 and 0.02 mM $HgCl_2$, respectively.

The mode of cleavage of pectin by the purified enzyme *A. niger* PGI was studied by comparison of the rate of reduction in viscosity with the corresponding increase in appearance of reducing groups in solution. The rate of the enzyme-catalyzed reduction of viscosity (7%) of solutions of pectin as a substrate was considerably lesser than the rate of release of reducing sugars (50%), confirming that this enzyme functioned in an exo-like manner. This result was similar to that reported for PGs from *B. cinerea* [44] and *Bacillus* [32] using PGA as a substrate. Furthermore, $PGIII_0$ and PG_5 enzymes purified from *F. oxysporum* f. sp. *ciceri* reduced the viscosity by 14 and 8%, respectively, after 4% hydrolysis of substrate suggesting that these two enzymes are exoenzymes. In contrast, to be classified as an endoenzyme, the rate of decrease in viscosity must exceed the rate of release of reducing sugars [45, 46]. For PG isolated from *M. flavus* [29], the specific viscosity of PGA and esterified pectin (DE 89%) were decreased by 50 and 40%, respectively, in 10 min at 30 °C but released only small amounts of reducing sugars suggesting that the enzyme acts in an endo-manner.

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