Separation, Purification and Characterization of Three Endo-polygalacturonases from a Newly Isolated *Penicillum oxalicum*

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Abstract: Three endo-polygalacturonases (endoPGs) from a newly isolated *Penicillum oxalicum* (CGMCC 0907) capable of utilizing waste biomass as growth substrate were separated and purified to homogeneity by ultra-filtration, affinity adsorption chromatography, CM-cellulose column chromatography, and Sephadex G-100 gel filtration chromatography with the overall yield of 64.5% from the crude enzyme. The specific activities and recovery rates of endoPG-1, endoPG-2 and endoPG-3 were 1120 U/mg and 21.6%, 1350 U/mg and 25.9%, and 1560 U/mg and 17.0%, respectively. The three purified endoPGs had a close molecular weight to 41 kDa as estimated by SDS–PAGE. The optimum temperature and pH for the function of them were 65 ℃ and 5.0, 55 ℃ and 5.0, 50 ℃ and 5.5, respectively. Their pI and K_m values were 5.9 and 0.78 mg/mL, 6.0 and 1.2 mg/mL, and 6.1 and 2.0 mg/mL, respectively.

Key words: separation; characterization; *Penicillum oxalicum*; endo-polygalacturonase
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1 INTRODUCTION

Pectinases comprise a heterogeneous group of enzymes that catalyze the breakdown of pectin-containing substrates. The major types of pectinases include endo-polygalacturonase (endoPG, EC 3.2.1.15), exo-polygalacturonase (exoPG, EC 3.2.1.67), pectin lyase (EC 4.2.2.10), pectate lyase (EC 4.2.2.2), and pectinesterase (EC 3.1.1.11)[1–4]. The endoPGs cause random hydrolysis of 1,4-α-D-galactosiduronic linkages in pectate molecules and are generally used for increasing the yield of fruit juices, as filter aid in their processing and for clarification[2,5]. Pectinases are now an integral part of fruit juice and textile industries with various biotechnological applications[2,6].

To date, many microorganisms have been studied for producing pectinases, and commercial pectinases preparations are obtained mainly from *Aspergillus* and *Penicillium*[7–13]. Pectinases from various microorganisms have also been purified and characterized. Gummadi et al.[14] reviewed the purification and biochemical properties of microbial pectinases. Many pectinases from various microorganisms were purified to homogeneity with a combination of ion exchange and gel filtration chromatograph as main procedures[9,14–18], while few purification procedures of pectinases contained affinity adsorption chromatography which was an efficient method for purification of enzyme from microbes. Most of the literature reported that only two polygalacturonases were purified from one strain of different microbes respectively[19–21].

Zhang et al.[22] isolated and identified a new strain of *P. oxalicum* with a high level yield of pectinases from waste biomass, and pectinases extract (PE) from the fermentation product of the strain of *P. oxalicum* was tested for its ability to induce protection against scab caused by *Cladosporium cucumerinum* on cucumber (*Cucumis sativus* L.) plants[23]. However, to the best of our knowledge, few studies on purification and characterization of endo-polygalacturonases from strains of *Penicillum oxalicum* have appeared in the literature.

Separation and purification of proteins and enzymes, which account for a major fraction of the overall production cost, in an efficient way and at a desired level of enzyme activity over a long period of time are important parameters for selection and design of pectinases, and an improved knowledge of the properties of microbial pectinases is important in commercialization of industrial production and application of these enzymes in various potential fields.

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In this work, the separation and purification of three endoPGs from *P. oxalicum* with great efficiency and characterization of the three purified endoPGs for potential applications were investigated.

2 MATERIALS AND METHODS

2.1 Microorganism and Enzyme Production

P. oxalicum (CGMCC0907) was isolated by our laboratory from waste biomass for pectinases production[22]. The strain was maintained on potato glucose agar slants in a refrigerator, and subcultured every six months. The fermentation medium in a 500 mL Erlenmeyer flask contained: dry sugar beet pulp 10 g, $(NH_4)_2SO_4$ 1.5 g, $Na_3PO_4{\cdot}12H_2O$ 0.15 g, and tap water 30 mL. The medium was mixed and inoculated with 107 spores per gram of wet substrate. The flasks were placed in a humid cultivation chamber with a gentle circulation of air at 30℃ under static condition for 72 h. After cultivation, the crude pectinases were extracted by mixing every flask of fermented materials with 50 mL of distilled water, squeezing at 30 kg/cm^2 in a hydraulic press and filtering through a 0.2 μm membrane filter.

2.2 Ultra-filtration

After filtering, 1600 mL crude solution of pectinases was concentrated to 300 mL using the hollow fibre cross-flow ultra-filtration module fit with a polysulfone membrane (10000 molecular weight cut-off) under trans-membrane pressure of 0.8 kg/cm^2. The concentrate was dialyzed by adding 500 mL pure water to it, and then concentrated to 300 mL by the ultra-filtration again, in 6 times repetition. The concentrated and dialyzed crude pectinases by ultra-filtration, which was designated as the crude enzyme, was stored at 4℃ in the presence of sodium azide (0.1 g/L).

2.3 Affinity Chromatography

The crude enzyme was purified by affinity chromatography on a column (1.6 cm×20 cm) packed with pectic biomass powder cross-linked by epichlorhydrin[24]. The column was equilibrated with 20 mmol/L acetate buffer at pH 4.2, and then the enzyme solution was added. After washing the column with the same buffer (60 mL), the enzyme was eluted with 50 mmol/L acetate buffer, which includes 1.0 mol/L NaCl, with pH 5.6 at a flow rate of 30 mL/h. The active fraction was collected, concentrated by vacuum evaporator, and dialyzed against three changes of 20 mmol/L acetate buffer (pH 5.0) for 36 h.

2.4 CM-cellulose Chromatography

The dialysate after affinity chromatography was applied to a column (1.6 cm×30 cm) of CM-cellulose (Whatman CM-52) that had been equilibrated with 20 mmol/L acetate buffer (pH 5.0). The column was washed with 30 mL equilibration buffer and then eluted with a linear gradient of 0 to 0.6 mol/L NaCl in the same buffer at a flow rate of 20 mL/h. The active fractions were collected, concentrated and dialyzed.

2.5 Sephadex G-100 Gel Filtration

The dialysates after CM-cellulose chromatography were separately applied to a Sephadex G-100 column (1.6 cm×80 cm) that had been equilibrated with 50 mmol/L acetate buffer (pH 5.0). The column was eluted at a flow rate of 20 mL/h with the same buffer. The active fractions were collected, concentrated and dialyzed.

2.6 Assay of Pectinases

The activity of endo-pectinases was measured by the AJDA method according to Friedrich et al.[25] and Gu et al[26]. One unit of endopectinases activity was defined as the amount of enzyme which degrades 1 mg pectin (from citrus fruits, methoxy content 8%, Sigma) at pH 5.0 and 40℃ in half an hour.

The activity of polygalacturonases (PGs) was assayed by measuring the reducing groups released from polygalacturonic acid (Sigma) using the 3,5-dinitrosalicylic acid (DNS) method with D-galacturonic acid monohydrate (Sigma) as the standard[27]. One unit of PGs activity was defined as the amount of enzyme which yielded 1 μmol reducing ends per minute at 40℃ and pH 5.0.

Pectin and pectate lyases were assayed spectrophotometrically by measuring the increase in absorbance at 235 nm as described by Brühlmann[28]. The reaction substrate of pectin lyase was pectin (Sigma) and pectate lyase was sodium polygalacturonate (Sigma).

The results of enzyme assay were the means of duplicate determination of two independent samples. The standard derivations were less than 5%.

2.7 Determination of Protein

The protein was determined by the method of Lowry et al.[29] with bovine serum albumin (Pharmacia) as a standard. Protein in the column effluents was monitored by an online UV detector at 280 nm.

2.8 SDS–Polyacrylamide Gel Electrophoresis (PAGE)

SDS–PAGE was performed on a 10% gel according to Laemmli[30] to check the homogeneity of the enzyme and determine the molecular weight. Protein bands were visualized by staining with coomassie brilliant blue R250. Low range (14.4~97.4 kDa) molecular weight markers (Pharmacia) were used for

the estimation of molecular weight of purified endo-polygalacturonase.

2.9 Isoelectric Focusing

The isoelectric points (pI) of the three purified endoPGs were determined by Beijing University by isoelectric focusing (IEF).

2.10 Optimum pH

The influence of pH on endo-polygalacturonase activity was measured by performing the activity assay at pH values ranging from 4.0 to 6.0 at a constant temperature of 40℃.

2.11 Optimum Temperature

Optimum temperature for enzyme activity was determined by varying the assay temperature from 30 to 70℃ at optimum pH.

2.12 Kinetic Analysis

The kinetic constant, K_m, of enzyme was determined by the double-reciprocal plot method of Lineweaver Burk with polygalacturonic acid (Sigma) as substrate at optimum pH and 40℃.

2.13 Substrate Specificity

Substrate specificity of the enzyme was studied by varying the assay substrates (pectin, esterized pectin and polygalacturonic acid) at a fixed concentration (0.25%) and conducting enzyme assays by measuring the reducing groups released using the DNS method.

3 RESULTS AND DISCUSSION

3.1 Production and Concentration of Pectinases from *P. oxalicum*

P. oxalicum (CGMCC0907) isolated by our laboratory from waste biomass for pectinases production was identified by China General Microbiological Culture Collection Center (CGMCC)[22]. The colonial morphology of *P. oxalicum* (CGMCC0907) after growth at 30℃ for 5 d on PDA was shown in Fig.1.

The newly isolated fungus *P. oxalicum* (CGMCC0907) grew well at 30 ºC for 72 h and secreted 1.1×10^5 U of endo-pectinases, 6.5×10^3 U of PGs per gram sugar beet pulp[22], which was much higher than the maximum production of polygalacturonase from *Aspergillus sojae* ATCC 20235 (29.093 U/g solid)[31] and exo-pectinase from *Aspergillus niger* DMF 45 (45.9 U/g)[32], respectively. But pectin and pectate lyases were not found in the crude solution of pectinases. This result showed that the pectinases secreted by the strain mainly comprised endoPGs. The concentrated and dialyzed crude pectinases by ultra-filtration, which were designated as the crude enzyme, retained 1.2×10^5 U/mL of endopectinases activity at a recovery rate of 95%.

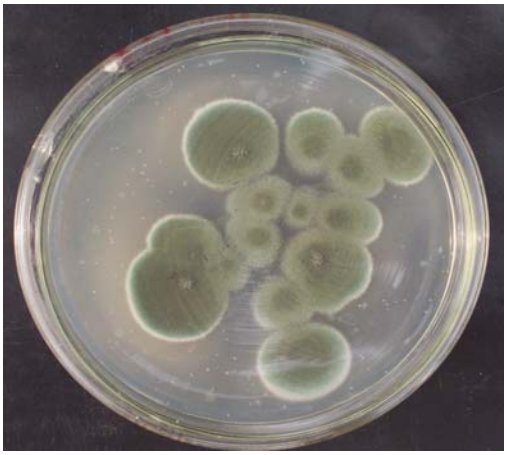

Fig.1 Colonial morphology of *P. oxalicum* (CGMCC0907) after growth at 30℃ for 5 d on potato dextrose agar (PDA)

3.2 Affinity, Cation Exchange and Gel Filtration Chromatography

Affinity chromatography on a column (1.6 cm×20 cm) packed with pectic biomass powder cross-linked by epichlorhydrin[24] was used to purify the crude enzyme, and by which a few of pectinases were purified from microbe[14]. The activity of endo-pectinases was monitored in the chromatography process. The affinity chromatography elution profile is shown in Fig.2.

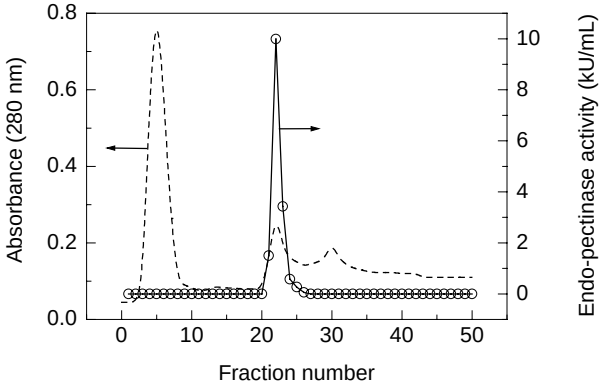

Fig.2 Affinity adsorption chromatography of crude pectinase from *P. oxalicum*

It is an efficient method for removing other substances from the crude enzyme. The active fraction was collected. Its total protein and activities of endo-pectinases and PGs were measured, and then the specific activities and recovery were calculated respectively. The specific activity and recovery rate of endo-pectinases and PGs were 13400 U/mg and 100%, 801 U/mg and 100%, respectively (Table 1).

Table 1 Purification of three endoPGs from *Penicillum oxalicum*

Step	PG		Endo-pectinase	
	Specific activity (U/mg)	Recovery rate (%)	Specific activity (U/mg)	Recovery rate (%)
Crude enzyme	172	100	2900	100
Affinity	801	100	13400	100
CM-cellulose		84.0		80.3
P1	1090	26.2	20900	25.0
P2	1280	35.4	23700	33.4
P3	1130	22.4	32500	21.9
Sephadex G-100		64.5		60.0
EndoPG-1	1120	21.6	23600	20.0
EndoPG-2	1350	25.9	25800	23.8
EndoPG-3	1560	17.0	33200	16.2

The cation exchange chromatography elution profile is shown in Fig.3. Three peaks of activity were obtained, and designated as P1, P2 and P3. Their proteinand activities of endo-pectinases and PGs were measured, then the specific activity and recovery rate were calculated. PGs of P1, P2 and P3 were purified to the yields of 26.2%, 35.4%, and 22.4% with their specific activities (Table 1). Meanwhile, the specific activities of endo-pectinases of P1, P2 and P3 were 20900, 23700 and 32500 U/mg, with the recovery rates of 25.0%, 33.4% and 21.9%, respectively. Their overall yields of PGs and endo-pectinases were 84.0% and 80.3% from the crude enzyme, respectively (Table 1). The anion exchange chromatography was also tested for the separation process, but it could not adsorb the pectinases from *P. oxalicum* (CGMCC0907) at all.

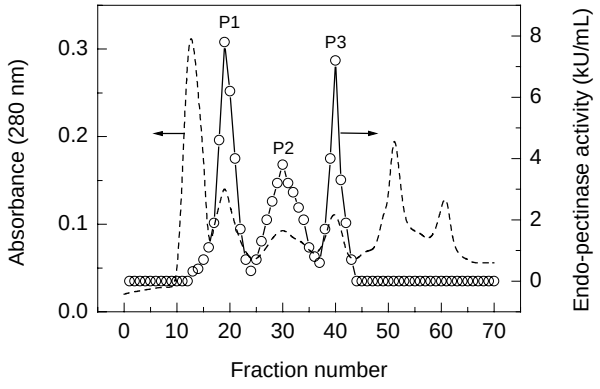

Fig.3 CM-cellulose chromatography of the endo-pectinase fractions from the affinity chromatography

The tree active fractions (P1, P2 and P3) were applied to gel filtration column separately. The elution profiles are shown in Fig.4. Each active fraction eluted obtained a sharp peak of activity, coinciding with the major protein peak. P1 obtained endoPG-1, P2 endoPG-2 and P3 endoPG-3. The three active fractions were endoPGs because all of them had both endo-pectinase and PG activities. The results are shown in Table 1. The PG and endo-pectinase of endoPG-1 were purified to the yields of 21.6% and 20.0% with the specific activities of 1120 and 23600 U/mg, respectively. The specific activities of PG and endo-pectinases of endoPG-2 were 13500 and 25800 U/mg with the recovery rates of 25.9% and 23.8%, respectively. The PG and endo-pectinase of endoPG-3 were purified to the yields of 17.0% and 16.2% with the specific activities of 1560 and 33200 U/mg, respectively. Their overall yields of PGs and endo-pectinases were 64.5% and 60.0% from the crude enzyme, respectively (Table 1), which were much higher than those of pectate lyase from *Bacillus pumilus* DKS1 (36%)[18], endopolygalacturonase from *Mucor rouxii* NRRL 1894 (1.87%)[15], exo-polygalacturonase from *Penicillium viridicatum* RFC3 (6.5%)[9], and exo-polygalacturonase from *Aspergillus sojae* (25.5%)[8].

Ion exchange and gel filtration chromatograph were often used to purify pectinases from various microorganisms. Polygalacturonase produced by *Streptomyces lydicus* was purified to homogeneity by Ultra-filtration and a combination of ion exchange chromatography (CM-cellulose column, 10 cm×2.8 cm)

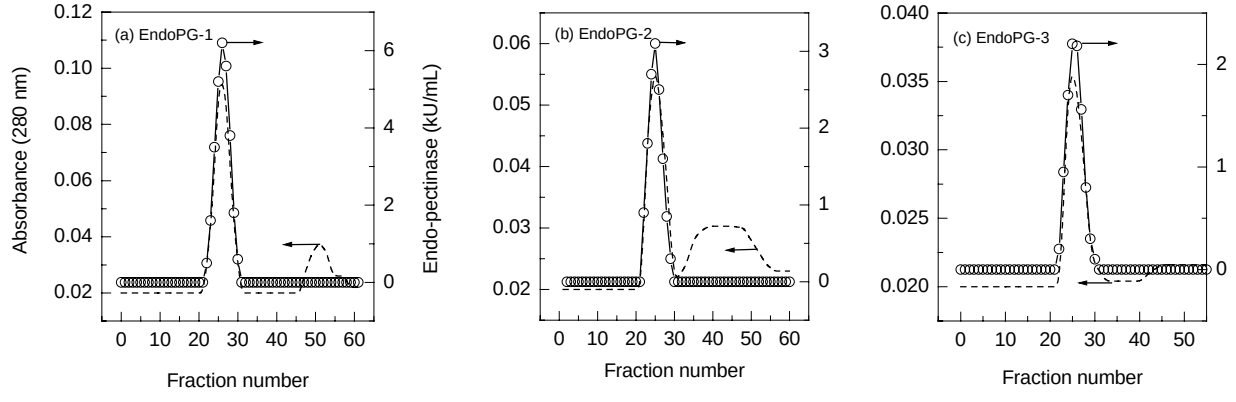

Fig.4 Sephadex G-100 gel filtration chromatography of the endo-pectinase fractions from CM-cellulose chromatography

and gel filtration chromatography (Sephadex G-100 column, 30 cm×3 cm) with the specific activity of 504.8 U/mg[16] which was lower than that of the purified endo-PGs (endoPG-1 1120 U/mg, endoPG-2 1350 U/mg, and endoPG-3 1560 U/mg) from *P. oxalicum* (CGMCC0907). An extracellular pectate lyase was purified from the culture filtrate of a newly isolated *Bacillus pumilus* DKS1 by ion-exchange chromatography (CM-Sepharose column, 5 mL bed volume) and gel filtration chromatography (Sephadex G-75, 30 mL bed volume) with the specific activity of 6200 U/mg[18] which was higher than that of the three purified endo-PGs from *P. oxalicum* (CGMCC0907), respectively. An extracellular polygalacturonase (PGase) from *Mucor rouxii* NRRL 1894 was purified to homogeneity by two chromatographic steps using CM-Sepharose and Superdex 75 with the specific activity of 1372.5 U/mg[15] which was similar to that of the three purified endo-PGs from *P. oxalicum* (CGMCC0907), respectively.

3.3 Characterization of Three Purified EndoPGs

3.3.1 Homogeneity, molecular weight and pI

Upon SDS–PAGE analysis of the three purified endoPGs, each of them had a single band visualized when stained with Coomassie Brilliant Blue (Fig.5). So they were homogeneous. Two polygalacturonases (PGs) from *Sclerotium rolfsii* (strain CBS 350.80) were purified by a recently introduced preparative electrophoretic device with quite similar properties[19], while two exo-polygalacturonases (EC 3.2.1.67) were purified from a commercial *Aspergillus niger* enzyme preparation by ammonium sulfate precipitation, preparative electrofocusing, anion-exchange and size-exclusion chromatographies[10].

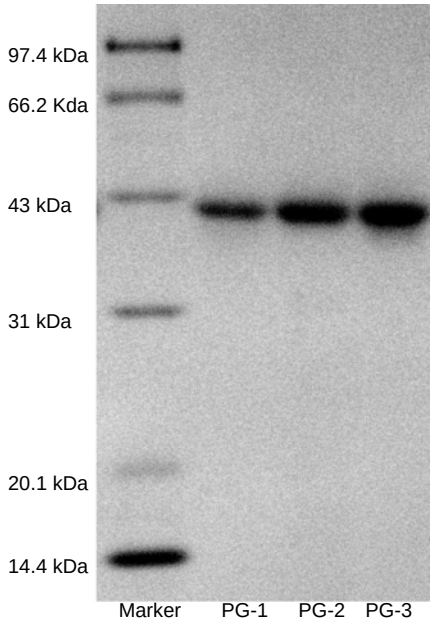

Fig. 5 SDS–PAGE analysis of three endoPGs from *Penicillum oxalicum*

To our knowledge, few pectinases from one strain of different microbes were separated with three purified parts checked by SDS–PAGE. The three purified endoPGs (endoPG-1, endoPG-2 and endoPG-3) had the same molecular weight of 41 kDa, as estimated by SDS–PAGE, which was close to that reported for two polygalacturonases from *Sclerotium rolfsii* (PG1 39.5 kDa, PG2 38 kDa)[19], endopolygalacturonase from *Mucor rouxii* NRRL 1894 (43.1 kDa)[15], polygalacturonase from *Streptomyces lydicus* (43 kDa)[16], and endo-polygalacturonase from *Mucor flavus* (40 kDa)[33], but quite different from that of exo-polygalacturonase from *Penicillium viridicatum* RFC3 (24 kDa)[9], two exo-polygalacturonases from *Aspergillus niger* (exo-PG1 82 kDa, exo-PG2 56 kDa)[10], exo-polygalacturonase I from *Penicillium frequentans* (74 kDa)[11], and polygalacturonase from *Aspergillus kawachii* (60 kDa)[12], polygalacturonase from *Trichoderma harzianum* (31 kDa)[34].

The pI value of endoPG-1 was 5.9, endoPG-2 6.0 and endoPG-3 6.1. On the contrast, the pI values of two polygalacturonases from *Sclerotium rolfsii* were 6.5 and 5.4[19], and the pI values of endopolygalacturonase from *Mucor rouxii* NRRL 1894[15], polygalacturonase from *Aspergillus kawachii*[12], and endo-polygalacturonase from *Mucor flavus*[33] were 6.0[15], 3.55[12] and above 8.3[33], respectively. The pI of polygalacturonase from *Streptomyces lydicus*[16] was not tested.

3.3.2 Optimum temperature and pH

The PG activity of the three purified endoPGs was assayed at various temperatures. The results are shown in Fig.6. As shown in Fig.6, the three endoPGs (endoPG-1, endoPG-2 and endoPG-3) were acidic endoPGs and performed the best enzymatic activities at an optimal pH of 5.0, 5.0 and 5.5, respectively. This finding is similar to what is reported for polygalacturonases from *Sclerotium rolfsii* (PG1 pH 5.0, PG2 pH 4.5)[19], endopolygalacturonase from *Mucor rouxii* NRRL 1894 (pH 4.5)[15], and endo-polygalacturonase from *Mucor flavus* (pH 3.5~5.5)[33], but quite different from what is reported for polygalacturonase from *Streptomyces lydicus* (pH 6.0)[16], pectinase from *Acrophialophora nainiana* (pH 8.0)[17], and exopolygalacturonase I from *Penicillium frequentans* (pH 3.9)[11].

The optimal temperatures for the activities of endoPG-1, endoPG-2 and endoPG-3 were 65, 55, and

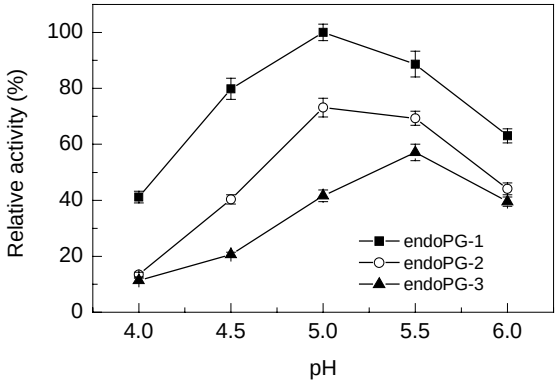

Fig.6 Influence of pH on the PG activity of purified endoPGs from *Penicillum oxalicum*

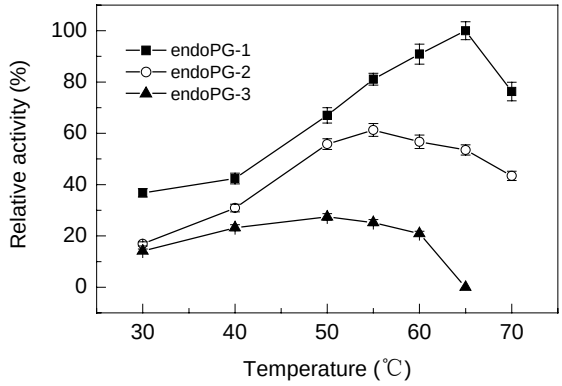

Fig.7 Influence of temperature on the PG activity of purified endoPGs from *Penicillum oxalicum*

50℃, respectively (Fig.7), which were close to those reported for polygalacturonases from *Sclerotium rolfsii* (PG1 64℃, and PG2 55℃)[19], exo-polygalacturonase from *Aspergillus sojae* (55℃)[8], exo-polygalacturonase from *Penicillium viridicatum* RFC3 (60 ℃)[9], exo-polygalacturonase I from *Penicillium frequentans* (50℃)[11], polygalacturonase from *Aspergillus kawachii* (50 ℃)[12], and endo-polygalacturonase from *Mucor flavus* (45℃)[33], but quite different from those reported for endopolygalacturonase from *Mucor rouxii* NRRL 1894 (35 ℃)[15], and polygalacturonase from *Trichoderma harzianum* (40℃)[34].

3.3.3 Substrate specificity and kinetic analysis

The degradation of pectin, esterized pectin and polygalacturonic acid by the three purified endoPGs was assayed by measuring the reducing groups released using the DNS method respectively. The degradation rate of substrate decreased with the increase of esterization of substrate. The same result was reported for PGs from different sources, whereas the hydrolysis of pectin decreased with increasing the degree of esterification[15,33,34].

The rate dependence of enzymatic reaction on the polygalacturonic acid concentration followed Michaelis–Menten kinetics. The K_m values of endoPG-1, endoPG-2 and endoPG-3 were 0.78 (galacturonic acid, pH 5.0, 40℃), 1.2 (galacturonic acid, pH 5.0, 40℃) and 2.0 mg/mL (galacturonic acid, pH 5.5, and 40℃), respectively. The K_m value of endoPG-1 was close to those of exo-polygalacturonase from *Aspergillus sojae* (0.75 mg/mL)[8] and *Penicillium frequentans* (0.68 mg/mL)[11], but the optimum temperature and pH for the action of the two exo-polygalacturonases were 55℃ and 4.0[8], 50℃ and 3.9[11], respectively. The K_m value of endoPG-2 was close to that of polygalacturonase from *Streptomyces lydicus* (1.63 mg/mL)[16], while the optimum temperature and pH for the action of the polygalacturonase were 50℃ and 6.0[16]. The K_m value of endoPG-3 was close to those of endopolygalacturonase from *Mucor rouxii* NRRL 1894 (1.88 mg/mL)[15] and exo-polygalacturonase from *Penicillium viridicatum* RFC3 (1.82 mg/mL)[9], but the optimum temperature and pH for the action of the former were 35℃ and 4.5[15], while the molecular weight of the latter was 24 kDa[9]. It was reported that the K_m value of 4.22 mg/mL was measured for pectinase from *Acrophialophora nainiana*[17], the K_m values of 5.77 and 11.55 mg/mL were tested for polygalacturonases from *Sclerotium rolfsii*[19], and the K_m value of 0.424 mg/mL was tested for exo-polygalacturonase from *Aspergillus sojae*[13], the K_m value of 3.4 mg/mL was measured for polygalacturonase from *Trichoderma harzianum*[34], which were quite different from those of endoPG-1, endoPG-2 and endoPG-3 reported in this study, respectively.

4 CONCLUSIONS

A newly isolated fungus *P. oxalicum* (CGMCC0907) grew well at 30℃ for 72 h and secreted 1.1×10^5 U of endo-pectinases, 6500 U of PGs per gram sugar beet pulp. The activities of pectin and pectate lyases were not found in the crude pectinases produced by *P. oxalicum*. The separation, purification and properties of three endoPGs from *P. oxalicum* (CGMCC0907) were studied in this work, and some conclusions could be drawn as follows:

(1) There were three endo-polygalacturonases separated and purified to homogeneity with good effect from the crude pectinases extract of *P. oxalicum* (CGMCC0907) by affinity adsorption chromatography, CM-cellulose (CM-52) column chromatography and Sephadex G-100 gel filtration chromatography. The

three purified endoPGs, designated as endoPG-1, endoPG-2 and endoPG-3, had the specific activities of 1120, 1350 and 1560 U/mg, with the recovery rates of 21.6%, 25.9% and 17.0%, respectively. The overall recovery rate of endoPGs was about 64.5% from the crude enzyme. The molecular weight of three purified endoPGs was very close to about 41 kDa as determined by SDS−PAGE.

(2) The three purified endoPGs were optimally active at pH 5.0 and 65 ℃, pH 5.0 and 55 ℃, and pH 5.5 and 50 ℃, respectively. The pI and K_m values of endoPG-1, endoPG-2 and endoPG-3 were 5.9 and 0.78 mg/mL, 6.0 and 1.2 mg/mL, and 6.1 and 2.0 mg/mL, respectively. As it is known that discovering new enzymes with novel properties is a tedious and difficult task to perform, therefore enzymes from new sources with unique properties such as the three here may have enormous economic significance which should not be overlooked.

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