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A Novel Acid-Stable Endo-Polygalacturonase from *Penicillium oxalicum* CZ1028: Purification, Characterization, and Application in the Beverage Industry

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Acidic endo-polygalacturonases are the major part of pectinase preparations and extensively applied in the clarification of fruits juice, vegetables extracts, and wines. However, most of the reported fungal endo-polygalacturonases are active and stable under narrow pH range and low temperatures. In this study, an acidic endo-polygalacturonase (EPG4) was purified and characterized from a mutant strain of *Penicillium oxalicum*. The N-terminal amino acid sequence of EPG4 (ATTCTFSGSNGAASASKSQT) was different from those of reported endo-polygalacturonases. EPG4 displayed optimal pH and temperature at 5.0 and 60–70°C towards polygalacturonic acid (PGA), respectively, and was notably stable at pH 2.2–7.0. When tested against pectins, EPG4 showed enzyme activity over a broad acidic pH range (>15.0% activity at pH 2.2–6.0 towards citrus pectin; and >26.6% activity at pH 2.2–7.0 towards apple pectin). The K_m and V_{max} values were determined as 1.27 mg/ml and 5,504.6 U/mg, respectively. The enzyme hydrolyzed PGA in endo-manner, releasing oligo-galacturonates from PGA, as determined by TLC. Addition of EPG4 (3.6 U/ml) significantly reduced the viscosity (by 42.4%) and increased the light transmittance (by 29.5%) of the papaya pulp, and increased the recovery (by 24.4%) of the papaya extraction. All of these properties make the enzyme a potential application in the beverage industry.

Keywords: *Penicillium oxalicum*, endo-polygalacturonase, purification, acid stablity, application

Introduction

Pectin, one of the most important parts of plant tissue, consists of a mainly linear backbone composed of α-1,4-linked D-galacturonic acid residues that are partially methylated [34]. Pectinases degrade pectin by various mechanisms and were classified as de-esterifying enzymes and depolymering enzymes [1]. De-esterifying enzymes catalyze the de-esterification of pectin, releasing protons and methanol [26]. Depolymering enzymes consist of polygalacturonase (including endo-polygalacturonase (E.C. 3.2.1.15, EPG) and exo-polygalacturonase), pectin hydrolase, pectate lyase, and pectin lyase. Among these enzymes, EPG is a major one that randomly cleaves the α-1,4 glycosidic linkages between two nonmethylated residues [25].

Pectinases have been widely used in various bioprocesses like food, feed, pectic wastewater treatment, paper, and textile industries [22]. Acidic EPGs are the major component of fungi source pectinase preparations, which are extensively applied in the clarification of fruit juices, vegetable extracts, and wines, and the digestibility of animal feed [1, 22, 48]. Currently, most commercial pectinases are produced by *Aspergillus niger*, which is safe in industrial-scale production [4, 5, 38]. Several EPGs have been reported from *Aspergillus niger*, and they are stable at acidic pH, and not stable above 50°C [13, 28, 41] or below pH 4.0 [28].

Many EPGs have been purified and characterized from fungi, yeasts, and bacteria [18, 31], among which fungal EPGs are considered to be good candidates for the industrial production due to their stability at pH 4.0–6.0 [22, 35]. However, a robust EPG for bioprocesses involves not only a high catalytic activity but also acid- and thermo-stability against different physiochemical conditions.

In this study, an extracellular EPG was purified from *Penicillium oxalicum* CZ1028, which is a highly efficient polygalacturonase-producing mutant strain (39.9 U/ml in the culture broth). The purified enzyme showed excellent acid stability and better thermostability than most reported fungal counterparts, and had potential application in the beverage industries.

Materials and Methods

Materials

Microorganism-containing soils were collected from the fruit growing and processing areas of Guilin City (China).

The D-(+)-galacturonic acid, trigalacturonic acid, polygalacturonic acid (PGA), citrus pectin, apple pectin, carboxymethylcellulose sodium salt (CMC), and xylan from beechwood were purchased from Sigma-Aldrich (MO, USA). The protein marker was supplied by TaKaRa Bio (China). Protein assay kits (Lowry method) were obtained from Sangon Biotech (China). All other chemicals were of analytical grade and commercially available.

Screening and Identification of Pectolytic Microorganisms

The isolating medium contained (g/l) pectin (30), $K_2HPO_4{\cdot}3H_2O$ (3.3), KH_2PO_4 (3.8), $NaNO_3$ (3), $FeSO_4$ (0.01), $MgSO_4$ (0.24), KCl (0.5), $(NH_4)_2SO_4$ (20), $CaCl_2$ (0.15), bromophenol blue (0.2), and agar (20) at pH 6.0. The liquid cultivation medium (LCM) was prepared as described by Hadj-Taieb *et al.* [14] and the pH value was maintained at 5.5 by using 50 mM sodium-citrate buffer. One gram of soil was suspended in 25 ml of sterile distilled water and serially diluted, and then each dilution (100 µl) was spread on isolating medium. After being cultivated at 30°C for 3 days, the isolates encircled with a big yellow halo were inoculated into 1 L Erlenmeyer flasks with 100 ml of LCM. After 2–5 days of cultivation at 30°C with shaking (150 rpm), the polygalacturonase (PG) activity of the culture broth was determined at 50°C and pH 5.0 by using the dinitrosalicylic acid (DNS) method [32]. Among these microorganisms, the fungal strain PC1 displayed the highest PG activity and was deposited at the China Center for Type Culture Collection (China) under the accession number of M2013627.

To identify the PC1, the partial gene sequences of ITS, β-tubulin, and calmodulin were amplified using the corresponding universal primer sets of ITS1 and ITS4 [46], Bt2a and Bt2b [11], and CMD5 [17] and PoCMDR1 [47], respectively. The polymerase chain reaction (PCR) was conducted as described by Lin *et al.* [27]. The amplified PCR products were sequenced by Genscript Corporation (China), and then the sequences were aligned against those reported in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Biolog GEN III MicroStation System (Biolog Inc., CA, USA) was used to assay the biochemical reactions of 95 substrates.

Mutagenesis and Rational Screening

The PC1 strain was grown on potato dextrose agar (PDA) slants for 5 days, and then the spores were eluted by 0.1% Triton X100 [14] and treated with HNO_2 following the method described by Smokvina *et al.* [42]. The treated spores were serially diluted and plated on PDA plates. After being cultivated at 30°C for 2 days, the mycelia of mutants were inoculated into a basic screening medium (BSM) [6, 15]. The inoculated plates were incubated at 30°C for 3 days. Pectin utilization was detected by flooding the BSM plates with freshly prepared 1% hexadecyl-trimethyl-ammonium bromide (CTAB) and allowed to stand for 20–30 min [15]. The ratio of the diameter of the zone of clearance to the diameter of the colony indicates the ability of an isolate to produce pectinase.

For enzyme production, 100 ml of LCM in 1 L Erlenmeyer flasks was inoculated with spores (10^5/ml), and then the cultures were incubated at 30°C on a rotatory shaker at 150 rpm for 2 days. The culture supernatant acquired by centrifugation was used for the analysis of PG activity.

Enzyme Assay

The PG activity was determined using the DNS method with a standard curve constructed using galacturonic acid. The reaction system containing 0.05 ml of enzyme solution and 0.45 ml of 0.5% (w/v) PGA in 0.1 M sodium acetate buffer (pH 5.0) was incubated at 60°C for 15 min, and then was terminated by adding 1 ml of DNS reagent. The mixture was boiled for 10 min, followed by being cooled to room temperature, and then the absorption of the mixture at 540 nm was measured. One unit (1U) of PG activity was defined as the amount of enzyme releasing 1 µmol reducing sugars (D-(+)-galacturonic acid equivalent) per minute under assay conditions.

Enzyme Purification Procedure

The cell-free culture supernatant from *P. oxalicum* CZ1028 was concentrated 10-fold using a FD 5-5 vacuum freeze drier (SIM, USA), and then the concentrated supernatant was precipitated overnight with 50% saturated ammonium sulfate at 4°C. The precipitated protein was removed by centrifugation at 1,1325 ×*g* for 20 min at 4°C and the supernatant was applied to hydrophobic interaction chromatography using a HiLoad 16/10 Phenyl Sepharose HP column (GE, Sweden) that had been equilibrated with 20 mM phosphate buffer (pH 6.5) containing 1.8 M ammonium sulfate. The proteins were eluted using a linear gradient of $(NH_4)_2SO_4$ (1.8–0 M) in the same buffer. The fractions containing highest PG activity were pooled and desalted by using a HiPrep 26/10 desalting column (GE Healthcare). After that, the low salt solution was subjected to anion exchang chromatography using a HiLoad

16/10 Q Sepharose HP column (GE Healthcare) that had been equilibrated with 20 mM Tris-HCl (pH 7.5). Proteins were eluted with a NaCl linear gradient (0–1.0 M) in the same buffer. The fractions with highest PG activity were pooled and then subjected to size exclusion chromotography using a HiLoad 16/600 superdex 75 column (GE Healthcare) equilibrated with deionized water and eluted with the same water at a flow rate of 1 ml/min. The collected enzyme preparation was stored at 4°C for further use.

Protein Determination and Gel Electrophoresis

The protein concentration was determined using a protein assay kit based on the method described by Lowry *et al.* [29]. The homogeneity of the enzyme preparation was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5% stacking gel and a 10% separating gel [24]. Proteins were visualized by Coomassie Brilliant Blue R-250 staining. The molecular mass of the purified enzyme (EPG4) was estimated using a standard protein marker containing proteins with known molecular mass of 14.4–116 kDa for SDS-PAGE use.

Detection of PG Activity by Zymography

The purified samples of EPG4 were separated on a native polyacrylamide gel. The gel was immersed in 0.7% PGA solution prepared in 0.1 M sodium acetate buffer (pH 5.0) and incubated at 50°C for 30 min, followed by being immersed in 1% CTAB for 10 min. Clear areas with a dark background indicated PG activity.

N-Terminal Sequencing

The NH_2-terminal sequence of the protein was determined by using the Edman degradation method with a Shimadzu Automated Protein Sequencer PPSQ-33A (Japan) at Sangon Biotech. (China). Alignment of the partial amino acid sequence of EPG4 with other highly homologous known PGs was carried out through a BLASTP search of the NCBI protein sequence database.

Substrate Specificity and Action Mode of EPG4

To determine the substrate specificity, the enzyme activities on 0.5% (w/v) of galacturonic acid polymers (PGA, citrus pectin, and apple pectin), CMC, and xylan were tested.

Thin-layer chromatography (TLC) was used to determine the action mode of EPG4 on oligo-galacturonates. The hydrolysis reaction system consisted of 1% (w/v) PGA with 2.5 U/ml of the purified enzyme, and the reaction was performed at pH 5.0 (in 0.1 M sodium acetate buffer) and at 40°C, and then the aliquots were withdrawn at different time intervals. The reaction mixtures were spotted (1 µl) on aluminum sheets (Silica gel 60 F254; Merck) and were developed with a solution containing *n*-butanol:acetic acid:water at a ratio of 9:4:7 (v/v/v). Products were visualized by heating at 105°C for 5 min after spraying the plates with 3% phosphomolybdic acid dissolved in 10% sulfuric acid in ethanol [7].

Characterization of Purified EPG4

The optimal pH was determined at 60°C for 15 min in 0.1 M Na_2HPO_4-citric acid buffer with pHs ranging from 2.2 to 7.0. The optimal temperature was measured at temperatures ranging from 20°C to 80°C at optimal pH. In order to determine the pH stability, the enzyme was preincubated in 0.1 M Na_2HPO_4-citric acid buffer with different pHs (from 2.0 to 7.0) and 0.1 M Tris-HCl buffer with different pHs (from 7.0 to 9.0) at 25°C for 1 h, and then the residual PG activities were assayed under standard condition (pH 5.0, 60°C, 15 min). Thermostability was determined by assessing the residual enzyme activities under standard condition after incubation of the enzyme at 45°C, 50°C, 55°C, and 60°C for various periods with 0.5% (w/v) PGA (pH 5.0).

The effects of metal ions and detergents on PG activity were determined via the addition of various metal salts (1 and 2 mM of NaCl, KCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, $BaCl_2$, $CoCl_2$, $ZnCl_2$, $FeCl_2$, and $CuCl_2$) and other reagents (10 and 20 mM of SDS, Tween-20, Tween-80, and Triton X-100) under standard assay condition.

The K_m and V_{max} values were determined using the Lineweaver-Burk double-reciprocal plots method, which was generated by plotting the reciprocal of reaction velocity (1/V) against the reciprocal of the corresponding substrate concentrations (1/S).

Application of Purified Enzyme in Juice Extraction

Papaya fruits (purchased from the local market) were smashed by using a laboratory mixer, and then the homogeneous fruit pulp was diluted with distilled water at a ratio of 1:1 (w/v). Enzyme doses of 0, 1.8, and 3.6 U/ml of the purified enzyme were used to degrade the papaya pulp at 50°C for 2 h. The resultant pulps were centrifuged at 2,370 ×*g* for 10 min at 25°C, and then the viscosity, transmittance, and juice yield of the supernatants were analyzed. The juice viscosity was measured using a glass Ostwald viscometer (Shanghai Liang Jing Glass Instrument Factory, China), and the viscosity reduction was calculated using the equation described by Celestino *et al.* [3]. The light transmittance was determined using a cuvette and the absorbance was read at 660 nm, with water used as a control.

Supplement of 0.5 ml of acetonitrile (6 g/l in water), as an internal standard, was added to 4.5 ml of the sample. The analysis of methanol was conducted in a 7890A gas chromatograph system (Agilent Technologies, CA, USA). After the injection of 0.2 µl of a treated pulp sample, the methanol content was determined using a ZB-WAX plus polar capillary column (30 m × 0.25 mm × 0.25 µm; Phenomenex, CA, USA) and a flame ionizing detector (H_2: 40 ml/min, and air: 450 ml/min). The flow rate of the carrier gas nitrogen was set at 1.3 ml/min. Before the injection, the temperatures at the injector port and detector were set at 200°C and 220°C, respectively. The temperature program employed for this analysis was set at 65°C for 2 min and then gradually increased to 85°C at a 5°C/min gradient. All determinations were performed using a standard curve.

Nucleotide Sequence Accession Number

The partial nucleotide sequences of the ITS, β-tubulin, and calmodulin genes of *P. oxalicum* PC1 have been deposited in the

Fig. 1. Screening of pectolytic mutants, after HNO_2 mutagenesis of strain PC1, by covering the petri dishes with CTAB.
A zone of clearance surrounding a colony is indicative of pectin hydrolysis by secreted pectinase.

GenBank database under accession numbers KR080372, KT734770, and KT734771, respectively.

Results and Discussion

Screening and Identification of the Fungal Strain PC1

Among 196 microbial strains showing a yellow hydrolyzing halo around the colony, fungal strain PC1 showing the highest PG activity of 23.6 U/ml was used for further study. The ITS sequence of strain PC1 showed 99–100% identity with a number of ITS sequences of *Penicillium oxalicum* strains. The partial sequence of the β-tubulin gene of strain PC1 showed 99% identity with the β-tubulin genes of six strains of *P. oxalicum* (GenBank Accession No. KC344992, KC344990, KC344989, KP735936, KP334129, and KC344993). The partial sequence of the calmodulin gene of strain PC1 showed 99–100% identity with the calmodulin genes of three strains of *P. oxalicum* (GenBank Accession No. KP735937, KP284553, and AY678546). Moreover, the result from the Biolog GEN III MicroStation system also showed that fungal strain PC1 belonged to *P. oxalicum*. Based on the molecular data and physiology characteristics, fungal strain PC1 was identified as *P. oxalicum*.

Selection of the Mutant *P. oxalicum* CZ1028 by Nitrous Acid

Based on the ratio of the diameter of the clear zone to the colony, after two rounds of nitrous acid treatment, a polygalacturonase-producing mutant, CZ1028 (Fig. 1), was selected from around 2,300 colonies. After 2 days of cultivation in liquid medium containing citrus pectin, the mutant CZ1028 exhibited PG activity of 39.9 U/ml. Under similar liquid fermentation conditions, the reported PG activity of *Fusarium moniliforme* NCIM 1276 was 0.3 U/ml [36], *Mucor circinelloides* ITCC 6025 was 10.0 U/ml [43], and *Rhizomucor pusillus* was 31.7 U/ml [40]. We can therefore conclude that *P. oxalicum* CZ1028 is a highly efficient polygalacturonase-producing strain and is suitable for further studies.

Enzyme Purification and Analytical Electrophoresis

After a three-step chromatography combination, an extracellular PG (EPG4) was purified from the culture broth of *P. oxalicum* CZ1028 (Table 1). The purified enzyme showed a single band with an apparent molecular mass of approximately 38.0 kDa on SDS-PAGE (Fig. 2A). The molecular mass of EPG4 was similar to EPGs from *Fusarium moniliforme* NCIM 1276 (38.0 kDa) [36], *Aspergillus niger* (40.4 kDa) [13], and *Saccharomyces cerevisiae* ATCC 28583 (42.0 kDa) [10]. Activity staining of the purified EPG4 revealed a single band corresponding to a clear band of PGA hydrolysis, as shown in Fig. 2B.

Analyses of the N-Terminal Amino Acid Sequence of EPG4

Edman degradation of EPG4 yielded a single N-terminal amino acid sequence (20 residues) of ATTCTFSGSNGA ASASKSQT. This sequence was identical to the putative EPG in the genome of *Penicillium oxalicum* 114-2 (GenBank Accession No. EPS29213) and displayed homology with other fungal PGs (Fig. 3) when analyzed using the BLASTN

Table 1. Purification steps of EPG4 from *P. oxalicum* CZ1028 culture supernatant.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	21,731.0	280.4	77.5	1.0	100.0
$(NH_4)_2SO_4$	17,650.1	175.1	100.8	1.3	81.2
HIC	13,230.7	7.0	1,890.1	24.4	60.9
AEC	7,723.1	3.5	2,206.6	28.5	35.5
SEC	3,712.2	1.6	2,320.1	29.9	17.1

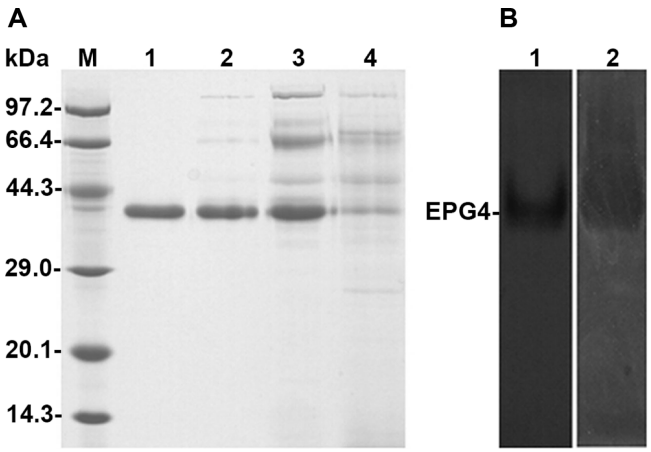

Fig. 2. Electrophoresis of the purified EPG4.
(**A**) SDS-PAGE. Lane M: standard proteins markers; lane 1: purified enzyme from Superdex 75 column; lane 2: the pooled proteins from Q Sepharose column; lane 3: the pooled proteins after precipitation of ammonium sulfate and Phenyl Sepharose column; lane 4: collected culture supernatant. (**B**) Native-PAGE. Lanes 1: purified enzyme stained by Coomassie Brilliant Blue in native electrophoresis; lanes 2: zymogram of the purified enzyme.

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                          **:*****:***:*****:.
Penicillium oxalicum      ATTCTFSGSNGAASASKSQT
Trichoderma reesei        ATTCTFSGSNGASSASKSQK
Trichoderma atroviride    ATTCTFSGSNGASSASKSQK
Aspergillus aculeatus     ATTCTFSGSNGASSASKSKT
Talaromyces marneffei     ATSCTFSGSNGAASASKSKT
Trichoderma harzianum     ATTCTFSGANGASSASKSQK
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Fig. 3. N-Terminal amino acid sequence alignment of EPG4 from *P. oxalicum* CZ1028 with other fungal PGs from *Trichoderma reesei* QM6a (XP_006961351), *Trichoderma atroviride* IMI 206040 (XP_013949139), *Aspergillus aculeatus* (1IA5_A), *Talaromyces marneffei* ATCC 18224 (XP_002149326), and *Trichoderma harzianum* (CAM07141).

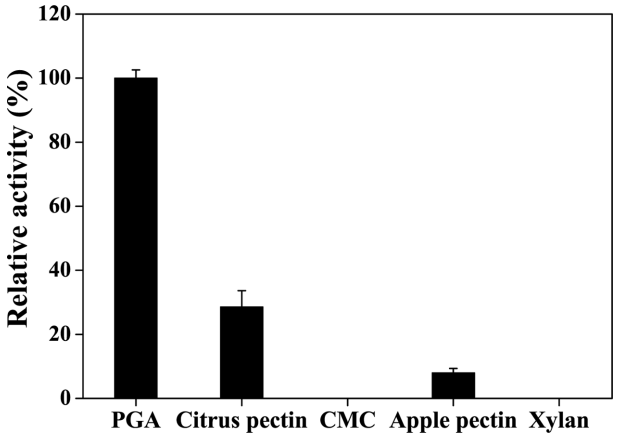

Fig. 4. Substrate specificity of purified EPG4 from *P. oxalicum* CZ1028.
The mean (SD) was calculated from three independent replicates.

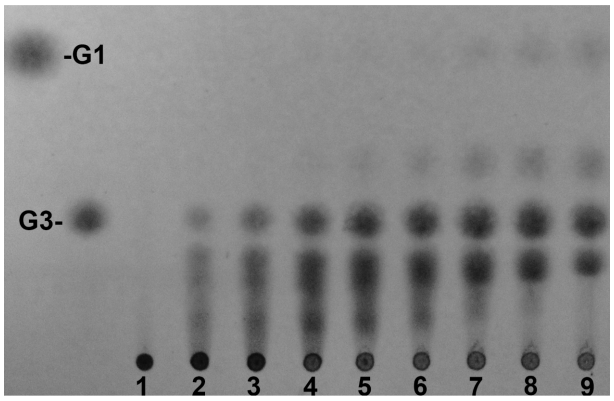

Fig. 5. TLC analysis of the products after hydrolysis of PGA (1%) by EPG4 (2.5 U/ml) in 0.1 M sodium acetate buffer (pH 5.0) at 40°C.
G1 and G3 indicate the galacturonic acid and trigalacturonic acid, respectively; Lanes 1–3: enzymatic hydrolysates of PGA incubated for 0, 15, and 30 min, respectively; Lanes 4–9: enzymatic hydrolysates of PGA incubated for 1, 2, 4, 8, 12, and 24 h, respectively.

online tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Substrate Specificity and Action Mode of EPG4

Purified EPG4 exhibited high specific activity (2,320.1 U/mg) on PGA (100%), followed by on citrus pectin (28.6%) and on apple pectin (8.0%), and showed no activity on CMC and xylan (Fig. 4). These results suggested that EPG4 was specifically active towards the α-1,4-galacturonic acid linkages of galactopolysaccharides.

TLC analysis of the products of PGA hydrolysis indicated that trigalacturonates and oligosaccharides were produced in the initial hydrolysis (15 min) and accumulated when the hydrolysis process was continued (Fig. 5). During the hydrolysis process, only a few monogalacturonates could be detected. EPG4 could degrade oligosaccharides rapidly and did not seem to attack trimers, as trimers accumulated throughout the incubation period. This behavior is consistent with the fact that digalacturonate is not hydrolyzed by EPGs, and trigalacturonate is usually a very poor substrate for EPGs [7], indicating that the action mode of EPG4 was endo-acting.

Emzymatic Characterization of Polygalacturonase

The purified EPG4 exhibited optimal activity at pH 5.0

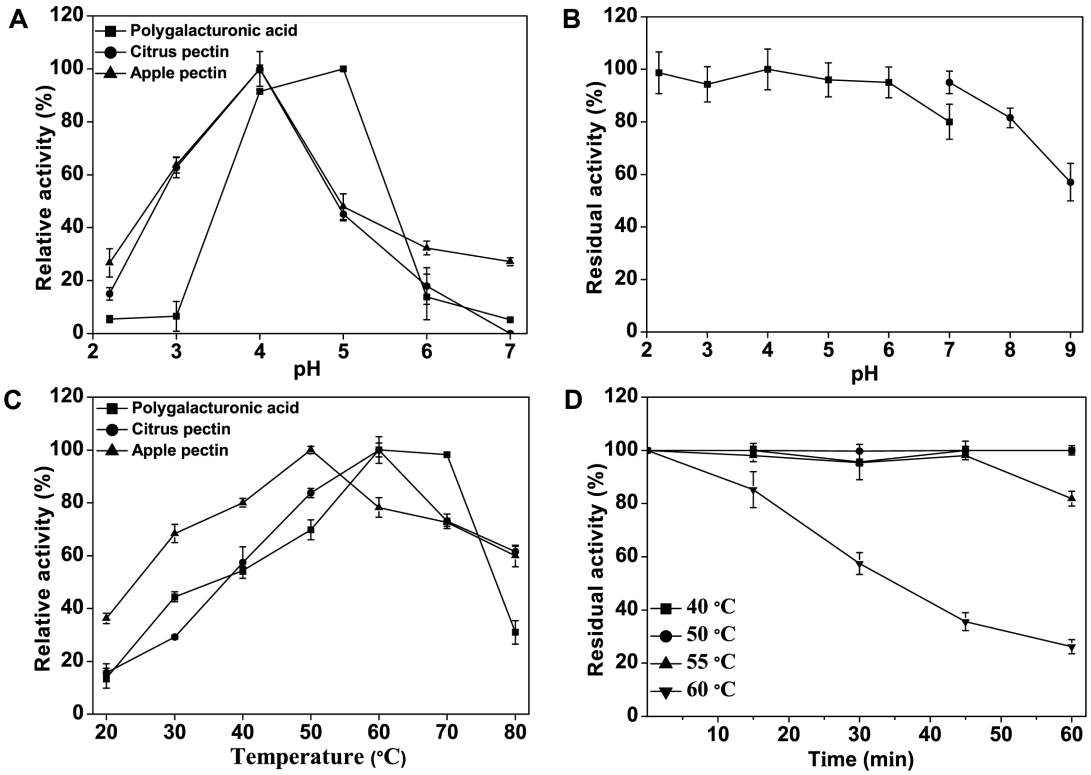

Fig. 6. Characteristics of the EPG4 enzyme activity.

The optimum pH (**A**) and temperature (**C**) for the activity of the purified EPG4 against polygalacturonic acid (PGA), citrus pectin, and apple pectin. The pH stability (**B**) and thermal stability (**D**) of the purified EPG4 for PGA. Error bars represent the standard deviation of three repeats.

and 4.0 when PGA and pectins were used as substrates, respectively (Fig. 6A). The optimal pH characteristic of EPG4 was similar to most acidic fungal PGs (pH 3.0–6.0) [18], but the enzyme activity was still observed at pH 2.2 (5.5% relative activity) with a solution of PGA precipitates. When tested against soluble pectin, EPG4 showed activity over a wide acidic pH range (>15.0% activity at pH 2.2–6.0 towards citrus pectin; and >26.6% activity at pH 2.2–7.0 towards apple pectin). After incubation at pH 2.2–7.0, 25°C for 1 h without substrate, EPG4 retained >94.2% of the initial activity (Fig. 6B). Few fungal EPGs remain both active and stable under such a wide acidic pH range. For example, the stability of EPG4 at extremely low pH was higher than those of rePgaA from *Aspergillus niger* JL-15 [28], endo-PG I from *Achaetomium* sp. Xz8 [44], and endo-PG from *Cryptococcus albidus* var. *albidus* IMAT-4735 [8]. In agriculture and food industries, a low pH of the extraction process can reduce the risk of microbial contamination, minimize the action of native de-esterifying enzyme, and stabilize pectin in solution [39, 45]. Endo-PG1 from *Bispora* sp. MEY-1 [48] was stable over a similar pH range (pH 2.0–7.0), but it had almost no activity above pH 5.0. Some EPGs, such as PGL from *Mucor flavus* IMI 197788 [9] and endo-PG I from *Penicillium* sp. CGMCC 1669 [49], were acidophilic and acidic-stable endo-PGs, but retained their stability only within a relatively narrow pH range. Pgase-X2 and Pgase-X2-R from *Aspergillus awamori* IFO4033 [33] had the same pH optimum (pH 5.0), but were not stable below pH 4.0 or above pH 6.0. Compared with these enzymes, EPG4 had good activity and stability under acidic pHs. These excellent characteristics make EPG4 a potential application in agriculture and food industries.

EPG4 exhibited optimal activity at 60–70°C for PGA, 50°C for apple pectin, and 60°C for citrus pectin (Fig. 6C). The temperature optima of EPG4 were different from most reported fungal PGs, which showed the temperature optima between 30°C and 50°C [18], like *Saccharomyces cerevisiae* SSM52 (45°C) [16], *Penicillium* sp. CGMCC 1669 (40°C) [49], *Aspergillus awamori* IFO4033 (40°C) [33], *Penicillium solitum* (50°C, 20–37°C) [19-20], and *Phomopsis cucurbitae* Pc-1062 (40–45°C) [51]. When the purified enzyme was exposed to 50°C and 55°C for 60 min (with substrate), it was found to retain 100% and 81.8% of the initial activity, respectively (Fig. 6D). Great efforts have been attempted to improve the

Table 2. Effects of metal ions on the activity of EPG4.

Metal ions	Relative activity[a] (%)	
	1 mM	2 mM
Control	100.0 ± 6.9	100.0 ± 1.3
NaCl	110.1 ± 2.0	101.1 ± 2.3
KCl	103.2 ± 2.3	98.7 ± 2.1
$MgCl_2$	102.0 ± 0.6	96.5 ± 0.6
$CaCl_2$	101.8 ± 0.6	71.7 ± 1.7
$MnCl_2$	47.9 ± 4.5	40.4 ± 3.7
$BaCl_2$	104.1 ± 1.4	98.1 ± 2.0
$CoCl_2$	93.5 ± 3.0	70.6 ± 1.6
$ZnCl_2$	92.2 ± 2.7	77.4 ± 0.8
$FeCl_2$	101.7 ± 4.2	91.0 ± 1.2
$CuCl_2$	12.4 ± 8.5	10.3 ± 9.1

[a]Values represent the mean ± SD from three independent replicates.

thermostability performance of acidic PGs, and thermophilic fungi represent an ideal source for thermostable PGs with high yield. So far, several thermophilic PGs have been identified in *Rhizomucor pusillus* [40], *Thermoascus aurantiacus* CBMAI-756 [30], and *Thermomyces lanuginosus* [23]. These PGs usually show high temperature optima (55–65°C) and thermostability (50°C), but there are still many obstacles to be overcome, such as cultivation of thermophilic fungi with acidic stability of enzyme acitivity.

In the beverage industry, different fruits have different pH values and require different EPGs with different pH optima and stability. EPG4 is promising for application in the beverage industry owing to its pH stability and enzyme activity over a broader acidic pH range.

The effects of metal ions on EPG4 activity were determined (Table 2). The enzyme activity was slightly inhibited by 2 mM of K^+, Mg^{2+}, Ba^{2+}, and Fe^{2+}. Mn^{2+} and Cu^{2+} strongly inhibited the enzyme activity, which were similar to the EPG from *Fusarium graminearum* [37]. Partial inhibition was observed in the presence of 2 mM of Zn^{2+}, Ca^{2+}, and Co^{2+}. Thus, the enzyme did not require any metal ions to express its activity. Tween-20, Tween-80, and Triton X-100 did not significantly affect the PG activity (Table 3). However, SDS strongly inhibited the PG activity, which was similar to the EPG from *Bispora* sp. MEY-1 [48].

Table 3. Effects of detergents on the activity of EPG4.

Detergents	Relative activity [a] (%)	
	10 mM	20 mM
Control	100.0 ± 1.6	100.0 ± 2.3
SDS	45.1 ± 2.5	30.2 ± 2.7
Tween-20	104.8 ± 2.6	101.8 ± 3.7
Tween-80	102.4 ± 3.3	102.8 ± 6.3
Triton X-100	102.4 ± 2.2	101.8 ± 2.0

[a]Values represent the mean ± SD from three independent replicates.

The K_m value for EPG4 (1.27 mg/ml) was higher than those for Pgase-X2-R (0.35 mg/ml) from *Aspergillus awamori* IFO 4033 [33] and endo-PG I (0.32 mg/ml) from *Achaetomium* sp. Xz8 [44], but lower than those for PG IV (7.2 mg/ml) from *Aspergillus niger* [41] and endo-PG I (19.5 mg/ml) from *Penicillium* sp. CGMCC 1669 [49]. The detected V_{max} (5,504.6 U/mg) value was lower than the EPG of *Achaetomium* sp. Xz8 (97,951 U/mg) [44], but significantly higher than those of the EPGs from *Aspergillus kawachii* IFO 4033 [7], *Bispora* sp. MEY-1 [48], and *Penicillium* sp. CGMCC 1669 [49], which were 1,500 U/min/mg, 2,526 U/min/mg, and 909.1 U/min/mg, respectively.

Application of the Purified Enzyme in Papaya Juice Processing

EPG4 had a significant effect on the extraction of papaya juice (Table 4). After treatment with 3.6 U/ml EPG4, the yield of papaya juice was increased by 24.4%, the intrinsic viscosity was reduced by 42.4%, and the light transmittance was increased by 29.5%. The commercial compound pectinase (3.6 U/ml) increased the yield by 25.6%, reduced the intrinsic viscosity by 42.9%, and increased the transmittance by more than 29.9%. In addition to pectin, plant tissue also contains cellulose, hemicellulose, and starch, so EPG4 was a little less effective than the commercial pectinase that had cellulase and xylanase.

Table 4. Effect of amount of enzyme on yield, viscosity, light transmittance, pH, and methanol content of papaya juice.

Enzymes units (U/ml)	Yield (%)	Viscosity (mPas)	Light transmittance (%)	pH	Methanol content (mg/l)
Control	37.0	2.31	61.6	4.91	276
1.8 U/ml EPG4	59.2	1.36	90.1	4.86	302
3.6 U/ml EPG4	61.4	1.33	91.1	4.82	308
1.8 U/ml commercial pectinase	61.8	1.34	90.1	4.62	449
3.6 U/ml commercial pectinase	62.6	1.32	91.5	4.56	506

Compared with EPG4, the methanol content was higher in the commercial pectinase-treated pulp. It could be due to the presence of de-esterifying enzyme, which hydrolyzes the pectin by releasing pectic acid and methanol. The higher the concentration of commercial pectinase used, the more methanol was produced. At the same amount of the enzyme (3.6 U/ml), the methanol content of the commercial pectinase-treated pulp was 506 mg/l, whereas the EPG4-treated pulp was 308 mg/l, which was slightly higher than that of the pulp itself (276 mg/l). This may be the result of decreased viscosity promoting the activity of the fruit's own de-esterifying enzyme [12]. Because of the released carboxylic acid, the pH of commercial pectinase-treated pulp decreased from 4.91 to 4.56 with increase in amount of the enzyme, but the pH of EPG4-treated pulp (pH 4.82) was almost the same as the control (pH 4.91).

Accidental intake of methanol will cause severe intoxication and even death owing to accumulation of extremely toxic metabolites such as formaldehyde and formic acid [2]. Recently, people in China have been drinking more juices for improving physical function. The use of commercial pectinases in the processing of raw materials will result in a significant increase of methanol content of juices [50]. To avoid a risk to consumers' health, this is an attractive way to reduce methanol content and enhance the yield of juices via replacing commercial pectinases by EPG4 combined with other hydrolases (such as cellulase, hemicellulase, and amylase) in the beverage industry.

In the present study, we identified an extracellular EPG (EPG4) from *P. oxalicum* CZ1028. The enzyme exhibited optimum activity at pH 5.0 and 60–70°C towards PGA, and had excellent stability over a broad acidic pH range (2.2–7.0) and good thermostability at 55°C. All of these characteristics make the enzyme an interesting biocatalyst for juice clarification. It also can be considered as a potential candidate in food, paper, and textile industries.

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