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Production optimization and expression of pectin releasing enzyme from *Aspergillus oryzae* PO

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ABSTRACT

Protopectinase is an enzyme that solubilizes protopectin forming highly polymerized soluble pectin. Protopectinase activity was detected from *Aspergillus oryzae* PO isolated from soil of persimmon orchard. Response surface methodology of Box–Behnken Design with three fermentation variables (temperature, $NaNO_3$ and apple pomace concentration) was used to optimize protopectinase production of *A. oryzae* PO, and protopectinase activity was improved to 270.0 U/ml. Endo-polygalacturonase belonged to A-type PPase from *A. oryzae* PO was cloned and expressed in *Pichia pastoris* GS115. The endo-polygalacturonase expression was 0.418 mg/ml and the specific activity of purified recombinant endo-polygalacturonase was 7520 U/mg toward polygalacturonic acid. The optimal temperature and pH of recombinant endo-polygalacturonase were 45 °C and 5.0, respectively. The recombinant endo-polygalacturonase activity was enhanced by the presence of Mg^{2+}, while Ca^{2+}, Ni^{2+} Mn^{2+}, Cu^{2+} and SDS strongly inhibited the enzyme activity. The apparent K_m value and V_{max} value were 5.59 mg/ml and 1.01 μmol/(min ml), respectively.

1. Introduction

Almost 600 million tons of fruit were gathered throughout the world in 2010. China is the world's largest fruit producer with an average annual growth rate of above 8% over the previous 20 years (FAOSTAT, 2012). Fruit processing industries generate large amount of solid residues resulting in potentially severe environment pollution. Only a small part of solid residue is used as animal feed, while others are dealt with landfill, compost and incineration (Min, Lim, Ko, Lee, Lee, 2011). Apple is one of the most widely produced fruit in the world. In food industry, apple processing generates 30% apple pomace. Annual apple pomace is estimated to be 3–4.2 × 10^6 tons (Vendruscolo, Albuquerque, Streit, Esposito, & Ninow, 2008).

These by-products are valuable resources, which consist of carbohydrates, crude fibers, fermentable sugars, pectin, minerals, etc. (Bhushan, Kalia, Sharma, Singh, & Ahuja, 2008). Rational use of apple pomace to turn the wastes into the valuables is not only benefit to environment protection, but also favorable for improving the added values of apples (Qiu, Tian, Qiao, & Deng, 2009). Apple pomace is an excellent substrate of microorganism growth for producing enzymes, ethanol, organic acids, heteropolysaccharides, aroma compounds and edible mushrooms (Vendruscolo et al., 2008). It consists of 10–15% dried pectin which is widely used as gelling, thickening and stabilizing agents in the food industry (Endreß, 2000; Theuwissen & Mensink, 2008). Due to be rich in pectin, apple pomace is mainly raw materials for pectin production (Willats, Knox, & Mikkelsen, 2006).

Most commercial pectin is produced from apple pomace and citrus peels by chemical extraction (Contreras Esquivel, Hours, Voget, & Mignone, 1999). However, the method is energy intensive and produces large amounts of industrial waste (Lim, Yoo, Ko, & Lee, 2012). Enzymatic extraction is an environment- and human-friendly technology (Min et al., 2011). Shkodina et al. studied enzymatic pectin extraction with cellulose, hemicellulase and glycolytic complex. They found that the pectin samples obtained by enzymatic method had lower molecular weights and viscosities, and high degrees of acetylation, which resulted in the pectin samples did not form gels (Shkodina, Zeltser, Selivanov, & Ignatov, 1998). Sakamoto et al. reported enzymatic pectin extraction using five kinds of protopectinase (PPase). The pectin yields of enzymatic extraction from water-washed protopectin were lower than acid

Abbreviations: PPase, protopectinase; RSM, response surface methodology; BBD, Box–Behnken Design; PGase, polygalacturonase; PGA, polygalacturonic acid; MD, minimal dextrose medium; BMGY, buffered dextrose-complex medium; BMMY, buffered methanol-complex medium.

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extraction (Sakamoto, Hours, & Sakai, 1995). Thus, searching for the enzymes releasing more and good quality pectin is significant for the industrialization of enzymatic extraction pectin.

PPase catalyzes the water-insoluble protopectin to release water-soluble pectic substance (Matsumoto, Sugiura, Kondo, & Fukuda, 2000). The enzymatic solubilization of pectin from protopectin is carried out by the action of a system of enzymes, including endo-polygalacturonase, pectin lyase, pectate lyase, arabinanase and rhamnogalacturonase (Sakai, Sakamoto, Hallaert, & Vandamme, 1993).

In view of the potential industrial application of PPase, the present study was carried out to isolate strains capable of producing PPase from soil and rotten fruits, and optimized fermentation conditions for maximum PPase production using Response surface methodology (RSM) in semisolid fermentation. Moreover, the gene of endo-polygalacturonase was cloned and expressed in *Pichia pastoris* GS115.

2. Materials and methods

2.1. Strains and plasmids

Aspergillus oryzae PO was isolated from a soil sample from persimmon orchard (Shanghai China), and cultured on potato dextrose agar slants at 30 °C. Spores suspension was prepared by washing slants using sterile water. *Escherichia coli* DH5α competent cell (Tiangen Biotech Co., Ltd., Shanghai, China) was used for plasmid manipulation. *P. pastoris* GS115 (Invitrogen, San Diego, CA, USA) was used as host for endo-polygalacturonase expression.pMD19-T vector Simple used as vector for the cloning was purchased from TaKaRa Biotech Co. Ltd, Japan. pPIC9K used as vector for the expression was purchased from Invitrogen (San Diego, CA, USA).

2.2. Media, enzymes and reagents

The media used in gene expression were the minimal dextrose medium (MD), the buffered dextrose-complex medium (BMGY) and the buffered methanol-complex medium (BMMY), which were prepared according to the manual of *Pichia* Expression Kit (Invitrogen, San Diego, CA, USA).

Restriction endonuclease and T_4 DNA ligase was purchased from Fermentas, Thermo Fisher Scientific Inc, USA. PCR reagents were purchased from TaKaRa Biotech Co. Ltd, Japan. Polygalacturonic acid from apple, red debranched arabinan and rhamnogalacturonan from potato pectic fiber were purchased from Megazyme International Ireland. D-Galacturonic acid and L-(+)-arabinose were purchased from Sigma–Aldrich, Co. Ltd, Germany. Others reagents were all analytical grade.

2.3. Enzyme and protein assay

Protopectinase activity was assayed in a mixture containing 100 mg of apple protopectin, 2.95 ml of 50 mM phosphoric acid buffer (pH 6.0), and 50 μl of enzyme solution at 45 °C for 1 h. The pectic substances liberated from protopectin were measured by the carbazole–H_2SO_4 method (Sakai & Yoshitake, 1984). One unit of enzyme activity was defined as the activity that liberates pectic substances corresponding to 1 μmol of D-galacturonic acid. Apple protopectin used as a substrate was prepared by the following procedure: apple pomace was firstly washed with hot water (90–100 °C) for 10 minutes and repeatedly washed with warm water until the soluble substances that reacted with carazole–H_2SO_4 were washed off. Then, the water-washed protopectin was washed with 2% sodium hexametaphosphate solution (pH 4.0) until the soluble substances that reacted with carbazole–H_2SO_4 were washed off, and then freeze-dried (Sakai & Okushima, 1982).

Table 1
Box–Behnken design for PPase production and experimental results.

Run	Temperature*A* (°C)	$NaNO_3$ *B* (g/l)	Apple pomace *C* (g/l)	PPase activity (U/ml)
1	35	3	20	82.6
2	25	4	30	231.5
3	30	3	30	258.4
4	30	2	40	205.5
5	30	2	20	211.0
6	25	2	30	223.3
7	25	3	40	155.0
8	30	4	40	192.7
9	30	3	30	264.5
10	25	3	20	243.8
11	30	3	30	264.0
12	30	3	30	254.0
13	30	4	20	243.9
14	35	4	30	90.3
15	35	2	30	128.0
16	30	3	30	271.0
17	35	3	40	124.8

Endo-polygalacturonases (endo-PGase) activity was measured by determination of reducing sugars released as a result of hydrolysis of polygalacturonic acid (PGA) by a dinitrosalicylic acid (DNS) reagent (Schnitzhofer et al., 2007). The mixture containing 450 μl 0.25% PGA solution in buffer pH 4.0 and 50 μl of appropriately diluted enzyme solution was incubated at 50 °C for 10 min and the reaction was stopped by addition of 750 μl of DNS. After boiling for 5 min, subsequent cooling and centrifugation the absorbance was read at 540 nm. The reducing sugars formed were quantified using D-galacturonic acid as a standard. One unit (U) was defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute.

Endo-1,5-α-arabinanase activity was assayed by measuring the release of reducing groups according to the Somogyi method with red debranched arabinan used as substrate (Takao, Akiyama, & Sakai, 2002). Rhamnogalacturonase activity was determined by formation of reducing groups using the dinitrosalicylic acid (DNS) assays with rhamnogalacturonan as substrate (Normand et al., 2010). Lyase activity was assayed by measurement of the increase in absorbance at 235 nm resulted from the generated unsaturated galacturonate, and polygalacturonic acid and pectin with 22, 67, and 87% esterification used as substrates were purchased from Sigma (Sakamoto, Hours, & Sakai, 1994).

The protein concentration was determined using the method described by Bradford (1976). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a stacking gel of 5% acrylamide and a separation gel of 12% acrylamide as described by Laemmli (1970).

2.4. Optimization of PPase production

2.4.1. Production medium

The basal medium consisted of apple pomace, $NaNO_3$, $MgSO_4{\cdot}7H_2O$ (0.5 g/l), KCl (0.5 g/l), K_2HPO_4 (1.0 g/l) and $FeSO_4{\cdot}7H_2O$ (0.01 g/l). The concentrations of apple pomace, $NaNO_3$ and fermentation temperature were shown in Table 1. Every experiment was conducted in shake flasks media (50 ml in 250 ml Erlenmeyer) for 48 h with an initial pH of 5.0. Enzyme activity was determined on supernatant obtained after the centrifugation of the broth at 13,000 × *g* for 10 min at 4 °C.

2.4.2. Box–Behnken design (BBD)

Temperature, $NaNO_3$ and apple pomace were chosen to optimize by BBD. These three independent variables were evaluated at

three different levels (−1, 0, +1). The PPase production was analyzed by using a second order polynomial equation; and the data were fitted into the equation by multiple regression procedure. The model equation for analysis was given below

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i<j}^{n} \beta_{ij} x_i x_j + \sum_{j=1}^{n} \beta_{jj} x_j{}^2$$

where Y is the predicted response; β_0 is the model constant; β_i is the linear coefficient; β_{jj} is the quadratic coefficient; β_{ij} is the interaction coefficient.

For three variable systems the model equation was

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

where A, B and C are three most significant various. With the help of Design Expert Software, the three-dimensional response surface curves were generated.

2.5. Expression of endo-PGase gene from A. oryzae PO

2.5.1. Cloning of endo-PGase gene from strain A. oryzae PO

The endo-PGase and endo-PGase gene from *A. oryzae* PO were named as PGPO and *pgaPO*, respectively. The *pgaPO* that consists of three exons was cloned from genomic DNA by overlap-PCR (Horton, Hunt, Ho, Pullen, & Pease, 1989). The primers 1F (5′-AATACGTAGCTCCCGCCCCTTCTCGCAC-3′) and 1R (5′-CGAAGGTGATGGTGGCACCGTCCTTGGCCT-3′) were used to amplify the modified exon1 (without signal peptide). The primers 2F (5′-CGGTGCCACCATCACCTTCGAGGGCACCAC-3′) and 2R (5′-ATTCG ATGTTCTCACCAGAGTTGATGGCCA-3′) were used for exon2. The exon3 of *pgaPO* were amplified using the primers 3F (5′-CTCTGGTGAGAACATCGAATTCACCGGC GC-3′) and 3R (5′-AAGCGGCCGCTTAGCAAGAAGCGCCAGAAG-3′). The fragment of exon1, exon2 and exon3 were mixed to serve as templates with the outermost primer pair (1F and 3R) and then the three exons were combined. The CDS of *pgaPO* was cloned precisely by overlap-PCR.

2.5.2. Plasmid construction and expression of recombinant PGPO

The *pgaPO* without signal peptide and introns was digested with *Sna*BI and *Not*I, and the digestion product was inserted into the vector pPIC9 K. The recombinant plasmid was linearized with *Sac*I, and then transformed into *P. pastoris* GS115 by electroporation. Positive transformants were screened on MD plates. Colonies that grew on MD agar medium were pooled and streaked on YPD-Geneticin plates.

Colonies growing on YPD-Geneticin plates with higher Geneticin concentration were conserved and confirmed by restriction digestion and DNA sequencing. The transformant was inoculated in 10 ml BMGY at 30 °C for 24 h with shaking at 250 rpm. The cells were harvested by centrifugation (3000 × *g*, 5 min at room temperature) and resuspended in 30 ml BMMY. Cultures were grown at 30 °C at 250 rpm. Methanol was added to a final concentration of 0.5% every 24 h. All these operations followed the *Pichia* Expression Kit manufacturer's instructions (Invitrogen, San Diego, CA, USA).

2.6. Purification of recombinant PGPO

To purify the recombinant PGPO, the culture was centrifuged at 13,000 × *g*, 4 °C for 10 min. The supernatant was applied to a Mono Q™ 5/50GL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) that had been equilibrated with 50 mM phosphate buffer (pH 8.0). The column was eluted with a NaCl linear gradient (0–1.0 M) in the same buffer. Fractions showing endo-PGase activity were pooled. All purification steps were carried out at 4 °C.

2.7. Characterization of purified recombinant PGPO

The optimal pH of the purified recombinant PGPO was determined at 50 °C in various pH values (1.9–8.2). To evaluate the pH stability, the purified recombinant PGPO was incubated in different pH (2.1–10.3) at 30 °C for 30 min. Thereafter, the remaining activity was determined at pH 4.0 at 50 °C.

The effect of temperature on purified recombinant PGPO was measured by incubating the enzyme at different temperatures (30–75 °C) at pH 4.0. The temperature stability of the purified PGPO was evaluated by measuring the residual activity after a period of time (15, 30 and 60 min) incubation of the enzyme at temperatures ranging from 30 to 60 °C.

The purified recombinant PGPO was incubated for 30 min with various metal ions (1, 5 and 10 mM) and surfactants (0.1%) at 30 °C. Then the residual activity was measured.

The Michaelis constant (K_m) and V_{max} values were determined from Lineweaver–Burk plots of enzyme activity measured with PGA as substrate (0.2–3.2 mg/ml) at optimal pH and temperature.

3. Results and discussion

3.1. Optimization with Box–Behnken design (BBD)

The single factor method ignores the interaction between different variables involved (Raza, Makeen, Wang, Xu, & Shen, 2011). Alternatively, RSM is a powerful and practicable technique that is often used to identify the relationships and interactions between multiple variables. Based on the results obtained from the one-factor-at-a-time experiments (data not shown), three variables (temperature, $NaNO_3$ and apple pomace concentration) that exhibited great effects on PPase production were chosen for BBD. The actual values of PPase production based on BBD experimental design were shown in Table 1. The following quadratic regression equation was obtained to describe the PPase production.

$$\text{PPase} = 262.37 - 53.48A - 1.19B - 12.92C - 11.49AB + 32.73AC - 11.43BC - 77.93A^2 - 16.19B^2 - 32.91C^2$$

where A, B and C are the coded values of temperature, $NaNO_3$ and apple pomace concentration, respectively.

ANOVA for PPase production revealed that the F-value of the model was 66.16, and the values of "Prob > F" less than 0.05, indicating model terms were significant. A, C, A^2, B^2, C^2 and AC were significant model terms in present study. The coefficient of determination (R^2) was 0.9734 for PPase production, suggesting a good agreement between experimental and predicted values and can explain up to 97.34% variability of the response. The model's ratio of 23.333 indicated an adequate signal, and the model can be used to navigate the design space.

The three-dimensional response surface curves were plotted to explain the interaction of three independent variables and to determine the optimum condition (Fig. 1). Each curve represented a combined effect of two tested variables on the response with the other variable maintained at its zero level. The model predicted that the maximum PPase activity (277.8 U/ml) was appeared with fermentation temperature of 27.7 °C, $NaNO_3$ concentration of 3.30 g/l and apple pomace concentration of 25.2 g/l. PPase production was exerted under the conditions predicted by RSM to evaluate the model. The PPase production attained 270.0 U/ml, which was similar to the predicted one and was about 2.38 times more than that of the fermentation with unoptimized medium (113.3 U/ml).

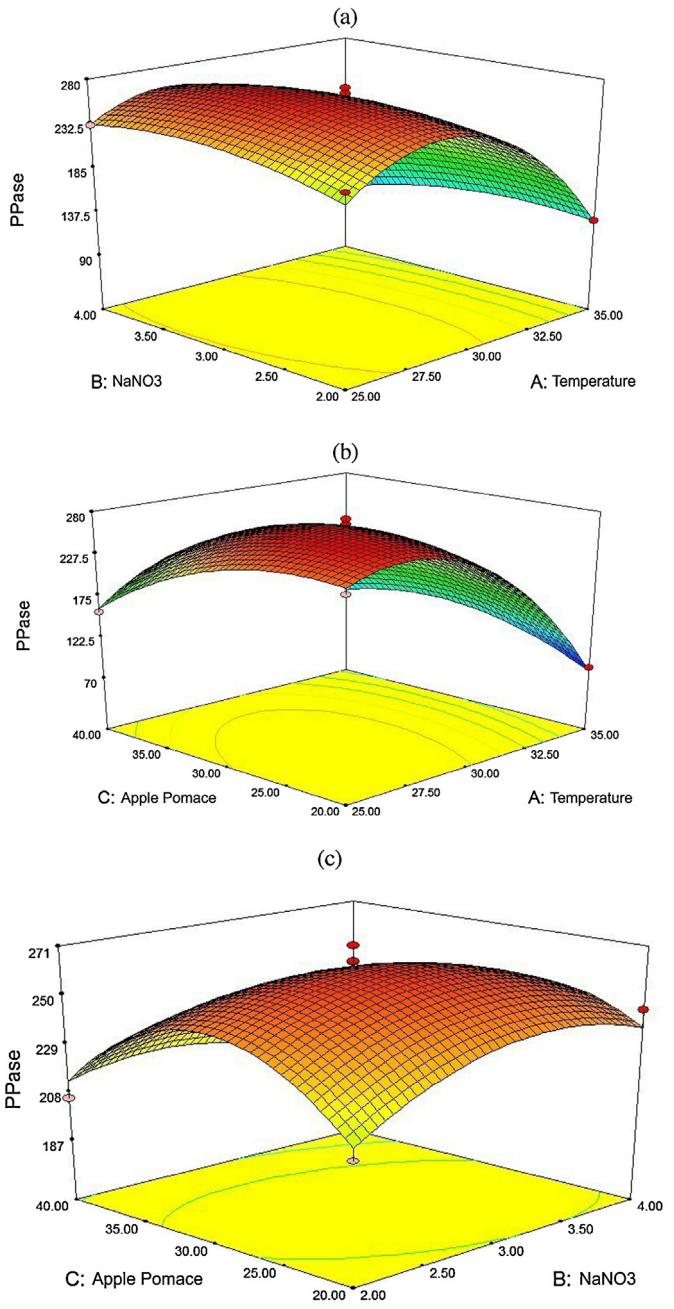

Fig. 1. The three-dimensional response surface curves. (a) The effect of temperature and $NaNO_3$. (b) The effect of temperature and apple pomace. (c) The effect of $NaNO_3$ and apple pomace.

PPases are heterogeneous groups of enzymes with different catalytic activities (Sakamoto, Yamada, Kawasaki, & Sakai, 1997). The present PPase of *A. oryzae* PO was found to contain endo-polygalacturonase and endo-1,5-α-arabinanase but without rhamnogalacturonase and lyase activity by the enzyme activity assay on their specific substrates respectively. Endo-polygalacturonase is not only a kind of A-type PPase but also can catalyze the hydrolysis of PGA. Pectin is released by restricted hydrolysis of the PGA's (smooth) region in protopectin (Yoshitake, Numata, Katsuragi, Hours, & Sakai, 1994). Sakai et al. have reported the mechanism of the endo-PGase with PPase activity, that the enzyme hydrolyzes more than three nonmethozylated-galacturonic acid chains (1999). However, 50% galacturonic acids of protopectin are methylated, which resulted in that the enzyme only cleaves at the restricted sites to release highly polymerized pectin from protopectin. In addition, galacturonic oligomers inhibit the hydrolysis of soluble pectin, but have no effect on protopectin. Consequently, the pectin released from protopecitn could not be subsequently hydrolyzed and remains a highly polymerized mode (Sakai, Takao, & Nagai, 1999).

3.2. Cloning of the pgaPO and sequence analysis

The nucleotide sequence of *pgaPO* from *A. oryzae* PO was submitted to GeneBank database under Accession Number KF499033. The complete open reading frame consists of 1226 bp. Two introns, 226–290 bp and 711–767 bp, were interrupted the *pgaPO* coding sequence. SignalP (http://www.cbs.dtu.dk/services/SignalP/) predicted the presence of a putative signal peptide at residue 1–27. The molecular mass of PGPO was estimated to be 37.8 kDa.

The deduced PGPO was same with the endo-polygalacturonase C from *A. oryzae* RIB40 and similar to polygalacturonase B from *A. oryzae* KBN616 (99% identity), polygalacturonase from *A. flavus* (95% identity) and extracellular polygalactronase from *Neosartorya fischeri* NRRL 181 (74% identity).

3.3. Expression of recombinant PGPO in Pichia pastoris

Recombinant PGPO was successfully expressed in *P. pastoris* GS115 and secreted into the culture medium. The endo-PGase activity reached up to about 1674 U/ml after methanol induction of 96 h in shaker flask fermentation. The PGPO yield was 0.418 mg/ml,

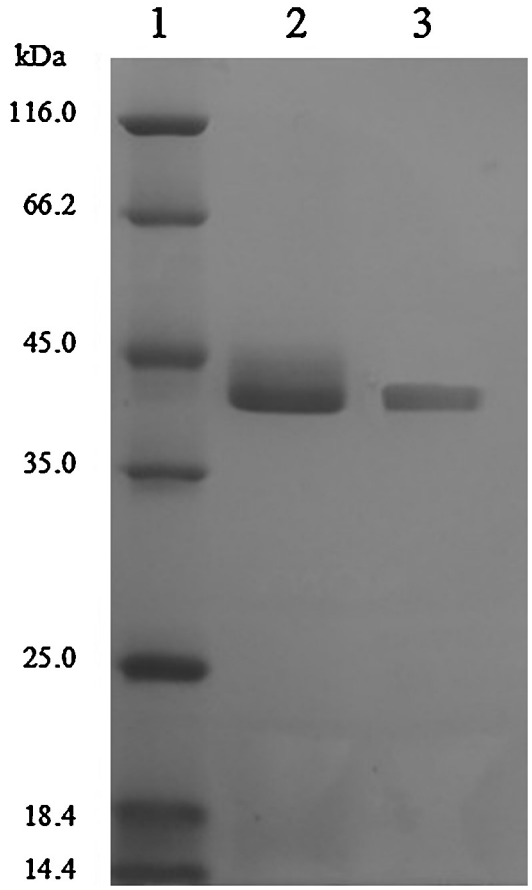

Fig. 2. SDS-PAGE of purified the recombinant PGPO. *Line 1*: Unstained Protein Molecular Weight Marker: β-galactosidase – 116.0; bovine serum albumin – 66.2; ovalbumin – 45.0; lactate dehydrogenase – 35.0; REase Bsp981 – 25.0; β-lactoglobulin – 18.4; lysozyme – 14.4. *Line 2*: Recombinant PGPO before purification. *Line 3*: Purified recombinant PGPO.

which was 4–6 times higher than previous report (Kitamoto et al., 1998). The results indicated that *P. pastoris* is an excellent expression system for efficient production of endo-PGase.

Although the recombinant PGPO showed endo-PGase activity of 1674 U/ml toward PGA, which was significantly higher than crude extract from *A. oryzae* PO (3.0 U/ml), it had lower PPase activity of 125.6 U/ml toward protopectin than crude extract (270.0 U/ml). A series of enzymes, such as endo-polygalacturonase, pectate lyase, arabinanase, rhamnogalacturonase were required to effectively degrade protopectin for pectin release. As mentioned above, crude enzyme from *A. oryzae* PO contains not only endo-polygalacturonase but also endo-1,5-α-arabinanase. Endo-polygalacturonase and endo-1,5-α-arabinanase degrade smooth and hairy region of protopectin, respectively. These two enzymes played roles together in the protopectin degradation making the crude enzyme from *A. oryzae* PO show higher PPase activity than recombinant PGPO used alone.

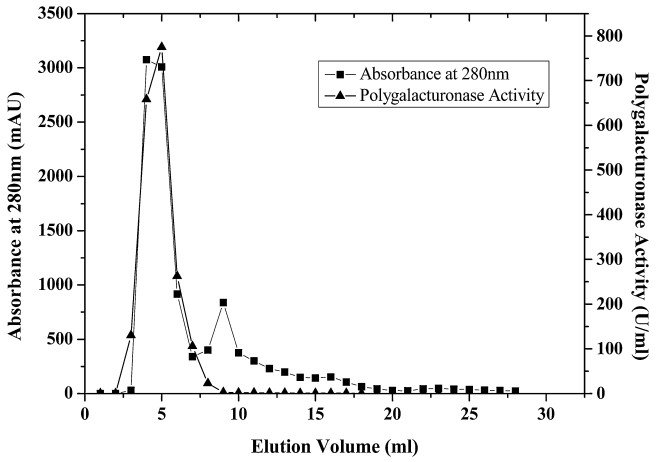

Fig. 3. Elution profile of recombinant PGPO by anion exchange chromatograpgy. Absorbance at 280 nm (-■-) and polygalacturonase activity (-▲-).

3.4. Purification of recombination PGPO

The purification of PGPO was summarized in Table 2. The elution profile of the chromatography only exhibited one endo-PGase peak (Fig. 2). Approximately 1.3-fold purification of the crude enzyme was achieved with a recovery of 87.14%. Purified PGPO migrated as a single band in SDS-PAGE and the apparent molecular mass was about 41 kDa (Fig. 3), which was much higher than the predicted

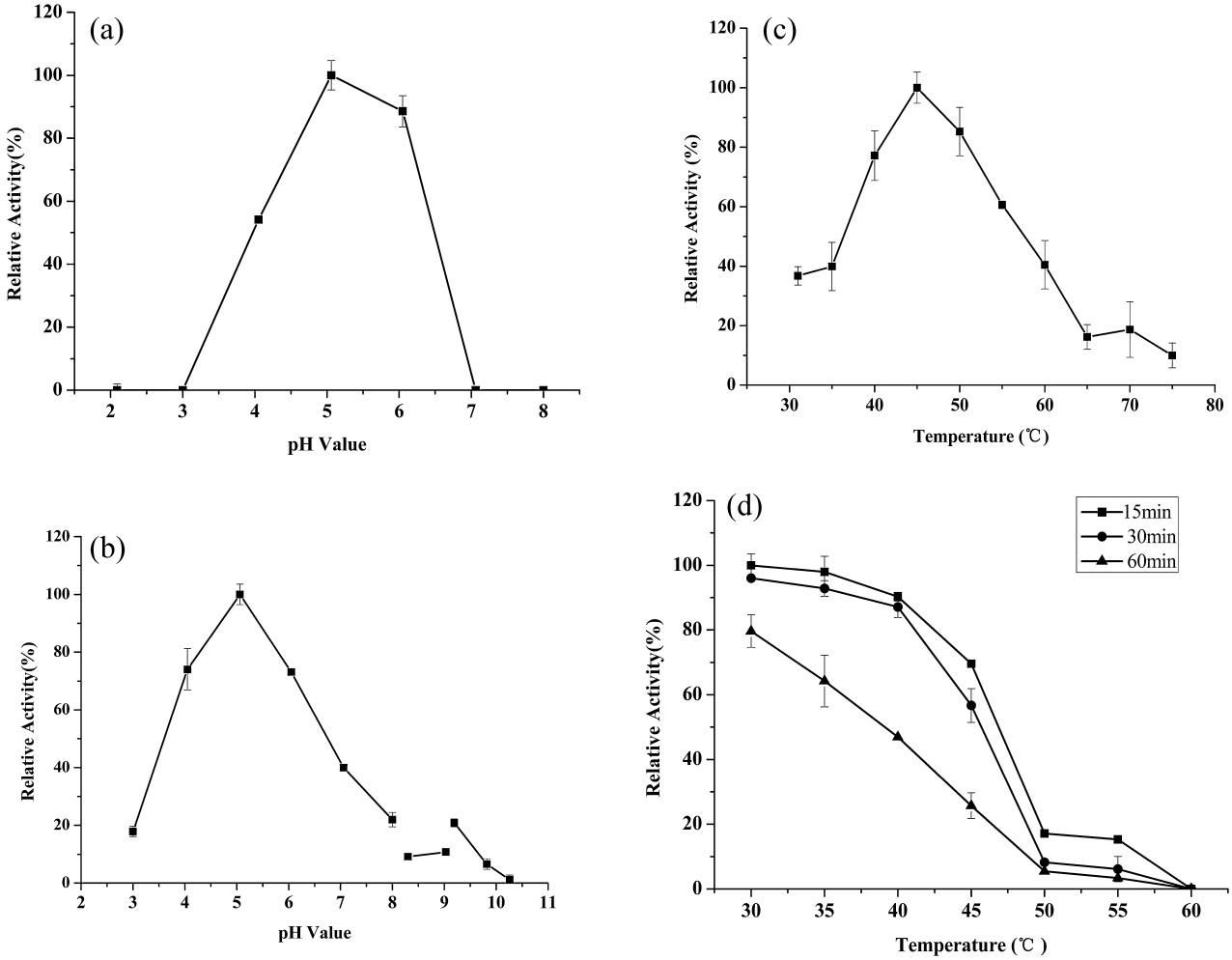

Fig. 4. Enzyme properties. (a) Effect of pH on activity. The optimal pH of the purified recombinant PGPO was determined at 50 °C in various pH values. (b) pH stability of recombinant PGPO. To evaluate the pH stability, the purified recombinant PGPO was incubated in different pH (2.1–10.3) at 30 °C for 30 min. Thereafter, the remaining activity was determined at pH 4.0 at 50 °C. (c) Effect of temperature on activity. The effect of temperature on purified recombinant PGPO was measured by incubating the enzyme at different temperatures (30–75 °C) at pH 4.0. (d) The temperature stability of the purified PGPO. The temperature stability was evaluated by measuring the residual activity after a period of time (15 min, 30 min and 60 min) incubation of the enzyme at temperatures ranging from 30 to 60 °C (1.9 to 8.2).

Table 2
Purification of the recombinant PGPO.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude	0.415	2386	5749	100	1
Q-anion exchange chromatograph	0.277	2079	7520	87.14	1.31

molecular weight (36.7 kDa). The result was analogous to polygalacturonase B reported by Kitamoto et al. (1998). It might due to the multiple glycosylation of recombinant PGPO by the *Pichia* host.

3.5. Characterization of the purified recombination PGPO

The optimal pH and temperature of endo-PGases from various strains were at the ranges of 3.5–5.5 and 30–50 °C, respectively (Jayani, Saxena, & Gupta, 2005). The optimal pH and temperature of present recombinant PGPO was 5.0 and 45 °C (Fig. 4a and c). After incubating at 30 °C for 30 min, less than 20% of maximum activity was maintained when the pH values below 3.0 and above 8.0, whereas the purified recombinant PGPO was stable at pH range from 4.0 to 6.0 (Fig. 4b). The pH stability of PGPO makes it an excellent candidate for potential application in pectin extraction from apple pomace, whose native pH was range from 4.0 to 5.0 (Vendruscolo et al., 2008). Thus, the present recombinant PGPO could be a prospecting pectin extraction enzyme.

The enzyme retained 96% and 80% activity after 30 min and 60 min at 30 °C, respectively. However, the enzyme retained 70%, 56% and 26% activity after incubation at the optimal temperature respectively for 15 min, 30 min and 60 min. Compared with PGases from *Bispora* sp. MEY-1 and *Trichoderma harzianum* that retained 50% activity for 15 min at 55 °C and 30% activity for 30 min at 40 °C, respectively (Yang et al., 2011; Mohamed, Farid, Hossiny, & Bassuiny, 2006), recombinant PGPO was heat labile, with losing total activity at 60 °C within 15 min (Fig. 4d).

The effects of various metal ions (1 mM, 5 mM and 10 mM) and chemicals on PGPO activity were investigated (Table 3). The enzyme activity was enhanced by the presence of Mg^{2+}. The presence of Co^{2+} at less than 5 mM could enhance PGPO activity, while 10 mM Co^{2+} resulted in inhabitation of activity. Na^{1+}, K^{1+}, Li^{1+}, Zn^{2+}, Tween and Triton X-100 had no significant effect on activity of the recombinant PGPO. Ca^{2+}, Ni^{2+}, Mn^{2+}, Cu^{2+} and SDS strongly inhibited the enzyme activity.

The kinetic parameters of recombinant PGPO for hydrolysis toward PGA at pH 5.0 and 45 °C was obtained by a typical double reciprocal Lineweaver–Burk plot (Fig. 5). The apparent K_m and V_{max} values are 5.59 mg/ml and 1.01 μmol/(ml min) respectively. The affinity of recombinant PGPO for PGA is lower than the reported PGases, whose K_m values vary between 0.12 and 4.7 mg/ml (Jayani et al., 2005).

Table 3
The effects of metal ions and surfactants on the activity of recombinant PGPO.

Metal ions	Relative activity (%)		
	1 mM	5 mM	10 mM
Control	100	100	100
Na^{1+}	101.5	90.4	119.6
K^{1+}	84.3	99.6	131.8
Li^{1+}	103.5	91.6	73.7
Zn^{2+}	94.2	117.1	64
Mg^{2+}	161.5	126.1	121.3
Co^{2+}	174.9	149.3	50.4
Ca^{2+}	66.6	18.9	20.5
Ni^{2+}	65.7	3.4	0
Mn^{2+}	62.4	0	0
Cu^{2+}	49.6	2.4	0
Surfactants			
Tween 20 (0.1%, v/v)	109.4		
Tween 60 (0.1%, v/v)	97.5		
Tween 80 (0.1%, v/v)	99.4		
Triton X-100 (0.1%, v/v)	89.4		
SDS (0.1%, w/v)	0		

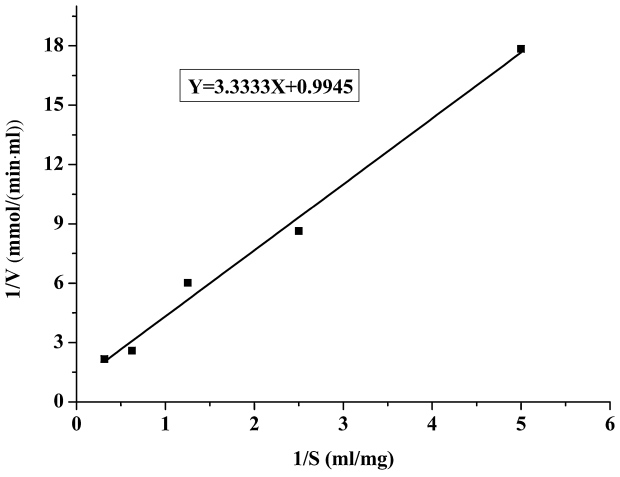

Fig. 5. Lineweaver–Burk plot for kinetics determinations of purified recombinant PGPO using PGA as the substrate.

Endo-PGases have been reported in many microorganisms, yet endo-PGase with PPase activity was rarely reported. Recombinant PGPO displayed the activities, 1674 U/ml toward PGA and 125.6 U/ml toward protopectin, respectively. The results revealed that the recombinant enzyme gained lower activity on PGA located in the smooth region of insoluble protopectin than soluble PGA, since protopectin is an insoluble complex substrate, which limits enzyme to reach reaction sites (Sakamoto et al., 1995). Accordingly, protopectin can be treated by mild acid to make protopectin loose to let the enzyme reach the reaction site more easily and to improve reaction rate, or with combined PPases, which reacted with different regions of protopectin for pectin release. Consequently, combination of chemical and enzymatic treatment or all enzymatic treatment might be potential applications in pectin extraction for more mild reaction condition and controllable pectin quality.

4. Conclusion

In the current study seven filamentous fungi strains were isolated from various sources. One of these isolates showed higher PPase activity (113.3 U/ml) was identified as *A. oryzae* PO on the basis of ITS-5.8S rDNA sequences analysis. *A. oryzae* PO was used for the further study.

On the base of single factor investigation, effects of temperature, $NaNO_3$ and apple pomace concentration were studied by response surface methodology. The optimal fermentation conditions were obtained by RSM as: apple pomace 25.2 g/l, $NaNO_3$ 3.30 g/l, $MgSO_4{\cdot}7H_2O$ 0.5 g/l, KCl 0.5 g/l, K_2HPO_4 1.0 g/l, $FeSO_4{\cdot}7H_2O$ 0.01 g/l, 27.7 °C and natural pH. Under the optimal fermentation conditions, 270.0 U/ml of PPase was achieved at 48 h, which was closely agreed with the predicted value (277.8 U/ml).

The PGPO gene was cloned and expressed in *P. pastoris* GS115. The specific activity of purified recombinant PGPO was 7520 U/mg

toward polygalacturonic acid. The purified recombinant PGPO displayed 125.6 U/ml of PPase activity, which was lower than crude enzyme from *A. oryzae* PO. Protopectin is solid complex variable polysaccharides, in order to obtain high quality pectin, PGPO might be combined with other PPase (such as pectin lyase, pectate lyase, arabinanase and rhamnogalacturonase, ect) for potential applications in pectin extraction. Our future study will focus on fermentation production of PGPO and the development of other PPases.

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