Partial purification and physicochemical characterization of polygalacturonase from *Aspergillus awamori*

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Abstract: In the present study, polygalacturonase from *Aspergillus awamori* was purified partially. The elution profile of the chromatography on DEAE-Sepharose column showed that polygalacturonase activity was detected in two peaks PGase 1 and PGase 2. PGase 1 was found to have optimum pH of 6.5, while optimum pH was 5.5 for PGase 2. The same temperature optima for *Aspergillus awamori* PGase 1 and PGase 2 were detected at 50˚C. The temperature stability for *Aspergillus awamori* PGase 1 and PGase 2 was detected up to 40 and 50˚C, respectively. The *Aspergillus awamori* PGase 1 and PGase 2 activities were compared to the activity with polygalacturonic acid which was regarded as 100% activity. The relative rate of hydrolysis of the pectins decreased with decreasing percentage of esterification: pectin 8% > pectin 26% > pectin 67% > pectin 87%. The apparent K_m and V_{max} values were reported for PGase1 (1.75 mg/ml and 14 units/ml) and PGase2 (1.5 mg/ml and 15.4 units/ml). The most of metal ions tested had no significant effect on *Aspergillus awamori* PGase 1 and PGase 2, except of Hg^{+2} caused strong inhibitory effect. The results information will be helpful to inhibit the fungal polygalacturonase caused spoilage for fruit and also useful for various biotechnological applications specially food processing.
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1.Introduction:

The plant cell wall consists of cellulose, hemicellulose, lignin and pectin. Pectin is a family of complex polysaccharides found in the middle lamella of primary cell walls of plants. Pectin and complex hemicelluloses are responsible for the integrity and coherence of plant tissues as well as for the texture of vegetables and fruits (Pilnik and Voragen, 1991). Pectins are an extremely complex and structurally diverse group of polymers. The fine structures of pectins can be widely heterogeneous between plants, between tissues, and even within a single cell wall. The composition of pectin is different in different plant species and dependent on the age and maturity of the plant parts (Niture, 2008).

Several phyto-pathogenic fungi secrete pectin-degrading enzymes during plant pathogenesis. Pectinases are the first enzymes secreted by fungal pathogens when they attack plant cell walls (Collmer and Keen, 1986; Idnurm and Howlett, 2001). These enzymes are essential for fungal pathogens that do not have specialized penetration structures as well as for necrotrophic pathogens during the late stages of the invasion process (De Lorenzo *et al.,* 1997). Among the pectinases, polygalacturonases are the important virulence factor in Botrytis cinerea-tomato interaction (ten Have *et al.,* 1998) and in the Claviceps purpurea-rye interaction (Oeser *et al.,* 2002). PGs are also crucial for citrus black rot caused by Alternariacitri (Isshiki *et al.,* 2001) and for lesion development on cotton caused by *Aspergillus flavus* (Shieh *et al.,* 1997).

On the other hand, pectolytic enzymes of fungal origin attract the most attention since they offer tremendous potential to the industry. Some of their applications are in retting of flax and vegetable fibres, de-pectinisation and clarification of fruit juices, extraction of oils from vegetables and citrus peels, manufacturing of paper and pulp and pretreatment of pectic waste water (Moyo *et al.,* 2003; Saito *et al.,* 2004; Jayani *et al.,* 2005). This study is aimed to partial purify and characterize of polygalacturonase from *Aspergillus awamori* which isolated previously from date palm cv. Mabrooma (Al-Najada, 2014).

2. Materials and methods

Date palm cv. Mabrooma Peel

Date palm cv. Mabrooma peel was dried in an oven at 60ºC for 48 h. The solid was then milled in a commercial mill and sieved. The mean diameter of the solid was 0.7 mm.

Aspergillus awamori

Aspergillus awamori was isolated and identified from spoilage date palm cv. Mabrooma (Al-Najada, 2014).

Production of polygalacturonase by *Aspergillus awamori* using solid state fermentation

A. awamori was inoculated under aseptic conditions in 50-ml Erlenmeyer flasks contained sterilized date palm cv. Mabrooma peel (1g/1 ml distilled water). The inoculated flasks were incubated at 28ºC for 5 days. Then add 5 ml distilled water to the flask, which subjected to rotary shaker at 180 rpm/min overnight. The suspension is then centrifuged at 7000

rpm for 10 min and the supernatant is designated as a crude extract. The crude extract was subjected to dialysis against 20 mM Tris-HCl buffer, pH 7.2 over night. The dialyzate was centrifuged at 10,000 rpm for 12 min and the supernatant was designed as crude extract.

Partial purification of polygalaturonase from *A. awamori*

Crude extract from *A. awamori* was loaded on a DEAE- Sepharose CL-6B column (10 x 1.6 cm i.d.) equilibrated with 50 mM Tris-HCl buffer, pH 7.2. The enzyme was eluted with a stepwise gradient from 0.0 to 0.3 M NaCl in the same buffer. Fractions in 3 ml volume were collected at a flow rate of 60 ml/h. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity. Protein fractions exhibiting enzyme activity were pooled.

Polygalacturonase assay

Polygalacturonase (EC 3.2.1.15) activity was assayed by determining the liberated reducing end products using galacturonic acid as standards, respectively (Miller, 1959). The reaction mixture (0.5 ml) contained 1% polygalacturonic acid, 0.05 M sodium acetate buffer pH 5.5 and a suitable amount of crude extract. Assays were carried out at 37°C for 1 h. Then 0.5 ml dinitrosalicylic acid reagent was added to each tube. The tubes were heated in a boiling water bath for 10 min. After cooling to room temperature, the absorbance was measured at 560 nm. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 µmol of reducing sugar per h under standard assay conditions.

Protein determination

Protein was determined according to Bradford (1976) and bovine serum albumin was used as standard.

Characterization of polygalacturonase

Optimum pH

Polygalacturonase activity was determined at various pH using different buffers, sodium acetate (pH 4.0-6.0) and Tris-HCl (6.5-8.5) at 50 mM concentration. The maximum activity was taken as 100% and % relative activity was plotted against different pH values.

Optimum Temperature

Polygalacturonase activity was determined at a temperature range of 20-80ºC. The maximum activity was taken as 100% and % relative activity was plotted against different temperatures.

Thermal stability

The enzyme was incubated at a temperature range of 20-80ºC for 30 min prior to substrate addition. The % relative activity was plotted against different temperatures.

Km

The Km values were determined from Lineweaver-Burk plots by using polygalacturonic acid concentrations from 1-3 mg/ml.

Effect of metal ions

The enzyme was incubated with 2 mM solution of Ni^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+} Cu^{2+}, pb^{2+} and Hg^{2+} for 30 min prior to substrate addition. The enzyme activity without metal ions was taken as 100% and % relative activity was determined in the presence of metal ions.

3. Results and Discussion

The purification of polygalacturonase (PGase) from *Aspergillus awamori* cultured on Mabrooma Dates was summarized in Table 1. The specific activity of crude extract was 77.7 units/mg protein. The crude extract was applied onto DEAE-Sepharose column (10 x 1.6 cm i.d.). The elution profile of the chromatography on DEAE-Sepharose column showed that polygalacturonase activity was detected in two peaks PGase 1 and PGase 2 (Fig. 1). The specific activities of PGase 1 and PGase 2 were 460 and units/mg protein that represented 5.9 and 3.8 fold purification over the crude extract with 33 and 26% recovery, respectively. Most fungal organisms produce multiple forms of the enzyme (Devi and AppuRao, 1996; Mikhailova *et al.,* 2000) suggesting either that several genes are involved, or that post-translational modification results in different physical properties of the enzymes (Caprari *et al.,* 1993). Most PGs reported so far have several isoenzymes (Cabanne and Doneche 2002; Mohamed *et al.,* 2006; Fahmy *et al.,* 2008).

Different pH's were observed for *Aspergillus awamori* PGase 1 and PGase 2 (Fig. 2). PGase 1 was found to have optimum pH of 6.5, while optimum pH was 5.5 for PGase 2. These findings were similar to several studies where a pH optimum of 4.8 for polygalacturonase from *Aspergillus niger*, a pH optimum of 5.0 for *Aspergillus awamori* and a pH optimum of 4–5.5 for *Aspergillus japonicus* was reported (Gummadi and Panda, 2003; Jayani *et al.,* 2005). It has been indicated that, among the polygalacturonases obtained from different microbial sources, most have an optimal pH range of 3.5–5.5 (Jayani *et al.,* 2005). With an optimum pH of 5.0, *A. sojae* polygalacturonase could be applicable to fruit juice industries and wine making (Tari *et al.,* 2008). It is well known that acidic pectinases mostly originate from fungal sources, especially from *A. niger* (Kashyap *et al.,* 2001).

The same temperature optima for *Aspergillus awamori* PGase 1 and PGase 2 were detected at 50˚C (Fig. 3). Several PGs show temperature optima between 40– 60◦C (Ortega *et al.,* 2004; Tari *et al.,* 2008). Acidic PG produced by Saccharomyces

cerevisiae shows maximum activity at 25◦C (Gainvors *et al.,* 2000). Thermophilic fungal strains such as Aspergillus aculeatus and Fusarium oxysporum produce different PGs with optimal activity at temperatures between 60–65◦C (Vazquez *et al.,* 1993; Martins *et al.,* 2002). Thermostability of an enzyme is defined as the ability to resist thermal unfolding in the absence of substrates (Bhatti *et al.,* 2006). The temperature stability for *Aspergillus awamori* PGase 1 and PGase 2 was detected up to 40 and 50˚C, respectively (Fig. 4).

With regard to substrate specificity, polygalacturonic acid and pectins with 8, 26, 67 and 87% degree estrification have been tested as substrates (Table 2). The *Aspergillus awamori* PGase 1 and PGase 2 activities were compared to the activity with polygalacturonic acid which was regarded as 100% activity. The relative rate of hydrolysis of the pectins decreased with decreasing percentage of esterification: pectin 8% > pectin 26% > pectin 67% > pectin 87%. The specific activity of fungal PG is dependent on the nature and affinity towards the substrate (Niture, 2008). Among different substrates studied the polygalacturonase from *Aspergillus niger* showed maximum activity towards polygalacturonic acid, followed by apple pectin and citrus pectin. Polygalacturonase showed minimum activity towards potato dextrose agar (Kant *et al.,* 2013). The polygalacturonase from *M. circinelloides* ITCC 6025 (Thakur *et al.,* 2010) showed maximum activity with PGA (0.1% w/v), but it decreased with all other substrates tested.

The kinetic parameters of polygalacturonase, describing its affinity towards polygalacturonic acid were obtained by a typical double reciprocal Lineweaver Burk plot. The apparent K_m and V_{max} values were reported for PGase1 (1.75 mg/ml and 14 units/ml) and PGase2 (1.5 mg/ml and 15.4 units/ml) (Fig. 5). Most fungal PGs have Km values between 0.19–20 mg/ml (Niture, 2008). A low Km values was reported for polygalacturonase from *Sporotrichum thermophile* (0.416mg/ml) and *Aspergillus sojae* (0.424 mg/ml) (Kaur *et al.,* 2004; Tari *et al.,* 2008).

The effect of metal ions showed that Ca^{+2}, Pb^{+2}, Ni^{+2}, Zn^{+2} and Co^{+2} had no significant effect on *Aspergillus awamori* PGase 1 and PGase 2 (Table 3). While Cu^{+2} caused moderate inhibitory effect on PGase 1 and PGase 2, Hg^{+2} caused strong inhibitory effect. The results indicated that *Aspergillus awamori* PGase 1 and PGase 2 had strong resistant toward some metal ions. Kant *et al.* (2013) reported that among various metal ions, Mn^{+2}, K^2, Zn^{+2}, Ca^{+2}, Fe^{+2} and Al^{+3} inhibited *Aspergillus niger* polygalacturonase activity, while activity increased in the presence of Mg^{+2} and Cu^{+2} ions But in case of Hg^{+2} the enzyme activity decreased to greater extent. The effects of different metal cations at the concentration of 1 mM on *P. ostreatus* PGs assay system were studied by Rashad *et al.* (2010). The effectiveness of metal cations as inhibitors for PG was in the order of Ba2+ < Co2+ < Zn2+ < Ni2+ < Ca2+ < Cu2+<Mg2+ < Hg2+ < Fe2+ with 6.5%, 15.3%, 26.5%, 29.4%, 33.0%, 48.2%, 56.3%, 90.4% and 95.82% inhibition, respectively.

Table 1. Purification of polygalacturonase from *Aspergillus awamori* cultured on Mabrooma Dates.

Purification steps	Total units	Total protein (mg)	Specific activity (unit/mg protein)	Fold purification	Recovery (%)
Crude extract	136	1.75	77.7	1	100
DEAE-Sepharose					
PGase 1	46	0.1	460	5.9	33
PGase 2	36	0.12	300	3.8	26

One unit of enzyme activity was defined as the amount of enzyme which produced one µmol glucoronic acid per h under standard assay conditions.

Table 2. Substrate specificity of *Aspergillus awamori* PGase1 and PGase2.

Substrate	Relative activity (%)	
	PGase1	PGase2
Polygalacturonic acid	100	100
Citrus pectin (8% esterification)	73	78
Citrus pectin (26% esterification)	70	73
Citrus pectin 67% esterification)	51	55
Citrus pectin (87% esterification)	35	40

Each value represents the average of two experiments.

Table 3. Effect of 2 mM metal cations on *Aspergillus awamori* PGase1 and PGase2.

Metal cations	Relative activity (%)	
	PGase1	PGase2
Zn^{+2}	100	100
Pb^{+2}	88	100
Ca^{+2}	100	103
Hg^{+2}	17	15
Cu^{+2}	40	40
Co^{+2}	87	94
Ni^{+2}	90	100

The enzyme activity without metal ions was taken as 100%.
Each value represents the average of two experiments.

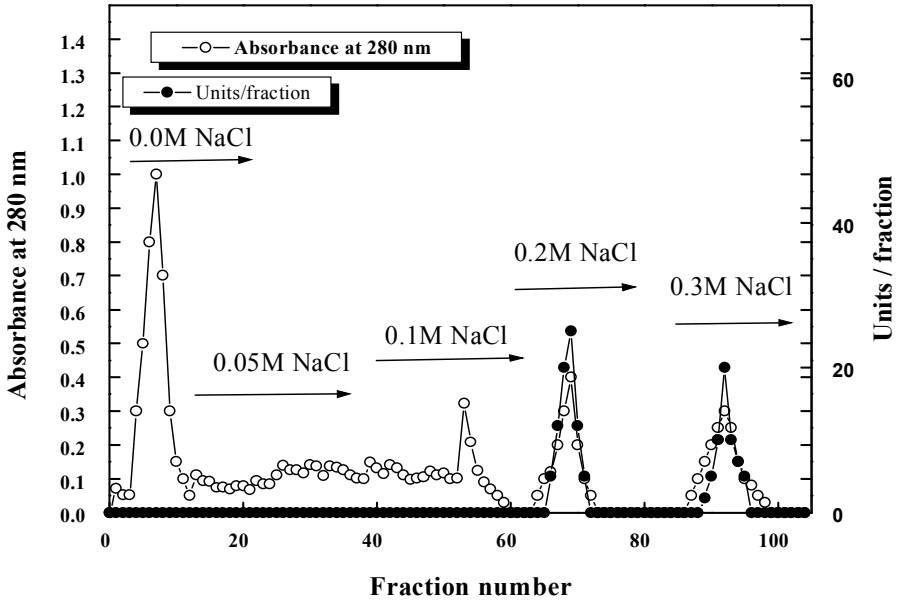


Fig. 1. A typical elution profile for the chromatography of polygalacturonase from *Aspergillus awamori* cultured on Mabrooma Dates on DEAE-Sepharose column (10 x 1.6 cm i.d.) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.2 at a flow rate of 60 ml/h and 3 ml fractions

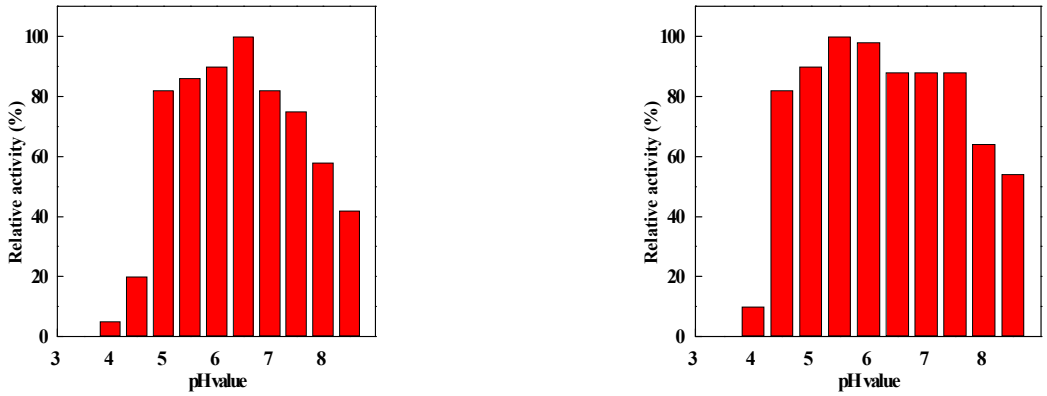
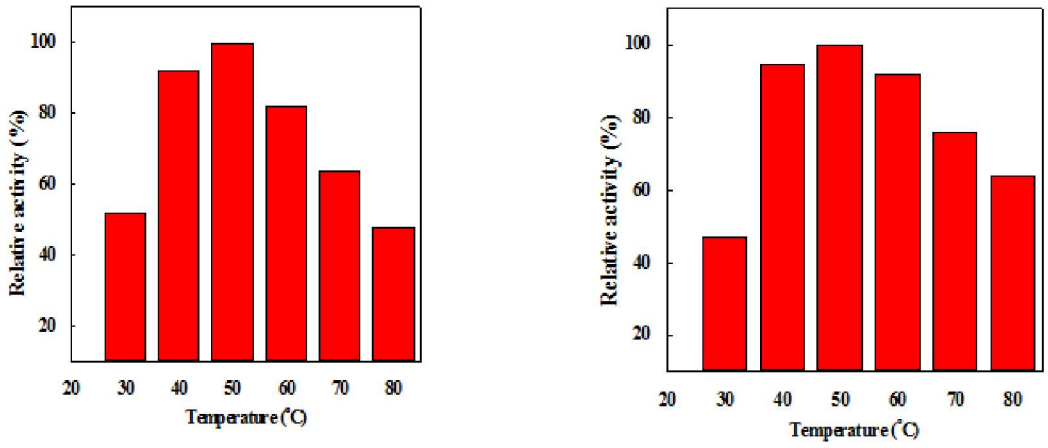
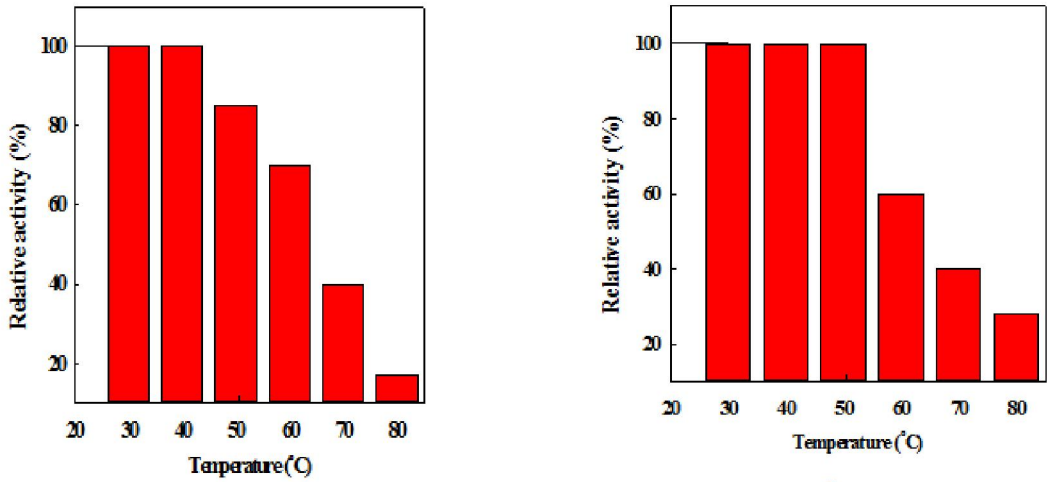


Fig. 2. Optimum pH of *Aspergillus awamori* PGase1 (a) and PGase2 (b). Each point represents the average of two experiments.



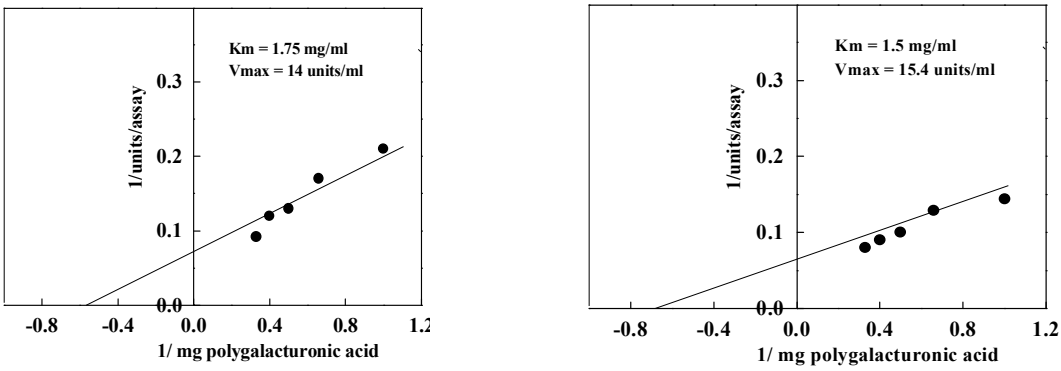
a b

Fig. 3. Optimum temperature of *Aspergillus awamori* PGase1 (a) and PGase2 (b). Each point represents the average of two experiments.



a b

Fig. 4. Temperature stability of *Aspergillus awamori* PGase1(a) and PGase2 (b). Each point represents the average of two experiments.



a b

Fig. 5. Lineweaver-Burk plot relating *Aspergillus awamori* PGase1(a) and Pgase2 (b) reaction velocities to polygalacturonic acid as substarte concentrations. Each point represents the average of two experiments.

Conclusion

This information will be helpful to contribute to the knowledge on *A. awamori* polygalacturonase, helpful for inhibiting the fungal polygalacturonase and also useful from the point of view of various biotechnological applications specially food processing.

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