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Short communication

Purification and partial characterization of an acidic polygalacturonase from *Aspergillus kawachii*

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Abstract

An endo-polygalacturonase, named PGI, was purified to homogeneity from the culture filtrate of *Aspergillus kawachii* IFO 4033 grown in a glucose-tryptone medium. The molecular mass of PGI was estimated to be 60 kDa by SDS–PAGE and 40 kDa by gel filtration on Sephacryl S-100. The isoelectric point was 3.55 as determined by isoelectic focusing. PGI exhibited binding properties to ConA–Sepharose suggesting that the protein is glycosylated. The N-terminal amino acid sequence was also determined as S-T-C-T-F-T-D-A-A-T-A-S-E-S-K. The remarkable property of PGI was its high activity in the pH range 2.0–3.0 towards soluble and insoluble substrates, while being inactive at pH 5.0. Enzyme stability at low pHs was markedly enhanced by different compounds, such as proteins, polysaccharides, simple sugars and the substrate pectin. PGI was very efficient to extract pectin from lemmon protopectin and to macerate carrot tissues at pH 2.0. These properties make PGI an interesting biocatalyst for industrial applications under highly acidic conditions.

Keywords: Pectin; Acidic enzymes; Endo-polygalacturonase; *Aspergillus kawachii*

1. Introduction

Aspergillus kawachii has been extensily used in the Japanese food industry, especially in *shochu* brewing for the preparation of *shochu-koji* (Iwano et al., 1986). Due to the low pH of the fermentation process, the fungus releases several hydrolases that are more acid stable than similar enzymes produced by other fungal species (Mikami et al., 1987; Ito et al., 1992). In a previous work (Contreras-Esquivel et al., 1999), it was reported that *A. kawachii* (IFO 4308), grown in a glucose-tryptone (GT) medium, produces an enzyme activity that releases pectin from protopectin and lemmon peels at low pH (∼2.0–3.0). In view of its potential applicability, we have undertaken the characterization of the acidic pectinases produced by *A. kawachii*, as hardly any studies have been carried out on this subject. The present paper reports the purification and some relevant physicochemical properties of an *A. kawachii* endo-polygalacturonase (endo-PG, poly(1,4-α-D-galacturonide glycanohydrolase, E.C 3.2.1.15) which showed pectin-releasing activity at

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pH 2.0. This enzyme, herein referred as PGI, may be considered the main acidic pectinase produced by *A. kawachii* in GT medium.

2. Materials and methods

2.1. Chemicals

Galacturonic acid, trigalacturonic acid, polygalacturonic acid (PGA), citrus pectin (DM: degree of methylation, 53%) were from Sigma Chemicals (St. Louis, MO, USA). Lemmon protopectin was prepared as described by Cavallito et al. (1997). The anhydrogalacturonic acid content of protopectin was 35% (w/w) and the DM 58%. Chemicals for column chromatography and electrophoresis were from Amersham Pharmacia Biotech.

NaCP and NaOAc buffers contained 50 mM citric acid/25 mM Na_2HPO_4 and 50 mM acetic acid respectively. The pH of both buffers were initially adjusted to pH 5.5 with NaOH and then recalibrated to the desired pH with ClH.

2.2. Microorganism and culture conditions

A. kawachii IFO 4308 was cultured at 30 °C in a 1-l flask containing 100 ml of GT medium (Contreras-Esquivel et al., 1999). The concentration of glucose and tryptone used were 10 and 5 g/l respectively. The initial pH was adjusted to 4.0 and the cultivation carried out until the glucose was entirely consumed (∼30–35 h). Separation of the fungal mycelium was achieved by filtration of the whole broth through a cheese cloth. The filtrated culture obtained was frozen at −20 °C until use.

2.3. Enzyme purification

Column chromatography was performed at room temperature using an FPLC system. The filtrated culture was thawed, centrifuged (5000 × *g* × 20 min) and clarified by refiltering at pH 3.0 through a 0.45 μm glass fiber prefilter (Osmonics) using a 47 mm polysulfone vacuum filtration unit. The amount of centrifuged medium filtered through each prefilter was 300 ml. The clarified medium was concentrated under reduced pressure at 40 °C for 8 h at pH 3.0. Two and a half volumes of acetone (−70 °C) were added to the concentrate and the mixture stirred in an ice bath for 3 h. The resulting precipitate was collected by centrifugation (7000 × *g* × 20 min), resuspended in NaCP buffer (1/4 strength), pH 5.0, and the solution dialyzed overnight at 4 °C against the same buffer. The dialyzate was loaded on a Sepharose Q column (XK 26/20, 60 ml gel) equilibrated in NaCP buffer (1/4 strength), pH 5.0. After washing with 150 ml of 0.3 M NaCl in the buffer, the bound PG was eluted by a linear gradient of 0.3–0.5 M NaCl in the buffer. The flow rate was 3 ml/min and fractions of 5 ml were collected. Active fractions were pooled, dialyzed overnight against NaCP buffer (1/4 strength) pH 5.0 and freeze-dried. This preparation was dissolved in a minimum amount of deionized water and poured over a Sephacryl S-100 column (XK 16/70, 100 ml gel) equilibrated in NaCP buffer (1/4 strength), pH 5.0. The column was eluted with the same buffer at a flow rate of 3 ml/min and fractions of 2 ml were collected. Active fractions were pooled, dialyzed overnight against NaCP buffer (1/10 strength), pH 5.0, freeze-dried and kept refrigerated until use. For the characterization of the purified enzyme, stock solution was prepared by disolving the final solid preparation in deionized water (∼130 μg protein/ml). This enzyme solution was stable, at least for 15 days at 5 °C.

2.4. Analytical methods

Protein concentration was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as standard. Methylation of protopectin was determined with alcohol oxidase after alkaline de-esterification (Klavons and Bennet, 1986). SDS–PAGE was carried out using a Mighty Small II Unit (Hoefer SE 260) according to Smith (1984). Isoelectric focusing was performed with an LKB 2117 Multiphor II on a 7.5% polyacrylamide gel containing 5% Pharmalyte (pH 2.5–5.0) according to the instructions of the supplier. The gel was silver stained for p*I* determination. Polygalacturonase activity was visualized by a pectate–agarose overlay gel with ruthenium red as the stain. Protein glycosylation was assessed by determining the binding properties of the protein to Concavaline A–Sepharose under the conditions described by Catley (1988). N-terminal aminoacid sequencing was conducted by the protein microsequencing

facilities at LANAIS-PRO (CONICET-UBA). The purified enzyme was dissolved in 0.1% trifluoracetic acid, applied to a reverse-phase HPLC column (Aquapore, C-4, 30 × 2.1 mm, Brownlee, Perkin-Elmer, Inc) and eluted with a linear gradient of acetonitrile (0–80% in 0.08% trifluoroacetic acid). The protein in the peak (∼10 pmoles) was sequenced with a gas phase protein sequencer (Model 477, Applied Biosystem).

2.5. Enzyme assays

Pectin-releasing activity was assayed using lemmon protopectin as substrate as previously described (Contreras-Esquivel et al., 1999). Activity towards PGA and pectin was determined by formation of reducing groups using the DNS (Miller, 1959) or the Somogyi–Nelson (Herber et al., 1971) assays with galacturonic acid as standard. One unit of enzyme activity corresponded to the amount of enzyme that produces one μmol of reducing sugar as galacturonic acid per minute. Viscosimetric assays were done in an Ostwald viscosimeter containing 7 ml of reaction mixture. The relative decrease of substrate viscosity was calculated according to Tutobello and Mill (1961). All enzyme activities were determined at 37 °C.

2.6. Enzyme properties

The influence of pH on the enzyme activity was determined with PGA, pectin and protopectin using NaCP buffer in the pH range of 2.0–5.5. Enzyme stability was evaluated in NaCP buffer at different pHs at 50 °C. The enzymatic degradation of PGA and pectin was carried out with 1% of PGA or pectin solution in NaOAc buffer at pH 4.5 and 2.5 respectively. Samples for analysis were heated in a boiling water bath for 5 min and kept frozen at −20 °C. The extent of hydrolysis was calculated according to Tam (1983). For TLC analysis of PGA degradation products, heat inactivated samples previously diluted 10-fold with NaOAc buffer were spotted (2 μl) on aluminium sheets (silica gel 60 F254, Merck), and the chromatography performed twice by using the ascending method with *n*-butanol:acetic acid:water (9:4:7, v/v/v) as the solvent system. Detection was accomplished by spraying the dried plate with 3% phosphomolybdic acid dissolved in 10% sulfuric acid in ethanol followed by heating at 105 °C for 5 min. The endo or exo mode of action of the enzyme was determined by measuring the formation of reducing groups together with changes in viscosity of a 1% PGA or pectin solution in NaCP buffer at pH 4.5 and 2.5 respectively. Kinetic parameters of PGA hydrolisis were determined from initial rate measurements employing PGA concentrations (0.01 to 1.0 mg/ml) in NaCP buffer, pH 4.5. The K_m and V_{max} were calculated using the Leverberg–Marquard non-linear regression algorithm.

2.7. Enzymatic pectin extraction and macerating activity

Pectin extraction with PGI was carried out under shaking at 37 °C in sealed glass tubes containing 30 mg protopectin, 1.5 ml NaCP buffer (final pH 2.0–2.2) and 0.025 μg/ml of PGI. A blank without the enzyme was also incubated. Released pectic substances were determined by the *m*-hydroxydiphenyl-sulphuric acid technique (Blumenkrantz and Asboe-Hansen, 1973) and expressed as galacturonic acid. High molecular weight pectin was determined by the same technique after ethanol precipitation (Contreras-Esquivel et al., 1999). Macerating activity was determined on carrot (*Daucus carota* L.) cylinders ($\phi = 5$ mm, length 5 mm) previously blanched at 100 °C for 5 min to inactivate native enzymes. Maceration was carried out in 50 ml erlenmeyer flasks containing 2.2 g carrot cylinders, 7.5 ml of 20 mM citric acid (final pH 2.0–2.2) and 0.03 μg/ml of PGI. A blank without enzyme was also incubated. The flasks were agitated at 37 °C in a reciprocating shaker (200 strokes/min) for 8 h. Maceration was assesed by measuring the amount of released cells and by microscopic observation (Nakamura et al., 1995).

3. Results

3.1. Purification of enzyme

During the purification process, the enzyme activity measured with protopectin at pH 2.0 correlated with the PG activity measured at either pH 2.0 or 2.5, thus indicating that the acidic pectin-releasing activity produced by *A. kawachii* in GT medium was carried out by a specific type of polygalacturonase. The results of purification of PGI from 3300 ml CM are

Table 1
Purification of PGI from *A. kawachii*

Purification step	Volume (ml)	Total protein (mg)	Specific activity (U/mg protein)[a]	Recovery (%)	Purification (-fold)
Clarified medium[b]	3300	561	1.11	100	–
Vacuum concentration	410	520	1.03	85	1
Acetone precipitation	40	108	4.62	82	4.5
Sepharose Q	10	0.65	483	50	470
Sephacryl S-100[c]	8	0.46	430	40	–

[a] PG activity was determined by measuring the release of reducing groups (DNS assay) in a reaction mixture containing 1.45 ml of 0.5% PGA solution in NaCP buffer, pH 2.5 and 50 μl of enzyme sample.
[b] Mycelium-free culture medium filtered at pH 3.0 through a 0.45 μm glass fiber prefilter.
[c] Active fractions were pooled, dialyzed overnight against NaCP buffer (1/10 strength) pH 5.0, freeze-dried and kept refrigerated until use.

summarized in Table 1. The activity present in the concentrate was recovered as a single and simmetric peak during anion-exchange chromatography on Sepharose Q (at 0.42 M NaCl). Minor contaminants were further removed by gel filtration, although some activity was lost during this separation step. PGI was purified more than 400-fold, with a final yield of 40%. The homogeneity of the purified enzyme preparation was confirmed by SDS–PAGE (Fig. 1A).

3.2. Enzyme characterization

The molecular mass of PGI was estimated to be 60 ± 1.15 kDa by SDS–PAGE and ∼ 40 ± 1.04 kDa by gel filtration on Sephacryl S-100. The lower values obtained by gel filtration may result from interactions between the protein and the gel (Kester and Visser, 1990). Accordingly, 60 kDa seems to be the most reliable value for the molecular mass of PGI.

PGI gave an almost single band of protein and activity, p*I* 3.55, on isoelectric focusing (Fig. 1B). A tiny band, p*I* ∼3.7, also stained for PG activity at pH 2.5, while other minor band, p*I* ∼3.8, did not show PG activity. The origin of these bands was not determined.

PGI bound to ConA–Sepharose which suggests that the protein is glycosylated. The N-terminal amino acid sequence was determined as S-T-C-T-F-T-D-A-A-T-A-S-E-S-K.

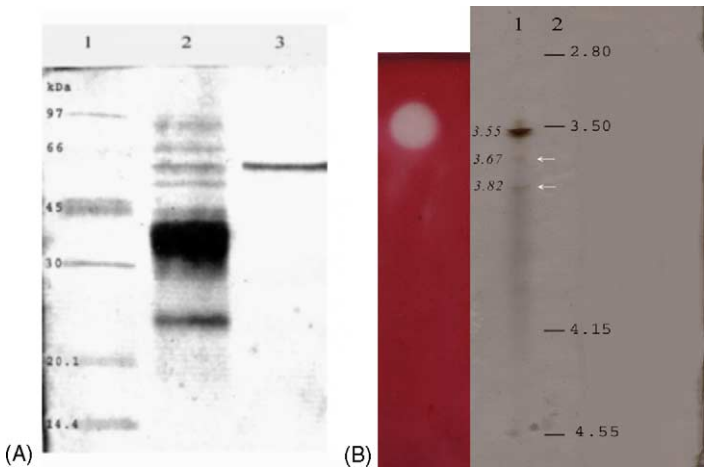

Fig. 1. Analysis of *A. kawachii* PGI purification by SDS–PAGE and isoelectric focusing SDS–PAGE (left): lane 1, molecular mass standard proteins; lane 2, protein from the vacuum concentration step (12 μg); lane 3, protein from gel filtration on Sephacryl S-100 (1.2 μg). Isoelectric focusing (right): lane 1, protein from gel filtration on Sephacryl S-100 (3.0 μg). Lane 2, p*I* standard proteins. PG activity was demonstrated by overlaying the focusing gel with a thin layer of pectate–agarose gel (60 min incubation in NaCP buffer, pH 2.5) with ruthenium red (0.02% in water) as the stain.

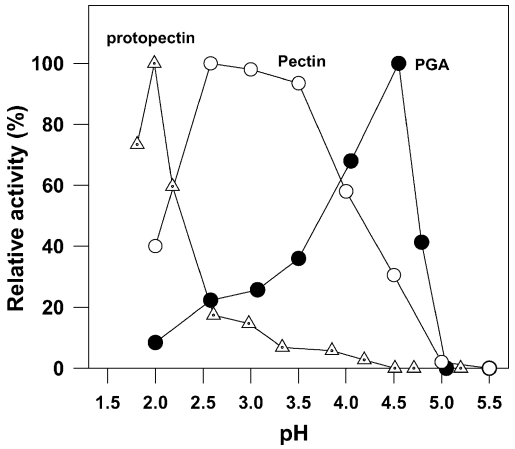

Fig. 2. Effect of pH on *A. kawachii* PGI activity. Activity towards PGA and citrus pectin (DM 53%) was determined by measuring the release of reducing groups (DNS assay) in a reaction mixture containing 1.45 ml of 0.5% substrate solution in NaCP buffer and 50 μl of enzyme solution. Activity towards lemmon protopectin was evaluated as indicated in Section 2. PGI concentration in the reaction mixture was in the range of 0.04–0.20 μg/ml.

3.3. Enzyme properties

The pH ranges for optimum activity depended on the substrate (Fig. 2). With protopectin a peak of activity occured at pH around 2.0 while for soluble pectin the optimal activity was observed in the pH range 2.5–3.0. For PGA the optimal pH was 4.5, but the enzyme activity was still observed at pH 2.0 (10% relative activity) where a solution of PGA precipitates. PGI did not show activity at pH 5.0 in any of the substrates tested. PGI activity towards PGA and pectin was similar regardless of anion (citrate/phosphate, acetate) or cation (Na^+ or K^+) in the reaction media.

The purified enzyme was relatively thermostable at pH values higher than 4.0 whereas at lower pH values the enzyme was readily inactivated (Fig. 3A). However, when albumin was incorporated to the incubation mixture, PGI was stable at pH 2.0. The minimum amount of albumin required to stabilize the enzyme was ∼100 ppm (Fig. 3B). Sucrose (500 ppm) and alginate (500 ppm) also protected PGI against thermal inactivation at pH 2.0 (50 and 36% of residual activity after 30 min incubation at 50 °C). Stabilization of the enzyme by the substrate pectin was also evident as judged by the fact that hydrolysis of this substate at pH 2.0 and 50 °C was not affected by the presence of albumin. The enzyme in NaCP buffer, pH 5.0, without any protective agent, was not inactivated after two cycles of freeze-thawing or after freeze-drying.

Analysis of substrate specificity showed that PGI hydrolyze faster and more extensively PGA than the partially esterified pectin (Fig. 4A) and was practically inactive against highly esterified pectin (DM 92%). Pretreatment of pectins with pectin methylesterase (PE, pectin methylhydrolase E.C. 3.1.1.11), increased both the rate of hydrolysis and the extent of substrate

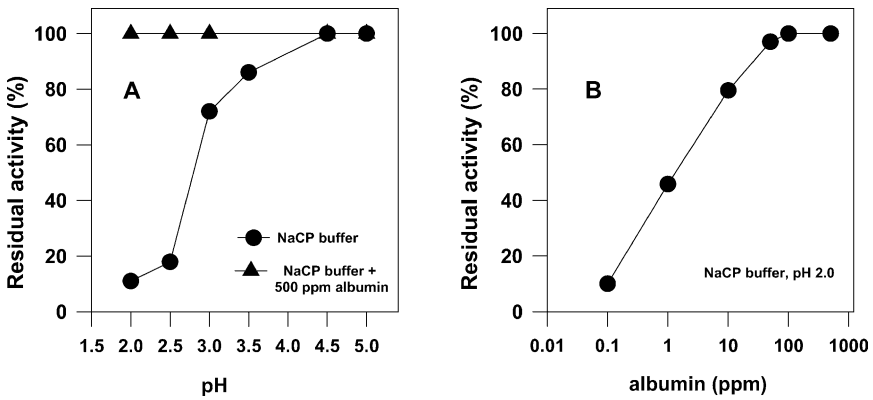

Fig. 3. Effect of pH and albumin on the stability of *A. kawachii* PGI. A NaCP buffer at various pHs in absence or presence of 500 ppm bovine serum albumin B NaCP buffer, pH 2.0 at various bovine serum albumin concentrations. Incubation was at 50 °C for 30 min in a total volume of 100 μl containing 0.6–1.0 μg/ml of PGI. After the incubation period, the sample was quickly cooled in an ice bath, mixed with 400 μl of NaCP buffer, pH 4.5 and incubated 10 min at 37 °C. The remaining activity was determined by adding 500 μl of 1% PGA solution in NaCP buffer, pH 4.5, and measuring the release of reducing groups (DNS assay).

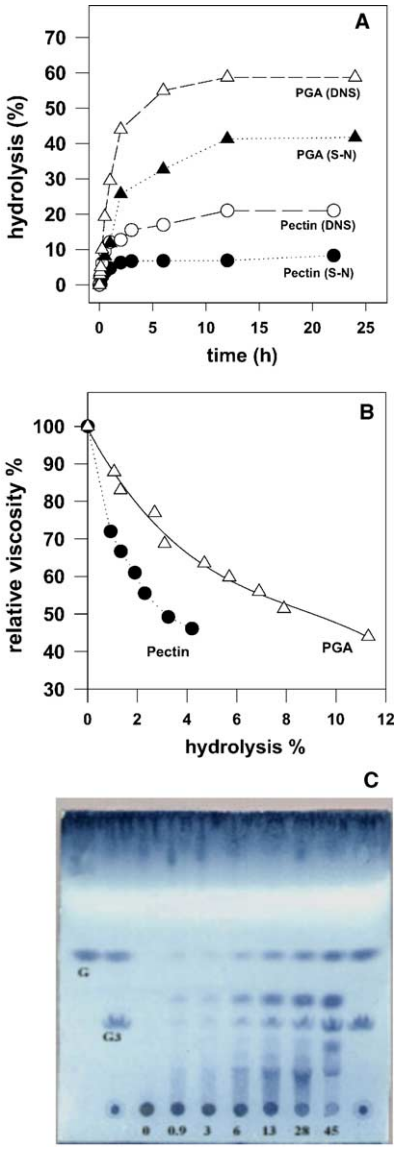

Fig. 4. Degradation of PGA and pectin by *A. kawachii* PGI. A Time-course of substrate hydrolysis determined by formation of reducing groups using the DNS and the Somogyi–Nelson (S–N) assays. The reaction conditions were: 1% PGA in NaOAc buffer, pH 4.5, PGI 0.08 μg/ml and 1% citrus pectin (DM 53%) in NaOAc buffer, pH 2.5, PGI 0.24 μg/ml. B Viscosity reduction and percent hydrolysis (S–N assay) of PGA and pectin solutions. The reaction conditions were: 1% PGA in NaCP buffer, pH 4.5, PGI 0.04 μg/ml and 1% citrus pectin (DM 53%) in NaCP buffer, pH 2.5, PGI 0.11 μg/ml. C TLC analysis of PGA hydrolysis reaction products. Numbers below each line indicate the percent of hydrolysis (S–N assay). G galacturonic acid; G3 trigalacturonic acid.

degradation, thus confirming that methyesters limit the action of PGI. The extent of hydrolysis of PGA and pectin obtained with the DNS assay was much higher than the values obtained by the Somogyi–Nelson assay. The greatest differences were found at the initial stage of hydrolysis. An explanation for this would be an increasing response with increasing degree of polymerization of the oligalacturonates in the DNS assay (Kormenlink et al., 1991).

The action mode of the enzyme (exoPG or endoPG) was determined by using viscosimetric assays. At the time PGI effected 50% decrease in the viscosity of PGA or pectin solutions, the extent of hydrolysis was less than 10% (Fig. 4B). This indicates an endo-action (Rombouts and Pilnik, 1980).

The initial rate of endo-PG activity (Somogyi–Nelson assay) increased with increasing PGA concentration in the range 0.01–1.0 mg/ml when assayed in NaCP buffer, pH 4.5 at 37 °C. Under these conditions, K_m was found to be 0.192 ± 0.028 mg/ml and V_{max} 1500 μmol/(min mg protein).

TLC analysis of the products of PGA hydrolysis indicated that mono-, di- and tri-galacturonates were produced from the initial stages of the hydrolysis and accumulated throughout the incubation period (Fig. 4C). Higher oligosaccharides were detected transitorily since 45% hydrolysis showed that most of these oligosaccharides were degraded and tetra-galacturonic acid, as well as mono-, di- and tri-galacturonates were mainly found in the reaction mixture.

3.4. Enzymatic pectin extraction and macerating activity

Pectin extraction by PGI was evaluated by using protopectin at the optimal pH for the enzyme activity. In absence of enzyme, about 8% of the galacturonic acid content of protopectin was solubilized after 12 h incubation at 37 °C in NaCP buffer, pH 2.0. In the presence of PGI, the release of galacturonic acid increased to 57%, most of which (90%) was present in the alcohol insoluble fraction. Thus, it may be concluded that PGI hydrolizes protopectin at pH 2.0 liberating polymeric, water-soluble pectic substances. PGI also showed a high macerating activity at pH 2.0, resulting in the complete degradation of carrot tissues. Microscopic examination of the maceration products showed a high percentage of single cells, most of them

remained undamaged. Microbial contamination during pectin extraction or maceration was not observed.

4. Discussion

An endo-polygalacturonase (PGI) was purified from the culture filtrate of *A. kawachii* grown in GT medium. The final enzyme preparation was highly homogeneous when analyzed by SDS–PAGE or isoelectric focusing. The results of the purification process and some kinetic properties of PGI, in particular those related with protopectin hydrolysis, indicate that the acidic pectin-releasing activity produced by *A. kawachii* in GT medium was certainly carried out by PGI.

Some characteristics of PGI such as p*I*, molecular mass, kinetic parameters with PGA as substrate and its glycoprotein nature are fairly typical of fungal endo-PGs (Rombouts and Pilnik, 1980; Whitaker, 1990). The remarkable property of PGI was its high activity in the pH range 2.0–3.0 towards soluble and insoluble substrates, while being inactive at pH 5.0. Moreover, PGI was able to release pectin from protopectin and to macerate plant tissues with high efficiency at pH 2.0. This condition makes the enzymatic process very attractive since the low pH of the extraction process minimizes the action of native PE, reduces the risk of microbial contamination and stabilizes pectin in solution (Sakai et al., 1993; Voragen et al., 2001).

Fungal endo-PGs exhibit different substrate degradation patterns. Results obtained with polygalacturonate showed that tri-, di- and mono-galacturonates were released at the initial stages of the incubation period, which suggests that PGI degraded the substrate with a certain degree of multiple attack. In this mechanism, the enzyme may catalyze the hydrolysis of several bonds before it dissociates and forms a new active complex with another polymer chain, resulting in the liberation of oligogalacturonates (Robyt and French, 1967; Benen et al., 1999). This degradation pattern may be similar to the mixed endo/exo mode of PGA cleavage described by Cook et al. (1999). PGI did not seem able to attack dimers and trimers as these products were accumulated throughout the incubation period. This behaviour is consistent with the fact that digalacturonate is not hydrolysed by endo-PGs and trigalacturonate is usually a very poor substrate for this kind of enzymes (Rombouts and Pilnik, 1980).

PGI was unstable when incubated at 50 °C in NaCP buffer at pH below 4.0, but their stability was dramatically improved by the addition of proteins, polysaccharides, simple sugars or pectin. This result may explain the high stability of the pectin-releasing activity observed in the culture filtrate at pH 3.0, whose protein concentration was around 100 ppm (Contreras-Esquivel et al., 1999).

There are few reports dealing with acidic fungal PGs. Kaji and Okada (1969) reported an unusually acid-stable endo-PG produced by *Corticium rolfsii* which was still active at pH 1.5–2.0 on various pectic substances. An enzyme with optimum activity on protopectin at pH 2.3 was described from a mutant *Aspergillus niger* strain (Leuchtenberger et al., 1992) and more recently an endo-PG (PPase-AS) with optimum activity on protopectin at pH 2.0 was purified from extracts of wheat bran cultures of *Aspergillus awamori* (IFO 4033) and its gene isolated and cloned (Nagai et al., 2000a,b). PGI and PPase-AS are glycoproteins, shows identical N-terminal aminoacid sequence and their molecular masses and p*I* are comparable. Considering that *A. kawachii* and *A. awamori* are closely related fungal species, it seems probable that both proteins are encoded by highly similar genes.

On the other hand, properties of PGI were not coincident with those reported by Kojima et al. (1999) for three PGs produced by *A. kawachii* IFO 4033, grown in a soybean flour/peptone medium. Further research is needed to determine whether this *A. kawachii* strain is able to produce different PGs based on the environmental conditions.

In conclusion, *A. kawachii* seems to be a good source of acidic pectinases. The extent to which PGI is, in fact, of industrial interest is at present being investigated (pectin extraction from citrus wastes, preparation of puress and nectars, etc.).

Acknowledgements

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