Cloning and Expression of *Pectobacterium carotovorum* Endo-polygalacturonase Gene in *Pichia pastoris* for Production of Oligogalacturonates

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A bacterial endo-polygalacturonase (endo-PGase) gene from the plant pathogen *Pectobacterium carotovorum* was cloned into pGAPZαA vector and constitutively expressed in *Pichia pastoris*. The recombinant endo-PGase secreted by the *Pichia* clone showed a 1.7 fold increase when the culture medium included glycerol in replacement of glucose as the carbon source. The enzyme had optimum activity at pH 5.5 and 40 °C with stability between pH 5.0 and 8.0 and at temperatures up to 50 °C. The enzyme activity was enhanced by 41% with the addition of 1 mM Co^{++}, and inhibited by Fe^{++} with a 63% reduction. The mode of the enzyme action showed internal cleavage of α-1,4 glycoside bonds of polygalacturonic acid and citrus peel pectin. Trigalacturonate and hexagalacturonate were the main hydrolysis products, with a yield of 0.44±0.01 and 0.21±0.01 mg released per mg polygalacturonic acid substrate, respectively. This represents the first report of a microbial endo-PGase that produced trimer and hexamer uniquely as the end products of hydrolysis, in contrast to mixtures of mono-, di-, and trigalacturonates commonly observed for the action of fungal enzymes. Pectic oligosaccharides generated from native carbohydrate polymers offer the potential application as building blocks for value-added products.

Keywords: Endo-polygalacturonase; Oligogalacturonate; Pectobacterium carotovorum; Pichia pastoris; Gene cloning

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INTRODUCTION

Pectic substances are heterogeneous mixtures of branched and highly hydrated polysaccharides present as one of the major components in plant cell wall. They account for about one third of all macromolecules in the primary cell wall. Pectin can comprise a significant portion of the lignocellulose structure in some biomass types, such as sugar beet pulp and citrus peel. Pectin plays an important physiological role in controlling cell porosity and permeability of the cell, regulating cell growth and differentiation, and providing rigidity to the plant tissue. The weight-average molecular mass of pectin varies from 41 to 307 kD depending on the source (Fishman *et al.* 2001). The structure of pectin consists of "smooth" regions of homogalacturonan (HG), and "hairy" or "ramified" regions of xylogalacturonan (XGA), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) (Sakamoto and Sakai 1995; Schols and Voragen 2003; Wong 2008).

Pectinolytic enzymes that degrade various forms of pectins are widely distributed in plants and microorganisms. The plant enzymes are involved in promoting cell wall extension, and softening of plant cell tissues during maturation and storage. Microbial enzymes, in contrast, cause the decomposition of pectin, utilizing it as nutrients to support growth of the organism. Microbial pectinases are the source of enzymes for commercial applications in fruit and vegetable juice processing and also in the paper and pulp industry (Jayani *et al.* 2005). Enzymatic hydrolysis of pectin can also be used to engineer texture and rheological properties of biomass fibers, and offer potential application in the formation of biobased value products (Ebringerova 2012). The enzyme of focus for these potential applications has been the polygalacturonases. Pectic enzymes can be classified into three groups (1) 6 hydrolases, (2) 4 lyases, and (3) 3 esterases, for a total of 13 enzyme groups, plus a number of auxiliary enzymes (Benen and Voragen 2003). Together with pectin methyl esterase, pectin lyase, and other pectin-degrading enzymes, it forms an enzyme mixture that enhances solubilization and fragmentation of cell wall (Sila *et al.* 2009).

Endo-polygalacturonases (EC 3.2.1.15) belong to the glycoside hydrolase family (GH28), and hydrolyze the polymeric substrate at the α-1,4-D-galacturonosidic linkages randomly. Endo-polygalacturonases (endo-PGases) from a number of fungi have been studied and some have been cloned and utilized (Cho *et al.* 2012; Liu *et al.* 2014). The enzymes of bacterial origin, however, have not received as much attention in cloning for large scale using GRAS microorganisms. The study of bacterial enzymes for pectin degradation may extend the classic industrial repertoire of pectinolytic enzymes, which are mostly of fungal origin. *Pichia pastoris* is an excellent host system with high growth rate, high yield expression, and vector constructions designed for constitutive secretion. In this study, the endo-polygalacturonase *peh* gene (AAA57139) of *Pectobacterium carotovorum* was heterologously cloned and expressed in *P. pastoris*. The properties of the recombinant enzyme were analyzed, and the oligogalacuronate reaction products were determined and quantified.

EXPERIMENTAL

Materials

Pichia pastoris strain SMD1168H, vector pGAPZαA, *Pichia* Easy Comp Kit, and precast SDS-PAGE gels were purchased from Invitrogen (Carlsbad, CA). Q-Sepharose was obtained from GE Healthcare (Piscataway, NJ). Restriction and modifying enzymes were from New England Biolabs (Beverly, MA). Microbiological culture medium components and agar were from Difco Laboratories (Detroit, MI). Thin-layer plates and substrates were purchased from Sigma. All chemicals and reagents were of analytical grade.

Gene Synthesis and Plasmid Construction

The gene encoding *peh* was synthesized by GenScript (Piscataway, NJ) as a 1,209 bp open reading frame ligated in the *Hind*III site of a modified pUC57 plasmid. The gene was sub-cloned as a fusion to the α-mating factor signal peptide in the *Pichia* expression vector pGAPZαA (Invitrogen), and a stop codon was inserted 5' to the C-terminal myc epitope. The gene-vector construct was transformed into *Pichia* strain SDM1168H using the *Pichia* Easy Comp Kit (Invitrogen). Although several combinations for the construct

were possible, activity was observed in the construct that had the α-mating factor signal, the catalytic enzyme and the C-terminus without His-tag.

Expression and Purification

A single colony of the transformants was inoculated in 5 mL YEPD medium and cultured at 225 rpm and 30 °C overnight. The culture was diluted 1:50 in 500 mL YEPG (1% Yeast Extract, 2% Peptone, 2% glycerol) in 2-L baffled flasks and grown under the same conditions for 3 days. The cultures were harvested by centrifugation twice at 6,000 x g and the supernatant filtered through 0.45 μm MachV Supor device (Nalgene). The supernatant was concentrated and buffer exchanged to 25 mM Tris buffer, 50 mM NaCl, pH 7.5 (buffer A), with a PelliconXL filter (10 kDa MWCO) on a TFF Labscale pump.

The enzyme was purified by anion exchange (Q-Sepharose FF) column (2.5 cm x 40 cm) equilibrated in buffer A at a flow rate of 30 mL/hr. The concentrated sample was loaded onto the column and washed with 380 mL buffer A. The enzyme was eluted in a gradient of 50 mM to 0.5 M NaCl in buffer A for a total volume of 500 mL. The enzyme protein was eluted at ~320 mM NaCl concentration. Elution fractions were pooled, concentrated and buffer exchanged to 50 mM phosphate buffer, pH 6.0. The concentrated sample was applied onto a TSK-GEL G2000SW (TosoHaas, Germany) HPLC column (7.5 mm x 60 cm, 10 μ), and isocratically eluted in 0.1 M sodium phosphate, pH 7.0 containing 0.3 M NaCl at ambient temperature with a flow rate of 0.5 mL/min.

Electrophoresis

The purified and concentrated enzyme was run on a 4 to 12% Tris-Glycine PAGE gel using denaturing and reducing conditions, at 100 V constant for 2 h, and stained with SimplyBlue Safe Stain (Invitrogen, CA). For molecular weight determination, the protein bands were analyzed by image analysis software (Alpha Innotech, CA).

Enzyme Activity

Enzyme activity expressed in the transformants was detected by plating the colonies or culture liquid on agarose plate containing 0.5% citrus peel pectin substrate. Plates incubated overnight were stained with 0.05% ruthenium red. Active clones were identified by the formation of clear halo against a pinkish red color background. Enzyme activity of the purified or partially purified enzyme on pectin was measured by liquid assay using the dinitrosalicylic acid (DNSA) method, which determines the number of reducing ends as galacturonic acid equivalent (Wong *et al.* 2005). Typically, the enzyme reaction mixture consisted of 75 μL of 1% PGA (polygalacturonic acid, Na salt, dissolved in water), 75 μL of 0.2 M NaOAc, pH 5.5, and various concentrations of 150 μL enzyme preparation. The mixture was incubated at 40 °C for 1 h. Enzyme activity unit was expressed as μg galacturonic acid per min at pH 5.5 and 37 °C.

Effect of pH and Temperature on Optimum Activity and Stability

For the measurement of pH optimum, the reaction mixture was incubated for 1 h at 40 °C, with PGA in pH of 5 to 9 buffers (sodium citric pH 5, sodium phosphate pH 6 to 8, glycine pH 9). For pH stability, the enzyme was incubated in various pH buffers at 25 °C for 4 h, reconstituted to pH 5.5 with 0.1 M NaOAc buffer, and the residual activity was measured by adding the substrate for 1 h incubation at 40 °C.

To determine the temperature optimum, the reaction mixture was incubated for 1 h in 0.1 M NaOAc, pH 5.5. Temperature stability was measured by incubating the enzyme

at various temperatures from 30 to 70 °C for 25 min, after which the substrate was added, and the residual activity was determined.

Effect of Metal Ions

To determine effects of various metal ions, the enzyme was incubated for 1 h at 4 °C with 1 mM concentrations of either $CaCl_2.2H_2O$, $CoCl_2.6H_2O$, $CuCl_2.2H_2O$, $FeSO_4.7H_2O$, KCl, $MgSO_4.7H_2O$, $MnCl_2.4H_2O$, $NiSO_4.6H_2O$, or $ZnSO_4.7H_2O$. The samples were assayed for activity by adding the substrate, incubated for 1 h at 40 °C. The reaction products were measured as reducing sugars using the DNSA method.

Analysis of Hydrolysis Products

Hydrolysis of PGA was measured by monitoring the formation of oligomeric products using thin-layer chromatography. TLC separation was performed on silica gel plates, developed twice with a mobile phase of n-BuOH/HCOOH/H_2O (2:3:1) (Koller and Neukom 1964). The sugars were detected by spraying the developed plate with 10% H_2SO_4 in methanol with 1 mg/mL orcinol.

The ability of the enzyme to hydrolyze PGA was analyzed by monitoring the release of oligogalacturonates. The analysis was performed on a Shimadzu LC-10AD delivery system using a Zorbax-SAX column (200 x 10.0 mm, Agilent) with 0.3 M NaOAc, pH 5.4 as the solvent, at a flow rate of 0.9 ml/min at 40 °C (Voragen *et al.* 1982). The eluant was monitored with a refractive index detector. The oligogalacturonates were identified by retention time and spiking with standards.

Bioinformatics and Graphics

Geneious was used for sequence analysis and vector construction (http://www.geneious.com, Kearse *et al.* 2012). Multiple sequence alignment was performed using ClustalW and presented by Boxshade (Chenna *et al.* 2003). For graphics and statistics, KaleidaGraph software was used for calculating standard errors and for plotting. All experimental points represent the working of at least duplicate samples.

RESULTS AND DISCUSSION

The endo-PGase gene of 1209 bp, encoding 402 amino acids, (with the N-terminal 26 residues as the signal peptide), originally in pUC57, was subcloned into the *Pichia* expression vector pGAPZαA, and transformed into strain SMD1168H. BLAST search revealed the amino acid sequence closely related to the primary structures of endo-PGases from *Erwinia tasmaniensis* (61.5% identities), *Zymobacter palmae* (57.8%), and *Pantoea ananatis* PA13 (49.8%) (Fig. 1).

Endo-PGase enzymes hydrolyze α-1,4-D-galacturonosidic linkages of deesterified ("smooth" or homogalacturonan) regions of pectin. The enzymes are classified as family GH28, and act by a single displacement mechanism with inversion of anomeric configuration (McCarter and Withers 1994).

Sequence alignment suggests that the catalytic residues consisted of Asp202 (numbering in the mature protein) as the general acid and an activated water molecule bound between Asp202 and Asp223 as the nucleophile.

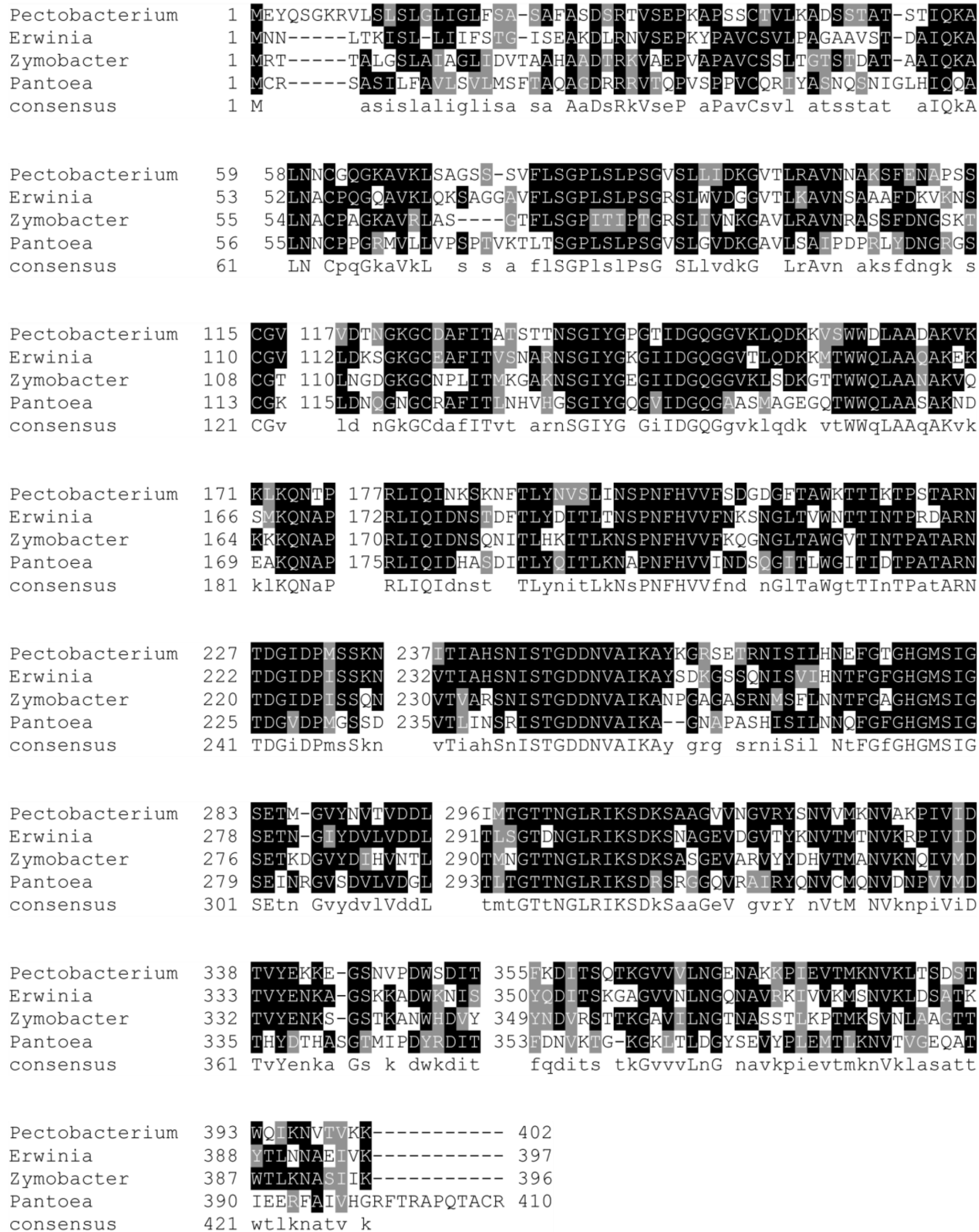

Fig. 1. Multiple sequence alignment of *Pectobacterium carotovorum* (WP_039543807), *Erwinia tasmaniensis* (WP_042958805), *Zymobacter palmae* (WP_027705705), *Pantoea ananatis* PA13 (AER33559), calculated with ClustalW (Chenna *et al.* 2003) and presented by Boxshade.

The medium YEPD (1% yeast extract, 2% peptone, 2% dextrose) commonly used for gene expression in *P. pastoris* contains reducing sugars, which would interfere with the DNSA method for direct measurement of the enzyme activity in the culture. Our previous report showed substituting glucose with glycerol (YEPG) would alleviate this problem, and also result in a 4.5 fold increase in expression and secretion of recombinant α-amylase

in *Saccharomyces cerevisiae* (Wong *et al.* 2002). The transcript level of the α-amylase gene was also significantly increased in the YEPG culture. The present study with the *Pichia* clone showed similar results of higher activity and yield, with ~1.7 fold increase comparing YEPG with YEPD (Fig. 2). Recently, Varnai *et al.* (2014) also observed increased expression rate and yield of endoglucanases in *Pichia pastoris* by substituting glycerol for glucose. Glucose is a fermentative carbon source for yeast, with ethanol being the product of the fermentative process. In contrast, glycerol is a non-fermentative carbon source, which enables respiratory growth under aerobic conditions. High-density cell mass has been observed using glycerol with controlled pH on-line feeding (Raj *et al.* 2002). Glycerol has been known to exhibit osmoprotection and stabilization effects on yeast cells (Brisson *et al.* 2001).

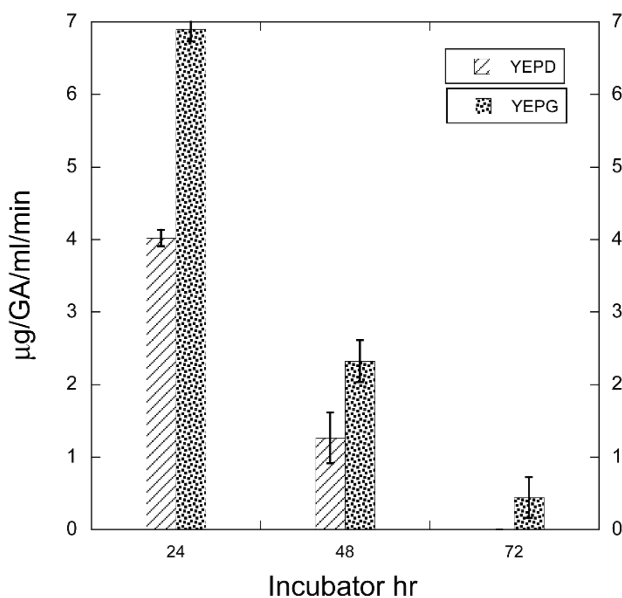

Fig. 2. Recombinant endo-PGase activity in YEPD and YEPG cultures of *Pichia pastoris* clone. Cultures were concentrated and buffer exchanged with 25 mM NaOAc, pH 6.0 using Amicon ultra-4 (10K MWCO) before application for activity assay with the DNSA method.

The purified protein was estimated by SDS-PAGE with a molecular mass of 48 kD, compared to the calculated value of 42.63 (Fig. 3). Its activity on PGA and citrus peel pectin was confirmed by the formation of halos on agarose-substrate plate (Fig. 4), as well as by measurement of reducing sugar as galacturonic acid equivalent using the DNSA method. A very light band of 50 kD was visible with less than 1% of the total protein. This band was most likely attributed to glycosylation, which has been shown to commonly occur in yeast expression of recombinant proteins (Wong *et al.* 2005, 2012). TLC and HPLC also confirmed the formation of oligogalacturonates in the enzyme-catalyzed hydrolysis of polygalacturonic acid. The specific activity of the purified enzyme on polygalacturonic acid was 203±11.7 units per mg (pH 5.5, 40 °C).

The recombinant enzyme was most active at pH 5.5, and relatively stable at pH 5.0 to 8.0 (Fig. 5A). More than 80% of the initial enzyme activity remained at this stability range (at 40 °C). The enzyme had a temperature optimum at 40 °C (at pH 5.5), was stable up to 50 °C, and completely inactivated at 70 °C incubation (Fig. 5B). The enzyme showed a wide pH range of stability, but a narrow range for temperature stability.

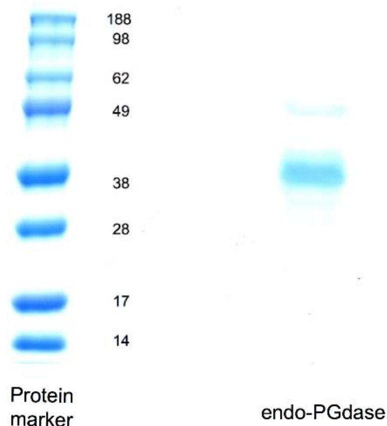

Fig. 3. SDS-PAGE of endo-PGase in purification steps: lane 1, protein marker, lane 2, anion-exchange and gel chromatography

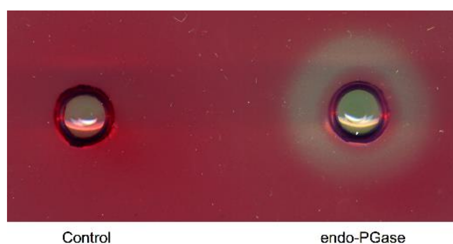

Fig. 4. Detection of activity by plate assay. A mixture of 0.5% citrus peel pectin and 0.8% agarose in 0.2 mM NaOAc, pH 5.5 buffer was microwaved to complete dissolution. Enzyme and control solutions were spotted into separate wells, incubated at 37 °C overnight, and stained with 0.02% ruthenium red. Left halo = control. Right halo = endo-PGase

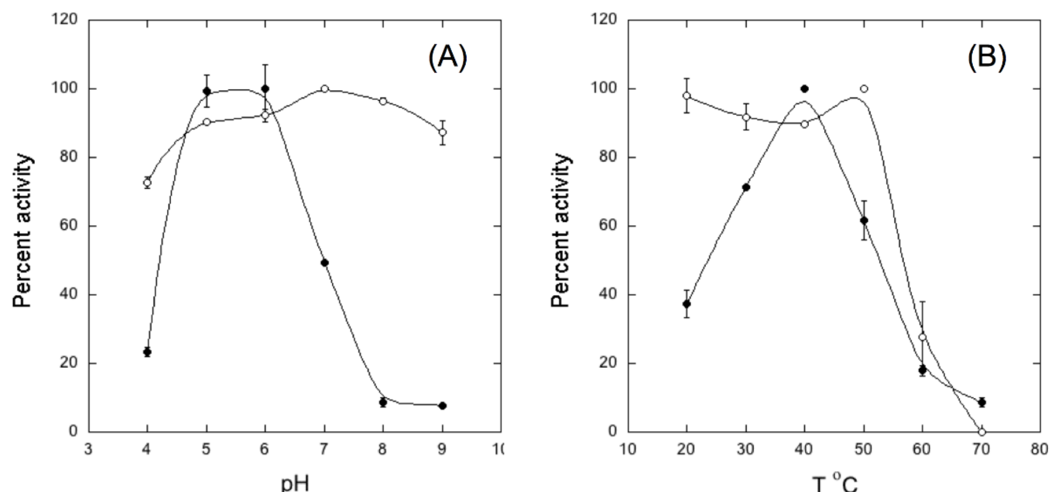

Fig. 5. (A) Effect of pH on optimum activity and stability. Reactions were incubated for 1 h at 37 °C using PGA as the substrate in variable pH buffers. For pH stability measurement, the enzyme was preincubated at various pH buffers at 25 ºC for 4 h, reconstituted to pH 5.5 and assayed for residual activity. (B) Effect of temperature on optimum activity and stability. The reaction mixture was incubated for 1 h in 0.1 M NaOAc, pH 5.5 at various incubation temperatures. For stability measurement, the enzyme was incubated at various temperatures for 25 min, before assay for residual activity. Solid circle: optimum pH or `temperature, hollow circle: pH or temperature stability

The enzyme was inhibited by 1 mM concentration of $FeSO_4$ with 63% reduction of activity. At the same concentration, $CoCl_2$ enhanced the activity by 41% (Fig. 6). EDTA did not affect the activity, suggesting metal ions was not involved in catalysis. The same concentration of Ca^{++}, Cu^{++}, K^{+}, Mg^{++}, Mn^{++}, Ni^{++}, and Zn^{++} did not affect the enzyme activity. It has been reported that the addition of 1 mM Mn^{++} enhanced the activity of *Galactomyces citri-aurantii* polygalacturonase by 161% of normal level, whereas Fe^{3+} acted as a strong inhibitor (Cho *et al.* 2012). The effect of metal ions appears with varying results depending on the source and the fine structure of the endo-PG enzyme.

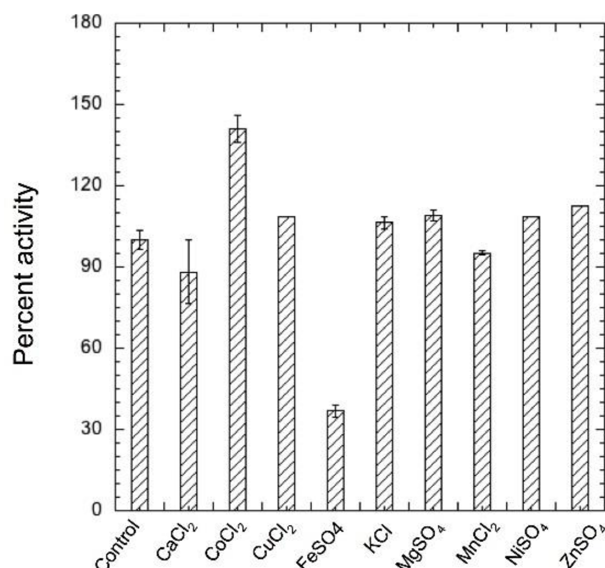

Fig. 6. Effect of metal ions on enzyme activity. Concentration of metal ion used in the reaction was 1 mM. Enzyme-metal mixtures were incubated for 1 h at 4 °C, before substrate was added for the reaction to proceed. Residual activity was measured after incubation for 1 h at 40 °C.

The mode of enzyme action was investigated by analyzing the products of hydrolysis using polygalacturonan as the substrate. The major products were trigalacturonate and the hexagalacturonate, which suggested endo-acting reaction (Fig. 7). The yield was 0.44±0.01 and 0.21±0.01 mg for trimer and hexamer per mg of polygalacturonate, respectively, under the reaction conditions described in the "Methods" section. The smallest end product was trigalacturonate, but hexamer seemed to accumulate in the enzyme reaction without further hydrolyzed to the trimer. In contrast, fungal enzymes and commercial enzyme preparations produce mono-, di-, and trigalacturonate as final products (Rexova-Benkova 1970; Armand *et al.* 2000; Combo *et al.* 2012). The present result suggests that the recombinant enzyme might require substrates longer than hexameric to form a productive binding. The active site of *endopolygalacturonases* in *Aspergillus* contains at least seven subsites (Benen *et al.* 1999). Although the subsite topology of the recombinant enzyme is not available, it is likely that in the recombinant enzyme, a hexameric substrate might not cover all the subsites in the correct binding mode to register a productive EA complex for hydrolysis. It has also been known that for many hydrolytic enzymes, transglycosylation may occur at high substrate concentration, where in this case, the triGA instead of water as an acceptor to yield a hexamer (Baumann *et al.* 2007).

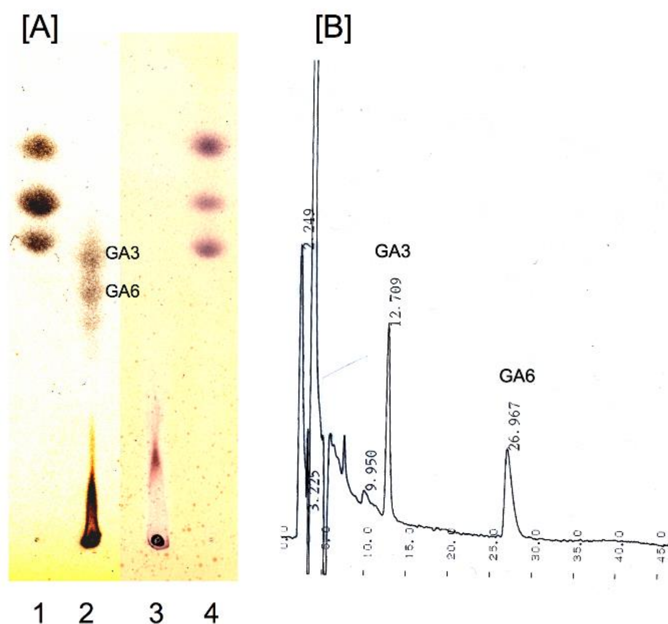

Fig. 7. Analysis of oligogalacturonates in the enzymatic hydrolysis of PGA. (A) TLC separation: lane 1 & 4, mixture of mono-, di-, and trigalacturonate; lane 2, endo-PGase hydrolysis of PGA; lane 3, control: reaction without gene/enzyme; (B) HPLC chromatograms: endo-PGase catalyzed hydrolysis of PGA

CONCLUSION

A bacterial endo-polygalacturonase gene from *Pectobacterium carotovorum* was successfully cloned and expressed in *P. pastoris*, and the enzyme was characterized. Its mode of action on the hydrolysis of PGA produces trigalacturonate and hexagalacturonate as the main end-products. This action pattern is unique compared to those observed for a number of fungal endo-PGases. The recombinant enzyme might provide a route for the production of the types of pectic oligosaccharides with functional characteristics that are not commonly found in pectic hydrolysis products using fungal endo-polygalacturonases or commercial enzyme preparations. These unique oligo-galacturonate products might provide potential applications. Oligosaccharides are of value as building blocks for pharmaceutical, cosmetic, and other bioproduct manufacturing receiving increased attention in recent years. Oligosaccharides carrying functional side group (such as uronic acids in pectic oligosaccharides) can be further modified as base materials for formulating surfactants, biodegradable plastics, films, coatings, capsules, and tablets (Ebringerova 2012).

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