Research Article

Isolation and Partial Characterization of a Bacterial Thermostable Polymethyl Galacturonase from a Newly Isolated *Bacillus* sp. strain BR1390

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Background: Pectinases are pectin degrading class of enzymes including polygalacturonase (PG), polymethyl galacturonase (PMG), pectate lyase (PEL), and pectin esterase (PE) that are commonly used in processes involving the degradation of plant materials, such as speeding up the extraction of fruit juices.
Objectives: A highly methylated pectin degrading bacterium from soil covered with fruit waste was isolated and its extracellular pectinase as a novel polymethyl galacturonase was characterized.
Materials and Methods: Pectin-degrading microorganism screening was performed using agar plates containing pectin as the sole carbon source. The biochemical studies were used to characterize the enzyme.
Results: Bacterium with greater PMG activity was a *Bacillus* sp. based on 16S rDNA sequence analysis and named as a *Bacillus* sp. strain BR1390. Two steps column chromatography showed a dimeric protein with apparent molecular masses of 104 and 56 kDa, evident by gel filtration and SDS-PAGE. Substrate specificity analysis using various polygalacturonic acid compounds revealed its polymethyl galacturonase (PMG, EC 3.2.1.-) activity. Biochemical studies represent the thermophilic characteristics and reasonable pH stability of the polymethyl galacturonase when using pectin as substrate. The PMG activity significantly enhanced in the presence of most divalent cations such as Ca^{2+} and Mg^{2+}, but Hg^{2+} and Fe^{3+} served as strong inhibitor.
Conclusions: Overall, regarding to have suitable activity in acidic conditions and high operational stability of the purified pectinase, the introduced PMG can be an ideal functional substitute for applications in the fruit juice industry, especially in citrus fruits extraction and clarification.

Keywords: Dimeric pectinase; Fruit juice industry; Polymethyl galacturonase; Thermostable pectinase

1. Background

Pectic polymers are amongst abundant carbohydrates present in cell wall of terrestrial plants. Pectins are composed of a long chain of D-galacturonic acid linked by α-1,4-glycosidic linkages with 60-90% esterification by methanol (1). Pectinases are classified into polygalacturonase, PG, (EC 3.2.1.15), polymethyl galacturonase, PMG, (EC 3.2.1.-), polygalacturonate lyase, PGL, (EC 4.2.2.9), pectate lyase, PEL, (EC 4.2.2.2), pectin lyase, PNL, (EC 4.2.2.10) and pectin esterase, PE, (EC 3.1.1.11) according to the substrate specificity and mode of action. Except animals, almost all other organisms encode for pectinases. In addition to pathophysiological and physiological importance, pectinases have widespread biotechnological applications (2-5). Acidic pectinases are widely used for extraction and clarification of fruit juice (6-9), whereas alkaline counterparts have found their ways in textile and pulp and paper industries, plant protoplast isolation and pectic waste water treatment (4, 6, 7).

Pectin lyase and polymethyl galacturonase cleave highly esterified pectin by hydrolysis or β-elimination mechanism, producing oligo or monomethyl galacruronate based on exo/endo mode of action, with no need of prior methyl esterase activity. PLs and PMGs are capable of cleaving the linear backbone of pectic substances without affecting the ester content of remaining polymer. Pectin esters provides specific

aroma to the fruits, helps in stabilizing juice cloudiness and also prevents toxic methanol formation during fruit processing (7, 8). To the best of our knowledge, this is the first report on a bacterial PMG.

2. Objectives

A bacterial thermotolerant PMG is purified and partially characterized via biochemical means and its biotechnological potential is discussed.

2. Materials and Methods

3.1. Materials

Polygalacturonic acid, sodium polypectate with esterification degree of 20-35% (ED 20-35%), citrus pectin (ED ~ 70%), thiobarbituric acid (TBA), ruthenium red and dinitrosalicylic acid (DNS) were purchased from Sigma (St. Louis, Missouri, USA). DEAE-Cellulose and Sephacryl S-200 were obtained from Pharmacia (Uppsala, Sweden) and all other chemicals were reagents of analytical grade supplied by Merck (Darmstadt, Hesse, Germany).

3.2. Bacterial Strain Isolation and Culture Conditions

Pectin-degrading microorganism screening was performed using agar plates containing pectin as the sole carbon source aiming to find a suitable enzyme for highly esterified pectin degradation. The selective medium contained 5 $g.l^{-1}$ citrus pectin with approximately 70% esterification, 1 $g.l^{-1}$ yeast extract, 3 $g.l^{-1}$ KH_2PO_4, 6 $g.l^{-1}$ Na_2HPO_4, 0.1 $g.l^{-1}$ $MgSO_4.7H_2O$, 0.11 $g.l^{-1}$ $CaCl_2$, and 20 $g.l^{-1}$ agar, (pH 7.0). After colonies were appeared, 10 ml of 0.05% (v/v) ruthenium red aqueous solution was added, incubate at room temperature for 1 h and rinsed with deionized water. Colonies with more than 5 mm clear zone selected for further analysis. Erlenmeyer flasks (250 ml) containing 50 ml of pectinase production medium (1 $g.l^{-1}$ citrus pectin, 10 $g.l^{-1}$ yeast extract, 10 $g.l^{-1}$ pepton, 3 $g.l^{-1}$ KH_2PO_4, 0.1 $g.l^{-1}$ $MgSO_4.7H_2O$, 0.11 $g.l^{-1}$ $CaCl_2$, (pH 7.0)) with 1% (v/v) inoculation, at 30°C under shaking condition (180 rpm) were used. Bacterial growth and extracellular pectinase production was monitored up to 6 days (Data not shown).

3.4. Enzyme Purification

At the first step, the cell free supernatant dialyzed against 50 mM phosphate buffer (pH ~ 7.0) for 24 h. The dialyzed supernatant was mixed with DEAE-Cellulose resin for 1 h to remove the soluble pectin. The supernatant was loaded on a DEAE-Cellulose column (20 × 2.5 cm) and equilibrated with the same phosphate buffer at 1 $ml.min^{-1}$ flow rate. The bounded proteins were eluted by applying a 0-0.5 M NaCl gradient and fractions with pectinase activity were concentrated. Concentrated fraction (1 ml) was loaded on a Sephacryl S-200 gel filtration column (70 × 1 cm) equilibrated with 0.1 M potassium phosphate, (pH 7.0) containing 50 mM NaCl at 0.5 $ml.min^{-1}$ flow rate. Active fractions were concentrated and stored at -20°C.

The molecular mass of the purified enzyme was determined using the same column calibrated previously with a range of reference proteins: BSA (67,000 Da), bovine carbonic anhydrase (29,000 Da) and cytochrome C (12,400 Da). Blue Dextran was used to determine the void volume of the column.

3.5. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed on a 10% resolving and 5% stacking gel by the method described by Laemmli (9). A ready to use molecular marker (Fermentase; SM#0661) was used as a standard.

3.6. Enzymatic Assay and Substrate specificity

Pectinase activity and substrate specificity assay were conducted using polygalacturonic acid, sodium polypectate (Esterification Degree 20-35%) and citrus pectin (ED~70%) as different substrates. Suitably diluted enzyme (100 µl) was added to 400 µl 1% (w/v) of substrates and incubated at 60°C for 30 min at pH ~ 6.0. Pectinase activity was monitored by DNS method (10). One unit (1 U) of enzyme activity was defined as the amount of enzyme that release reducing sugar equivalent to 1 µmol galacturonic acid per min under standard assay conditions. Lyase activity was also measured by thiobarbituric acid (TBA) method (11). The kinetic constants (K_m and V_{max}) were measured using pectin as substrate at optimum condition of temperature and pH with applying Hanes-Woolf equation (12). Protein concentration was determined using the Bradford method with bovine serum albumin as standard (13).

3.7. Temperature and pH Effect on PMG Activity and Stability

The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures from 30 to 90°C. The effect of pH on PMG activity was determined by incubating the reaction mixture at pH values ranging from 4.0 to 10.0, in the following buffer systems: 30 mM sodium acetate (pH 3.5-5.5); 30 mM sodium phosphate (pH 6.0-7.5); 30

Table 1. Purification steps of PMG from *Bacillus* sp. strain BR1390

Purification steps	Total protein(mg)	Total activity (U)	Specific activity ($U.mg^{-1}$)	Yield (%)	Fold
Crude extract	37.68	1017	26.99	100	1.0
DEAE-Cellulose (I)	17.23	486	28.20	47.8	1.04
DEAE-Cellulose (II)	3.08	305.8	99.28	30.1	3.68
Sephacryl S-200	1.31	291.6	222.6	28.7	8.25

mM Tris-HCl (pH 8.0-9.0); 30 mM glycine-NaOH (pH 9.5-12.0) in constant temperature of 60°C. To study the irreversible thermal denaturation, diluted enzyme was pre-incubated for 30 and 60 min at 60 to 90°C, cooled on ice for 15 min and assayed under standard reaction conditions. To check the pH stability, the enzyme solution (10 µl) was mixed with 190 µl of each buffer solution and after incubation for 1 h at 25°C, pectinase activity was measured under standard assay conditions.

3.8. Effect of chemicals on pectinase stability

The effect of various metal ions, enzyme inhibitor (EDTA), and surfactants (SDS and Tween 80) on the PMG activity was investigated at various final concentrations (See Table 2). The enzyme was pre-incubated with the selected chemicals for 30 min at 25°C and then assayed in optimum conditions. The residual pectinase activity was measured against control in three independent measurements.

4. Results

4.1. Bacterial Characterization and Culture Condition

More than 67 bacterial isolates were collected from 7 different soil and water samples from fruit gardens and fruit local shops. One isolate from soil covered with fruit waste, produced significant amounts of pectinase on pectin-agar plate. The bacterium was rod-shaped, Gram-positive, mesophilic, exhibiting an optimum growth temperature of 30ºC. The bacterium was able to grow between 10 and 50ºC and in a wide range of pH (5.0-10.0), with optimal pH of 7.0. Bacterial growth and pectinase production were measured during 6 days with maximum PMG 6.21 $U.ml^{-1}$ production of the enzyme (Data not shown).

The 1016-bp partial sequence of 16S ribosomal RNA was deposited in GenBank (accession number JQ417650). The phylogenetic tree constructed by the neighbor-joining method indicated that the isolate BR1390 was part of the cluster of *Bacillus licheniformis* (Data not shown).

4.2. Purification and Properties of the PMG

An extracellular pectinase from the culture media was purified about 8.25-fold, specific activity of 222.6 $U.mg^{-1}$ on pectin and a final yield of 28% by applying a combination of two-step batch and column anion exchange chromatography followed by a Sephacryl S-200 gel filtration step (Table 1). The purified enzyme migrated as a single band on SDS-PAGE and apparent molecular mass of the pectinase was about 56 kDa (Figure 1), whereas it was estimated 104 kDa by Sephacryl S-200 gel filtration chromatography.

Substrate specificity analysis, hydrolytic and lyase activities were monitored by DNS and TBA assay methods using three substrates: polygalacturonic acid, sodium polypectate and pectin. Amongst which, the

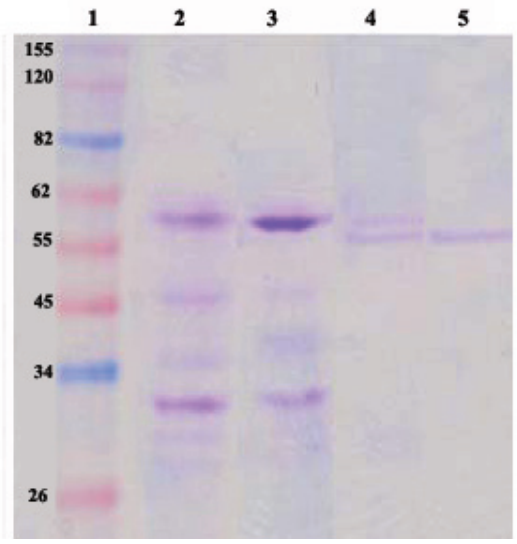

Figure 1. SDS-PAGE pattern of various purification steps of BR1390 extracellular PMG. Lanes: 1, molecular markers; 2, Supernatant of culture medium; 3, batched DEAE-cellulose; 4, columned DEAE-cellulose; 5, the purified PMG (Further details are described in Materials and Methods)

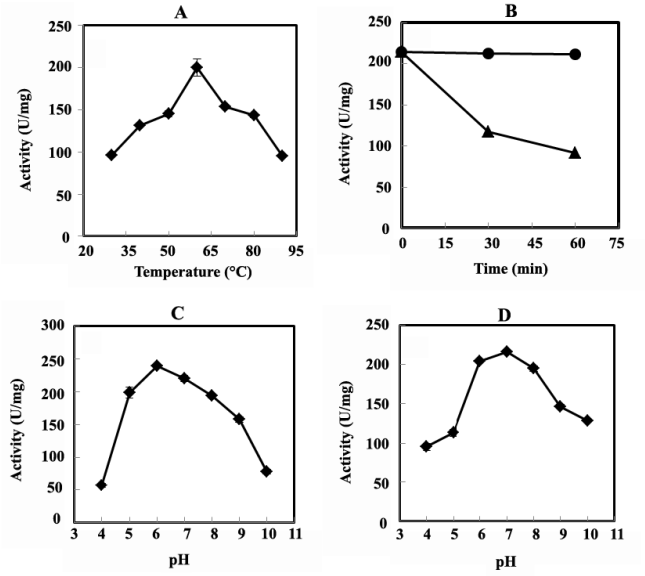

Figure 2. The effect of temperature and pH on activity and stability of the PMG. A: The temperature profile and B: stability of the purified PMG. For determining thermostability, the pectinase was incubated for 30 min and 60 min at 60 ºC (●) and 90ºC (▲) and cooled for 30 min on ice. C: Effect of pH on PMG activity and D: pH stability profile. Data were presented as the mean ± standard deviation (SD) of triplicate experiments

Table 2. Effect of chemicals on polymethyl galacturonase activity

Chemicals	Final Concentrations	Residual Activity (%)[a]
None	-	100 ± 2.0
$CuCl_2$	5 mM	111.70 ± 1.8
$CaCl_2$	5 mM	111.60 ± 2.3
$MgCl_2$	5 mM	108.93 ± 2.8
$MnCl_2$	5 mM	105.16 ± 3.8
$CdCl_2$	5 mM	61.51 ± 1.6
$CoCl_2$	5 mM	107.04 ± 2.5
$ZnCl_2$	5 mM	78.57 ± 1.2
$NiCl_2$	5 mM	103.87 ± 1.7
$FeCl_2$	5 mM	25.20 ± 1.4
$HgCl_2$	5 mM	0
EDTA	1 mM	52.88 ± 2.1
	5 mM	20.93 ± 1.5
SDS	0.1%	98.31 ± 3.9
TWEEN 80	0.1%	107.24 ± 2.7

[a]The PMG was incubated with various metal ions (5 mM) in 0.05 M phosphate buffer (pH 7.0) at room temperature for 30 min. The activities are shown as values relative to that measured without addition of any metal ions (control). Data were presented as the mean ± standard deviation (SD) of triplicate experiments

enzyme was only active on pectin and not other substrates. Accordingly, it can be stated that the enzyme is a PMG. The enzyme showed a Michaelis-Menten kinetic behavior on pectin and the V_{max} and K_m values were calculated from Hanes-Woolf plot to be 0.066 µmol.min^{-1} and 2.51 mg.ml^{-1}, respectively.

Isolated PMG exhibited optimum temperature for maximum pectinase activity at 60ºC, pH 6.0 (Figure 2A) and showed a thermal activation above 30ºC. The enzyme was active at higher temperatures and kept more than 40% of its initial activity at 90ºC. The thermal stability assay was also shown 100% remaining activity at 60°C after 60 min and even almost 50% of its activity at 90°C after 30 min of incubation (Figure 2B). The enzyme exhibited more than 70% of its initial activity in pH range of 5.0-8.0 with an optimum pH of 6.0. The enzyme kept 80% of its initial activity at pH ~ 5.0 (Figure 2C). Moreover, pH stability pattern was very similar to pH activity (Figure 2D).

4.5. Effect of Chemicals on Pectinolytic Activity

Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Co^{2+} were slightly stimulated the enzyme activity, while Hg^{2+} and Fe^{3+} strongly inhibited the PMG activity and Zn^{2+} and Cd^{2+} having minimal inhibitory effects. Although, surfactants like SDS and Tween 80 (0.1%) did not show destabilizing effect on the PMG activity but EDTA, a metal ion chelator, at 5 mM concentration decreased 80% of the PMG activity (Table 2).

5. Discussion

Fruit juice industry requires acidic pectinases for extraction, clarification and improving the cloud stability of juices and nectars. In cloudy juices and nectars like citrus, carrot, and tomato, pectin degradation is considered, without removing the methyl that maintains stability of cloud and fruit odor, reducing the viscosity (3, 7). Although the ideal enzyme should have no methyl esterase (ME) activity on pectin, commercial pectinases are mainly mixes of several such enzymes, including polygalacturonase, pectate lyase along with methyl esterase. Among all pectinases, pectin lyase and polymethyl galacturonase are pectin degrading enzymes that cleave main chain without removing methyl and subsequently preventing toxic methanol formation (7, 14).

Here, a bacterial strain was isolated from soil covered with fruit waste and characterized as *Bacillus* sp. strain BR1390. Exclusive degradation of citrus pectin by strain BR1390 pectinase, with no activity on polygalacturonic acid, sodium polypectate with 35% DE,

and also no lyase activity on pectin indicates that like PMG from *Sclerotinia sclerotiorum*, suggests to be a polymethyl galacturonase (5, 15, 16). Although there are no reports on biochemical properties of bacterial PMGs in literature, our findings are similar to pectin lyase A characteristics which is not able to cleave pectin with lower than 40 % degree of esterification (5, 15). Based on our knowledge, like all bacteria, *Bacillus* strains such as *Bacillus macerans*, *Bacillus* sp. KSM-P7, *B. subtilis* & *Bacillus* sp. TS44 mainly produce polygalacturonase lyase (5, 17, 18), whereas *Bacillus gibsonii*, *B. licheniformis*, and *Bacillus* sp. SC-H express polygalacturonase and in a fewer extent other lyases like pectin lyase of *Bacillus polymixa* & *B. pumilus* and pectate lyase of *B. licheniformis* SVD1 (19-24). In contrast to many pectinolytic enzymes from *Bacillus* species, the PMG from strain BR1390 is secreted largely at the end of the stationary phase of growth, reaching to maximum after 5 days of cultivation (Data not shown). The only similar secretion pattern report was for a pectate lyase from *B. pumilus* BK2 (23). The PMG purification was carried out using a combination of anion exchange and size exclusion chromatography. The molecular mass was 104 kDa with a unique dimeric structure among *Bacillus* sp. pectinases (ranging from 25-115 kDa), but it is smaller than the only purified 140 kDa dimeric PMG from *Sclerotinia sclerotiorum* (16-23).

The biochemical properties of microbial pectinases are important in their industrial applications. Optimum temperature of the reported PMGs is similar in most thermophilic *Bacillus* pectinases. However, in our study the optimum pH was 6.0 that is different from most reported bacterial pectinases which show the alkaline properties. In an overall view, all reported pectinases have extremely acidic or alkaline pH optimum and only a few reports for PGs and PMGs from fungi show moderate acidic to neutral optimum pH behavior. Reported fungal PGs and PMGs have mesophilic characteristics with optimum temperatures around 30-45°C. However, the present introduced PMG has a unique feature among bacterial pectinases with an optimum pH and temperature of 6.0 and 60ºC, respectively. The kinetic parameters of the enzyme were calculated as 0.066 µmol.min^{-1} (V_{max}) and 2.51 mg.ml^{-1} (K_m) using Hanes-Woolf linearized plot. The K_m value of the PMG was lower than any *Bacillus* species pectinases reported so far (17-23). Among the different ions tested, Ca^{2+}, Cu^{2+}, Mn^{2+} and Mg^{2+} stimulated the PMG activity that is in contrast with pectin lyase from *B. pumilus* (21). The current PMG requires Ca^{2+} for its activity that is different from what have been reported for fungal and tomato pectinases. Hg^{2+} and Zn^{2+} ions significantly inhibit the PMG activity, possibly due to their blocking function on thiol-groups. Based on our results, involvement of thiol-group in PMG active site would be a possibility that is in agreement with *B. subtilis* polygalacturonase (25). The enzyme was identified as a metal-dependent pectinase, which was inhibited by 5 mM EDTA. SDS had no effect and Tween 80 stimulated the PMG activity. These data are indicative of high detergent stability similar to *Tetracoccosporium* sp. polygalacturonase (26).

Overall, the present report has introduced the purification of the first bacterial polymethyl galacturonase activity from an environmental isolate, *Bacillus* sp. strain BR1390. High yield of polymethyl galacturonase secretion, acidophilic, thermo and detergent tolerant properties qualified the PMG BR1390 to be used as a cloud stabilizer of citrus juices. On the basis of beneficial properties, further work on production and industrial facility optimization is currently underway.

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Authors' Contribution

B. Rastegari performed the experiments, analyzed data and wrote the manuscript. H.R. Karbalaei-Heidari designed, provided consultation, supervised the study, analyzed data and wrote the manuscript.

Financial Disclosure

There is no financial interest.

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