Purification and Characterization of Three Alkaline Endopolygalacturonases from a Newly Isolated *Bacillus gibsonii*

LI Zu-ming (李祖明)[1,3,4], JIN Bo (金 波)[2], ZHANG Hong-xun (张洪勋)[3],
BAI Zhi-hui (白志辉)[2,3], XUE Wen-tong (薛文通)[1], LI Hong-yu (李鸿玉)[4]

(*1. Teacher's College of Beijing Union University, Beijing 100011, China;*
2. School of Earth and Environmental Science, University of Adelaide, Adelaide, SA 5005, Australia;
3. Research Center for Eco-environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China;
4. College of Food Science & Nutritional Engineering, China Agricultural University, Beijing 100083, China)

Abstract: A newly isolated *Bacillus gibsonii*, designated as S-2 (CGMCC1215), was cultivated for production of alkaline pectinases utilizing sugar beet pulp as growth substrate. Purification of three alkaline endopolygalacturonases (endoPGs) from the crude pectinases extract was carried out by ultra-filtration, ammonium sulphate fractionation and ion-exchange chromatography, and their enzyme activities characterized. The three purified alkaline endoPGs, designated as S-I, S-II, and S-III, had a molecular weight about 38 kDa as determined by SDS−PAGE. The K_m value and optimal temperature for optimal enzyme activities of S-I, S-II and S-III were 1.2 mg/mL and 60 ℃, 0.9 mg/mL and 55 ℃, 1.1 mg/mL and 60 ℃, respectively. Their best performances were given at an optimal pH 10.5, and sodium polygalacturonate was found to be the best substrate. The isoelectric points of S-I, S-II and S-III were 5.4, 7.4, and 8.2, respectively. Surfactants of Tween-80 and Tween-20 and metal ions such as Mg^{2+} and Ca^{2+} stimulated the activity of S-I, S-II and S-III, whereas S-III was inhibited by Ca^{2+}, and Mn^{2+} and Zn^{2+} ions inhibited the activity of the three enzymes.

Key words: alkaline endopolygalacturonases; *Bacillus gibsonii*; purification; enzyme activity
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1 INTRODUCTION

Pectic substances are complex colloidal acid polysaccharides, which have a backbone of galacturonic acid residues linked by α (1~4) linkage. The enzymes hydrolyzing these pectic substances are broadly known as pectinases, including endopolygalacturonase (endoPG), exopolygalacturonase (exoPG), pectin lyase, pectate lyase and pectinesterase, depending on their mode of action[1−3]. Based on pH requirement for optimum enzymatic activity, pectinases can also be classified into acidic and alkaline pectinases. The endoPGs which cause random hydrolysis of 1,4-α-D-galactosiduronic linkages in pectate molecules are the key enzymes to degrade the plant cell wall and release pectic oligomers which can stimulate a wide array of plant defence responses[4−6].

Alkaline pectinases have been used in many industrial and biotechnological processes, such as textile and plant fibre processing, coffee and tea fermentation, oil extraction, treatment of industrial wastewater containing pectinacious material, purification of plant viruses, and paper making[7]. Especially in textile, cotton bioscouring with the alkaline pectinases would not affect the cellulose backbone and thus avoid fiber damage without pollution to environment in contrast to drastic alkaline conditions conventionally used[8]. Now, alkaline pectinases have proved to be the most effective and suitable enzymes for cotton bioscouring[9].

Up to now, some microorganisms have been studied for producing alkaline pectinases. The alkaline pectinases are produced predominantly from the genus *Bacillus* sp. and *Pseudomonas* sp.[7]. Although a few alkaline pectinases from various microbes have also been purified and characterized[10−12], there is still a demand for the alkaline pectinases with high enzymatic activities and stable properties at alkaline conditions for a wide application[8]. To our knowledge, no reports have been published on the purification and characterization of the alkaline pectinases produced from *Bacillus gibsonii*. A newly isolated *Bacillus gibsonii* was confirmed to be a suitable strain for the production of a high yield of alkaline pectinases with the ability to

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Biography: LI Zu-ming (1968−), male, native of Shaoyang City, Hunan Province, Ph.D., docent, major in biotechnology, Tel: 010-64249434, E-mail: zml05666@yahoo.com.cn.

induce cucumber disease resistance was isolated in our laboratory[13].

Although the alkaline pectinases are used in industrial processes in crude form, their purification and knowledge of the biochemical properties of these enzymes are essential for a better understanding of their mechanisms of action. In this work, we employed ultra-filtration, ammonium sulphate fractionation and ion-exchange chromatography to purify and characterize the three alkaline endoPGs from the *Bacillus gibsonii*, designated as S-2.

2 EXPERIMENTAL

2.1 Microorganism and Enzyme Production

Bacillus gibsonii S-2 (CGMCC1215) was isolated from a soil sample in our laboratory for alkaline pectinases production[14]. The strain was maintained in a refrigerator on solid medium slants which consisted of (g/L): peptone 15, yeast extract 3, glucose 2, NaCl 2, K_2HPO_4 1.2, Na_2CO_3 3, agar 15, and subcultured every six months. The medium used for liquid inoculum culture was sterilized at 115℃ for 15 min. The media (60 mL) contained in 250 mL Erlenmeyer flasks were inoculated and cultivated in a shake incubator at 150 rev/min and 35℃ for 24 h.

The medium used for alkaline pectinases production contained 10 g dry sugar beet pulp, which acts as the carbon source as well as the pectinases inducer[13], and 30 mL liquid medium (comprising KH_2PO_4 1.5 g/L, yeast extract 10 g/L, Na_2CO_3 6 g/L). The combined medium was sterilized in a 500 mL Erlenmeyer flask at 115℃ for 15 min. The sterilized medium was inoculated using 3 mL of a 24-h-old inoculum culture and cultivated at 35℃ for 48 h. Enzyme was extracted with 100 mL 0.05 mol/L Na_2CO_3–$NaHCO_3$ buffer (pH 10.5) for 30 min, and centrifuged at 9000 r/min for 20 min to remove the cells and debris, and supernatant was designated as the crude enzyme. The crude enzyme was stored at 4℃ in the presence of sodium azide (0.1 g/L).

2.2 Purification of the Enzyme

2.2.1 Ultra-filtration

The crude enzyme (0.5 L) was concentrated to 180 mL using the hollow fibre cross-flow ultra-filtration module fitted with a polysulfone membrane (10000 M_r cut-off) and trans-membrane pressure of 0.10 kg/cm^2. The concentrate was dialyzed by adding 500 mL 2.5 mmol/L Na_2CO_3–$NaHCO_3$ buffer (pH 10.5) to it, and concentrated to 180 mL by the ultra-filtration again, 8 times repetitions.

2.2.2 Ammonium sulphate fractionation

Powdered ammonium sulfate was gradually added up to 400 g/L saturation with stirring at 4℃ to the concentrated and dialyzed crude pectinase solution which was allowed to stand up to 1 h in a refrigerator. The pectinase solution was centrifuged at 9000 r/min for 20 min to separate the pellets. The supernatant was again saturated with ammonium sulphate up to 600 g/L of saturation and was allowed to stand overnight and then centrifuged at 9000 r/min for 20 min. The active fraction (400~600 g/L) was dissolved in 10 mmol/L Na_2HPO_4–KH_2PO_4 buffer (pH 8.0) and was dialysed against the same buffer.

2.2.3 Fast flow express-ion D (anion exchange) chromatography

The dialysed enzyme solution was loaded on fast flow express-ion D (Whatman) column (1.8 cm×30 cm), which was equilibrated with 10 mmol/L Na_2HPO_4–KH_2PO_4 buffer (pH 8.0). The protein adsorbed on the column was washed with the same buffer, and then eluted with a linear gradient of 0~0.6 mol/L NaCl in the same buffer at a flow rate of 18 mL/h. The eluted active fractions were pooled, concentrated and dialyzed against 10 mmol/L Na_2HPO_4–KH_2PO_4 buffer (pH 7.0). As the major active fraction, the fist retentate was re-chromatographed using the same column that had been equilibrated with 10 mmol/L Na_2HPO_4–KH_2PO_4 buffer (pH 7.0). After washing the column with the equilibration buffer, proteins were eluted with a linear gradient of 0~0.6 mol/L NaCl in the same buffer at a flow rate of 18 mL/h. The fractions containing alkaline PG activity were collected, concentrated and dialyzed against 20 mmol/L Na_2HPO_4–KH_2PO_4 buffer (pH 6.0).

2.2.4 SP sepharose fast flow (cation exchange) chromatography

The dialysed enzyme solution was applied to a column (1.0 cm×20 cm) of SP sepharose fast flow (Pharmacia) that had been equilibrated with 20 mmol/L Na_2HPO_4–KH_2PO_4 buffer (pH 6.0). After washing the column with the equilibration buffer at a flow rate of 12 mL/h, proteins were eluted with a linear gradient of 0~0.6 mol/L NaCl in the same buffer at a flow rate of 18 mL/h. At this purification step, alkaline PG activity was resolved as three peaks designated as S-I, S-II and S-III. The three active fractions were collected and adjusted to pH 10.5 by adding 1 mol/L sodium carbonate separately, then concentrated and dialyzed against 2.5 mmol/L Na_2CO_3–$NaHCO_3$ buffer (pH 10.5) respectively. Homogeneity of the three fractions containing alkaline PG activity was checked by SDS–PAGE as described

below.

2.3 Enzyme Assays

The activity of endopectinases was measured by the AJDA method according to Friedrich et al.[15] and Gu et al.[16]. One unit of endopectinase activity was defined as that amount of enzyme which would degrade 1 mg pectin (from citrus fruits, methoxy content 8%(ω), Sigma) at pH 10.5 and 40℃ in half an hour.

The activity of the polygalacturonase (PG, a major type of pectinases) was assayed by measuring the reducing groups released from sodium polygalacturonate (Sigma) using the 3,5-dinitrosalicylic acid (DNS) method with D-galacturonic acid monohydrate (Sigma) as the standard[13]. One unit of PG activity was defined as that amount of enzyme which releases 1 μmol galacturonic acid per minute at pH 10.5 and 40℃.

The results of the enzyme assay were the means of duplicate determination of two independent samples. The standard derivations were less than 5%.

2.4 Analytical Methods

SDS–PAGE was performed on a 120 g/L gel according to Laemmli[17], and an SDS–PAGE low-molecular-weight standard (Pharmacia) was used as the marker proteins. Protein was determined by the method of Lowry et al.[18] with bovine serum albumin as a standard. The elution was monitored by an online UV detector at 280 nm. The isoelectric points (pI) of the three purified alkaline endoPGs were determined by IEF–PAGE. The K_m values were determined by the double-reciprocal plot method of Lineweaver–Burk. All assays were done at the pH optimum of the enzyme being tested.

3 RESULTS AND DISCUSSION

3.1 Production of Alkaline Endopolygalacturonases Using *Bacillus gibsonii* S-2

The newly isolated *Bacillus gibsonii* S-2 (CGMCC0907) grew well at 35℃ and secreted maximum alkaline PGs yield of 3 600 U/g dry substrate in 48 h was described previously[13]. The crude enzyme was concentrated and dialyzed by ultra-filtration with a recovery rate of 95%.

3.2 Purificaiton of Alkaline Endopolygalacturonases

The 400~600 g/L ammonium sulfate precipitate was collected, dissolved in 10 mmol/L Na_2HPO_4–KH_2PO_4 buffer (pH 8.0), and dialyzed against the same buffer. The PGs was purified 4.5 fold to a yield of 99.2% to the PGs activity applied with the specific activity of 61.8 U/mg (see Table 1).

Table 1 Purification of three alkaline endoPGs from *Bacillus gibsonii* S-2

Purification step	Specific activity (U/mg)	Recovery rate (%)	Fold
Crude enzyme of alkaline PGs after ultra-filtration	13.9	100	1.0
Ammonium sulphate fractionation (400~600 g/L)	61.8	99.2	4.5
Fast flow express-ion D chromatography (pH 8.0)	363.4	58.1	26.2
Fast flow express-ion D chromatography (pH 7.0)	401.3	55.2	29.0
SP sepharose fast flow chromatography (pH 6.0)		50.3	
EndoPG S-I	294.2	3.7	21.2
EndoPG S-II	719.8	30.4	52.0
EndoPG S-III	670.5	16.2	48.4

Following the precipitation with ammonium sulfate, the salt-extracted and dialyzed proteins were subjected to fast flow express-ion D (anion exchange) chromatography (pH 8.0). The elution profile is shown in Fig.1. The first PG activity peak contained a majority of PG activity was collected with a recovery of 58.1% to the total activity applied. The PGS were purified 26.2 fold with the specific activity of 363.4 U/mg (Table 1).

The second fast flow express-ion D chromatography (pH 7.0) of the major PG with gradient elution is showed in Fig.2. One PG activity peak was obtained. The major PG was purified 29.0 fold to a yield of 55.2% from the crude enzymes with the specific activity of 401.3 U/mg (Table 1).

PG activity of the retentate from the second fast flow express-ion D chromatography (pH 7.0) was separated to three peaks by SP sepharose fast flow chromatography (pH 6.0), designated as S-I, S-II and

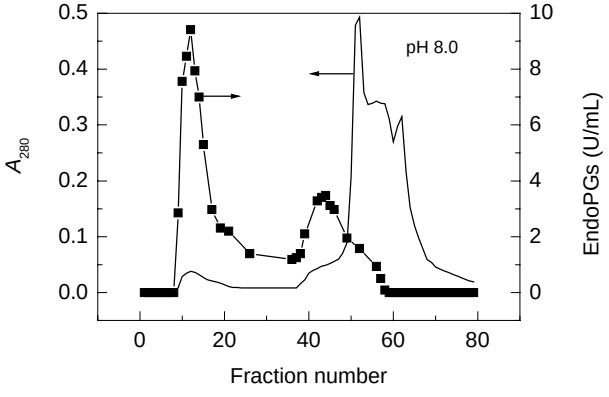

Fig.1 First anion exchange chromatography of alkaline endoPGs on fast flow express-ion D

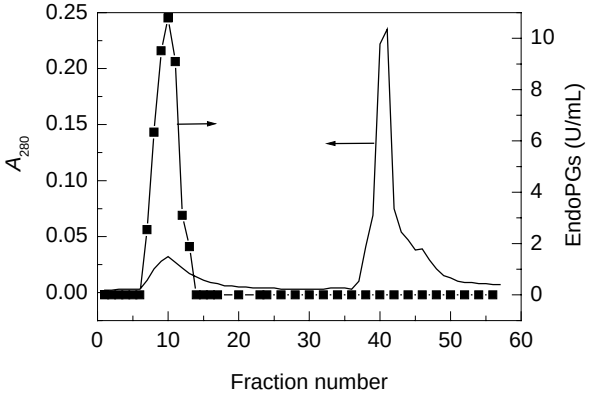

Fig.2 Second anion exchange chromatography of alkaline endoPGs on fast flow express-ion D (pH 7.0)

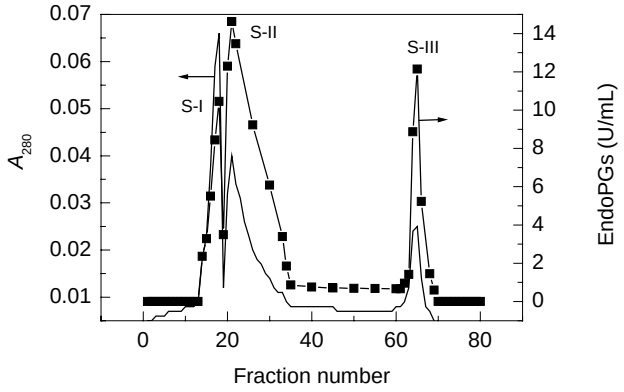

Fig.3 Cation exchange chromatography of alkaline endoPGs on SP sepharose fast flow (pH 6.0)

S-III (Fig.3). Their pH value was separately adjusted to 10.5.

The protein in the S-I, S-II and S-III preparations was electrophoretically homogeneous with a single band respectively on SDS–PAGE with Coomassie brilliant blue staining (Fig.4). The overall purification of the three PGs is summarized in Table 1. PGs of S-I, S-II, and S-III were purified for 21.2, 52.0 and 48.4 fold with the specific activity of 294.2, 719.8 and 670.5 U/mg respectively. Their overall yield was 50.3% from the crude enzyme (Table 1). The three active fractions were determined as alkaline endoPGs, because all of them not only demonstrated alkaline PGs activities, but also had alkaline endopectinases activities.

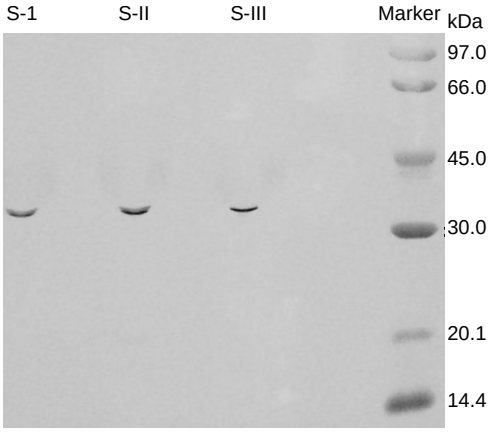

Fig.4 SDS-PAGE of the three purified alkaline endoPGs from *Bacillus gibsonii* S-2

3.3 Properties of the Alkaline EndoPGs

3.3.1 Molecular weight and PI

The homogeneity of the three purified endoPGs (S-I, S-II and S-III) was judged by SDS–PAGE. It was found that the three endoPGs had a molecular weight about 38 kDa (Fig.4), which is similar to the value reported for both pectin lyases of *Aspergillus flavus* MTCC 7589 (38 kDa)[3] and pectate lyase of *Bacillus pumilus* BK2 (37.3 kDa)[8], but quite different from that of exopolygalacturonase from *Bacillus* sp. KSM-P576 (115 kDa)[10].

The isoelectric points (PI) of S-I and S-II were 5.4 and 7.4, respectively, and S-III showed a majority band, PI 8.2, with a tiny band of PI 8.4, as estimated by IEF–PAGE. On the contrast, the PI of exopolygalacturonase from *Bacillus* sp. KSM-P576 is 4.6[10], and the PI of pectate lyase from *Bacillus* sp. KSM-P7[12] was around pH 10.5. The PI of both pectin lyase from *Aspergillus flavus* MTCC 7589[3] and pectate lyase from *Bacillus pumilus* BK2[8] was not tested.

3.3.2 Effects of pH and temperature

The effect of pH on the three endoPGs was examined at 40℃ in 50 mmol/L Na_2CO_3–$NaHCO_3$ buffer. As shown in Fig.5, the three endoPGs (S-I, S-II and S-III) were alkaline endoPGs and performed the best enzymatic activities at an optimal pH of 10.5. This finding is similar to what is reported for *Bacillus* sp. KSM-P7 (pH 10.5)[12] and *Bacillus* sp. P-4-N (pH 11.5)[11], but quite different from what is reported for *Aspergillus flavus* MTCC 7589 (pH 8.0)[3], *Bacillus pumilus* BK2 (pH 8.5) [8], and *Bacillus* sp. KSM-P576 (pH 8.0)[10].

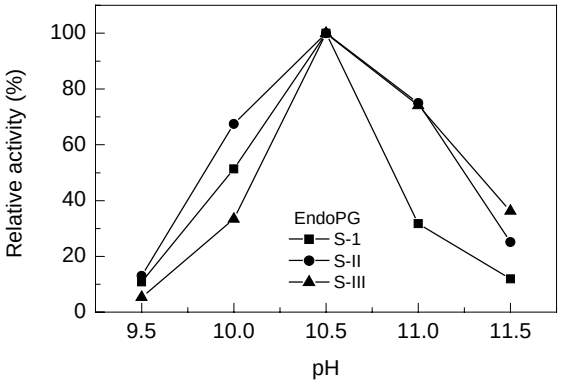

Fig.5 Effect of pH on the activity of purified alkaline endoPGs

The effect of temperature on the three endoPGs was examined at pH 10.5 in 50 mmol/L Na_2CO_3–$NaHCO_3$ buffer. The optimal temperature for activity of S-I, S-II and S-III was 60, 55, and 60℃ respectively (Fig.6), which is similar to what is reported for *Aspergillus flavus* MTCC 7589 (50℃)[3], *Bacillus* sp. KSM-P576 (55 ℃)[10], and *Bacillus* sp. KSM-P7 (60~65 ℃)[12], but quite different from what is reported for *Bacillus* sp. P-4-N (70℃)[10], and *Bacillus pumilus* BK2 (70℃)[8].

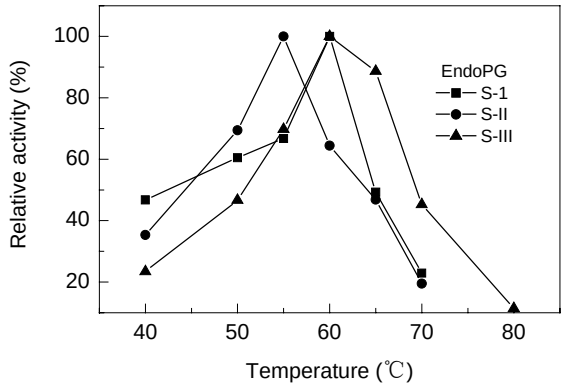

Fig.6 Effect of temperature on the activity of purified alkaline endoPGs

3.3.3 Effects of metal ions and surfactants

The three endoPGs were incubated with 0.1 mmol/L of various metal ions in 50 mmol/L Na_2CO_3–$NaHCO_3$ buffer (pH 10.5) at 40 ℃ for 15 min. Analytical results revealed that Mg^{2+} (0.1 mmol/L $MgSO_4$) ion stimulated the PG activity, while Ca^{2+} (0.1 mmol/L $CaCl_2$) ion also stimulated the PG activity of S-I and S-II, but inhibited the activity of S-III. On the other hand, Mn^{2+} (0.1 mmol/L $MnSO_4$) and Zn^{2+} (0.1 mmol/L $ZnSO_4$) ions inhibited the PG activity of all the three endoPGs (Table 2). This discrepancy in the divalent metal ion preference suggested that these enzymes might have differential flexibility in the active site. Metal ions such as Mg^{2+} and Ca^{2+} might play a vital role in maintaining the active confirmations of the alkaline endoPGs to stimulate the activity[19,20].

It is interesting to observe that different results were reported in the literature regarding the effects of metal ions on the activity of alkaline pectinase. Zn^{2+}, Mg^{2+} and Ca^{2+} partially inhibited the activity of pectin lyase from *Aspergillus flavus* MTCC 7589 using 1.0 mmol/L concentrations respectively, while Mn^{2+} ion had no effect on the activity of the pectin lyase at the same concentration[3]. Metal ions such as Mg^{2+}, Mn^{2+} and Ca^{2+} stimulated the activity of exopolygalacturonase from *Bacillus* sp. KSM-P576 at 0.4 mmol/L, while Zn^{2+} ion inhibited the enzyme activity at the same concentration[4].

Table 2 Effects of metal ions and surfactants on the activity of three alkaline endoPGs from *Bacillus gibsonii* S-2

Metal ion or surfactant	Relative activity of alkaline polygalacturonases (%)		
	S-I	S-II	S-III
No addition	100	100	100
Mg^{2+}	107	122	113
Ca^{2+}	111	110	66
Mn^{2+}	58	60	57
Zn^{2+}	61	42	26
Tween-80	129	118	108
Tween-20	114	117	101

The effects of surfactants (1 g/L Tween-80, and 1 g/L Tween-20) on the three endoPGs were examined at 40 ℃ for 15 min in 50 mmol/L Na_2CO_3–$NaHCO_3$ buffer (pH 10.5). It was interesting to note that both of them stimulated the PG activity of the three endoPGs (Table 2), which is similar to what is reported for *Bacillus* sp. MG-cp-2[21]. The reason is probably that the surfactants might improve the turnover number of PGs by increasing the contact frequency between the active site of the enzyme and the substrate by lowering the surface tension of the aqueous medium[21].

3.3.4 Substrate specificity and kinetic analysis

All of the three purified alkaline endoPGs could depolymerize pectins. The hydrolysis rate was given in an order: sodium polygalacturonate > pectin > esterified pectin, respectively. When the degradation rate of sodium polygalacturonate at pH 10.5 and 40℃ was taken as 100%, the relative rates of S-I toward pectin was 74.0%, S-II 87.3%, and S-III 89.0%. The relative rates of S-I, S-II and S-III toward pectin with the degree of methylation of 8% was 46.9%, 52.5%, and 62.3%, respectively.

The kinetic parameters of the purified alkaline endoPGs for hydrolysis toward sodium polygalacturonate at pH 10.5 and 40℃ were obtained by the double-reciprocal plot method of Lineweaver–Burk. The apparent K_m value of S-I, S-II and S-III was 1.2, 0.9 and 1.1 mg/mL, respectively. The K_m value of S-II is similar to the K_m (0.86 mg/mL) of an exopolygalacturonase from *Bacillus* sp. KSM-P576, but the maximum activity of the exopolygalacturonase is at pH 8.0 and 55℃[10]. It was reported that a K_m value of 0.59 mg/mL was measured for *Aspergillus flavus* MTCC 7589[3] and a K_m value 0.24 mg/mL was tested for *Bacillus pumilus* BK2[8], which were quite different from that of S-I, S-II or S-III reported in this study.

4 CONCLUSIONS

Alkaline pectinases have been used in many industrial and biotechnological processes. A newly isolated *Bacillus gibsonii* S-2 was capable of utilizing sugar beet pulp as growth substrate and secreted the maximum alkaline PGs yield of 3600 U/g substrate in 48 h at 35℃. In this experimental work, the purification and properties of three alkaline endoPGs from *Bacillus gibsonii* S-2 were studied. Some conclusions can be drawn as follows:

(1) Three alkaline endopolygalacturonases (endoPGs) from the crude pectinases extract of *Bacillus gibsonii* S-2 were purified to homogeneity by ultra-filtration, ammonium sulphate fractionation, and ion-exchange chromatography with the overall yield of 50.3%. The three purified alkaline endoPGs, designated as S-I, S-II and S-III, had a molecular weight about 38 kDa as determined by SDS–PAGE.

(2) The K_m value and optimal temperature for activity of S-I, S-II and S-III were 1.2 mg/mL and 60℃, 0.9 mg/mL and 55℃, 1.1 mg/mL and 60℃, respectively. They demonstrated the best enzymatic activity at an optimal pH value of 10.5. Sodium polygalacturonate was found to be the best substrate. The isoelectric points of S-I, S-II and S-III were 5.4, 7.4, and 8.2, respectively. Both surfactants, such as Tween-80 and Tween-20, and metal ions, such as Mg^{2+} and Ca^{2+}, stimulated the activity of S-I, S-II and S-III except that S-III was inhibited by Ca^{2+}, whereas Mn^{2+} and Zn^{2+} ions inhibited the activity of the three enzymes.

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