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An alkaline-active and alkali-stable pectate lyase from *Streptomyces* sp. S27 with potential in textile industry

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Abstract A pectate lyase gene (*pl-str*) was cloned from *Streptomyces* sp. S27 and expressed in *Escherichia coli* Rosetta. The full-length *pl-str* consists of 972 bp and encodes for a protein of 323 amino acids without signal peptide that belongs to family PF00544. The recombinant enzyme (r-PL-STR) was purified to electrophoretic homogeneity using Ni^{2+}–NTA chromatography and showed apparent molecular mass of ~35 kDa. The pH optimum of r-PL-STR was found to be 10.0, and it exhibited >70% of the maximal activity at pH 12.0. After incubation at 37°C for 1 h without substrate, the enzyme retained more than 55% activity at pH 7.0–12.0. Compared with the commercial complex enzyme Scourzyme®301L from Novozymes, purified r-PL-STR showed similar efficacy in reducing the intrinsic viscosity of polygalacturonic acid (49.0 vs. 49.7%). When combined with cellulase and α-amylase, r-PL-STR had comparable performance in bioscouring of jute fabric (22.39 vs. 22.99%). Thus, r-PL-STR might represent a good candidate for use in alkaline industries such as textile.

Keywords Alkaline pectate lyase · *Streptomyces* sp. S27 · *Escherichia coli* Rosetta (DE3) · Bioscouring

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Introduction

Pectins are polysaccharides that are present in all plant primary cell walls, being involved in plant growth and development [19]. Pectinases that have capacity to degrade pectins are ubiquitous in the plant and microorganism kingdoms. Pectate lyase is one type of pectinase that degrades the pectin component of plant cell wall and even leads to plant soft rot [1, 28]. The activity of pectate lyase was first discovered by Starr and Moran [23] in cultures of *Erwinia carotovora* and *Bacillus polymyxa*. Further studies indicated that pectate lyases are widely distributed in plants [15], Gram-positive bacteria *Bacillus* sp. [30] and *Cellvibrio* sp. [27], Gram-negative bacteria *Erwinia* sp. [20] and *Pseudomonas* sp. [13], and fungi *Penicillium* sp. [26] and *Mrakia* sp. [14].

Pectate lyase breaks the glycosidic bond via β-elimination and generates 4,5-unsaturated oligogalacturonates [3, 29]. It is the most crucial enzyme in depolymerization of pectic substances and shows a requirement for calcium ions. Pectate lyases are also called *trans*-eliminases and have alkaline pH optima, generally from 8.0 to 10.0 [3]. Recently, alkaline pectate lyases have received more attention due to their extensive applications in many areas, such as paper-making, coffee and tea fermentation, textile and plant fiber processing, oil extraction, and treatment of industrial wastewater [5, 7, 18, 24]; For example, endo-Pel of *Bacillus pumilus* BK2 has a high isoelectric point (p*I*) and an alkaline pH optimum, which are highly valued properties for bioscouring of cotton [8]. Not all alkaline pectate lyases are from bacteria. A thermoactive pectate lyase from *Penicillium occitanis* exhibits maximum activity at pH 9.0 [26].

Enzymatic scouring of textiles is more advantageous than with chemical reagents and is replacing the

conventional and environmentally harmful chemical process [22]. Thus, more economical and environmentally responsible pectin-degrading enzymes are needed to improve textile pretreatment. Herein we report gene cloning, expression, and characterization of an alkaline pectate lyase from *Streptomyces* sp. S27. Its ability to reduce the intrinsic viscosity of pectate and to bioscour jute fabric was also assessed.

Materials and methods

Bacterial strains, plasmids, and chemicals

Streptomyces sp. S27 (ACCC41168) from soil of Flaming Mountain in the Turpan Basin of Xinjiang, China has been reported to be an excellent enzyme producer [12, 21]. *Escherichia coli* JM109 (TaKaRa, Japan) and the pGEM-T Easy vector (Promega, USA) were used for gene cloning and sequencing, respectively. *E. coli* Rosetta (DE3) (TaKaRa) and vector pET-22b(+) (Merck KGaA, Germany) were used for gene expression. Polygalacturonic acid and citrus pectin P9311 [degree of esterification (DE): 34%], P9436 (DE: 70%), and P9561 (DE: 85%) were purchased from Sigma (USA). The DNA Purification Kit, Genome Walking Kit, LA *Taq* DNA polymerase, and T4 DNA ligase and restriction endonucleases were supplied by TaKaRa. DNA polymerase FastPfu mix from Transgen (China) and Protein Assay Kit from Bio-Rad (USA) were obtained. All other chemicals were of analytical grade and commercially available. The commercial complex enzyme Scourzyme®301L (Novozymes, Denmark) is composed of pectate lyase, cellulase, and α-amylase.

Gene cloning

A degenerate primer set (PF00544F and PF00544R; Table 1) was designed based on the conserved motifs GTHV(I)WI(V)DH and HV(A)Y(V)NNYYE of family PF00544 from the Pfam website (http://pfam.janelia.org/search/sequence) and used to clone the core region of pectate lyase genes. Genomic DNA of *Streptomyces* sp. S27 was obtained and used as the template. Polymerase chain reaction (PCR) was performed using LA *Taq* DNA polymerase with high-GC buffer. The PCR reaction was performed as follows: 5 min at 95°C, 10 cycles of 95°C for 30 s, 51 to 46°C (decreasing 0.5°C after each cycle) for 30 s, and 72°C for 1 min, followed by 30 cycles of 95°C for 30 s, 46°C for 30 s, and 72°C for 1 min, and final extension at 72°C for 10 min. The PCR product was purified and ligated with pGEM-T Easy vector for sequencing. The flanking region of the gene fragment was amplified from *Streptomyces* sp. S27 genomic DNA by thermal asymmetric interlaced (TAIL)-PCR [6] with the following nested insertion-specific primers: D1, D2, D3 and U1, U2, U3 (Table 1). The PCR products with appropriate sizes were gel purified, ligated into the pGEM-T Easy vector, sequenced, and then assembled with the known fragment to give the whole pectate lyase gene *pl-str*.

Analysis of nucleotide and protein sequences

The nucleotide and amino acid sequences of *pl-str* were assessed by using the BLASTx and BLASTp programs (http://www.ncbi.nlm.nih.gov/BLAST/), respectively. The promoter was predicted using the promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html). The signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Vector NTI 10.0 software was used to assemble and analyze the nucleotide sequence, evaluate the identity and similarity of amino acid sequences with other known proteins, and predict the molecular mass and p*I* value of the mature protein.

Enzyme expression and purification

The gene fragment encoding the mature protein was amplified by PCR using primers pl-str-petF and pl-str-petR

Table 1 Primers used in this study

Primer name	Sequence (5′ → 3′)[a]	Size (bp)
PF00544F	GGTACGCACRTNTGGRTNGAYCA	23
PF00544R	CTCGTAGTARTTRTTRTAIRCRTG	24
D1	GACATCTCGAACGCCAACGACGGCGCC	27
D2	GCCAACGACGGCGCCCTCGACATCAAG	27
D3	GTTCGACGGGACCAACCAGCGCCACC	26
U1	TTGCCGAAGCGGACCCGCGGGTG	23
U2	GTGGCGCTGGTTGGTCCCGTCGAACCA	27
U3	CGCGCTTGATGTCGAGGGCGCCGTC	25
pl-str-petF	GAACGCCATGGATATGCGCAGGACGAGCGCAC	32
pl-str-petR	GATACGAATGCGGCCGCGATCTTCCCGACGCCCGC	35

[a] Y = C/T, K = T/G, R = A/G, N = A/T/G/C; restriction sites are underlined

(Table 1). The PCR product was purified from an 1.2% agarose gel, digested with *Nco*I and *Not*I, and cloned into pET-22b(+). The recombinant plasmid, pET-*pl-str*, was transformed into *E. coli* Rosetta (DE3) competent cells and confirmed by sequencing. The positive transformant harboring pET-*pl-str* was grown in LB medium containing 100 μg ml^{-1} ampicillin at 37°C to A_{600} of ~0.6. Protein expression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) at final concentration of 0.5 mM at 20°C for additional 48 h.

To purify the His-tagged recombinant protein, cells were harvested (4,000×*g*, 4°C, 10 min), sonicated on ice, and centrifuged at 12,000×*g* and 4°C for 10 min to remove cell debris. The concentrated cell lysate (crude enzyme) was loaded onto a Ni^{2+}–NTA agarose gel column that had been previously equilibrated with buffer A (20 mM Tris–HCl, 500 mM NaCl, 10% glycerol, pH 7.6). The protein was eluted with a linear gradient of imidazole (20–200 mM). Fractions with enzymatic activity were pooled and dialyzed against 100 mM Tris–HCl (pH 9.0) with 10 mM ethylenediamine tetraacetic acid (EDTA) followed by dialysis in 100 mM Tris–HCl (pH 9.0) to remove EDTA. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of the purified r-PL-STR was performed as described by Laemmli [11]. The protein concentration was determined using a protein assay kit (Bio-Rad). Liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS) was performed at the Institute of Zoology, Chinese Academy of Sciences.

Enzyme assay

Pectate lyase activity was determined by measuring the absorbance change at 235 nm after incubation with 0.2% (w/v) polygalacturonic acid in 100 mM glycine–NaOH (pH 10.0) containing 50 μM $CaCl_2$ at 60°C for 30 min. One unit (U) of pectate lyase activity was defined as the amount of enzyme which produces 1 μmol of unsaturated galacturonide per minute using a molecular extinction coefficient of 4,600 M^{-1} cm^{-1} at 235 nm [4].

Enzymatic characterization

Soluble polygalacturonic acid was used as the substrate for enzymatic characterization. The optimal pH for pectate lyase activity was determined at 60°C in buffers of pH 8.0–12.0 for 30 min. The buffers used were 100 mM Tris–HCl for pH 7.0–8.5 and 100 mM glycine–NaOH for pH 9.0–12.0. The enzyme stability was assessed by measuring the residual activity under standard conditions (pH 10.0, 60°C, 30 min) after incubating the enzyme in buffers ranging from pH 7.0 to 12.0 at 37°C for 1 h without substrate. To determine the optimal temperature for r-PL-STR activity, its pectate lyase activity was measured at temperatures ranging from 0 to 80°C and at pH 10.0 for 30 min. The thermostability of r-PL-STR was investigated by measuring the residual activity under standard conditions as described above after pre-incubation of the enzyme at temperatures of 50 and 60°C for various times.

The effect of different metal ions and chemical reagents on the activity of purified r-PL-STR was determined in the presence of 1 or 5 mM of different metal salts (NaCl, KCl, LiCl, $CoCl_2$, $CrCl_3$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, $Pb(CH_3COO)_2$, SDS, EDTA, and β-mercaptoethanol as described by Yuan et al. [29]. The Ca^{2+} requirement for r-PL-STR activity was determined in the presence of 0–1 mM $CaCl_2$.

The K_m and V_{max} values of r-PL-STR were determined in 100 mM glycine–NaOH (pH 10.0) containing 1–10 mg ml^{-1} polygalacturonic acid at 60°C, respectively. A Lineweaver–Burk plot was constructed using the nonlinear regression computer program GraphPad Prism 5. Three independent experiments were averaged, with each experiment including three replicates.

Analysis of substrate specificity and hydrolysis products

The substrate specificity of purified r-PL-STR was assayed by incubating the enzyme solution with 0.2% (w/v) substrate (polygalacturonic acid and citrus pectin P9311, P9436, and P9561) under standard conditions (pH 10.0, 60°C, 30 min).

The reaction mixture containing 2.0 U purified r-PL-STR and 40 mg polygalacturonic acid in 20 ml of 100 mM glycine–NaOH (pH 10.0) was incubated at 50°C for 12 h. Extra enzyme was removed from the reaction system using the Nanosep Centrifugal Device (Pall, USA). Analysis of hydrolysis products was carried out using time-of-flight mass spectrometry (TOF-MS) in Chinese Academy of Medical Sciences.

Effect of r-PL-STR on viscosity reduction and bioscouring of jute fabric

Mixtures of polygalacturonic acid (0.2%, w/v) and purified r-PL-STR (1.0 U) or Scourzyme®301L (1.0 U) in 10 ml of 100 mM glycine–NaOH (pH 10.0) were incubated at 50°C for 1 h. The reduction in viscosity was assessed by using a glass capillary viscometer (Julabo Tech., China). The viscosity reduction was calculated as described before [29].

r-PL-STR alone (0.2 U ml^{-1}) or its combination with commercial α-amylase DFM-ZW (Denykem, England; 2.0 U ml^{-1}) and cellulase Cellusoft L (Novozymes; 0.2 U ml^{-1}) or Scourzyme®301L (0.2 U ml^{-1}) were used for

bioscouring of jute fabric at pH 9.0 and 40°C for about 24 h, respectively. Then, the samples were air dried at 105°C for 16 h and climatized at 75% relative humidity for 24 h. The hygroscopicity of bioscoured jute was estimated [29].

Nucleotide sequence accession number

The nucleotide sequence of *Streptomyces* sp. S27 pectate lyse gene (*pl-str*) was deposited in the GenBank database under accession number JN969071.

Results

Gene cloning and sequence analysis

A 276-bp DNA fragment was amplified from *Streptomyces* sp. S27 by PCR using degenerate primers PF00544F and PF00544R. The fragment exhibited 80% identity with the pectate lyase from *Thermomonospora curvata* DSM 43183 (ACY97999). The 5′ and 3′ flanking regions were obtained by TAIL–PCR and assembled with the core sequence. The full-length gene was 972 bp in length with GC% content of 66.7% and encoded a polypeptide of 323 amino acids (see Supplementary Fig. S1). The deduced amino acid sequence of *pl-str* exhibited 72% identity with the hypothetical pectate lyase from *Actinosynnema mirum* DSM 43827 (ACU35796; identified based on whole genome sequence) and 70% with the characterized pectate lyase from *Pseudomonas* sp., both of which belong to family PF00544. SignalP analysis indicated the absence of signal peptide in deduced PL-STR. The molecular mass and p*I* value were estimated to be 33.5 kDa and 6.83, respectively.

Expression and purification of the recombinant pectate lyase (r-PL-STR)

The gene coding for the mature protein was expressed in *E. coli* Rosetta (DE3). After induction with 0.5 mM IPTG at 20°C for 48 h, significant pectate lyase activity was detected in the cell lysate. No pectate lyase activity was detected in the cultures of uninduced transformant or of induced transformant harboring the empty plasmid pET-22b(+).

r-PL-STR in the culture supernatant was purified to electrophoretic homogeneity by a single step of Ni^{2+}-affinity chromatography. The purified r-PL-STR migrated as a single band of ~35 kDa on SDS-PAGE, which was identical to the calculated value (Fig. 1). The specific activity of purified r-PL-STR towards polygalacturonic acid was 23.0 U mg^{-1}. To identify the purified protein, the band was cut off from the SDS-PAGE gel and digested with trypsin, and the resulting peptides were analyzed by LC-ESI-MS. Three peptides (ITGSGLNVSNVSNVIIR, ASDLI TVSWNR, and VTYDHNWFDGTNQR) were obtained and completely corresponded to the deduced amino acid sequence of PL-STR, confirming the purity of the band and the identity of r-PL-STR.

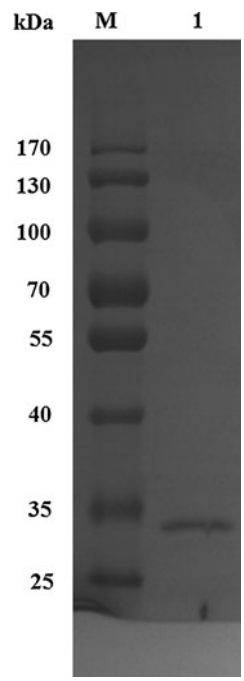

Fig. 1 SDS-PAGE analysis of purified r-PL-STR. The gel was stained with Coomassie Brilliant Blue R-250. *Lanes*: *M* protein molecular weight markers, *1* purified r-PL-STR after Ni^{2+}–affinity chromatography

Enzymatic properties of purified r-PL-STR

Purified r-PL-STR exhibited optimal activity at pH 10.0 (60°C) and retained 70% of maximum activity at pH 9.0–12.0 (Fig. 2a). Following incubation at pH 7.0–10.0 at 37°C for 1 h, the enzyme retained more than 80% activity, and even 58% activity at pH 12.0 (Fig. 2b). The optimal temperature of r-PL-STR was 60°C at pH 10.0 (Fig. 2c). The enzyme was stable at 50°C, and lost 70% of the initial activity after incubation at 60°C for 45 min (Fig. 2d).

The effect of metal ions and other chemical reagents on r-PL-STR activity was determined (Table 2). Pb^{2+}, Cr^{3+}, and EDTA at both concentrations (1 and 5 mM) were strong inhibitors of enzymatic activity. Other chemicals enhanced or had no significant effect on the enzymatic activity at 1 mM. On increasing the concentration to 5 mM, Li^{+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{3+} had negative effects on the enzymatic activity.

The same as other reported pectate lyases, r-PL-STR was also activated in the presence of Ca^{2+}. The optimal Ca^{2+} concentration was determined to be about 0.05 mM (Fig. 3).

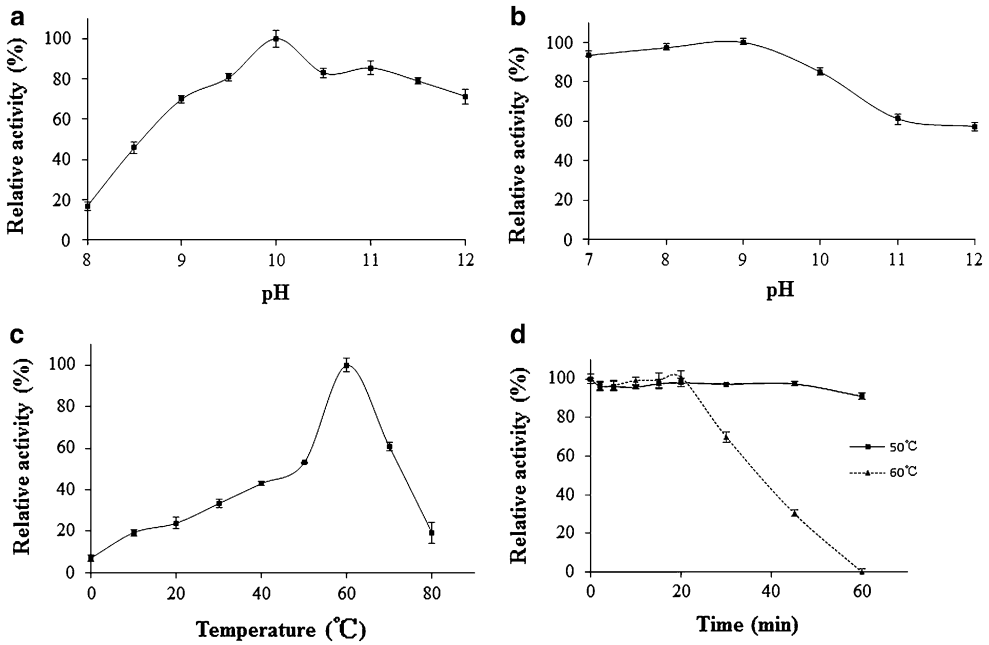

Fig. 2 Characterization of purified r-PL-STR. **a** Effect of pH on r-PL-STR activity. The r-PL-STR activity was assayed at 60°C in buffers from pH 8.0 to 12.0. **b** pH stability of r-PL-STR activity. Following incubation at 37°C for 1 h in buffers ranging from pH 7.0 to 12.0, activity was determined in 100 mM glycine–NaOH (pH 10.0) at 60°C for 30 min. **c** Effect of temperature on r-PL-STR activity measured in 100 mM glycine–NaOH (pH 10.0). **d** Thermostability of r-PL-STR. Thermostability of r-PL-STR was determined by measuring residual activity after pre-incubation at 50°C (*filled squares*) and 60°C (*filled triangles*) for various periods. Each value in the panel represents the mean ± standard deviation (SD) ($n = 3$)

Table 2 Effect of metal ions and chemical reagents on the activity of purified r-PL-STR

Chemical	Relative activity (%)[a]	
	1 mM	5 mM
None	100.0	100.0
Mn^{2+}	129.4 ± 1.9	107.5 ± 2.8
Zn^{2+}	113.0 ± 2.1	54.0 ± 1.9
Cu^{2+}	110.9 ± 4.7	58.7 ± 0.9
Na^{+}	109.8 ± 2.4	103.1 ± 4.5
K^{+}	109.2 ± 0.6	96.4 ± 0.2
Mg^{2+}	108.7 ± 1.7	78.1 ± 1.7
Co^{2+}	107.9 ± 1.5	21.5 ± 4.6
Li^{+}	104.6 ± 1.3	38.3 ± 3.4
Ni^{2+}	95.7 ± 2.4	6.7 ± 1.7
Fe^{3+}	96.1 ± 1.0	0.6 ± 0.6
Pb^{2+}	69.4 ± 0.5	0
Cr^{3+}	0	0
β-Mercaptoethanol	129.5 ± 1.7	132.1 ± 2.9
SDS	105.8 ± 2.6	111.2 ± 0.7
EDTA	0	0

[a] Values represent means ± SD ($n = 3$) relative to untreated control samples

The K_m and V_{max} values of purified r-PL-STR for polygalacturonic acid were 7.9 mg ml^{-1} and 17.1 μmol min^{-1} mg^{-1}, respectively (Fig. 4).

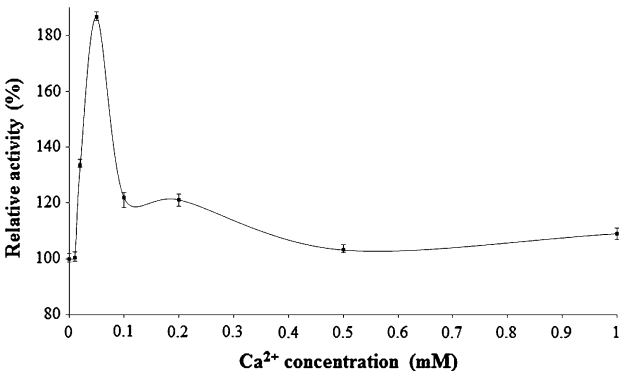

Fig. 3 Effect of Ca^{2+} on the activity of r-PL-STR

Substrate specificity and hydrolysis product analysis

The activity towards polygalacturonic acid (23.0 U mg^{-1}) was regarded as 100%. r-PL-STR exhibited 43.9, 22.9, and 19.3% activity on citrus pectin P9311 (DE 34%), P9436 (DE 70%), and P9561 (DE 85%), respectively.

The hydrolysis products of polygalacturonic acid by purified r-PL-STR were determined by TOF-MS. The major component was galacturonic acid with different kinds of side-chain carbohydrate (Fig. 5). The result indicated that r-PL-STR was a strict *exo*-type pectate lyase.

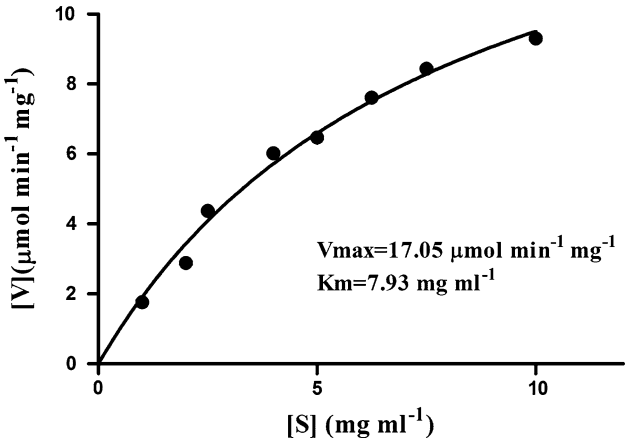

Fig. 4 Kinetic parameters of purified r-PL-STR towards polygalacturonic acid. Assay was performed in 100 mM glycine–NaOH (pH 10.0) at 50°C. Kinetic data were fitted using GraphPad Prism 5 software

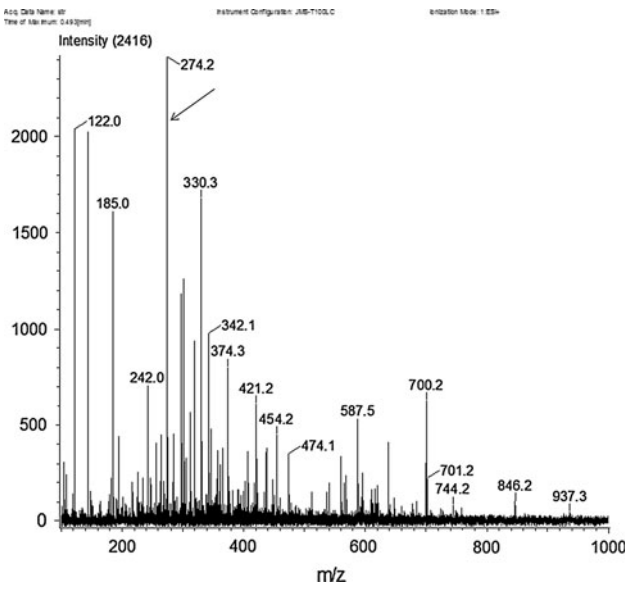

Fig. 5 The hydrolysis products of polygalacturonic acid by purified r-PL-STR analyzed using TOF-MS. The *arrow* indicates the main product information

Effect of r-PL-STR on viscosity reduction and hygroscopicity of jute fabric

Purified r-PL-STR (1.0 U) and commercial complex enzyme Scourzyme®301L (1.0 U) reduced the viscosity of polygalacturonic acid by 49.0 and 49.7%, respectively. In the bioscouring assay, r-PL-STR alone, r-PL-STR in combination with commercial α-amylase and cellulase, and Scourzyme®301L increased the moisture regain percentages of jute fabric by 19.63, 22.39, and 22.99%, respectively.

Discussion

In this study we identified a low-molecular-weight, high-alkaline pectate lyase from *Streptomyces* sp. S27 that belongs to family PF00544. Pectate lyases have been reported in *Streptomyces* sp. [10, 25], but their pH optima are generally within pH 8.0–9.0. Some *Bacillus* pectate lyases have been reported to be optimal at pH 10.5–11.5 [9, 16]. In comparison with the studied r-PL-STR, which was highly active over a wide pH range from 9.0 to 12.0 (>65% relative activity) and showed stability at pH 7.0–12.0, the pH profiles of those *Bacillus* pectate lyases are much narrower. Such alkaline-active and alkali-stable properties make r-PL-STR a good candidate for use in alkaline industries.

Almost all pectate lyases require Ca^{2+} for in vitro activity. The optimal Ca^{2+} concentration has been reported from 100 μM to 1.0 mM. In this study, the enzyme activity was elevated about 1.8-fold in the presence of 50 μM Ca^{2+}, much lower than the reported concentration. The reason might be that calcium ions bind the substrate and the enzyme and further increase the enzyme stability [17]. Some metal ions, such as Mn^{2+}, Zn^{2+}, and Cu^{2+}, enhanced the enzymatic activity, probably by forming the metal-binding pocket.

It is obvious that polygalacturonic acid rather than pectin of various methyl ester groups is the best substrate for r-PL-STR. Most pectate lyases efficiently degrade pectin with low or moderate degree of esterification, but are inactive on highly esterified pectin [2]. This character is suitable for pectate lyases that have potential for application in processing cotton fabric, because under alkaline conditions and elevated temperatures (the optimal conditions for scouring), methyl esters in pectin are rapidly hydrolyzed in the form of pectate, the favored substrate for pectate lyase. Therefore, pectate lyases that have alkaline pH optima such as r-PL-STR are more compatible with typical textile processing [22].

In the application experiment, purified r-PL-STR alone reduced the viscosity of polygalacturonic acid and increased the moisture regain of jute fabric. Compared with the commercial complex enzyme Scourzyme®301L that has activities of pectate lyase, α-amylase, and cellulase, combination with r-PL-STR showed comparable performance in bioscouring of jute fabric. Considering the fact that the three constituent enzymes in Scourzyme®301L are combined through systemic screening and r-PL-STR is randomly combined with commercial enzymes, r-PL-STR would be more efficient in many applications requiring alkaline milieu.

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