Biodegradation (2011) 22:153–161
DOI 10.1007/s10532-010-9384-6

ORIGINAL PAPER

Arg^{235} is an essential catalytic residue of *Bacillus pumilus* DKS1 pectate lyase to degum ramie fibre

Snehasish Basu · Arunava Roy · Abhrajyoti Ghosh · Amit Bera · Dhrubajyoti Chattopadhyay · Krishanu Chakrabarti

Received: 15 January 2010 / Accepted: 23 June 2010 / Published online: 2 July 2010

Abstract After 24 h of incubation with only purified pectate lyase isolated from *Bacillus pumilus* DKS1 (EF467045), the weight loss of the ramie fibre was found to be 25%. To know the catalytic residue of pectate lyase the *pel* gene encoding a pectate lyase from the strain *Bacillus pumilus* DKS1 was cloned in *E. coli* XL1Blue and expressed in *E. coli* BL21 (DE3) pLysS. The *pel* gene was sequenced and showed 1032 bp length. After purification using CM-Sepharose the enzyme showed molecular weight of 35 kDa and maximal enzymatic activity was observed at 60°C and a pH range of 8.5–9.0. Both Ca^{2+} and Mn^{2+} ions were required for activity on Na-pectate salt substrates, while the enzyme was strongly inhibited by Zn^{2+} and EDTA. The deduced nucleotide sequence of the DKS1 pectate lyase (EU652988) showed 90% homology to pectate lyases from *Bacillus pumilus* SAFR-032 (CP000813). The 3D structure as well as the catalytic residues was predicted using EasyPred software and Catalytic Site Atlas (CSA), respectively. Site directed mutagenesis confirmed that arginine is an essential catalytic residue of DKS1 pectate lyase.

Keywords *Bacillus pumilus* DKS1 · Ramie fibre degumming · *Pel* gene expression · Site-directed mutagenesis

S. Basu
Bose Institute, Centenary Building, Kolkata 700054, India

A. Roy · D. Chattopadhyay
Dr. B C Guha Centre for Genetic Engineering and Biotechnology, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700019, West Bengal, India

A. Ghosh
Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Straße, 35043 Marburg, Germany

A. Bera · D. Chattopadhyay · K. Chakrabarti (✉)
Department of Biochemistry, University College of Sciences, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700019, West Bengal, India
e-mail: kcbioc@gmail.com

Introduction

Pectin, the important structural constituent of plant cell walls, is composed essentially of long chains of (1,4)-α-D-polygalacturonate, which are partially esterified. Pectin degrading enzymes (pectinase) weaken the plant cell wall and expose other polymers to degradation by hemicellulases and cellulases. They are the first cell wall degrading enzymes that are secreted by pathogens and are important virulence factors. Among the best known microbial pectic enzymes are polygalacturonases, pectate lyases, pectin lyases and pectin methylesterases (Lagaert et al. 2009). Microbial pectin degradation is accomplished by methylesterases, which remove the methyl groups from pectin, and the depolymerases (hydrolases and lyases), which cleave the bonds between galacturonate units. Lyases cleave glycosidic bonds by β-elimination, giving rise to unsaturated products.

Among these enzymes, pectin lyases showed specificity for methyl esterified substrates (pectin) while pectate lyases are specific for unesterified polygalacturonate (pectate). Bacteria produce mainly pectate lyases, which require an alkaline pH and Ca^{2+} for their optimal enzyme activity. Pectate lyases are widely distributed among microbial plant pathogens and have been the focus of several studies to ascertain their role as virulence factors (Barras et al. 1994; Hugouvieux-Cotte-Pattat et al. 1996). They have also been found in saprophytic microorganisms, including the genus *Bacillus* (Nasser et al. 1990), and in some thermophilic bacteria (Kozianowski et al. 1997). Pectin-degrading enzymes are widely used in the food industry for improving the yield and the clarification of fruit juices (Alkorta et al. 1998). At present, pectinolytic enzymes are being introduced into the textile industry to release fibres from flax and ramie, as an alternative to conventional retting (Henriksson et al. 1999; Antonov et al. 2007; Basu et al. 2009a).

A number of pectate lyase genes from different *Bacillus* species have been cloned and characterized in recent years. PelA from *B. licheniformis* 14A (Berensmeier et al. 2004); Pel-103 from *Bacillus* sp. KSM-103 (Hatada et al. 1999) and Pel-4B from *Bacillus* sp. P4-N (Hatada et al. 2001), belongs to polysaccharide lyase family 1.

In previous papers (Basu et al. 2008, 2009a, b) we had reported the isolation and characterization of a theromoactive pectate lyase from a strain of *Bacillus pumilus* DKS1 and its potential use for degumming ramie fiber. This paper reports the cloning, expression, sequencing and site-directed mutagenesis of *pel* gene encoding pectate lyase from *Bacillus pumilus* DKS1 strain (Basu et al. 2008) to elucidate that Arg235 is an essential catalytic residue to degumming ramie fibre.

Materials and methods

Degumming of the ramie fibres using wild-type purified DKS1 pectate lyase

Degumming of ramie fibre (measured as % weight loss) in presence of wild-type purified DKS1 pectate lyase was estimated after 24 h of incubation. Degumming was done at 37°C. 1 ml of purified DKS1 pectate lyase was diluted to 10 ml in presence of 25 mM Tris–HCl (pH 8.5) containing 1 mM $CaCl_2$. The final activity of the enzyme was 93U/ml and weight of the ramie fibre was 1 g. After enzymatic treatment, fibres were washed twice with hot distilled water to remove residual gum from the surface of ramie fibre.

Bacterial strains and plasmid

The pectate lyase gene used in this study was isolated from *B. pumilus* DKS1 (EF467045) (Basu et al. 2008). To enhance the production of DKS1 pectate lyase for degumming ramie fibre more efficiently, the pET20b(+) plasmid (Novagen, USA) along with the host *E. coli* strain XL1Blue was used for cloning of DKS1 *pel* gene and the pET20b(+) plasmid along with the *E. coli* BL21 (DE3) pLysS cell (Novagen, USA) was used as the host for protein over expression using the T7 RNA polymerase expression system. Therefore pET20b(+) has been used both as a cloning as well as expression vector.

Construction of pET20b(+)-*pel*

To clone *pel* (pectate lyase) gene, total Genomic DNA was extracted from the *B. pumilus* DKS1 as described by Sambrook and Russel (2001). The gene encoding pectate lyase from *B. pumilus* DKS1 was amplified by PCR. PCR reaction was carried out in 50 µl containing 50 ng DNA template, 10 pmol of each primer, 200 µM dNTP, 1 × PCR buffer with 1.5 mM $MgCl_2$ and 2U FidelityTaq^{TM} DNA Polymerase (USB, USA) using the following program: 94°C, 30 s; 53°C, 35 s; 68°C, 60 s; 35 cycles. The forward primer PelF was used (5′–3′) GGAATTC CATATGTTGAAGAAAAAAGTTCC (Tm-59°C) and the reverse primer PelR was (5′–3′) CGGATCC TTAAGGGTTTACTTTTCC (Tm-57°C). The primer sequences were designed from the pectate lyase sequence of *Bacillus pumilus* SAFR-032 (Genbank accession no. ABV64163). A 1 kb PCR fragment was separated by agarose gel electrophoresis and purified using an agarose gel DNA extraction kit (Qiagen, USA). The purified fragments were digested for 60 min at 37°C with NdeI and BamHI, and then ligated into the NdeI, BamHI-digested pET20b(+) vector. The constructed plasmid was introduced into *E. coli* XL1Blue cells by heat shock at 37°C. A single transformant colony was selected and inoculated

into 3 ml LB broth with ampicillin (100 μg/ml) for overnight incubation with vigorous shaking (150 rev min^{-1}) at 30°C. The recombined plasmid was extracted according to the alkaline lysis procedure (Sambrook and Russel 2001). The recombinant plasmid was digested with NdeI to confirm the correct gene insertion. The plasmid obtained was designated pET20b(+)-*pel*.

Sequencing of the *pel* gene

After cloning, amplified and gel-eluted PCR fragment of the *pel* gene were sequenced in ABI 3100 Genetic Analyzer with four primers; PelF (5′-GGAATTCCA TATGTTGAAGAAAAAAGTTCC-3′), PelR (5′-CG GGATCCTTAAGGGTTTACTTTTCC-3′), T7 promoter (5′-TAATACGACTCACTATAGGG-3) and T7 terminator (5′-GCTAGTTATTGCTCAGCGG-3′). Sequencing reaction was performed using the Big Dye terminator cycle sequencing Kit V3.1 (Applied Biosystems, Foster City, USA) following the manufacturer's protocol. The nucleotide sequence of DKS1 *pel* gene was deposited in the GenBank under the accession no. EU652988.

Expression of *pel* gene

For expression, pET20b(+)-*pel* was transformed into *E. coli* BL21 (DE3) plysS cells (Novagen). The cells were grown in 5 ml LB medium (containing 100 μg/ml ampicillin). The transformants were cultivated at 37°C and 170 rev min^{-1} to an absorbance (at 600 nm) of 0.4. To the culture broth IPTG (0.5 mM final concentration) was added and growth was continued at 140 rev min^{-1} for another 16 h at 16°C, for induction of recombinant protein.

Extraction of recombinant DKS1 pectate lyase

After induction at 16°C for 16 h, the culture was centrifuged at 10,000 rpm at 4°C. The cells were washed twice with 25 mM Tris–HCl (pH 8.5) containing 25 mM NaCl buffer and then frozen at −20°C for more than 6 h. The frozen cells were thawed, sonicated in 25 mM Tris–HCl (pH 8.5) containing 25 mM NaCl buffer on ice and centrifuged; the resulting cell lysate was kept for assay of enzyme activity.

Activity assay of the recombinant pectate lyase

The pectate lyase activity was determined by the TBA (thiobarbituric acid) assay which measure absorbance at 550 nm (Roberts et al. 1986; Basu et al. 2008, 2009a, b). Suitable dilutions of the cell lysate (1 ml) were added to 5 ml of PGA (polygalacturonic acid, sodium salt, Sigma) solution (0.75%, w/v). The assay volumes were made up to 10.0 ml with Tris–HCl buffer (25 mM, pH 8.5) containing 1 mM $CaCl_2$ and incubated at 60°C for 2 h. About 0.6 ml zinc sulphate (9.0%, w/v) and 0.6 ml sodium hydroxide (0.5 M) were then added. The samples were centrifuged ($3000 \times g$, 10 min) and 5.0 ml of the clear supernatant was added to a mixture of thiobarbituric acid (3.0 ml, 0.04 M) and HCl (1.5 ml, 0.1 M). The mixture was heated in a boiling water bath for 30 min, and the absorbance of the coloured solution was measured at 550 nm against a reference cuvette which contains the same reagents as the experimental cuvette but for which the zinc sulphate and sodium hydroxide were added before adding the enzyme and substrate. One unit of activity was defined as the amount of enzyme that caused a change in absorbance of 0.01 under the condition of the assay.

Purification of recombinant pectate lyase

The cell lysate was loaded onto a CM-Sepharose column (Amersham) for purification of recombinant expressed *pel* gene. The cell lysate was dissolved in the minimum amount of Tris–HCl buffer (25 mM, pH 8.5) and dialysed overnight against the Tris–HCl buffer (25 mM, pH 8.5). 5 ml of dialysed sample was loaded onto a CM-Sepharose column (5 ml bed volume), equilibrated with Tris–HCl buffer (25 mM, pH 8.5). The column was washed with the Tris–HCl buffer containing (0–1 M) NaCl concentrations to elute the proteins. The protein content of each fraction was measured by the method of Lowry et al. (1951) and the pectate lyase activity was assayed by the method described earlier. 12% SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (1970) using Bio-Rad electrophoresis apparatus. Protein markers (Fermentas; #SM0431) and the protein bands were stained by silver staining according to Swain and Ross (1994).

Effect of temperature and pH on recombinant enzyme activity

The optimum temperature of pectate lyase was determined by carrying out the standard assay in Tris–HCl buffer (25 mM, pH 8.5), at temperatures ranging from 30 to 100°C. In each case, the substrate was pre-incubated at the desired temperature for 5 min.

The pH optimum of the pectate lyase was measured at a fixed assay temperature of 60°C at various pH values between 3 and 11, using buffers with the same ionic strength (25 mM). The buffers used were glycine–HCl (pH 3), sodium acetate (pH 4–5), potassium phosphate (pH 6–7), Tris–HCl (pH 8–9) and glycine–NaOH (pH 10–11).

Effect of metal ions on recombinant pectate lyase activity

The metal ions (Ca^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+}) as chloride salts were added to the substrate-buffer mixture (PGA 0.75%, 25 mM Tris–HCl, pH 8.5) to give a final concentration of 1 mM, and the pectate lyase activity was measured as previously described.

Site-directed mutagenesis

Mutagenesis was performed by the oligonucleotide-directed in vitro mutagenesis method (Kunkel et al. 1987) using the four primers, PelF, PelR, MutF (5′-CTGAACTCTGCTGTACCGTCTATGC-3′; Tm-56°C) and MutR (3′-TTTGGACTTGAGACGACA TGGCAGATA-5′; Tm-57°C), respectively. MutF and MutR were used to change the arginine (Arg) to alanine (Ala) residue. The plasmid pET-20b(+) was used as an expression vector. Mutation was verified by using the Big Dye terminator cycle sequencing Kit V3.1 (Applied Biosystems, Foster City, USA) following the manufacturer's protocol.

Results

Degumming of ramie fibres by purified wild-type DKS1 pectate lyase

Degumming of ramie fibre was done by only purified DKS1 wild-type pectate lyase in a single step process. The percentage decrease in the weight of ramie fibres by only enzymatic treatment was up to 25% after 24 h. Therefore, only single step degumming by purified enzyme showed same percentage of weight loss compare to combine process (enzyme + alkali) (Basu et al. 2009a) after 24 h. The degummed fibres exhibited more soft and smooth after treatment with wild-type purified pectate lyase (Fig. 1).

Purification and characterization of the recombinant pectate lyase

Purification of the recombinant pectate lyase was shown in Table 1. After ion-exchange chromatography, the purified enzyme showed a single band on 12% SDS-PAGE in Lane 2 (Fig. 2), corresponding to a molecular weight of around 35 kDa, which is similar to wild-type pectate lyase purified by gel

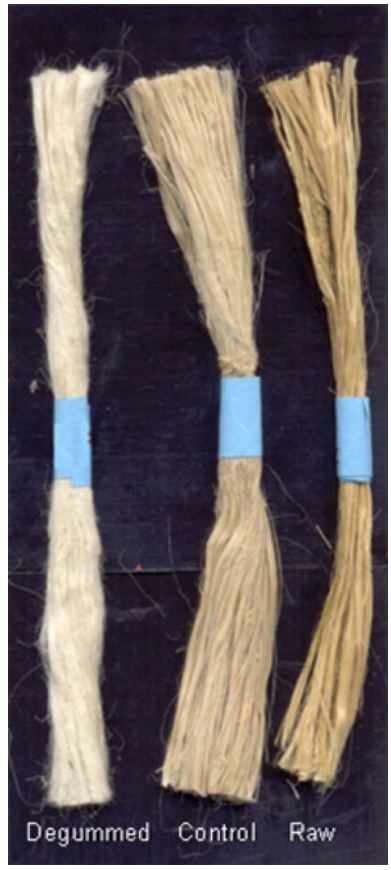

Fig. 1 **a** Degumming of ramie fibre using purified DKS1 pectate lyase

Table 1 Purification of the recombinant DKS1 pectate lyase

Procedure	Total protein (mg)	Total pectate lyase (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell lysate	6.1	2658	435.73	1	100
CM-Sepharose	0.46	1331	2893.47	6.64	50

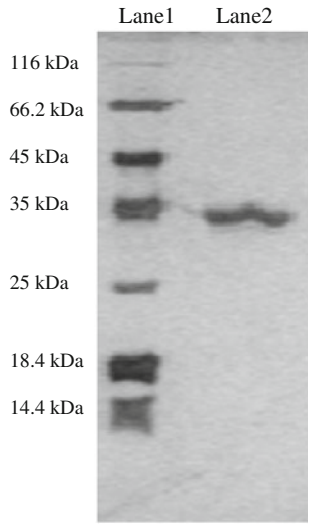

Fig. 2 12% SDS-PAGE; protein standards Lane 1, purified fraction Lane 2

filtration G-75 (Basu et al. 2008). Protein standards were used as shown in Lane 1 (Fig. 2). More than six fold purification was observed (Table 1).

Pectate lyase activity was optimally active between pH 8.5 and 9.0 and the optimal temperature for recombinant pectate lyase activity was around 60°C. The optimum pH range was quite similar to wild-type pectate lyase enzyme (Basu et al. 2008), but the recombinant pectate lyase enzyme shows less thermoactive than wild-type enzyme (Basu et al. 2008). The recombinant protein did not show any enzymatic activity above 85°C.

The recombinant pectate lyase activity in reaction mixtures with 1 mM $CaCl_2$ or 1 mM $MnCl_2$ was enhanced by 68 or 61%, respectively. These values were almost similar to those of the wild-type pectate lyase (Basu et al. 2008). Similarly, the Mg^{2+} stimulated enzyme activity up to 11%. However, metal ions $Ni^{2+}$and Zn^{2+} inhibited enzyme activity to 78 and 52%. Metal ions Na^{+} and K^{+} had no obvious effect on pectate lyase activity, while EDTA (1 mM) almost completely repressed pectate lyase enzyme activity by 95% (data not shown). Therefore, the effects of the different metal ions on recombinant pectate lyase activity were similar to wild-type pectate lyase activity (Basu et al. 2008).

Degumming of ramie fibre using purified recombinant pectate lyase

Degumming of ramie fibre was also done by purified recombinant pectate lyase in a single step process as same as purified wild-type pectate lyase. The degumming of ramie fibre by only purified recombinant DKS1 pectate lyase treatment showed lower percentage weight loss (21% weight loss after 24 h) compare to purified wild-type enzymatic treatment (25% weight loss after 24 h).

pel gene sequence of *B. pumilus* DKS1

After cloning, *pel* gene sequence was done by using four primers; PelF, PelR, T7 promoter (forward) and T7 terminator (reverse). The 1032 bp deduced nucleotide sequence (EU652988) from DKS1 *pel* gene was compared with those of other pectinolytic enzymes by homology analysis, using the BLAST in NCBI database. The amplified recombinant mature *pel* gene exhibited highest homology to *Bacillus pumilus* SAFR-032 (Gioia et al. 2007), *Bacillus* sp. KSM-103 (Hatada et al. 1999) and *Bacillus* sp. KSM-P7 (Kobayashi et al. 1999), showing 90, 96 and 89% identity, respectively.

Within the polysaccharide family 1, the crystal structures of BsPel from *B. subtilis* (Pickersgill et al. 1994), PelC from *E. chrysanthemi* (Yoder and Jurnak 1995; Scavetta et al. 1999) and of pectate lyase 47 from *Bacillus* sp. TS-47 (Nakaniwa et al. 2003) have been solved.

To predict the 3D structure of DKS1 pectate lyase, amino acid sequence was submitted to EasyPred3D Web Server (Lambert et al. 2002). The 3D template of pectate lyase 47 (Brookheven Protein data bank ID: 1VBL.pdb) shares 24% identities with DKS1

Fig. 3 3D structure amino acid sequence alignment between pectate lyase 47 (P47) and DKS1 pectate lyase (DKS1) belonging to class I family. Alignment was performed with the CLUSTAL W program. Numbering of the amino acids starts at the *N*-termini of the protein. This is the mature portion of the protein. Gaps are indicated by *dashes*. Asterisks show identical amino acids and *dots* show conserved residues. The sequences shown are; pectate lyase 47 from *Bacillus* sp. TS-47 and pectate lyase from *B. pumilus* DKS1

pectate lyase protein or amino acid sequence as shown in Fig. 3.

It has been already proved that the lyases cleave glycosidic bonds of pectin by β-elimination, giving rise to unsaturated products. During this β-elimination reaction, different catalytic residues were initiating the breakdown of pectin. A pectate lyase (PDB ID: 2bsp) from *B. subtilis* showed lysine (Lys) is a catalytic residue which can binds with calcium to initiate pectin breakdown (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/CSA/CSA_Site_Wrapper.pl?pdb=2bsp). Another pectate lyase (PDB ID: 1gxn) showed asparagine (Asn) acts as a catalytic residue (Charnock et al. 2002). According to Pickersgill et al. (1994), an aspartate residue (D) interacts with the Ca^{2+} to form the Ca^{2+}-pectate lyase protein complex. After the formation of Ca^{2+}-protein complex, Ca^{2+} and aspartate (D) interact with the substrate (pectin) carboxylate, making the C5 (C is the carbon atom) proton more acidic. Then arginine (R) deprotonates the C5 hydrogen atom from the axial position of the pectin, which leads to formation of a double bond between C4 and C5 carbon atoms (Herron et al. 2000). According to Nakaniwa et al. (2003), the two essential catalytic residues were identified in pectate lyase 47, which initiate the β-elimination reaction. These two residues were aspartate (D) at 190 positions and arginine (R) at 285 positions in protein sequence of pectate lyase 47 (Fig. 3). After alignment of two pectate lyase protein sequences (pectate lyase 47 and DKS1), it has been observed that the D^{190} and R^{285} of pectate lyase 47 completely aligned with D^{152} and R^{235} residues of DKS1 pectate lyase (aligned residues were marked by underline in Fig. 3). Therefore, from the above results, it could be predicted that the D^{152} and R^{235} were the two catalytic residues present in *B. pumilus* DKS1 pectate lyase.

A predicted 3D model of *B. pumilus* DKS1 pectate lyase enzyme has been built using the RASMOL web server shown in Fig. 4. From the figure it may be seen that the DKS1 pectate lyase contains a right-handed parallel beta-helix folding motif. The majority of the regular secondary structure was composed of parallel beta-sheets. The individual strands of the sheets are connected by unordered loops of varying length. The backbone was formed by a large helix composed of beta-sheets.

Arginine residue is present at the DKS1 pectate lyase catalytic site

Though Arg^{235} residue of DKS1 *pel* gene was completely aligned with the Arg^{285} residue of *Bacillus* sp. TS-47 *pel* gene, Arg^{235} residue of DKS1 *pel* gene was replaced with alanine (A) residue to generate variant/mutant (Arg^{235}Ala) to investigate whether the

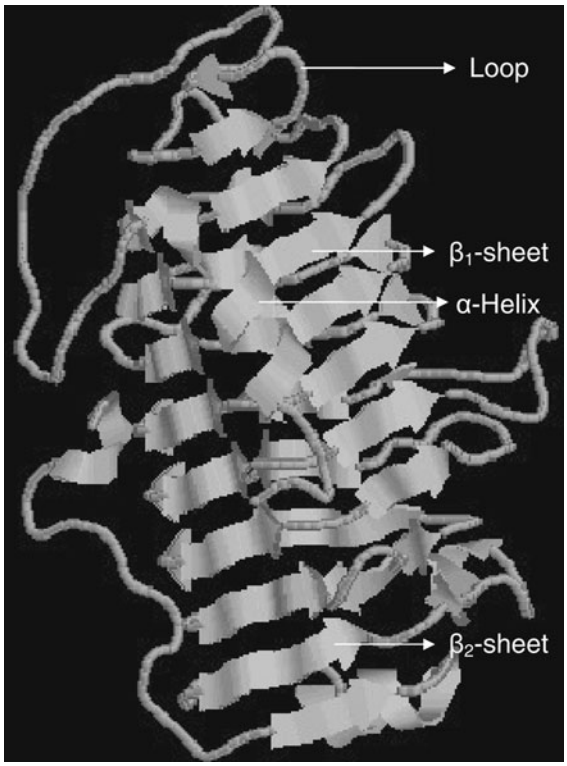

Fig. 4 Predicted 3D structure of *B. pumilus* DKS1 pectate lyase. β_1-sheet and β_2-sheet are right-handed parallel to each other

Arg^{235} is present at the catalytic site of the DKS1 pectate lyase or not. After enzymatic assay, it has been observed that the Arg^{235}Ala variant does not show any pectate lyase activity. Wild-type and variant enzyme was overexpressed in *E. coli* BL21 (DE3) pLysS and both enzymes showed apparently same molecular weight (around 35 kDa) as judged by 12% SDS-PAGE (data not shown). The yield of the mutated enzyme was similar to that of the wild-type enzyme. These results indicate that the variant/mutant was not compromised by major structural changes. Therefore, site-directed mutagenesis indicates that Arg^{235} is an essential catalytic residue of DKS1 pectate lyase to breakdown pectin substrate.

Discussion

The degumming of ramie fibre has been already done in two step process (Basu et al. 2009a). In laboratory scale (small scale), 17–18% weight loss was observed after treatment with only DKS1 crude pectate lyase (Basu et al. 2009a) compare to 25% in presence of only purified pectate lyase. Therefore, purified enzyme could degum fibre more efficiently than crude enzyme. This could be attributed to the higher activity per milligram of purified protein (6200 U/mg) compare to crude enzymes (433.33 U/mg). In addition, enzymes in such industries as food processing and medicine production being used a pure preparation in order to guarantee the quality of the final product and to avoid color and flavor changes due to contaminants in the enzyme extract itself. A pure preparation will also eliminate substances that could act as inhibitors of the enzyme activity and toxins (Pedrolli and Carmona 2010).

The sequence of the *B. pumilus* DKS1 *pel* gene corresponded to an open reading frame of 1032 bp and encodes a mature protein of 343 amino acids with calculated molecular weight of 37,556 Da and pI of 9.23 (using ExPasy software). By using SignalP 3.0 Server, DKS1 pectate lyase showed signal peptide sequence which is MLKKKVPMLAGVLAVGLVTS LFAPNGEAKA (1–30 residues).

The specific activity of wild-type extracellular pectate lyase in YP fermentation broth (Basu et al. 2008) reached as high as 433.33U/mg, which was quite similar to the recombinant intracellular pectate lyase in expression system (435.73U/mg). However, the enzyme activity (U/ml) in expression system (443U/ml) was more than eight times compared to that of pectate lyase produced (52U/ml) naturally in fermentation broth (Basu et al. 2008). The purified recombinant pectate lyase showed maximum activity (665.5 U/ml) at 60°C. But it showed only 303 U/ml activity at 75°C which is 67.42% less compare to the activity of wild-type protein (930 U/ml) at the same temperature (Basu et al. 2008). The cloning followed by expression of *pel* gene from *B. pumilus* in *E. coli* is being reported for the first time as per our knowledge.

Although some recombinant pectate lyases were difficult to purify (Zhai et al. 2003), the recombinant pectate lyase enzyme of *B. pumilus* DKS1 was completely purified by one-step purification using a CM-Sepharose cation-exchanger column. Recombinant DKS1 Pectate lyase had higher activity over a pH range of 8–10 and a temperature range of 40–70°C, as well as relative stability between pH 4.0 and 10.0.

The nucleotide sequence of *B. pumilus* DKS1 *pel* gene (EU652988) shows 90% homology to *B. pumilus*

SAFR-032 (CP000813), 96% to *Bacillus* sp. KSM-103 (AB015044) and 89% to *Bacillus* sp. KSM-P7 (AB015043) *pel* gene sequence in NCBI database. It has been also observed that the *pel* gene of *B. pumilus* DKS1 showed homology to enzymes belonging to the polysaccharide lyase family 1, which comprises pectate and pectin lyases, based on their primary sequence homology (Couthino and Henrissat 1999).

The predicted 3D structure of DKS1 pectate lyase was done using RASMOL bioinformatics tools as previously described by Sayle and Milner-White (1995). From the predicted 3D structure of DKS1 pectate lyase (Fig. 3), it was observed that the DKS1 pectate lyase falls in the β-class protein structure, which was in keeping with previously mentioned reports on this enzyme and its structure (Basu et al. 2008). Finally, site-directed mutagenesis indicates that Arg^{235} is essential catalytic residue of DKS1 pectate lyase to breakdown pectin substrate. After enzymatic assay, it has been observed that the Arg^{235}Ala variant/mutant does not show any pectate lyase activity. The aspartate residue (D^{152}) was also considered for site-directed mutagenesis. However Asp^{152}Ala mutant showed 48% of the total activity of recombinant protein.

Pectate lyase has immense applications in the paper and textile manufacturing industries. Large-scale production of recombinant pectate lyase from *E. coli*, as shown in our work, may be industrially utilized. Degumming of ramie fiber using purified pectate lyase has been better compared to bacterial crude enzyme (Basu et al. 2008) due to its (pure enzyme) higher specific activity and the lack of potential inhibitors. Such a single step degumming process using purified enzyme could further reduce the amounts of chemicals and energy used in the conventional chemical degumming process.

The thermostability and activity of DKS1 recombinant pectate lyase could be enhanced by single amino acid substitution as mentioned by Xiao et al. 2008. This process will enhance the importance of DKS1 recombinant pectate lyase in biotechnological application in future as thermostability of the enzyme has great importance in industry.

Acknowledgements The authors have acknowledged the support from Dr. Himadri Sekhar Sen (Former Director, CRIJAF) and Dr. Manabendra Nath Saha (Scientist, CRIJAF, West Bengal, India). This study was supported by grants from ICAR (Indian Council of Agricultural Research) and UGC (under department of special assistance grant to the Department of Biochemistry, University of Calcutta). Support for DNA sequencing was obtained from the NCMP grant to University of Calcutta, India.

References

Alkorta I, Garbisu C, Llama MJ, Serra JL (1998) Industrial applications of pectic enzymes: a review. Process Biochem 33:21–28

Antonov V, Msarek J, Bjelkova M, Smirous P, Fischer H (2007) Easily available enzymes as natural retting agents. Biotechnol. J 2:342–346

Barras F, Van GF, Chatterjee AK (1994) Extracellular enzymes and pathogenesis of soft-rot of erwinias. Annu Rev Phytopathol 32:201–234

Basu S, Ghosh A, Bera A, Saha NM, Chattopadhyay D, Chakrabarti K (2008) Thermodynamic characterization of a highly thermoactive extracellular pectate lyase from a new isolate *Bacillus pumilus* DKS1. Bioresour Technol 99(17):8088–8094

Basu S, Saha NM, Chattopadhyay D, Chakrabarti K (2009a) Large-scale degumming of ramie fibre using a newly isolated *Bacillus pumilus* DKS1 with high pectate lyase activity. J Ind Microbiol Biotechnol 36:239–245

Basu S, Saha NM, Chattopadhyay D, Chakrabarti K (2009b) Degumming and characterization of ramie fibre using pectate lyase from immobilized *Bacillus pumilus* DKS1. Lett Appl Microbiol 48(5):593–597

Berensmeier S, Singh SA, Meens J, Buchholz K (2004) Cloning of the pelA gene from *Bacillus licheniformis* 14A and biochemical characterization of recombinant, thermostable, high-alkaline pectate lyase. Appl Microbiol Biotechnol 64:560–567

Charnock JS, Brown EI, Turkenburg PJ, Black WG, Davies JG (2002) Convergent evolution sheds light on the anti-β-elimination mechanism common to family1 and 10 polysaccharide lyases. PNAS 99:12067–12072

Couthino PM, Henrissat B (1999) In: Gilbert H, Davies GJ, Henrissat B, Svenson B (eds) Recent advances in carbohydrate bioengineering. The Royal Society of Chemistry, Cambridge, UK, pp 3–12

Gioia J, Yerrapragada S, Qin X, Jiang H, Igboeli OC, Muzny D, Dugan-Rocha S, Ding Y, Hawes A, Liu W, Perez L, Kovar C, Dinh H, Lee S, Nazareth L, Blyth P, Holder M, Buhay C, Tirumalai MR, Liu Y, Dasgupta I, Bokhetache L, Fujita M, Karouia F, Eswara MP, Siefert J, Uzman A, Buzumbo P, Verma A, Zwiya H, McWilliams BD, Olowu A, Clinkenbeard KD, Newcombe D, Golebiewski L, Petrosino JF, Nicholson WL, Fox GE, Venkateswaran K, Highlander SK, Weinstock GM (2007) Paradoxical DNA repair and peroxide resistance gene conservation in *Bacillus pumilus* SAFR-032. PLoS ONE 2(9):e928

Hatada Y, Higaki N, Saito K, Ogawa A, Sawada K, Ozawa T, Hakamada Y, Kobayashi T, Ito S (1999) Cloning and sequencing of a high-alkaline pectate lyase gene from an alkaliphilic *Bacillus* isolate. Biosci Biotechnol Biochem 63:998–1005

Hatada Y, Kobayashi T, Ito S (2001) Enzymatic properties of the highly thermophilic and alkaline pectate lyase Pel-4B from alkaliphilic *Bacillus* sp. strain P-4-N and the entire nucleotide and amino acid sequences. Extremophiles 5:127–133

Henriksson G, Akin DE, Slomczynski D, Eriksson K-EL (1999) Production of highly efficient enzymes for flax retting by *Rhizomucor pusillus*. J Biotechnol 68:115–123

Herron RS, Benen AEJ, Scavetta DR, Visser J, Jurnak F (2000) Structure and function of pectic enzymes: virulence factors of plant pathogens. Proc Natl Acad Sci 97(16): 8762–8769

Hugouvieux-Cotte-Pattat N, Condemine G, Nasser W, Reverchon S (1996) Regulation of Pectinolysis In Erwinia Chrysanthem. Annu Rev Microbiol 50:213–257

Kobayashi T, Hatada Y, Higaki N, Lusterio DD, Ozawa T, Koike K, Kawai S, Ito S (1999) Enzymatic properties and deduced amino acid sequence of a high-alkaline pectate lyase from an alkaliphilic *Bacillus* isolate. Biochim Biophys Acta 1427(2):145–154

Kozianowski G, Canganella F, Rainey FA, Hippe H, Antranikian G (1997) Purification and characterization of thermostable pectate-lyases from a newly isolated thermophilic bacterium, *Thermoanaerobacter italicus* sp. nov. Extremophiles 1:171–182

Kunkel TA, Roberts JD, Zakour RA (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol 154:367–382

Laemmli (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nat 227:680–685

Lagaert S, Beliën T, Volckaert G (2009) Plant cell walls: protecting the barrier from degradation by microbial enzymes. Seminar Cell Dev Biol 20:1064–1073

Lambert C, Leonard N, De Bolle X, Depiereux E (2002) ESyPred3D: prediction of proteins 3D structures. Bioinformatics 18(9):1250–1256

Lowry OH, Rosebrough JN, Farr LA, Randall JR (1951) Protein measurement with the Folin-Phenol Reagent. J Biol Chem 193:265–275

Nakaniwa T, Tada T, Yamaguchi A, Kitatani T, Takao M, Sakai T, Nishimura K (2003) Crystallization and preliminary X-ray analysis of a thermostable pectate lyase PL 47 from *Bacillus* sp. TS 47. Acta Cryst D59:341–342

Nasser W, Chalet F, Robert-Baudouy J (1990) Purification and characterization of extracellular pectate lyase from *Bacillus subtilis*. Biochimie 72:689–695

Pedrolli BD, Carmona CE (2010) Purification and characterization of the exopolygalacturonase produced by *Aspergillus giganteus* in submerged cultures. J Ind Microbiol Biotechnol. doi:10.1007/s10295-010-0702-0

Pickersgill R, Jenkins J, Harris G, Nasser W, Robert-Baodouy J (1994) The structure of *Bacillus subtilis* pectate lyase in complex with calcium. Nat Struct Biol 1:717–723

Roberts PD, Berman MP, Allen C, Stromberg KY, Lacy HG, Mount SM (1986) Requirement for two or more *Erwinia carotovora* subsp. carotovora pectolytic gene products for maceration of potato tuber tissue by Escherichia coli. J Bacteriol 167:279–284

Sambrook J, Russel DW (2001) Molecular cloning: a laboratory manual, 3rd edn. CSH Laboratory Press, Cold Spring Harbor, NY

Sayle RA, Milner-White EJ (1995) RASMOL: biomolecular graphics for all. Trends Biochem Sci 20:374

Scavetta RD, Herron SR, Hotchkiss AT, Kita N, Keen NT, Benen JA, Kester HC, Visser J, Jurnak F (1999) Structure of a plant cell wall fragment complexed to pectate lyase C. Plant Cell 11:1081–1092

Swain M, Ross NW (1994) A silver stain protocol for proteins yielding high resolution and transparent background in sodium dodecyl sulfate-polyacrylamide gels. Electrophoresis 16:948–951

Xiao Z, Bergeron H, Grosse S, Beauchemin M, Garron M, Shaya D, Sulea T, Cygler M, Lau CKP (2008) Improvement of the thermostability and activity of a pectate lyase by single amino acid substitutions, using a strategy based on melting-temperature-guided sequence alignment. Appl Env Microbiol 74:1183–1189

Yoder MD, Jurnak F (1995) The parallel β-helix and other coiled folds. FASEB J 9:335–342

Zhai C, Cao J, Wang Y (2003) Cloning and expression of a pectate lyase gene from *B. alcalophillus* NTT33. Enzyme Microb Technol 33:173–178