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Research Article
Ricin Super Family Carbohydrate Binding Module 13 Containing Pectate Lyase 1B from *Bacillus licheniformis* Display Hyper Thermal Stability

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Abstract

Background and Objective: Carbohydrate Binding Module (CBMs), a non catalytic carbohydrate binding modules present in glycosyl hydrolases, are playing substantial role in localizing the enzyme to the target substrate, disrupting the structure of robust polymers and their presence in pectate lyase is rare. Deduced gene sequence of pectate lyase 1B (*pelB*) from *Bacillus licheniformis* (Bli PelB) shows unique structural features containing N-terminal signal peptide following with ricin super family CBM13 and C-terminal catalytic domain. The objective of the study was to characterize Bli PelB and evaluation of role of CBN13 present in Bli PelB sequence. **Methodology:** Full length and catalytic domain part of *pelB* was cloned and expressed. Protein was purified through Ni-NTA his tag affinity chromatography and characterized. **Results:** Biochemical characterization of Bli PelB revealed maximal activity at 50°C with pH 8.0. Enzyme retained more than 80% activity between pH 7.5-9.0. Bli PelB found to be thermostable at 50°C by maintaining more than 60% activity for 4 days. Bli PelB was 100% active on methylated (70-75%) apple pectin as compared to non methylated polygalacturonic acid (PGA) with which it was 29%. Specific activity was found to be 1451±2.0 and 423±3.5 U mg^{-1} for methylated pectin and PGA, respectively. k_{cat} and k_{cat}/K_m is markedly higher for methylated pectin than PGA. In comparison with Bli PelB, C-terminal catalytic domain of Bli PelB (CD Bli PelB) showed 60% decrease in specific activity while 70% decrease in thermal stability and kinetic efficiency. **Conclusion:** The characterized properties of Bli PelB are suitable for its industrial applications. The reduction in catalytic activity and thermal stability of Bli PelB without CBM13 could explain the role of CBM in enzyme efficiency.

Key words: Pectate lyase B, carbohydrate binding module, CBM13, methylated pectin, polygalacturonic acid, *Bacillus licheniformis*, thermal stability, ricin super family

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Primary cell wall of cotton fiber composed of non cellulosic materials such as polygalacturonic acid, proteins, cellulose, hemicelluloses and waxes[1] and secondary cell wall contain mainly cellulosic material. For efficient dye binding to the fabrics, enzymatic degradation of non cellulosic materials is potentially valuable because they could reduce the usage of toxic alkaline chemical. To improve the scouring pretreatment process of cotton fabric, it is necessary to treat with alkaline pectinase[2,3].

Pectinolytic enzymes are produced by plant pathogenic microbes like *Erwinia*[4-6] and saprophytic bacteria including *Bacillus* genus[7], which degrade complex pectin through concerted action of several enzymes. They are carbohydrate degrading enzymes which categorizes in various family (www.cazy.org/Polysaccharide-Lyases). Pectin esterase, pectate lyase, pectin lyase and polygalacturonase are the class of enzymes for degradation of pectin[8,9]. Pectate lyase and pectin lyase are specifically active towards the methoxylated pectin materials and produce unsaturated polygalacturonides[10,11]. Enzymatic mechanism is well established for complete degradation of pectin materials, (1) Cleavage of glycosidic bond and (2) β-elimination reaction at non reducing end resulting in Δ4,5 unsaturated oligomer residue. Pectate lyases of family 1, 2, 3, 9 and 10 carry the β-eliminative cleavage of α (1-4) glycosidic bond and generate Δ4, 5 unsaturated D-glucopyranosyluronic acid (Gal*p*A)[11].

Many glycoside hydrolases that attack cellulose and hemicelluloses have modular structure consisting catalytic module and ancillary non catalytic carbohydrate binding module (CBMs), which probably evolve to the specific sugar binding and facilitate the catalytic action of module[12-14]. Pectin degrading enzymes on the contrary, it has a relatively simple structure lacking CBMs, probably due to substrate accessibility is easier than cellulose and xylan[15,16]. Genome sequences show that some of the pectinases are with modular structure and two of them have been characterized which contain the CBM6 and CBM26[17]. While analyzing the pectate lyase sequence from the database, we encountered CBM with *Bacillus licheniformis* pectate lyase B of family 1 (*pelB*, GenBank accession No. AAU24559.1). Sequence analysis revealed that the CBM present in pectate lyase B of *Bacillus licheniformis* (Bli PelB) is of ricin super family 13 and C-terminal is the catalytic module. First, CBM13 has been characterized in xylanase of *Streptomyces*[18-20] and then many studies have shown its presence in endo-glucanase, chitinase, α-galactosidase and α-L-arabinofuranosidase[21].

Bacillus licheniformis is a soil bacterium and complete genome sequence analysis shows that it has extensive repertoire of glycoside hydrolase such as xylanase, cellulase, mannase and several pectin degrading enzymes[22]. The basic aim of this study includes the characterization of Bli PelB and evaluation of role of CBM13 present in Bli PelB sequence by comparing the data of two proteins, full length PelB protein (Bli PelB) and the catalytic module of PelB (CD Bli PelB).

MATERIALS AND METHODS

Bacteria, chemicals and media: *Bacillus licheniformis* DSM13/ATCC14580 was purchased from MTCC (MTTC No. 429). All molecular grade reagents required was purchased either from Takara Biomedical (Otsu, JP), New England Biolabs (UK), Invitrogen (Carlsbad, CA) or Bio-Rad (CA). The pET 21b vector was used for cloning and expression. Polygalacturonic acid (PGA) and pectin from apple (75% methyl esterified) was purchased from Sigma Aldrich. Metals, media and other chemicals were procured from Hi media Laboratories.

Recombinant DNA technique and sequence analysis: Total genomic DNA was isolated from *B. licheniformis* according to method described by Sambrook and Russell[23]. The *pelB* gene of 1485 bp (GenBank accession number AAU24559.1, Bli pelb) was amplified using FP 5'AGCTGCACG*Nhe*IATGAAACTGATCAAAAACGCA3' and RP 5'ACGTTCCAT*Xho*I GTCTTTTTTAAACTGGCTGTA3'. The catalytic domain of Bli pelb (CD Bli pelb, residue No. 173-494) was amplified using gene specific forward primer 5'AGCTGCACG*Nhe*IATGAGCGACGGGCTCGAAGGTTTC3'. An amplified fragment was restricted with *Nhe*I and *Xho*I restriction site and cloned in to pET-21b expression vector and positive clones were subjected for sequencing.

Protein expression and purification: Sequenced confirmed clones were transformed into *E. coli* BL21 for expression of Bli pelb and CD Bli pelb. Single isolated transformed colonies were inoculated in 200 mL LB medium and incubated at 30°C. After overnight incubation, cells were harvested by centrifugation at 4°C and dissolved in lysis buffer (50 mM tris-HCl, 20 mM imidazole, 300 mM NaCl). Cell lysis was carried out by sonication for 10 min with 30 mV amplitude with 15 sec of interval. Further, lysate was clarified by centrifugation (10,000×g, 30 min and 4°C) and allowed to pass through nickel-nitrilo triacetate acid (Ni-NTA) agarose matrix. After extensive washing with wash buffer, the protein was finally eluted in elution buffer containing 250 mM imidazole (pH 8.0) and eluted protein was dialyzed against 50 mM tris

(pH 8.0) using a 10 kDa ultra filter (Merck millipore, Germany). Dialyzed proteins were quantified using the Bio-Rad protein assay reagent (Bio-Rad, CA) against bovine serum albumin as a standard. The purified proteins were subjected to 12% SDS-PAGE and visualized with coomassie brilliant blue R-250 staining.

Enzyme activity measured spectrophotometrically using 1% methylated pectin as a substrate in 50 mM tris-HCl buffer and 0.25 mM Ca^{+2} and increase in released unsaturated product was measured at 235 nm[24]. The activity was expressed as 1 U of enzyme releasing the 1 μg of unsaturated product per minute per milliliter by using molar extinction coefficient 4600 M^{-1} cm^{-1}.

Biochemical characterization and thermal inactivation of Bli PelB: To test the effects of pH on Bli PelB, the pH was varied from 5.0-9.0 using 50 mM phosphate buffer (pH 5.0 and 7.0) and tris-HCl (pH 7.5-9.0) and enzyme activity was measured using 1% methylated pectin. For influence of temperature on Bli PelB, enzyme activity was measured on 1% methylated pectin at various temperatures ranging from 30.0-70°C. Activation of Bli PelB by various divalent metals was performed using 1.0 mM of EDTA, $CaCl_2$, $MnSO_4$, $ZnCl_2$, $CoCl_2$, $MgSO_4$, $CuSO_4$, $NiCl_2$ and apoenzyme in tris-HCl (pH 8.0) with 1% methylated pectin. Thermal stability of Bli PelB and CD Bli PelB was monitored at 50.0°C in 50 mM tris-HCl (pH 8.0). At different time intervals, samples were withdrawn and the relative activity was determined after the reaction.

Specific activity and catalytic efficiency: Approximately 1% of PGA and methylated pectin were treated with Bli PelB and CD Bli PelB under optimized conditions for 10 min and the release of unsaturated products was measured in UV-visible spectrophotometer at 235 nm. Specific activity was defined as the amount of unsaturated products produced per amount of protein per reaction time.

Kinetic parameters were analyzed at 50°C using a range of 0.01-1.5 mg of methylated pectin and PGA. Each concentration was treated with Bli PelB and CD Bli PelB containing 0.25 mM $CaCl_2$ in 50 mM tris-HCl, pH 8.0. Reactions were stopped after 10 min and the reaction mixtures were analyzed for release of unsaturated product at 235 nm. Kinetic parameters such as K_m (mg) and k_{cat} (min^{-1}) were determined by fitting the data to the Michaelis-Menten equation.

Statistical analysis: Simple statistical analysis was done for the results. All the results presented were average of triplicates and values shown were Mean ± SD. Kinetics data analysis was performed in Origin 6.0 software.

RESULTS

Recombinant DNA technique and nucleotide sequence analysis: Bli PelB sequence analysis revealed the downstream of N- terminal signal sequence is a ricin super family CBM13 with 136 residues which is followed by catalytic domain comprising total 322 residues (Fig. 1a). When the CBM13 portion of Bli PelB (residue No 36-172) was aligned with ricin binding domain of xylanase from *Streptomyces xiamenensis* (GenBank accession No. AKG42035.1) and alpha fucosidase from *Streptomyces* sp. MMG112 (GenBank accession No. KOV65226.1), it showed 22% identity. The Trp (W, residue No. 74, 121 and 169) in CBM is essential for substrate stacking is conserved in Bli PelB (Fig. 1b). C-terminal (residue No. 173-494) shows 53% identity with PelB of closely related species *B. subtilis* and 35% identity with *pelA* from *B. licheniformis* 14A[25]. Arginine (R376 for Bli PelB) working for substrate charge neutralization is conserved throughout the different family of bacterial pectate lyase B[26,27]. With limited knowledge on CBM13 in pectate lyase, it is very difficult to answer such evolution in enzyme. Possibly the amplification of CBM coding sequences throughout the genome in cellulosome containing organism conferred the advantage of CBM fusion with the pectate lyase[15]. To find out the role of CBM in Bli PelB, full length of pectate lyase (Bli pelb) and only the catalytic module of C-terminal Bli pelb (CD Bli pelb) was successfully cloned into pET 21b expression vector.

Protein expression and purification: Soluble forms of all recombinant proteins were obtained when they were expressed in *E. coli* strain BL21. The SDS-PAGE analysis of Ni-NTA-purified proteins resulted in apparent molecular masses of 54 kDa for Bli PelB and 36 kDa for CD Bli PelB (Fig. 2a, b). Total 4 and 7 fold purification was obtained for Bli PelB and CD Bli PelB respectively when methylated pectin used as a substrate (Table 1).

Biochemical characterization of Bli PelB: The enzyme showed broad range of pH working profile and optimal pH was found to be pH 8.0. It has retained 80% activity at pH 7.5, 8.5 and 9.0 (Fig. 3). To determine the temperature at which the highest activity occurs, the temperature was varied from 30-70°C. The enzyme activity increased as the temperature increased, showing higher activity at 50°C and found to be active over wide range of temperature (Fig. 4).

Like other pectate lyase, Bli PelB also increases the activity in the presence of metal ions (Fig. 5), with Ca^{+2} it is more active while Hg^{+2}, Cu^{+2} and Fe^{+2} inhibit the Bli PelB activity. The Ethylene Diamine Tetra Acetic Acid (EDTA) showed no

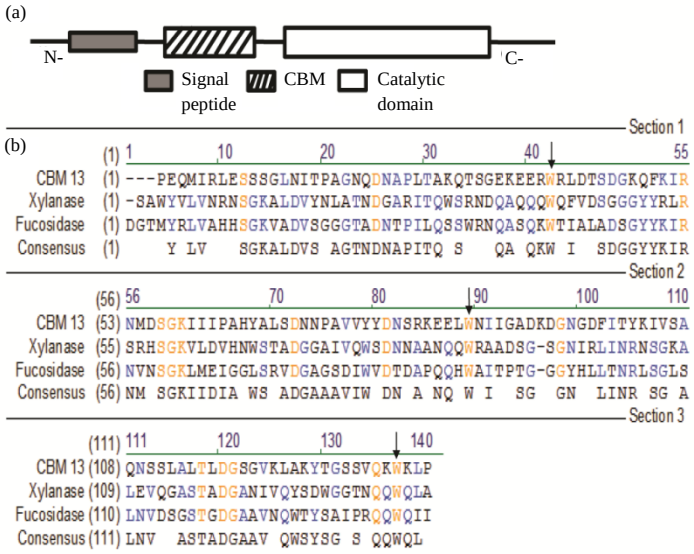

Fig. 1(a-b): (a) Schematic diagram of Bli PelB sequence, (b) Sequence alignment of CBM13 of Bli PelB with xylanase from *Streptomyces xiamenensis* and fucosidase of *Streptomyces* sp. MMG1121

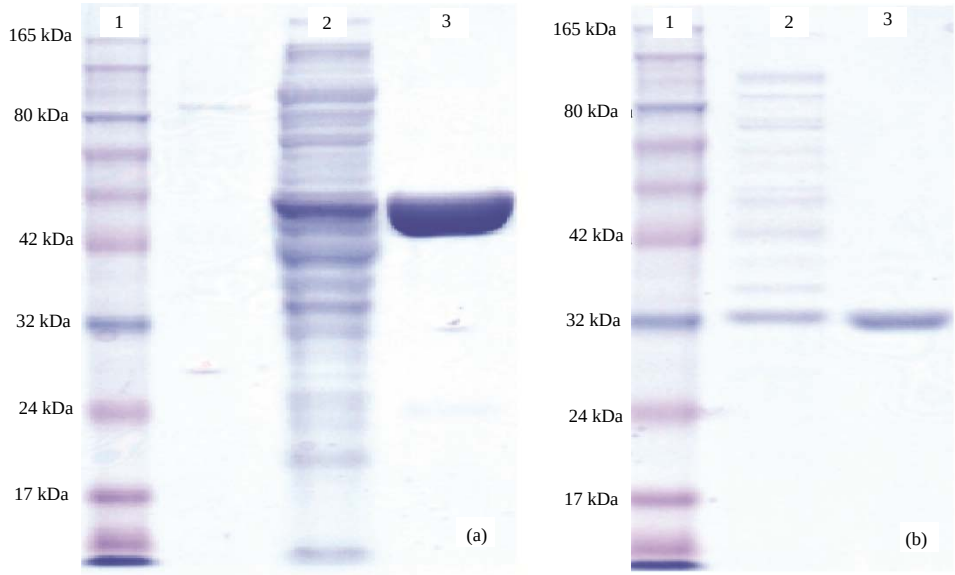

Fig. 2(a-b): 12% SDS PAGE analysis of Bli PelB and CD Bli PelB (a) Bli PelB SDS PAGE 1: Marker, 2: Bli PelB lysate, 3: Bli PelB purified, (b) CD Bli PelB SDS PAGE 1: Marker, 2: CD Bli PelB lysate, 3: CD Bli PelB purified

Table 1: Summary of purified Bli PelB and CD Bli PelB

Sample	[a]Protein (mg)	[a]Unit (μg mL^{-1} min^{-1})	Yield (%)	Specific activity (U mg^{-1})	Fold purification
Bli PelB lysate	15	6421	100	428±5.1	1
Bli PelB purified	2.1	3051	47	1451±2.0	4
CD Bli PelB lysate	13.5	1174	100	87±1.43	1
CD Bli PelB purified	0.75	434	37	578±2.6	7

[a]Total protein and units from 200 mL culture, ±represent SD of triplicate

influence on the activity of enzyme which indicates that the metal is not strict requirement for Bli PelB. Various concentrations of Ca^{+2} were tested to attain higher activity and 0.25 mM Ca^{+2} produced the most activity. Comparison of

thermal inactivation profile showed that more than 80% residual activity observed after 3 days for Bli PelB.

Specific activity and catalytic efficiency: Bli PelB showed higher activity on methylated pectin than non-methylated PGA. It is worth noting that the specific activity of Bli PelB is higher than the CD Bli PelB (Table 2). The Michaelis-Menten kinetic parameters (K_m and V_{max}) and catalytic efficiency k_{cat}/K_m of Bli PelB and CD Bli PelB were determined by plotting the reaction velocity (V_0) against the substrate concentration ([S]). Bli PelB showed higher turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m), at 6.8±0.2 and 15.5±0.82, respectively (Table 3). The k_{cat}/K_m of CD Bli PelB on methylated pectin was found to be 4.1±0.2 mg^{-1} min^{-1}. Removing CBM13 from Bli PelB showed 60% reduce in enzyme activity and 70% in thermal stability after 3 days which establishes the important role of CBM13 in Bli PelB functioning (Fig. 6).

DISCUSSION

The present study showed that *pelB* from *Bacillus licheniformis* DSM 13 is alkaline in nature and removal of CBM13 from the sequence decreases enzyme activity and thermal stability. Alkaline pectinases are mainly used in

Table 2: Substrate specificity of Bli PelB and CD Bli PelB

Substrate	Bli PelB Specific activity (U mg^{-1})	CD Bli PelB Specific activity (U mg^{-1})
PGA	423±3.5	263±1.5
Methylated pectin	1451±2.0	578±2.6

±represent SD of triplicate

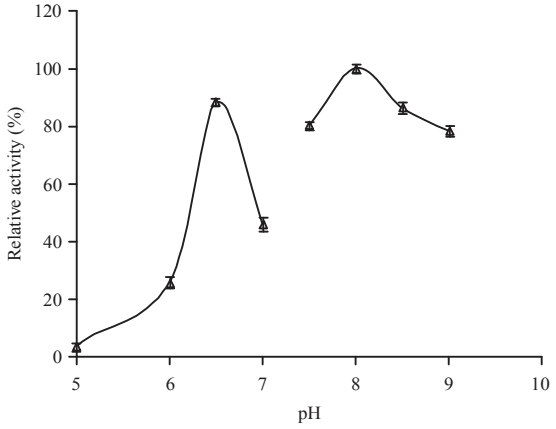

Fig. 3: pH optima of Bli PelB values represent the Mean±SD of triplicate samples

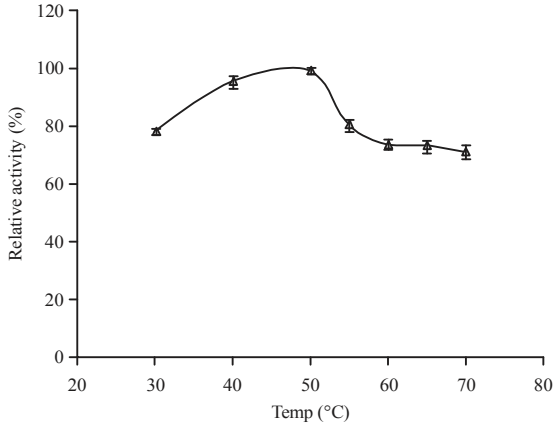

Fig. 4: Temperature optima of Bli PelB values represent the Mean±SD of triplicate samples

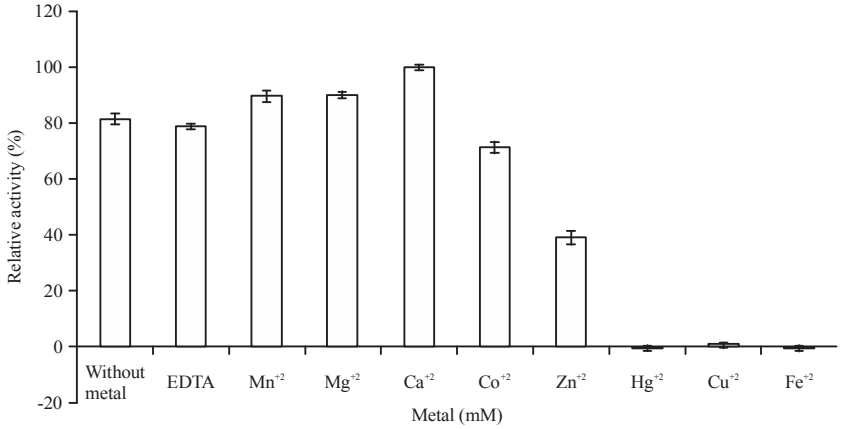

Fig. 5: Effect of various divalent metal ions on activity of Bli PelB values represents the Mean±SD of triplicate samples
(Relative activity for: Without metal 80% (SD±1.8), EDTA 79% (SD±1.5), Mn^{+2} 90% (SD±2.9), Mg^{+2} 90% (SD±2), Ca^{+2} 100% (SD±1.6), Co^{+2} 72% (SD±3), Zn^{+2} 39% (SD±2.4), Hg^{+2} 0% (SD±1), Cu^{+2} 1% (SD±1) and Fe^{+2} 0% (SD±1))

Table 3: Kinetic parameters of Bli PelB and CD Bli PelB for two different substrates

Enzyme	Substrate	K_m (mg)	V_{max} (μg min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (mg^{-1} min^{-1})
Bli PelB	PGA	2.0±0.01	6.4±2.1	3.17±0.34	1.5±1.0
	Methylated pectin	0.4±0.1	13.3±1.4	6.8±0.2	15.5±0.82
CD Bli PelB	PGA	0.48±0.04	4.7±1.7	2.3±0.1	4.8±0.32
	Methylated pectin	0.85±0.01	7.2±0.5	3.6±0.23	4.1±0.2

±represent SD of triplicate

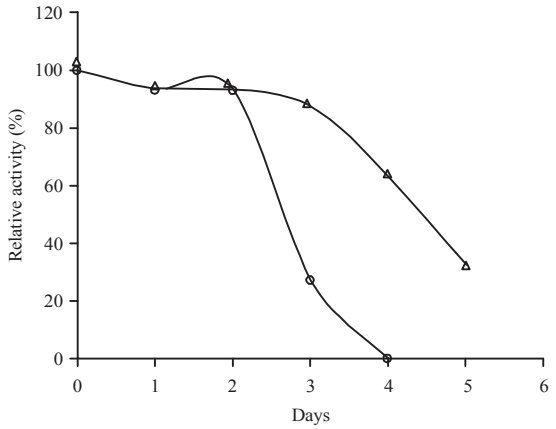

Fig. 6: Thermal stability profiles of Bli PelB and CD Bli PelB values represent the Mean±SD of triplicate samples (Bli PelB triangle, CD Bli PelB round)

degumming process and for pretreatment of pectin waste water[3,28,29]. It has been speculated that the higher pH is because of their substrates which is generally alkaline in nature. At high pH values, arginine involved in catalysis become deprotonated[17,27]. Reported pectate lyases from different *Bacillus* sp. showed optimum pH in alkaline side and temperature in range of 40-70°C[3,8]. Besides having its optimum pH in alkaline range, the Bli PelB displayed activity in acidic range too; hence it can be used in fruit juice extraction process[30,31]. Generally all pectate lyases are absolutely Ca^{+2} dependants for its catalysis that differentiate it from pectin lyase[32,33]. On mechanistic point, it has been proposed that calcium neutralize the charge of substrate (uronic acid) during catalysis[26,34]. However, Bli PelB is active without metal and presence of metal increased activity to 20% of the wild type, this feature makes this PelB different from others. Enzyme with high thermostability at moderate temperature (50-60°C) is an ideal candidate for various industrial applications. High thermal stability of Bli PelB at 50.0°C compared to the CD Bli PelB could insight the role of CBM13. This was also been reported for XynB from *Caldicellulosiruptor* sp. Strain F32, showed high thermostability as compared to catalytic domain and further analysis revealed that there is intramolecular interaction between CBM and catalytic domain[35]. The Bli PelB possibly evolved in similar fashion in order to adopt the changing environment. The role of CBM13 in increased thermal stability was also reported in alginate lyase from *Agarivorans* sp. L11[36], in contrast its presence in XynB decreased the thermostability of xylanase[37]. However, to further establish the role of CBM13 for thermo stability in Bli PelB, crystal structure analysis needs to done.

One of the most important notable features observed for Bli PelB was high substrate specificity towards the highly methylated pectin than PGA, similar to what was observed for pectate lyase from *Bacillus* sp. BP 23[38]. The PelB of *Paenibacillus amylolyticus* showed the maximum activity on 20-34% methylated pectin[39]. However, some of pectate lyase like from *Bacillus subtilis*[40] and *Bacillus* sp. RN1[41] exhibited most activity with substrate having very low or moderate degree of esterification. In contradiction, pectate lyase from *Bacillus* sp. N16-5[42], *B. licheniformis*[43], *Paenibacillus* sp. 0602[44] and *Erwinia carotovora*[45] showed highest activity with PGA. The reduction in thermal stability of CD Bli PelB is observed in many enzymes where removal of carbohydrate binding module influences the enzyme properties[46]. It may cause the alteration in activity or lose the affinity for crystalline substrate[46-51].

CONCLUSION

Thermo stability of Bli PelB coupled to its high specific activity on esterified substrate makes it an attractive candidate to be employed in textile industries for fiber degumming process. A hypothetical protein encoded by PelB of *B. licheniformis* was found to be mesophilic and alkaline. To date, this is first report to characterize the pectate lyase 1B which contains family 13 CBM. Further probe is requiring for establishing the role of CBM13 for pectin degradation.

SIGNIFICANCE STATEMENTS

- The CBM present in *pelB* is the first report for pectate lyase B having family 13 CBM
- Removing CBM13 decreased the thermal stability and enzyme activity shows that the CBM13 is essential part of the pectate lyase B
- These findings will further create base for establishing the exact mechanism of CBM13 in pectate lyase B

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