Amino groups of chitosan are crucial for binding to a family 32 carbohydrate binding module of a chitosanase from Paenibacillus elgii

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ABSTRACT

We report here the role and mechanism of specificity of a family 32 carbohydrate binding module (CBM32) of a glycoside hydrolase family 8 chitosanase from Paenibacillus elgii (PeCsn). Both activity and mode of action of PeCsn towards soluble chitosan polymers were not different with/without the CBM32 domain of P. elgii (PeCBM32). The decreased activity of PeCsn without PeCBM32 on chitosan powder suggested that PeCBM32 increases the relative concentration of enzyme on the substrate and thereby enhanced enzymatic activity. PeCBM32 specifically bound to polymeric and oligomeric chitosan, and showed very weak binding to chitin and cellulose. In isothermal titration calorimetry, the binding stoichiometry of 2 and 1 for glucosamine monosaccharide (GlcN) and disaccharide (GlcN)2, respectively, was indicative of two binding sites in PeCBM32. A 3D-model-guided site-directed mutagenesis (SDM) and the use of defined disaccharides varying in the pattern of acetylation suggested that the amino groups of chitosan, and the polar residues E16 and E38 of PeCBM32 play a crucial role for the observed binding. The specificity of CBM32 has been further elucidated by a generated fusion protein PeCBM32-eGFP that binds to the chitosan exposing endophytic infection structures of Puccinia graminis f. sp. tritici. Phylogenetic analysis showed that CBM32s appended to chitosanases are highly conserved across different chitosanase families suggesting their role in chitosan recognition and degradation. We have identified and characterized a chitosan-specific CBM32 useful for in situ staining of chitosans in the fungal cell wall during plant-fungus interaction.

INTRODUCTION

Carbohydrate binding modules (CBMs) are distinct structural folds of a stretch of amino acids within carbohydrate-active enzymes having carbohydrate-binding activity. Many carbohydrate-active enzymes belonging to glycoside hydrolases (GHs) are associated with non-catalytic CBMs that
A chitosan specific CBM32 from *Paenibacillus elgii*

CBMs increase the activity of endo-acting enzymes several fold, by increasing the concentration of appended enzymes in the vicinity of insoluble substrates and also by targeting the enzymes to its cognate substrates (1, 2). There are also instances where the ligand specificity of CBMs does not match to the activity of the catalytic module (3, 4, 5). Based on the nature of binding, inferred from structural and functional data, CBMs were classified into A, B and C types (1, 6).

CBMs of type A have a planar binding surface that is rich in aromatic amino acids, and bind to surfaces of crystalline chitin or cellulose. Binding sites of type B show extended clefts or grooves that bind internally on glycan chains and accommodate longer sugar chains, whereas type C CBMs bind to shorter oligosaccharides with a shallow surface site binding pocket.

Members grouped under CBM family 32 are more diverse, prevalent in the GHs of bacteria and bind to a variety of carbohydrate ligands (7). A few bacterial family 32 CBMs have been functionally characterized for their ligand specificities. CBMs of a GH89 from *Clostridium perfringens* binds to galactose, GalNAc and GlcNAc-α-1,4-Gal (8). CBM32 from an N-acetyl-β-hexosaminidase GH84C binds preferentially to β-D-galactosyl-1,4-β-D-N-acetylglucosamine (LacNAc) followed by lactose and galactose (5). A non-catalytic periplasmic protein belonging to CBM32 from *Yersinia enterocolitica* was shown to interact with polygalacturonic acid (9). CBM32 of sialidase from *Micromonospora viridifaciens* recognises galactose and lactose (10). Cocrystallisation of CBMs with their preferred ligands showed differences in the residues in the loop region of binding sites. A subtle variation in the substitution of amino acids resulted in a change in the pattern of molecular interaction of CBMs with their ligands (5). Multiple CBMs of family 32 appended to a single enzyme had different ligand specificities and were phylogenetically distinct, suggesting that enzymes containing multiple copies of CBMs possess complex mechanisms of ligand recognition (8). The members of CBM32 with low sequence identity are known to bind to one type of sugar moiety, suggesting an inability to predict binding specificity and affinity of family 32 CBMs from their primary structure. For example, CBM32-1 from *CpGH84A* lacks significant amino acid sequence identity to other galactose specific CBM32s, but yet preferred galactose-configured sugars with an unusual mode of recognition (11). The diversity and differential ligand binding specificity, and potential biotechnological applications reinforce the necessity to study CBM32-carbohydrate interactions to understand their specificities and binding mechanisms.

Chitosan is the most promising and advanced functional biopolymer in the family of polysaccharides obtained by partial de-N-acetylation from one of the most abundant renewable resources on earth, chitin. Chitin is a linear polysaccharide consisting of N-acetylglucosamine (GlcNAc) residues only, but chitosans are linear co-polymers of GlcNAc and glucosamine (GlcN) residues. In nature, chitosan is found in fungal cell walls of zygomycetes and in the cell walls of infection structures of certain fungi as part of their defence strategy against plants (12, 13). Chitosan being amorphous, compared to crystalline chitin, easily amenable to enzymatic hydrolysis by chitosanases (EC 3.2.1.132). Chitosanases occur in seven different GH families (14, 15). Often chitosanases have a single catalytic domain with no auxiliary domains, unlike chinases and cellulases which are frequently associated with CBMs. The mechanism of chitosan binding and degradation by chitosanases, in comparison to chitin and cellulose degradation, is not fully understood due to a lack of well defined chitosan substrates in terms of their degree of acetylation (DA) and pattern of acetylation (PA). Further, the amorphous nature of chitosan suggests that the pattern of
chitosan-binding by CBMs present in multidomain chitosanases may differ from CBMs that bind and enhance the degradation of crystalline chitin and cellulose.

To date, two tandemly occurring discoidin domains (DD1 and DD2) belonging to CBM32 from a chitosanase/glucanase of *Paenibacillus fukuinensis* are reported to have chitosan binding ability (16). Binding experiments performed by ITC revealed that irrespective of high protein sequence identity (74%), DD1 and DD2 (hereafter denoted as CBM32-1 and CBM32-2) displayed different binding affinity and specificity towards chitosan oligosaccharides. Although CBM32-1 and CBM32-2 bind chitosan oligosaccharides, the binding affinity of CBM32-1 was higher than CBM32-2. Furthermore, CBM32-2 appeared to be less specific to chitosan (in comparison to CBM32-1) and showed binding to laminarioligosaccharides and cello-oligosaccharides.

We assessed chitinolytic bacterial diversity from chitin-rich soil and identified a bacterium, namely *Paenibacillus elgii* SMA-1-SDCH02 with many chitin/chitosan-degrading enzymes (17, 18). Here, we present the role of a CBM32 in a GH8 chitosanase from *P. elgii* (PeCsn). Specificity and affinity of PeCBM32, associated with PeCsn, towards chitosan polymers and oligomers were studied by dot blot assay, CD spectroscopy-based thermal unfolding, and isothermal titration calorimetry. For a better understanding of the specific interaction, we have identified crucial amino acid residues by 3D-model-guided site-directed mutational approach. We also show that PeCBM32 is useful for *in situ* staining of chitosan of fungal cell wall during plant-fungus interaction.

**RESULTS**

**Effect of deletion of CBM32 from PeCsn on substrate hydrolysis**- The deduced polypeptide sequence of PeCsn, comprising 623 amino acids, was analysed for functional domains and motifs using the SMART (Simple Modular Architecture Research Tool) data base. PeCsn appeared as a multi-domain protein with an N-terminal GH8 catalytic domain and a C-terminal CBM32 linked by a fibronectin type III (FN3) domain (Fig. 1). To understand the role of PeCBM32 in chitosan degradation, truncated proteins viz. GH8 (lacking FN3 and CBM32) and GH8FN3 (lacking CBM32) were generated. The molecular masses calculated from the amino acid sequences of the truncated proteins were in reasonable agreement with those assessed by SDS-PAGE (Table S1).

The kinetics of hydrolysis of PeCsn and its truncated mutants was determined using 0% DA chitosan as substrate (Fig. 2A). The derived kinetic values (\(K_m\), \(k_{cat}\) and \(k_{cat}/K_m\)) are summarized in Table 1. The two truncated protein variants GH8FN3 and GH8 showed a minor increase in the \(K_m\) value in comparison to the wild type enzyme. The overall catalytic efficiency (\(k_{cat}/K_m\)) of PeCsn, GH8FN3 and GH8 remained the same suggesting no remarkable influence of PeCBM32 on the activity when 0% DA chitosan solution was the substrate (Table 1). The hydrolytic activity of both PeCsn and GH8 towards the chitosan polymers of different DAs decreased substantially when the DA of substrate increased. However, no significant difference in activity was observed between the wild type and the truncated protein (Fig. 2B). The activity of GH8 on 26% DA powdered chitosan was reduced more than three fold in comparison to the full length protein (Fig. 2C). To discern whether PeCBM32 influences the pattern of product formation, we analysed the formation of hydrolysed products both qualitatively and quantitatively using UHPLC-ELSD-ESI-MS. Hydrolysed products ranging from chitosan disaccharide to heptasaccharide were observed at the early phase of degradation (Fig. 3, A and B, Fig. S1). With increase in time, the quantity of all oligomeric products increased progressively both for the wild type and the truncated...
protein. No noticeable difference in the quantity and pattern of product formation was observed between GH8 and the wild type protein.

**Binding of PeCBM32 towards polymeric chitosan substrate**- The binding specificity of PeCBM32 was detected by a chemiluminescence assay using StrepII antibodies conjugated with HRP. Binding was detected up to 20% DA chitosan with the maximum signal observed for 0% DA chitosan polymer (Fig. 4). At a quantity of 0.2 µg, the signal was detected only for 0% DA chitosan polymer. We also clearly observed that the affinity of PeCBM32 decreased with increasing DA. PeCBM32 had no detectable affinity to fully acetylated glycol-chitin.

The binding of PeCBM32 to defined DA chitosan polymers was further investigated by measuring the unfolding transition temperatures \( (T_m) \) using CD spectroscopy at 218 nm as shown in Fig. 5. The transition temperature of thermal unfolding \( (T_m) \) of PeCBM32 was maximum in the presence of 0% DA chitosan \( (\Delta T_m = 7.2^\circ C) \) and 11% DA chitosan \( (\Delta T_m = 7.2^\circ C) \). However, elevation of \( T_m \) was less for 20% and 60% DA chitosan showing \( \Delta T_m \) values of 4.4ºC and 2.9ºC, respectively (Table 2). PeCBM32 bound to chitosan polymer with higher affinity; increase in DA of chitosan polymer resulted in decreased binding.

**Binding of PeCBM32 towards oligomeric substrates**- The binding of GlcN, (GlcN)\(_2\), (GlcN)\(_3\), (GlcN)\(_4\), (GlcNAc)\(_2\), (GlcNAc)\(_3\) and cellobiose to PeCBM32 was studied using ITC at pH 5.6 and 25ºC. Typical ITC thermograms and theoretical fits to the experimental data for chitosan oligosaccharides are shown in Fig. 6. For GlcN, the stoichiometry ‘n’ was 2.0, whereas for (GlcN)\(_2\), (GlcN)\(_3\) and (GlcN)\(_4\) the ‘n’ value was close to unity (Table 3). GlcN bound with a \( K_b \) of 4×10\(^5\) M\(^{-1}\) and the binding was enthalpy driven \( (\Delta H^o = -7.0 \text{ kcal.mol}^{-1}) \) with a very low entropy penalty \( (\Delta S^o = -6.4 \text{ cal.mol}^{-1}.K^{-1}) \). For (GlcN)\(_2\) binding, the \( K_b \) was 1.4×10\(^5\) M\(^{-1}\) and the process was also enthalpy driven \( (\Delta H^o = -11.6 \text{ kcal.mol}^{-1} \text{ and } \Delta S^o = -15.6 \text{ cal.mol}^{-1}.K^{-1}) \). When (GlcN)\(_3\) and (GlcN)\(_4\) were used as ligands, similar \( K_b \) values of 1.9×10\(^5\) M\(^{-1}\) and 2.2×10\(^5\) M\(^{-1}\) were observed. The binding of (GlcN)\(_3\) and (GlcN)\(_4\) had a favourable enthalpic contribution with \( \Delta H^o \) values of -11.4 and -13.5 kcal.mol\(^{-1}\), respectively with little entropic contribution (Table 3). Binding of (GlcNAc)\(_2\) and (GlcNAc)\(_3\) had a similar \( K_b \) value of 9.0×10\(^3\) M\(^{-1}\). However, the binding of (GlcNAc)\(_2\) and (GlcNAc)\(_3\) was at least 15 and 20-fold weaker than (GlcN)\(_2\) and (GlcN)\(_3\) respectively, clearly indicating that at least some of the amino groups of the chitosan oligomers are important for their association with PeCBM32 (Table 3).

Similarly, the binding of cellobiose was also low with a \( K_b \) of 7.0×10\(^3\) M\(^{-1}\) (Fig. 6). To know the effect of pH on the binding, ITC experiment was conducted using (GlcN)\(_3\) at pH 7.0 by assuming that the protonation state of the amino groups of glucosamine sugars could affect the thermodynamic parameters (Fig. 6, Table 3). The binding of (GlcN)\(_3\) at pH 7.0 was similar to the binding at pH 5.6 having a \( K_b \) value of 1.5×10\(^5\) M\(^{-1}\) with a favourable enthalpy contribution \( (\Delta H^o = -14.3 \text{ kcal.mol}^{-1} \text{ and } \Delta S^o = -24.3 \text{ cal.mol}^{-1}.K^{-1}) \).

To understand the influence of PA of chitosan oligomer on PeCBM32, we used chemically synthesised pure chitosan disaccharides having GlcN unit either at non-reducing (GlcN-GlcNAc) or reducing end (GlcNAc-GlcN). The ITC isotherms and theoretical fits to the experimental data for GlcN-GlcNAc and GlcNAc-GlcN are shown in Fig. 6. For both these disaccharides, the ‘n’ value was ~1 indicating an equimolar stoichiometry. The disaccharide GlcN-GlcNAc bound with a \( K_b \) of 2.0×10\(^5\) M\(^{-1}\) and the binding was enthalpy driven \( (\Delta H^o = -7.5 \text{ kcal.mol}^{-1}) \) with a very low entropic penalty \( (\Delta S^o = -5.7 \text{ cal.mol}^{-1}.K^{-1}) \). For GlcNAc-GlcN binding, the \( K_b \) value (1.0×10\(^4\) M\(^{-1}\)) was near to half of the \( K_b \) of GlcN-GlcNAc,
and the reaction was also enthalpy driven ($\Delta H^\circ = -8.6$ kcal.mol$^{-1}$ and $\Delta S^\circ = -10.3$ cal.mol$^{-1}$.K$^{-1}$). Overall, the affinities of PeCBM32 towards chitosan disaccharides with different PA was found in the order of GlcN-GlcN $>$ GlcN-GlcNAc $>$ GlcNAc-GlcN $>$ GlcNAc-GlcNAc.

**Identification of amino acid residues important for chitosan binding** - The binding studies performed by dot blot, circular dichroism (CD) spectroscopy and ITC confirmed the specific binding of PeCBM32 to both chitosan polymers and oligomers. To further understand the mechanism of interaction, particularly to identify the amino acid residues responsible for specific binding, we generated PeCBM32 variants by SDM and compared their binding with the wild type protein.

The modeled structure of PeCBM32 had one $\alpha$ helix and 8 $\beta$ strands connected by loops. Consistent with this, secondary structure analysis by CD spectroscopy revealed the presence of ~65% antiparallel $\beta$ strands and $\beta$ turns (data not shown). Docking of (GlcN)$_2$ with PeCBM32 revealed that interaction of all the ligand conformations was restricted to one shallow binding cleft on the protein. Out of ten, seven ligand conformations were associated with nearly the same binding free energies at one binding site. Among these, one conformation with the lowest binding free energy value of $-5.62$ kcal.mol$^{-1}$ (Table S2) was selected and analysed for interactions in PyMol and Discovery Studio. The residues forming interactions with ligand were E16 and S18 present in the loop between $\beta_1$ strand and $\alpha$ helix, and W34, S36 and K37 present in the loop between $\alpha$ helix and $\beta_2$ strand. The carboxylate group of S36 formed a hydrogen bond with the amino group of reducing end sugar moiety (Fig. 6B). The carboxylate group of E16 and the hydroxy group of S18 with 2.5 and 2.7 Å distances, respectively formed hydrogen bonds with the amino group of non-reducing end sugar moiety of the ligand (Fig. 7). The carboxylate group of E16 also formed a hydrogen bond with the hydroxy group on C3 of reducing end sugar moiety. The hydroxy group on C6 of reducing sugar moiety formed hydrogen bond with amino group of W34. Docking studies with (GlcN)$_4$ also showed the involvement hydrogen bonds with residues similar to (GlcN)$_2$ (except S18) with additional accessory residues viz. E38 (loop between $\alpha$ helix and $\beta_2$ strand), E63, Y66 (loop between $\beta_3$ and $\beta_4$ strand), T119 and S120 (loop between $\beta_7$ and $\beta_8$).

In addition to docking studies, we also considered the amino acid conservedness in PeCBM32 (Fig. 8) for selecting residues for SDM. Residues E16, S18, W34, S36 and E38 were substituted by Ala to know their role in binding. Amino acid sequence comparison of CBM32s appended to various chitosanases showed that E38 is highly conserved except a substitution of Tyr in CBM32-2 of *P. fukuinensis* (Fig. 8). Therefore, to know the influence of an aromatic amino acid at this position, E38 was substituted by Phe. The mutants E16A, S18A, E38A and E38F were produced and purified (Table S1). For W34 and S36, we couldn’t get sufficient protein in a soluble form to perform ITC-based interaction studies.

The binding of PeCBM32 variants to (GlcN)$_4$ was investigated using ITC at pH 5.6 and 25°C (Fig. 9). For all the mutants, the stoichiometry (n value) was ~1 (Table 4). The mutant E16A had a $K_b$ of $1.9 \times 10^4$ M$^{-1}$ and the binding was enthalpy driven ($\Delta H^\circ = -6.7$ kcal.mol$^{-1}$) with a very low entropy penalty ($\Delta S^\circ = -2.9$ cal.mol$^{-1}$.K$^{-1}$). For E38A binding, the $K_b$ was $1.8 \times 10^4$ M$^{-1}$ and the process was also enthalpy driven ($\Delta H^\circ = -7.0$ kcal.mol$^{-1}$ and $\Delta S^\circ = -3.8$ cal.mol$^{-1}$.K$^{-1}$). For the mutant S18A, the binding was similar to the wild type having $K_b$ value of $2.0 \times 10^3$ M$^{-1}$ with a favourable enthalpy contribution ($\Delta H^\circ = -11.8$ kcal.mol$^{-1}$ and $\Delta S^\circ = -15.5$ cal.mol$^{-1}$.K$^{-1}$). For E38F, we couldn’t obtain a considerable thermogram indicating very weak or no binding.
**Phylogenetic analysis of CBM32s:** A data set of 48 CBM32 sequences including many representative members of GHs was collected from the CAZy and NCBI database. Although chitosanases belong to several GH families, only family 8 and 46 chitosanases were found to have CBM32 sequences as their accessory domains. Multiple sequence alignment of CBM32s from different enzymes did not lead to a good alignment (data not shown) and alignment within the 18 chitosanase-appended CBM32s showed a good conservation of overall amino acid residues (Fig. 8). Among the chitosanase-appended CBM32s, the lowest identity was of 44.53% between PeCBM32 and GH46 CBM32 from *Streptomyces* sp. whereas the highest identity of 87.79% was observed between GH8 CBM32 sequence of *Streptomyces exfoliates* and *Streptomyces venezuelae*. Phylogenetic analysis also showed that CBM32 sequences connected to chitosanases were clustered together except a CBM32 sequence appended to a GH8 of *Kribbella catacumae* (Fig. 10).

**In situ staining of chitosan using PeCBM32-eGFP:** A fusion protein PeCBM32-eGFP was generated by fusing eGFP at the C-terminal of PeCBM32 with the expected size of 45 kDa on SDS-PAGE. Differentiation of infection structures was induced by giving mild heat shock to the germinating urediniospores of the wheat stem rust fungus. After induction, urediniospores of wheat rust fungus differentiate into infection structures, namely, germ tube, appressorium, substomatal vesicle, and infection hyphae (Fig. 11). The germ tube and appressorium expose mainly chitin whereas the endophytic structures like substomatal vesicle and infection hyphae expose chitosan on the surface of their cell walls (13). Simultaneous staining of germlings with PeCBM32-eGFP and chitin-specific lectin (WGA) coupled to Texas Red showed the binding of PeCBM32-eGFP to substomatal vesicle (green fluorescence) whereas WGA bound to appressoria (red fluorescence) as shown in Fig. 11. We also observed lower GFP fluorescence signal in the infection hypha and urediniospores.

**DISCUSSION**

**Role of CBM32 in PeCsn:** CBMs play an important role in hydrolytic enzymes that mediate the recycling of carbon and nitrogen in the biosphere. The diversity of CBMs in terms of their sequence, specificity and mechanism of binding with ligand, offers an attractive model for studying CBM-carbohydrate interaction (1). Here, we have investigated the function of a CBM32 appended to the GH8 family chitosanase (PeCsn) of *P. elgii* to elucidate ligand specificity using well defined chitosans. Steady state kinetic data suggested that there was no great difference in the *Km* and the overall catalytic efficiency on 0% DA chitosan polymer between the wild type and the truncated mutants, GH8 and GH8FN3 (Table 1). Deletion of CBMs from their catalytic modules belonging to different GHs decreases the activity of many enzymes on insoluble, but not on soluble substrates (1, 19). To check the influence of CBM32 on the hydrolytic acivity of PeCsn, we used both chitosan solution and chitosan powder as substrates. Chitosans are in general known to be soluble at acidic pH and the solubility depends on the DA and molecular weight. The solubility of chitosan decreases with increase in DA and *Mw* (20, 21). In the present study we used a higher molecular weight chitosan of moderate DA to minimize the solubility of chitosan powder. On polymeric chitosan solutions of DA 0, 11, 26, 37 and 61%, PeCsn and GH8 showed nearly similar activity (Fig. 2B). However, activity of PeCsn on powder chitosan of DA 26% was over three fold higher than GH8 (Fig. 2C), indicating that PeCBM32 possibly increased the concentration of enzyme in close proximity of powder chitosan and hence improved catalytic activity. The enhanced binding to amorphous chitosan may also lead to swelling of chitosan by disrupting interchain hydrogen bonding and thereby changing its...
bulk properties as observed for amorphous cellulose degradation (22). Reyes-Ortiz et al. (23) demonstrated that the fusion of a CBM increased binding and facilitated penetration of a chimeric cellulase into the bulk of the amorphous cellulose leading to enhanced cellulose activity.

CBMs enhance the activity of their cognate enzymes by either disruption and modification of polysaccharide structures (24) or increasing enzyme concentration in the vicinity of substrate and sometimes by targeting specific carbohydrate structures in a native biomass containing different polysaccharides (2, 25). Mizutani et al. (26) showed that a CBM32 appended to a GH5 from Clostridium thermocellum directly influenced the mode of catalysis, resulting in difference in product formation in the presence and absence of CBM32. However, comparison of the hydrolysed products generated by PeCsn and GH8 at a very early phase of degradation of 0% DA chitosan didn’t show any noticeable difference in the pattern and quantity of product formation (Fig. 3). Taken together, the soluble chitosan hydrolysis data suggested that PeCBM32 neither enhances the hydrolytic activity nor affects the mode of action of PeCsn. However, the affinity and specificity towards chitosan substrates may permit PeCBM32 to adhere and maintain proximity to chitosan, which occurs along with several other carbohydrate polymers in nature. Therefore, it was paramount for us to study the specificity of PeCBM32 in recognising carbohydrate ligands.

Specificity of PeCBM32- Many ligand molecules like galactose, lactose, sialic acid, LacNAc, chitosan oligomer and mannan oligomer are recognized by CBM32 appended to different enzymes (7, 16, 26). We studied the influence of DA of chitosan polymers on the binding of PeCBM32. Both dot blot and thermal denaturation study in presence of defined DA polymers showed a reduction in affinity or no affinity towards higher DA chitosan (Figs. 4 & 5), suggesting the specific binding of PeCBM32 to chitosan. PeCBM32 also didn’t show detectable binding to laminarin and dextran (data not shown), and its binding to (GlcNAc)_3 and cellobiose was about 20 times weaker than to (GlcN)_3 and (GlcN)_2, respectively (Table 2), suggesting specific binding of PeCBM32 towards chitosan polymers and oligomers. The specificity and affinity with which PeCBM32 binds to chitosan oligomers suggests its closeness to a chitosan specific CBM32-1 of P. fukuinensis. Consistent with this result, PeCBM32 showed 68 and 59% identity to CBM32-1 and CBM32-2 of P. fukuinensis, respectively. PeCBM32 is the second chitosan-specific CBM32 which bound to chitosan polymers and oligomers with greater affinity, and is consistent with the general postulation that CBM specificity matches with the function of the cognate catalytic module. Therefore, it is conceivable that the specificity of PeCBM32 assists in maintaining proximity of PeCsn to the chitosan polymer as observed with other CBMs (25, 27).

Binding sites in PeCBM32- The affinity of CBM32 towards chitosan oligomers, (GlcN)_2-(GlcN)_6 is reported using ITC (16). Here, we report the binding of GlcN with a stoichiometry of 2 and $K_b$ of $4 \times 10^3$ M$^{-1}$, clearly indicating the accommodation of two GlcN residues by PeCBM32 (Table 3). This observation taken together with the binding of longer chitosan oligomers, (GlcN)$_2$-(GlcN)$_4$ with an equimolar stoichiometry suggest PeCBM32 has either two different binding sites or has one binding site with 2 binding subsites. The decrease in the binding affinity of PeCBM32 in the order of GlcN-GlcN >> GlcN-GlcNAc > GlcNAc-GlcN > GlcNAc-GlcNAc is indicative of two subsites with higher selectivity for glucosamine sugars over N-acetyl glucosamine sugars. However, the possibility of two different binding sites with accommodation of only GlcN in one of the sites can’t be ruled out. Therefore, cocrystallisation of PeCBM32 with its preferred ligands could provide detail insight on the binding site of PeCBM32. The
binding affinities ($K_b$) increased marginally with the increase in chain length of chitosan oligomers (Table 3), indicating the extended part of longer oligomers, beyond the primary interaction site, may be involved in additional, minor interactions with the protein. This was further observed in our docking study, where residues such as E38, E63, Y66, T119 and S120 showed potential interaction with (GlcN)$_4$ in addition to the residues that interact with (GlcN)$_2$. The substitution of Ala in one of these accessory interacting residues, E38A weakly bound to (GlcN)$_4$ (12 times weaker) when compared to the wild type protein (Table 4), supporting the involvement of residues other than primary binding site for interaction of longer chain chitosan oligomers. In mammalian lectins ERGIC-53 and VIP36, additional secondary binding sites were shown to interact with additional sugar residues, apart from the common primary mannose binding site (28, 29). The extended binding site can also provide specificity for different sugar moieties.

**Binding mechanism**- Direct hydrogen bond formation in Type A CBMs had little effect in binding towards crystalline polysaccharide ligands (30). However, Ala substitution of amino acids, which make direct hydrogen-bonding in B Type and C Type CBMs resulted in a considerable loss of binding affinity towards their respective ligands (31, 32, 33). Our results provide compelling evidence that amino groups of chitosan participate in binding to PeCBM32. Molecular docking of both (GlcN)$_2$ and (GlcN)$_4$ ligands revealed that the carboxylate group of E16 formed one hydrogen bond with amino group of non-reducing end sugar moiety of the ligand (Fig. 7). The substitution of E16 by Ala (E16A) decreased the binding affinity by 11 times, suggesting the crucial role of E16 for chitosan binding. However, we didn’t observe any reduction in binding affinity in case of S18A mutant which showed a formation of hydrogen bond with the amino group of non-reducing sugar of (GlcN)$_2$ but not with (GlcN)$_4$. Therefore, we speculate that S18 may not be crucial for longer chain chitosan binding, and needs further structural evidence. The binding affinity for (GlcN)$_4$ was 12 times lesser for E38A which interacts with the hydroxy group on C4 of non-reducing end of (GlcN)$_4$ sugar (Table 4). Similar interaction between polar amino acids with the amino groups of GlcN was observed in a structural study of a chitosanase from Bacillus sp. K7 (34). Substitution of E38 by an aromatic amino acid Phe reduced the binding affinity drastically, suggesting the introduction of hydrophobic residues may alter the local conformation in the loop region affecting the chitosan-binding site. This finding was further supported by the observation that CBM32-2 of *P. fukuinensis* (16) which has a Tyr at this position showed lower binding affinity towards chitosan oligomers.

To obtain further evidence for the involvement of amino groups in the binding of chitosan oligosaccharides to PeCBM32, we have synthesised well defined chitosan disaccharides of two different patterns i.e. GlcNAc at non-reducing end (GlcNAc-GlcN) and at reducing end (GlcN-GlcNAc) and investigated their binding by ITC (Fig. 6). The binding affinities for GlcNAc-GlcN and GlcN-GlcNAc decreased substantially with the $K_b$ values being 12 and 7 times weaker in comparison to (GlcN)$_2$, respectively, indicating the acetylation of GlcN either at the non-reducing or reducing end decreased the binding affinity. The reduction of binding affinity suggests the disfavour of bulkier acetyl group of GlcNAc owing to the well-ordered ligand binding site topology specific for chitosan, but not for chitin. For instance, the positioning of acetamido group needs a hydrophobic pocket of aromatic amino acids for providing substantial van der Waals interactions to select GlcNAc over glucose (6).

Binding of all the chitosan oligomers investigated here was exothermic with very low entropic contribution (Table 3 & 4). High enthalpic contribution in PeCBM32-
A chitosan specific CBM32 from *Paenibacillus elgii*

Chitosan binding was presumably due to an increased number of hydrogen bonds and heteroatom mediated van der Waals interactions at the ligand-target interface, though the possibility of electrostatic interaction can’t be ruled out. Binding of (GlcN)$_3$ to PeCBM32 at pH 7.0 (deprotonated state as the $pK_a$ value of the amino group of glucosamine residue was reported to be 6.4) was not affected, indicating that electrostatic interactions may play a subordinate role in chitosan binding. This result is in accordance with the earlier report by Shinya et al. (16) wherein no considerable difference in binding affinity was observed at pH 5.0 and 7.0 for CBM32-1 and CBM32-2. In an analogous study on DNA-protein interaction, it was reported that both electrostatic and non-electrostatic forces are involved in binding, but the sequence specific binding occurred through non-electrostatic forces driven by enthalpic component (35). These observations suggest that PeCBM32-chitosan interaction may not be simply attributed to the positive charge which chitosan bear at acidic pH, rather a specific interaction mediated by the well ordered binding site topology of PeCBM32. Thus, our data on point mutation and use of specific pattern chitosan disaccharides suggested the crucial role of polar residues of PeCBM32 and amino groups of chitosan in providing specificity in PeCBM32-chitosan interaction.

**Potential application of PeCBM32** - Typically, fungal cell walls contain chitin as a structural element, but some plant-pathogenic fungi produce chitin deacetylase to convert the chitin into chitosan to protect their cell wall as part of their pathogenicity mechanism (13). Even a fungal pathogen of human also converts surface exposed chitin to chitosan to protect their cell wall as part of their pathogenicity mechanism (13). Even a fungal pathogen of human also converts surface exposed chitin to chitosan to protect their cell wall as part of their pathogenicity mechanism (13). Even a fungal pathogen of human also converts surface exposed chitin to chitosan to protect their cell wall as part of their pathogenicity mechanism (13). Even a fungal pathogen of human also converts surface exposed chitin to chitosan to protect their cell wall as part of their pathogenicity mechanism (13). Even a fungal pathogen of human also converts surface exposed chitin to chitosan to protect their cell wall as part of their pathogenicity mechanism (13).

Simultaneous double staining with PeCBM32-eGFP and WGA showed detection of chitosan on the surface of substomatal vesicles and infection hyphae, whereas chitin was detected in the appressoria (Fig. 11). The observation of stronger GFP signal from chitosan in the substomatal vesicle (strongly deacetylated than the one exposed on the surface of the infection hyphae) was consistent with the findings of Nampally et al. (47). Our result on simultaneous staining by two binding proteins suggested that PeCBM32-eGFP can be used with other carbohydrate specific proteins or lectins for localization studies in biological tissues.

**Conclusions** - Our results demonstrated that CBM32 of PeCsn displays specificity towards both polymeric and oligomeric chitosans. The PeCBM32 has two binding sites, and the amino groups of chitosan were found to be crucial for their interaction owing to the well-ordered substrate binding site topology specific for chitosan, but not for chitin. The module plays an important role in the hydrolysis of powder chitosans presumably by enhancing the relative concentration of enzyme on chitosan. Our findings also established that CBM32 of PeCsn would be useful for localization of chitosan in biological tissues. The phylogenetic analysis of CBM32s appended to GHs provides persuasive evidence for the evolutionary conservedness of chitosan specific CBM32 in bacterial chitosanases. This report highlights the biological importance of identifying CBMs and
understanding the mechanisms of their concomitant ligand binding.

**EXPERIMENTAL PROCEDURES**

**Materials**- Polymeric chitosan substrates with a DA of 0, 20 and 26% were kindly provided by Dr. Dominique Gillet, Mahtani Chitosan Pvt., Ltd. (Veraval, India). The rest of the polymeric chitosans with different DAs were prepared by partial re-N-acetylation using acetic anhydride in 1,2-propanediol (40) and the resulting chitosan polymer were analysed by $^1$H-nuclear magnetic resonance (NMR) spectroscopy (41). The properties of chitosan polymers used are given in Table S3. Chitosan monosaccharide (GlcN), disaccharide (GlcN)$_2$, trisaccharide (GlcN)$_3$, tetrasaccharide (GlcN)$_4$, pentasaccharide (GlcN)$_5$, hexasaccharide (GlcN)$_6$ and chitin disaccharide (GlcNAc)$_2$ and trisaccharide (GlcNAc)$_3$ were obtained from Megazyme (Wicklow, Ireland) and Seikagaku Corporation (Tokyo, Japan). Cellobiose was purchased from Sigma-Aldrich.

**Chemical synthesis of chitobioside derivatives (GlcNAc-GlcN and GlcN-GlcNAc)-** Chitobioside derivatives GlcNAc-GlcN and GlcN-GlcNAc were synthesized using phenyl 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-1-thio-$eta$-D-glucopyranoside and tert-butyldimethylsilyl 3,6-di-O-benzyl-2-deoxy-2-azido-$eta$-D-glucopyranoside monomer building blocks. The glycosylation of 4-free azido sugar derivative with phthalimido N-protected glucosamine derivative yielded tert-butyldimethylsilyl 3,4,6,tri-O-acetyl-2-deoxy-2-phthalimido-$eta$-D-glucopyranosyl-(1→4)-3,6-di-O-benzyl-2-deoxy-2-azido-$eta$-D-glucopyranoside. The thioglycoside donor was activated under N-iodosuccinimide (NIS)-trifluoromethanesulfonic acid (TIOH) reagent system to get protected disaccharide. Selective deprotection of two different N-protecting groups viz. phthalimide and azide at the C2 position followed by global deprotection were carried out sequentially to obtain 2-acetamido-2-deoxy-$eta$-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-$eta$-D-glucopyranose and 2-amino-2-deoxy-$eta$-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-$eta$-D-glucopyranose. The structure of the disaccharides produced was assigned by $^1$H-NMR and MALDI-MS analysis (Fig. S2) and by comparing the results obtained with literature (42, 43). $^1$H-NMR (400 MHz, D$_2$O) resonances of GlcNAc-GlcN (amorphous solid): $\delta$ 5.07 (s, 0.5H), 4.83 (d, $J = 4.1$ Hz, 0.6H), 4.59 (dd, $J = 2.0, 8.3$ Hz, 1.4H), 3.83-3.34 (m, 18H), 1.93 (s, 3H, NCOCH$_3$). $^1$H-NMR (400 MHz, D$_2$O) resonances of GlcN-GlcNAc (amorphous solid): $\delta$ 5.11 (s, 1H), 4.50-4.39 (m, 0.4H), 4.03-3.31 (m, 16H), 2.76-2.43 (m, 2H), 1.96 (s, 3H, NCOCH$_3$).

**Plasmid construction and generation of mutants of PeCBM32**- The draft genome sequence information of *Paenibacillus elgii* B69, a soil isolate having broad-spectrum antimicrobial activity (44), was used to amplify the *Pecsn* gene of *P. elgii* (18). The SignalP server (http://www.cbs.dtu.dk/services/SignalP/) predicted the *Pecsn* to contain an N-terminal signal peptide-encoding sequence (126 bp). Using gene-specific forward and reverse primers, *Pecsn* was amplified without the signal peptide coding sequence and cloned into pET-28a-His6 at the NcoI and XhoI sites to make pET-28a-PeCsn-His6 construct. The *Pecsn* was also cloned in pET-22b-StrepII using Gibson assembly master mix (NEB) to generate Strep-tag fused protein for efficient purification (45). To make truncated genes of *Pecsn* lacking the CBM32 and FN3 domain, pET-22b-PeCsn-StrepII construct was used as template for amplification by 5’ phosphorylated primers. Similarly, the pET-28a-PeCBM32-His6 construct was made and used as template to create mutations in the CBM32 domain using 5’ phosphorylated mutagenic primers. To fuse with the enhanced green fluorescent protein (eGFP) (46) to PeCBM32, the plasmids pET-22b-StrepII-CSN-eGFP-His6 (47) and pET-22b-PeCsn-StrepII (this study) were used to amplify the respective fragments using a set of overlapping primers as per Gibson.
assembly manual. Finally, the fragments were assembled yielding the pET-22b-StrepII-PeCBM32-eGFP-His6 construct. The details of plasmid constructs used in this study are given in Table S4.

Protein expression, purification, and measurement- E. coli Rosetta 2 (DE3) [pLysSRARE2] harbouring the generated plasmid constructs served as expression strain and was cultivated in auto-induction medium (48). E. coli BL21 (DE3) harbouring pET-28a-PeCBM32-His6 and the plasmids coding for the PeCBM32 variants were induced by isopropyl-β-D-thiogalactopyranoside (IPTG). In auto-induction media, E. coli Rosetta 2 (DE3) [pLysSRARE2] cells were grown at 37°C for 3 h and then at 26°C for 20 h. E. coli BL21 (DE3) cells were grown at 37°C until the optical density reached a value of 0.6. Then IPTG at a final concentration of 0.1 mM was added and the cells were grown at 18°C for 20 h. His6-tagged proteins were purified from the sonicated supernatant by gravity flow affinity chromatography using Ni-NTA resin (49). The C-terminally StrepII-tagged proteins were purified using an ÄKTApame 4.01 fast protein liquid chromatography system (GE Healthcare, Germany) fitted with a 1 ml Strep-Tactin Superflow Plus Cartridge (Qiagen, Germany). The protein concentration was determined by a BCA protein assay kit (Thermo Scientific) using a bovine serum albumin (BSA) standard calibration curve.

Steady-state kinetics- The maximum activity for PeCsn was at 60°C and in a pH range between 5 and 6 (data not shown). For all the enzymatic assays, a pH of 5.6 (the maximum buffering capacity range of sodium acetate buffer) was used. Kinetic parameters of PeCsn and its truncated mutant were determined by incubating 110 nM enzyme with varying concentration of 0% DA chitosan ranging from 0.01-1.5 mg.ml⁻¹ for 5 min. Buffer, enzyme and substrate alone were taken as control for reducing end assay. The amount of reducing groups generated was determined by the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method as described earlier (50). Enzyme activity was defined as the release of one micromole of glucosamine per second under standard experimental conditions. Kinetic parameters were obtained by fitting values to the Michaelis-Menten equation by nonlinear regression function available in GraphPad Prism version 5.01 (GraphPad Software Inc., San Diego, CA).

Enzyme assays on chitosan solution and powder chitosan- Chitosans of different DA (Table S3) were mixed to a concentration of 2-5 mg.ml⁻¹ (depending on DA) in 50 mM of sodium acetate buffer pH 5.6. To dissolve the chitosan fully without any observable flakes or particles, the mixed chitosan solution was kept at 37°C with slow stirring for overnight. The obtained chitosan solutions of different DA are used for enzyme assays with a final concentration of 1 mg.ml⁻¹. For enzyme assays with powder chitosan, a high molecular weight (M_w = 385,000 Da) chitosan with DA of 26% was used. Reaction mixture containing 5 mg.ml⁻¹ chitosan powder and enzyme (separate reaction for both PeCsn and GH8) in sodium acetate buffer pH 5.6 was incubated for 10 min. The reaction was centrifuged at 16,100×g at 4°C for 10 min and the supernatant was immediately transferred to a pre-cooled eppendorf and the generated reducing groups was determined by MBTH method as described earlier (50).

Analysis of hydrolysed products by UHPLC-ELSD-ESI-MS- To analyse the influence of CBM32 on the chitosanase activity in terms of product pattern formation, the reaction was set up with 5 nM of each PeCsn and GH8 with 0% DA chitosan. At different time points, 10 µl of the reaction mixture was collected and mixed immediately with equal volume of 1 N of ammonium hydroxide to stop the reaction. Aliquots of 2 µl were analysed by UHPLC-ELSD-ESI-MS as described elsewhere (51) with minor modifications. In brief, an Acquity UPLC BEH Amide column was used to separate chitosan
oligomers by hydrophilic interaction chromatography (HILIC). Samples were run at a flow rate of 0.4 ml.min\(^{-1}\) under isocratic conditions with the following eluent: 80:20 acetonitrile/water, 10 mM NH\(_4\)HCO\(_2\) and 0.1% (v/v) formic acid. Mass spectra were acquired over a scan range from m/z 50–2000 in positive scan mode. Internal standards ranging from GlcN-(GlcN)\(_6\) of known concentration were injected for quantification of oligomers generated from polymer.

**Dot blot assay of PeCBM32**- In order to determine the binding specificity to polymeric substrates, chitosans with a DA of 0%, 11%, 20%, 37% and 61%, and glycol-chitin were spotted at two different concentrations, i.e. 1.0 µg and 0.2 µg onto a nitrocellulose membrane. The membrane was incubated at 70°C for 30 min to allow the chitosans and glycol-chitin to stick to the membrane as described elsewhere (47). To block the membrane, 3% biotin free milk powder in 1x Tris-buffered saline (TBS) was used and incubated for 1 h at 25°C. The membrane was washed with TBS for 15 min and incubated with 100 µg.ml\(^{-1}\) PeCBM32 containing 3% milk powder in TBS for 1 h. After washing with 1x TBS containing 0.05% (v/v) of both Tween 20 and Triton X-100 (twice for 15 min each), the membrane was incubated with Strept-Tactin antibodies conjugated with horseradish peroxidase (HRP). In a parallel independent experiment, instead of adding protein and antibody, the membrane was stained with Ponceau stain to confirm that the chitosan substrates were bound to the membrane. The signal was detected by chemiluminescence.

**Circular dichroism measurements for studying thermal unfolding**- Circular dichroism (CD) measurements were recorded on a Jasco-J810 spectropolarimeter. A change in thermal stability of PeCBM32 was monitored using the ellipticity changes at 218 nm by increasing the temperature at a rate of 1°C.min\(^{-1}\). To verify the influence of chitosans on thermal unfolding, different DAs of polymeric chitosan substrates (1 mg.ml\(^{-1}\)) were used and the final concentration of protein was 35 µM. The ellipticity values were normalized between 0 (native) and 1 (unfolded). The fraction of unfolded protein at each temperature was calculated from the CD value by linearly extrapolating pre- and post-transition baselines into the transition zone and plotted against the temperature.

**Isothermal titration calorimetry (ITC)**- The binding affinity and stoichiometry of PeCBM32 and its variants towards chitosan oligomers and other carbohydrates were quantified by ITC. Titrations were performed on a VP-ITC isothermal titration calorimeter from MicroCal (Northampton, MA, USA) at pH 5.6, which is within the optimal pH range for PeCsn activity. Titration at pH 7.0 was done only for (GlcN)\(_3\) in 50 mM sodium phosphate buffer. The purified proteins used in ITC measurements were dialysed extensively against either 50 mM sodium acetate buffer (pH 5.6) or sodium phosphate buffer (pH 7.0). The buffer from the final dialysate was filtered through a 4 µm membrane, and used to dissolve ligands and also for titrations at 25°C. PeCBM32 and chitosan oligosaccharide solutions were degassed under vacuum. Titrations were performed by injecting 5-10 µl aliquots of the carbohydrate ligand from a 2-15 mM stock solution taken in the calorimeter syringe into the reaction cell containing 80-340 µM of PeCBM32. A constant stirring speed of 300 rpm was maintained to ensure uniform mixing of the reactants during the titration. The titration data were analyzed using MicroCal Origin ITC software. Thermodynamic parameters such as change in enthalpy (ΔH), association constant (K\(_a\)) and binding stoichiometry (n) were obtained by nonlinear least-squares fitting of experimental data using the one set sites binding model in the MicroCal Origin software provided by the ITC manufacturer (52).
Molecular docking of PeCBM32 with (GlcN)$_2$ and (GlcN)$_4$. Amino acid sequence homology search by BLAST against the protein databank (PDB) was performed to identify 3D structures that could share high sequence homology to PeCBM32. The crystal structure of a lectin binding domain (4GW1) from Streptococcus mitis showed 37% identity to PeCBM32. The 3D structure of the PeCBM32 (Fig. 7A) was generated using the MODELLER v9.12 (53) and validated by performing Verify_3D (92.42% of residues had an average 3D-1D score > 0.2) and Ramachandran plot generated by PROCHECK analysis (91.2% of amino acids in the most favoured regions) (54). Molecular docking for PeCBM32 with (GlcN)$_2$ and (GlcN)$_4$ were performed by Autodock 4.2 (55). (GlcN)$_2$ and (GlcN)$_4$ ligands were extracted from crystal structure of the LysM effector protein of Cladosporium fulvum (4B8V), using Discovery studio 4.0. Polar hydrogens and atomic charges by the Kollman method were added to the modeled receptor molecule by Auto Dock Tools (ADT) graphical user interface. Torsion and rotatable bonds were defined and Gasteiger charges were added to the ligand molecule using ADT graphical user interface. The ligand was allowed to dock to the receptor within a grid box space of 56 x 66 x 70 grid points along X, Y and Z axes with 0.46 Å grid spacing. The centre of the grid was set to -7.405, -14.449 and -8.849 on XYZ coordinates. Docking was employed by using the Lamarckian Genetic Algorithm (LGA) available in Autodock 4.2 with parameters set to LGA population size: 150; GA runs: 10 and maximum number of energy evolutions: 250,00,000. During docking, a maximum number of top 10 conformers were considered, and the root-mean-square (RMS) cluster tolerance was set to 0.2 nm. Amongst the docked conformations of all ten ligands, the conformation with the lowest binding energy was visualised for detailed interactions in PyMol Molecular Graphics System 1.3 and Discover Studio 4.0.

In situ staining of chitosan in the fungal cell wall by PeCBM32-eGFP-Urediniospores of the wheat stem rust fungus Puccinia graminis f. sp. tritici (Eriks. & E. Henn) were used for induction of infection structures as described by Nampally et al. (47). For differentiation of infection structures, spores were sown in poly-styrene petri dishes containing 5 ml of sterile milliQ water and incubated for 50-60 min at 23°C followed by a mild heat shock (30°C) for 2 h and finally spores were incubated at 23°C over night for the development of infection structures. After the differentiation of infection structures, checked using a light microscope, germlings were incubated with 2% (wt/v) BSA in 1x phosphate-buffered saline (PBS) for 2 h followed by repeated washing in 1x PBS containing 0.05% (v/v) Tween 20. Subsequently, spores were incubated with 0.1 mg.ml$^{-1}$ PeCBM32-eGFP and wheat germ agglutinin (WGA) conjugated with Texas Red in TBS containing 5% BSA for 1 h at 25°C. After washing with 1x PBS containing Tween 20, spores were observed under confocal laser scanning microscope. The excitation/emission wavelengths for eGFP and Texas Red were 488/595 nm and 500 to 545/608 to 700 nm, respectively.
A chitosan specific CBM32 from Paenibacillus elgii

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Author contributions: ARP, BMM and SND planned the experiments. SND, MW, PKN, BB and RN performed the experiments. ARP, MJS, SND, PKN, BMM and RB analysed and interpreted the data. SND, ARP and MJS wrote the paper.

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A chitosan specific CBM32 from *Paenibacillus elgii*

FOOTNOTES

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1Abbreviations: DA, degree of acetylation; eGFP, enhanced green fluorescent protein; GlcN, chitosan monosaccharide; (GlcN)$_2$, chitosan disaccharide; (GlcN)$_3$, chitosan trisaccharide; (GlcN)$_4$, chitosan tetrasaccharide; (GlcN)$_5$, chitosan pentasaccharide; (GlcN)$_6$, chitosan hexasaccharide; (GlcNAc)$_2$, chitin disaccharide; (GlcNAc)$_3$, chitin trisaccharide; PA, pattern of acetylation; PeCBM32, carbohydrate binding module 32 of *PeCsn*; PeCsn, chitosanase of *Paenibacillus elgii*; WGA, wheat germ agglutinin

FIGURE LEGENDS

FIGURE 1. Schematic representation of the modular structure of *PeCsn*. Multi-domain *PeCsn* showing an N-terminal glycoside hydrolase 8 (GH8) catalytic domain, fibronectin type III (FN3) domain and a C-terminal carbohydrate binding module belonging to family 32 (CBM32). Modular boundaries are given as amino acid numbers. Double arrow below the schematic shows the truncated proteins produced in this study.

FIGURE 2. Comparison of hydrolytic activity of *PeCsn* and its truncated mutants. A, Kinetic analysis of *PeCsn* and its truncated mutants performed on 0% DA chitosan substrate. Equimolar concentration (110 nM) of *PeCsn*, GH8 and GH8FN3 were used and the hydrolytic activity was measured as µmol of sugar generated per sec. The data was fitted to the Michaelis-Menten equation by nonlinear regression function using GraphPad Prism software version 5.0 to get the respective kinetic graphs and parameters. B, Polymeric chitosan solutions of different DAs used as substrates for *PeCsn* and GH8. C, Hydrolytic activity of *PeCsn* and GH8 on 26% DA chitosan powder. All the reactions were set up in sodium acetate buffer of pH 5.6.

FIGURE 3. Base peak chromatogram of hydrolysed products generated from *PeCsn* (A) and GH8 (B). Chitosan of 0% DA was incubated with 5 nM of *PeCsn* and GH8 in 50mM ammonium acetate pH 5.6 and at 60°C. At different time points 10 µl of reaction mixtures were collected and analysed by UHPLC-ELSD-ESI-MS.

FIGURE 4. Dot blot assay showing binding specificity of *PeCBM32* to polymeric chitosan. Chitosans of different DAs ranging from 0% to 60% and glycol-chitin of DA 100% were spotted in a concentration of 1 and 0.2 µg each onto nitrocellulose membrane. The membrane was blocked with BSA, washed, and incubated with 0.1 mg.ml$^{-1}$ *PeCBM32* in TBS containing 5% (wt/v) BSA for 1 h at 25°C. Bound *PeCBM32* was detected using StreptII antibody.
A chitosan specific CBM32 from *Paenibacillus elgii*

FIGURE 5. Thermal unfolding of *Pe*CBM32 monitored by CD in presence of polymeric chitosan. The final concentrations of 35 uM purified *Pe*CBM32 and 1 mg.ml\(^{-1}\) polymeric chitosans of different DAs in 50 mM Na acetate buffer pH 5.6 were used.

FIGURE 6. ITC binding studies of ligand binding to *Pe*CBM32. Upper panels show the raw ITC data obtained from successive automatic injections of the ligand from the syringe to the protein in the ITC cell. Lower panels show the integrated heats of binding obtained from raw data shown in the upper panels together with binding isotherms to one set of sites binding model. Results are shown for the binding of GlcN, (GlcN)\(_2\), (GlcN)\(_3\), (GlcN)\(_4\), (GlcNAc)\(_2\), (GlcNAc)\(_3\), cellobiose and chitosan disaccharide of different pattern of acetylation (GlcN-GlcNAc, GlcNAc-GlcN) to *Pe*CBM32. Titrations were performed at 25°C and pH 5.6 using a MicroCal VP-ITC System (Microcal, Northampton, MA, USA).

FIGURE 7. The 3D-model of *Pe*CBM32 showing interacting residues. A, Modeled CBM32 showed beta sandwich structure having one alpha helix and eight beta strands connected by loops. Binding site for (GlcN)\(_2\) is shown in magenta, and (GlcN)\(_4\) binds to the region similar to (GlcN)\(_2\) with additional accessory interacting site shown in blue. B, Closer view of interacting residues with (GlcN)\(_2\) targeted for mutation. Pictures used for representation were made with PyMol (www.pymol.org).

FIGURE 8. Amino acid sequence comparison of *Pe*CBM32 with the available CBM32 sequences of chitosanases. Among all the chitosanases belonging to different GH families, only GH8 and GH46 family chitosanases were found to have family 32 CBMs as accessory domain(s). CBM32 sequences of chitosanases with highly conserved residues shown in black shade. Based on docking and SDM study, crucial residues that are highlighted in red box are highly conserved in CBM32 sequences of chitosanases. Secondary structure for *Pe*CBM32 is shown below the alignment.

FIGURE 9. ITC binding studies for *Pe*CBM32 variants with (GlcN)\(_4\). Upper panels show the raw ITC data obtained from successive automatic injections of the ligand from the syringe to the protein in the ITC cell. Lower panels show the integrated heats of binding obtained from raw data shown in the upper panels together with binding isotherms to one set of sites binding model. Each isotherm is labelled with the *Pe*CBM32 variant investigated (viz. E16A, E38A and S18A).

FIGURE 10. Phylogenetic relationship of CBM32s. Phylogenetic tree was constructed in MEGA6 and the evolutionary history was inferred using the Maximum Parsimony method. CBM-associated enzymes with their known family are indicated on the right-hand side near the species names. CBMs appended to chitosanases are fall into a cluster highlighted in thick line branches.
A chitosan specific CBM32 from *Paenibacillus elgii*

FIGURE 11. *In situ* staining of fungal cell wall chitosan. Confocal microscope image of *in situ* staining of *in vitro*-induced infection structure of *Puccinia graminis* f. sp. *tritici* using PeCBM32-eGFP and WGA conjugated to Texas Red. Infection structures observed under microscope were labelled as; S-urediniospore, G-germ tube, A-appressorium, V-substomatal vesicle, I-infection hypha. Stronger green fluorescence coming from substomatal vesicle indicated presence of chitosan, while red fluorescence revealed the presence of chitin in the appressorium. Scale bar has been shifted close to the infection structure to reduce the size of image.
A chitosan specific CBM32 from *Paenibacillus elgii*

Table 1
Comparison of kinetic parameters of PeCsn with its truncated proteins

| Protein      | $K_m$ (mg.ml$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (mg$^{-1}$.ml.s$^{-1}$) |
|--------------|----------------------|----------------------|--------------------------------------|
| PeCsn        | 0.40 (±0.03)         | 77.21 (±5.03)        | 189.60 (±10.58)                      |
| GH8FN3       | 0.49 (±0.04)         | 91.51 (±5.33)        | 185.65 (±7.54)                       |
| GH8          | 0.45 (±0.07)         | 93.17 (±7.30)        | 211.35 (±19.59)                      |

Table 2
Thermal unfolding temperatures of PeCBM32 in presence and absence of various chitosan polymers

| Ligands          | $T_m$ (°C) | $\Delta T_m$ (°C) |
|------------------|------------|-------------------|
| None             | (70.9°C)   | -                 |
| 0% DA chitosan   | (78.1°C)   | 7.2               |
| 11% DA chitosan  | (78.1°C)   | 7.2               |
| 20% DA chitosan  | (75.3°C)   | 4.4               |
| 61% DA chitosan  | (73.8°C)   | 2.9               |

Table 3
Thermodynamic parameters of sugar binding to PeCBM32 derived from ITC. Values correspond to averages obtained from 2 or 3 independent titrations with standard deviations indicated in parentheses

| Ligand            | $n$     | $K_b$ (M$^{-1}$) | $\Delta G^\circ$ (kcal.mol$^{-1}$) | $\Delta H^\circ$ (kcal.mol$^{-1}$) | $\Delta S^\circ$ (cal.mol$^{-1}$.K$^{-1}$) |
|-------------------|---------|-----------------|-----------------------------------|-----------------------------------|---------------------------------------------|
| (GlcN)$_4$        | 1.0 (±0.02) | 2.2 × 10$^5$ (±0.11) | -7.3 | -11.4 (±0.31) | -13.9 (±1.1) |
| (GlcN)$_3$        | 1.1 (±0.09) | 1.9 × 10$^5$ (±0.01) | -6.8 | -13.5 (±0.29) | -15.4 (±1.02) |
| (GlcN)$_2$        | 1.0 (±0.05) | 1.4 × 10$^5$ (±0.02) | -6.9 | -11.6 (±0.12) | -15.6 (±0.14) |
| GlcN              | 2.0 (±0.03) | 4.0 × 10$^3$ (±0.4) | -5.0 | -7.0 (±0.28) | -6.4 (±0.04) |
| (GlcNAc)$_3$      | 1.0     | 9.0 × 10$^3$     | -4.0 | -14.1         | -33.9                      |
| (GlcNAc)$_2$      | 1.0     | 9.0 × 10$^3$     | -4.0 | -10.0 (±0.49) | -20.3 (±1.70) |
| Cellubiose        | 1.0     | 7.0 × 10$^3$     | -3.9 | -6.2 (±0.15) | -7.7 (±0.49)  |
| GlcNAc-GlcN       | 1.1 (±0.32) | 1.0 × 10$^4$ (±0.3) | -5.5 | -8.6 (±1.68) | -10.3 (±5.76) |
| GlcN-GlcNAc       | 1.0 (±0.10) | 2.0 × 10$^4$ (±0.3) | -6.0 | -7.5 (±0.51) | -5.7 (±1.58)  |
| *(GlcN)$_3$       | 0.8 (±0.05) | 1.5 (±0.13) | -7.0 | -14.3 (±0.69) | -24.3 (±2.19) |

* Titration performed at pH 7.0
Table 4
Thermodynamic parameters of (GlcN)₄ binding to PeCBM32 variants obtained from ITC. Values correspond to averages obtained from 2 or 3 independent titrations with standard deviations indicated in parentheses. NB, no binding detected

| PeCBM32 variants | n      | $K_b$ (M⁻¹) | $\Delta G^\circ$ (kcal.mol⁻¹) | $\Delta H^\circ$ (kcal.mol⁻¹) | $\Delta S^\circ$ (cal.mol⁻¹.K⁻¹) |
|------------------|--------|-------------|-------------------------------|------------------------------|-------------------------------|
| E16A             | 1.00   | $1.9 \times 10^4$ | -5.8                          | -6.7                         | -2.9                          |
| E38A             | 0.9 (±0.04) | $1.8 \times 10^4$ (±1.3) | -5.8                          | -7.0 (±0.11)                 | -3.8 (±0.44)                |
| E38F             | NB     | NB          | NB                            | NB                           | NB                            |
| S18A             | 0.9 (±0.05) | $2.0 \times 10^5$ (±0.07) | -7.2                          | 11.8 (±0.95)                 | -15.5 (±3.25)               |
A chitosan specific CBM32 from *Paenibacillus elgii*

**FIGURE 1.**

![Diagram showing GH8, FN3, and CBM32 domains](https://example.com/diagram)

**FIGURE 2.**

**Panel A:**

![Graph showing enzyme activity](https://example.com/graph)

**Panel B:**

![Bar chart comparing enzyme activities](https://example.com/bar_chart)

**Panel C:**

![Activity comparison chart](https://example.com/compare_chart)
A chitosan specific CBM32 from *Paenibacillus elgii*

FIGURE 3.
A chitosan specific CBM32 from *Paenibacillus elgii*

**FIGURE 4.**

**FIGURE 5.**
A chitosan specific CBM32 from *Paenibacillus elgii*

**FIGURE 6.**

- **GlcN**
- (GlcN)$_2$
- (GlcN)$_3$
- (GlcN)$_4$
- GlcN-GlcNAc
- GlcNAc-GlcN
- (GlcNAc)$_2$
- (GlcNAc)$_3$
- Cellobiose

[kcal/mole of injectant vs. molar ratio over time]
A chitosan specific CBM32 from *Paenibacillus eligii*
FIGURE 7.
A chitosan specific CBM32 from *Paenibacillus elgii*
A chitosan specific CBM32 from Paenibacillus elgii

FIGURE 9.
A chitosan specific CBM32 from *Paenibacillus elgii*
Amino groups of chitosan are crucial for binding to a family 32 carbohydrate binding module of a chitosanase from Paenibacillus elgii

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