Chitin Binding Proteins Act Synergistically with Chitinases in *Serratia proteamaculans* 568

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Abstract

Genome sequence of *Serratia proteamaculans* 568 revealed the presence of three family 33 chitin binding proteins (CBPs). The three *Sp* CBPs (*Sp* CBP21, *Sp* CBP28 and *Sp* CBP50) were heterologously expressed and purified. *Sp* CBP21 and *Sp* CBP50 showed binding preference to β-chitin, while *Sp* CBP28 did not bind to chitin and cellulose substrates. Both *Sp* CBP21 and *Sp* CBP50 were synergic with four chitinases from *S. proteamaculans* 568 (*Sp* ChiA, *Sp* ChiB, *Sp* ChiC and *Sp* ChiD) in degradation of α- and β-chitin, especially in the presence of external electron donor (reduced glutathione). *Sp* ChiD benefited most from *Sp* CBP21 or *Sp* CBP50 on α-chitin, while *Sp* ChiB and *Sp* ChiD had major advantage with these *Sp* CBPs on β-chitin. Dose responsive studies indicated that both the *Sp* CBPs exhibit synergism ≥0.2 μM. The addition of both *Sp* CBP21 and *Sp* CBP50 in different ratios to a synergistic mixture did not significantly increase the activity. Highly conserved polar residues, important in binding and activity of CBP21 from *S. marcescens* (Sm CBP21), were present in *Sp* CBP21 and *Sp* CBP50, while *Sp* CBP28 had only one such polar residue. The inability of *Sp* CBP28 to bind to the test substrates could be attributed to the absence of important polar residues.

Introduction

Chitin is a highly insoluble β-1, 4-linked polymer of N-acetylglucosamine (GlcNAc), and is the second most abundant polysaccharide (next only to cellulose) in nature. For the complete hydrolysis of chitin to GlcNAc, concerted action of chitinase ([EC 3.2.1.14] and β-N-acetylglucosaminidase [EC 3.2.1.30]) is essential. Chitin was extracted as two allomorphs, namely α- and β-forms [1]. The structures of α- and β-forms of chitin differ only in the arrangement of piles of chains. Alternate chains are antiparallel in α-chitin, whereas they are all parallel in β-chitin. Among the chitin variants, α-chitin is the most abundant biopolymer in the nature. It occurs in fungal and yeast cell walls, krill, lobster and crab tendons and shells, and in shrimp shells, as well as in insect cuticle. Chitinase cleaves the glycosidic linkages between the adjacent GlcNAc residues to produce soluble oligosaccharides, which are further hydrolysed to GlcNAc by β-N-acetylglucosaminidases. Chitinase genes from bacteria have been cloned from both terrestrial and marine environments [2,3]. Biochemical properties, catalytic mechanisms, and tertiary structures of chitinases were widely reported [4,5]. A processive mechanism that improves substrate accessibility is generally considered favourable. But, it might in fact slow down enzymes. Improving substrate accessibility has been a key issue because this might reduce the need for using processive enzymes, which are intrinsically slow. Furthermore, carefully selected substrate-disrupting accessory proteins or domains might provide novel tools to improve substrate accessibility, and thus contribute to more efficient enzymatic processes [6].

Efficient chitin degradation also depends on the action of a family 33 chitin binding proteins (CBPs). The CBPs bind to the insoluble crystalline chitin, leading to structural changes and increased accessibility of substrate. The function of family 33 CBPs was first demonstrated for *Sm* CBP21 [7]. The details of CBPs and their binding preferences are given in Table 1. Studies of *Sm* CBP21 revealed that the protein has a “bundled” fibronectin type 3-fold consisting of two β-sheets, arranged as a compact β-sheet sandwich-fold surface, having a at conserved region that binds chitin through interactions mediated mainly by polar amino acids [7,8]. Conserved aromatic residues that have been suggested previously to play a role in chitin binding [9] were found in the interior of the protein, seemingly incapable of interacting with chitin. *Sm* CBP21 was designated as “chitin oxidohydrolase” as it acts on the surface of crystalline chitin, to introduce chain breaks and generates oxidized chain ends, promoting further degradation by chitinases [10]. Swapping of the chitin-binding domain in Bacillus chitinases improved the substrate binding affinity, and conformational stability [11].

*S. proteamaculans* 568, a member of family Enterobacteriaceae, was isolated as a root endophyte from *Populus trichocarpa* [12]. According to the Carbohydrate Active *enz*yme data base (CAZy; http://www.cazy.org) [13] *S. proteamaculans* 568 has at least eight genes involved in chitin turnover, coding for four family 18 chitinases (*Sp* ChiA, *Sp* ChiB, *Sp* ChiC and *Sp* ChiD), three family 33 CBPs (*Sp* CBP21, *Sp* CBP28 and *Sp* CBP50), and a family 20 N-acetylhexosaminidase (*Sp* CHB). The present study, describes the cloning and characterization of three CBPs from *S. proteamaculans*...
568 and their synergy with Sp chitinases in degradation of natural chitin variants.

**Results**

**Amplification and cloning of CBPs from S. proteamaculans 568**

Three Sp cbp genes were amplified using gene specific primers with gDNA of S. proteamaculans 568 as template. The three Sp CBPs were predicted to contain N-terminal leader peptide directing secreted secretion. Signal peptide was predicted using the SignalP server (http://www.cbs.dtu.dk/services/SignalP/). The genes were cloned without the signal peptide-encoding portion. The amplicons were cloned in the Nco I and Xho I sites of pET 22b (+) and Eco RI and Xho I sites of pET-28a (+), respectively.

The three Sp CBPs were over expressed with a C-terminal Histag in E. coli. The expressed Sp CBPs were separated either from periplasmic fraction (Sp CBP21 and Sp CBP28) or from whole cell lysate (Sp CBP50) as soluble proteins, and purified using Ni-NTA agarose chromatography. The PelB signal sequence in pET-22 b (+) directs the expressed Sp CBP21 and Sp CBP28 proteins towards periplasmic space. SDS-PAGE analysis of the purified Sp CBPs revealed approximate molecular weight of 18.6, 28.0 and 50.0 kDa, which correspond to Sp CBP21, Sp CBP28 and Sp CBP50, respectively (Figure S1).

**Substrate binding preference of Sp CBPs**

The binding preference of Sp CBPs was assayed by incubating the protein with different insoluble polymeric substrates α-chitin, β-chitin and colloidal chitin and Avicel. The amount of Sp CBP bound to the respective substrate was analyzed by determining the protein concentration in the supernatant of the reaction mixture after 24 h of incubation. Sp CBP28 did not bind to any of the test substrates (data not shown). Both Sp CBP21 and Sp CBP50 bound equally high to β-chitin (86.2% and 77.0%), followed by colloidal chitin (68.8% and 65.6%), α-chitin (30.9% and 25.6%) and Avicel (25.9% and 19.3%) (Figures 1A and B).

The time course of binding was monitored for both the Sp CBPs as a function of time to find the time required for the Sp CBPs to get saturated with natural chitin variants. After separating the protein bound to chitin, the decrease in concentration of the unbound protein (remaining in the supernatant) was monitored at different time points up to 24 h. The binding of Sp CBP21 to β-chitin occurred rapidly and reached equilibrium within 6 h, while Sp CBP50 reached equilibrium by 12 h. On the other hand, Sp CBP21 and Sp CBP50 have established binding equilibrium to α-chitin by 12 h (Figures 1C and D).

Adsorption isotherms of Sp CBP21 and Sp CBP50 towards α- and β-chitin were estimated and plotted with fixed concentration of substrate and varied concentrations of the CBPs. The dissociation binding constants (K_d) of α- and β-chitin were estimated from the non-linear regression function. The K_d value of the Sp CBP21 to α-chitin (5.31±1.03 μM) was much lower than the K_d value of Sp CBP50 to α-chitin (9.34±1.67 μM), whereas the K_d value of Sp CBP21 to β-chitin (2.22±0.45 μM) was slightly lower than the K_d value of Sp CBP50 to β-chitin (2.37±0.5 μM) (Figure 2). Binding of Sp CBPs to the soluble substrates was also examined especially to investigate whether Sp CBP28 binds at least to soluble substrates. Electrophoretic mobility of Sp CBPs did not change in presence or absence of glycol chitin, CM cellulose and laminarin substrates (Figure S2).

**Homology modeling of Sp CBP21 and Sp CBP50**

Sequence alignment of Sp CBP21, Sp CBP28 and Sp CBP50 with Sm CBP21 displayed 93%, 55% and 18% identity, respectively. The surface-located polar residues of Sm CBP21 [7,8] were highly conserved in Sp CBP21 (Tyr-54, Glu-55, Glu-60, His-114, Asp-182, and Asn-185) and Sp CBP50 (Tyr-48, Glu-49, Glu-54, His-108, Asp-176, and Asn-179). Sp CBP28 had only one (Asp-176) matching residue (Figure 3A), out of six conserved polar residues. Both Sp CBP21 and Sp CBP50, showed similar β-chitin binding preference as shown by Sm CBP21, because of sequence homology and presence of conserved polar surface residues. The sequence alignment and the SMART domain search data base revealed that the Sp CBP50 has N-terminal ChBD domain consisting of only 192 amino acids and the large part of the remaining sequence did not give any functional domain (Figure 3B). Sp CBP21 displayed high sequence identity to the sequences of the Sm CBP21 (93%). Sp CBP50 displayed only 55% of identity to Sm CBP21. The 3D structure models of Sp CBP21 and Sp CBP50 were generated using the template structure of Sm CBP21 (PDB ID: 2BEI) [Figures 4A and B]. We have modeled only particularly ChBD region of Sp CBP50 protein. The superimposition Cα atoms of the final model, on the template

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Table 1. Details of bacterial CBPs and their binding preferences.

| CBP Name   | Source            | Binding substrates                      | References |
|------------|-------------------|-----------------------------------------|------------|
| CBP21      | S. marcescens     | High preference to β-chitin followed by regenerable chitin and colloidal chitin | [15]       |
| ChbB       | B. amyloliquefaciens | Binds to both α- and β-chitin but shows preference to β-chitin | [17]       |
| LCBP33A    | Lactobacillus lactis | Equally well to α- and β-chitin, followed by Avicel, colloidal chitin and chitin beads | [4]        |
| CHB1       | St. alveiocoviridis | Strictly to α-chitin                   | [25]       |
| CHB2       | St. reticuli      | Strictly to α-chitin                   | [26]       |
| CHB3       | St. coelicolor   | Most preferably to α-chitin followed by β-chitin | [27]       |
| CbpD       | P. aeruginosa    | Colloidal chitin                      | [28]       |
| E7         | Thermobifida fusca | Equally well to α- and β-chitin followed by bacterial microcrystalline cellulose | [29]       |
| E8         | Thermobifida fusca | Preferentially to β-chitin followed by α-chitin and microcrystalline cellulose | [29]       |
| Cbp50      | B. thuringiensis  | Preferentially to β-chitin followed by α-chitin, colloidal chitin and cellulose | [18]       |
| ECFB33A    | E. faecalis       | Binds both α- and β-chitin, but slightly more protein binds to β-chitin | [19]       |

*Details of binding to other substrates not available.

doi:10.1371/journal.pone.0036714.t001
structure, gave a root mean square deviation (RMSD) of 0.182 Å and 0.164 Å for Sp CBP21 and Sp CBP50. Closer inspection of these structures revealed that the conformations of the several regions (α5, β4, β5, β6 and β7) were differing from the template structure (Figures 4C and D). Superimposition of Sp CBP21 and Sp CBP50 with the template revealed that the difference was mostly in the β-sheets.

Synergism between Sp chitinases and Sp CBPs (Sp CBP21 & Sp CBP50) during degradation of α- and β-chitin

The hydrolytic efficiency of four Sp chitinases (Sp ChiA, Sp ChiB, Sp ChiC, and Sp ChiD) on natural chitin variants was estimated in the presence/absence of Sp CBP21/Sp CBP50 and/or an external electron donor (reduced glutathione). The results indicate that, upon addition of Sp CBP21/Sp CBP50, efficiency of hydrolysis of α- and β-chitin by all the four Sp chitinases increased, especially in the presence of a reduced glutathione. Combination of both enzymes and the external electron donor led to total solubilization of β-chitin within 24 h, whereas only small fraction of the α-chitin was solubilised under the same conditions. The reactions shown in Figure 5 were sampled for up to one week following the 24-h time point. All reactions containing β-chitin reached complete solubilization after about one week of incubation, whereas none of the samples containing α-chitin were degraded completely after one week (results not shown). All three Sp CBPs showed optimum binding at 35°C-40°C, while all four Sp chitinases were also optimally active at the same temperature range. So, the synergistic experiments were carried out at 37°C (data not shown).

Figure 5 shows that the synergism exhibited by Sp CBP50 with Sp chitinases was lower when compared to the Sp CBP21 in degrading natural chitin variants. This was almost compensated when reduced glutathione was supplemented to the Sp CBP50. The product formation efficiency by Sp ChiA, Sp ChiB, Sp ChiC, and Sp ChiD increased by 0.38, 1.04, 0.99 and 5.75-fold, respectively on α-chitin in presence of Sp CBP21 and reduced glutathione (Figure 5A). On the β-chitin substrate, the products formation increased by 0.44, 3.28, 1.20, and 7.50- folds in the presence of Sp CBP21 and reduced glutathione (Figure 5B). In the presence of Sp CBP50, Sp ChiA, Sp ChiB, Sp ChiC and Sp ChiD efficiency of α-chitin hydrolysis increased by 0.35, 0.88, 0.72 and 5.11-fold, respectively (Figure 5C) where as it was 0.42, 3.12, 0.76 and 7.43-fold higher, respectively on β-chitin (Figure 5D). The increased product formation in presence of Sp CBP21 and Sp CBP50 was relatively more with Sp ChiD on α-chitin compared to other Sp chitinases, while on β-chitin, Sp ChiD and Sp ChiB were having major advantage with both the Sp CBPs. Dose-response studies of the effect of Sp CBP21 and Sp CBP50 on Sp ChiD efficiency showed that Sp ChiD displayed maximum degradation rates at both the Sp CBP’s concentrations ≥ 0.2 μM (Figure S3). The addition of both Sp CBP21 and Sp CBP50 in different ratios to a synergistic mixture of varied concentrations of Sp ChiD did not significantly increase the activity. Sp CBP21 appears to be compensating Sp CBP50 activity (Figure 6). Sampling of the synergistic mixtures (as above) at regular intervals, up to 24 h, also

Figure 1. Binding of Sp CBPs to insoluble polymeric substrates. The reaction mixture (1 mL) containing 100 μg of Sp CBP21/Sp CBP50 and 1 mg of one of the insoluble substrates (α-chitin, β-chitin, colloidal chitin and Avicel) was incubated in 50 mM sodium phosphate buffer pH 7.0 under constant shaking at 1300 rpm at 37°C for 24 h. (A and B) The amount of bound protein was calculated as the difference in protein concentration before and after incubation with the insoluble substrates, (C and D) Decrease in free protein concentration after binding to α- and β-chitin was determined at different time points till 24 h. (A and C) Sp CBP21, (B and D) Sp CBP50. Vertical bars represent standard deviation of triplicate experiments.

doi:10.1371/journal.pone.0036714.g001
did not resulted improved product formation when compared to one time point sampling at 24 h (data not shown).

Discussion

The ChBMs (chitin binding modules) are known to occur as discrete domains in chitinases and also exist independently as CBPs grouped in families 14, 18, and 33. Families 14 and 18 constitute small anti-fungal proteins that share a structurally similar chitin-binding motif [14]. Family 33 CBPs are mainly found in bacteria and viruses. Bacterial family 33 CBPs are expressed and secreted during chitin degradation. Sm CBP21 invades the chitin matrix to dissolve individual polymers, and make them more accessible to degradation by chitinases [8].

Analysis of genome sequence of S. proteamaculans 568 revealed the presence of genes coding for three CBPs of family 33. The Sp CBP21 was designated according to its homology to the reported Sm CBP21. Sp CBP21 showed high sequence identity to Sm CBP21 (93%). There were no reports on the presence of additional CBPs in S. marcescens. The two additional CBPs of S. proteamaculans were designated as Sp CBP28 and Sp CBP50 according to the estimated molecular weights (without the signal peptide) of these two proteins. S. proteamaculans 568 codes for at least 3 CBPs, while others produce either one or two CBPs (Table 1). The three Sp CBPs were arranged distantly in the genome of S. proteamaculans 568 and shares conserve 8 bp regions (5’-C(C/T/A) C(G/T) (T/G) G (C/A) (C/G)-3’) in the upstream sequences with other Sp chitinolytic genes (data not shown). Therefore, these Sp cbp genes might be co-ordinately controlled by the same regulatory protein(s) along with other Sp chitinolytic genes. Since S. proteamaculans 568 produced additional CBPs, characterization of CBPs in terms of their binding properties as well as synergism with Sp chitinases in chitin degradation was investigated.

The amino acid sequence of Sp CBP21 was BLAST at NCBI database to search for homologs. The result displayed 93% identity to Sm CBP21 (BAA31569), 57% to CBP from Bacillus cereus G9241 (EAL13960) and CBP from B. thuringiensis serovar tochigiensis BGSC 4Y1, 44% to Cbp21 from from B. anthracis str. CDC 684 (ACP12567), 41% to Cbp21 from B. anthracis str. CDC 684 (ACP12567), 41% to CHB2 from Streptomyces reticuli (EEM22267), 30% to CbpD from Stenotrophomonas sp. SKA14 (EED38588) and 27% to CbpD from Pseudomonas aeruginosa (AF196565). Sp CBP21 contained a signal peptidase site between amino acid residues Ala-27 and His-28. ChBD (Chitin binding domain) was present from His-28 through Asn-194.
acid residues Ala-22 and Gln-23 of Sp CBP28. ChBD was present from His-39 through Asn-273.

The BLAST search for Sp CBP50 homologs displayed 62% identity to N-acetylglucosamine-binding protein A from Enterobacter cloacae subsp. cloacae ATCC 13047 (ADF60226), 57% to CBP21 from S. marcescens (BAA31569), 46% to CBP from Shewanella sp. HN-41 (ABK37291), 45% to CBP from Aeromonas hydrophila subsp. hydrophila ATCC 7966 (ABK37291), 40% to CHB2 from Streptomyces reticuli (CAA74695), and 24% to CbpD from Vibrio harveyi HY01 (EDL70242). Sp CBP50 contained a signal peptidase site between amino acid residues Ala-21 and His-22 and ChBD present from His-22 through Asp-188.

Binding studies of Sp CBPs to chitin variants and cellulosic substrates revealed that Sp CBP28 did not bind to the test substrates. Sp CBP21 and Sp CBP50 were similar to the Sm CBP21, with maximum binding to β-chitin followed by α-chitin, colloidal chitin and Avicel (Figures 1A and B). As α-chitin has strong intersheet and intrasheet hydrogen bonding, compared to
weak hydrogen bonding in intrasheets of β-chitin, the Sp CBP21 and Sp CBP50 preferably bound to β-chitin. The difference in substrate preference was mainly attributed to the difference in the amino acid sequence of respective CBPs. The only available three-dimensional structure of close to Sp CBPs was Sm CBP21, which binds exclusively to β-chitin [15]. The combination of sequence and structural information with the results of site-directed mutagenesis showed that the surface of family 33 CBPs contains a patch of highly conserved, mostly polar residues (Tyr54, Glu55, Glu60, His114, Asp182, and Asn185), important for binding to chitin, and also for a positive effect on the efficiency of chitinase [8]. Ll CBP33A which binds equally to α- and β-chitin had two substitutions in the conserved surface patch. Both these residues were known to be important for Sm CBP21 functionality [8]. Ser63 occurs at a position at which Sm CBP21 had a tyrosine (Tyr54), and other family 33 CBPs have tryptophan e.g. Trp57 in CHB1 from St. olivaceoviridis, which has been shown to be important for the ability of CHB1 to bind α-chitin [16]. Asn64 occurs instead of a Glu55 of Sm CBP21. The closest homologue of LCBP33A is ChbB from B. amyloliquefaciens (66% sequence identity), which binds both α- and β-chitin [17]. ChbB differs from Sm CBP21 in the same two positions as LCBP33A: Tyr54 is replaced by Asp62, and Glu55 is replaced by Asn63.

Alignment of the amino acid sequence of Sp CBPs with Sm CBP21 revealed that all the amino acid residues that are important in chitin binding [7,8] are conserved in Sp CBP21 (Tyr-54, Glu-55, Glu-60, His-114, Asp-182, and Asn-185) and Sp CBP50 (Tyr-48, Glu-49, Glu-54, His-108, Asp-176, and Asn-179), while Sp CBP28 showed only one conserved residue (Asp-176) (Figure 3A). Minimum homology and absence of important polar residues could be the reason for the inability of Sp CBP28 to bind to substrates. It remains to be confirmed whether Sp CBP28 has a role other than chitin binding. The presence or absence of conserved amino acid residues in CBPs, therefore, conferred substrate binding preference.

The binding of Sp CBP21 and Sp CBP50 to β-chitin occurred rapidly and reached equilibrium within 6 and 12 h, respectively. Sm CBP21 established binding equilibrium after 16 h of incubation [7]. Sp CBP21 and Sp CBP50 showed relatively slow binding to α-chitin and reached equilibrium by 12 h. LCBP33A from Lactococcus lactis subsp. lactis established binding equilibrium by approximately 24 h of incubation with both α- and β-chitin [4]. In agreement with the binding assay, the lower $K_d$ values of Sp CBP21 and Sp CBP50 indicate that both these CBPs have high binding strength towards the β-chitin in comparison with α-chitin. Sp CBP21 and Sp CBP50, $K_d$ values towards the β-chitin were relatively higher, while $B_{\text{max}}$ values were lower when compared to the $K_d$ and $B_{\text{max}}$ values of reported CBPs from S. marcescens and B. thuringiensis serovar konkukian [7,18]. None of the Sp CBPs bound to soluble substrates, as observed for Sm CBP21 by Vaaje-Kolstad et al., [7].

Figure 4. The 3D models of Sp CBP21 and Sp CBP50. (A and B) The models Sp CBP21 and ChBD region of Sp CBP50 were generated by Modeller9v8 (http://www.salilab.org/modeller/) using Sm CBP21 (PDB ID: 2BEM) as structure template. Residues important for chitin binding were shown in sticks representation with carbon, oxygen and nitrogen atoms colored light green, red and dark blue, respectively. The figures were prepared using PyMOL (http://www.pymol.org/). (C and D) Stereo view of the superimposed structure of Sp CBP21 and Sp CBP50 (green) with Sm CBP21 (red), respectively. doi:10.1371/journal.pone.0036714.g004
Figure 5. Degradation of α- and β-chitin by Sp chitinases in the absence or presence of Sp CBP21, Sp CBP50 and reduced glutathione. Reaction mixture (1 mL) containing 0.25 mg/mL of chitin substrates (α- and β-chitin), 1 μM of Sp chitinase (Sp ChiA/Sp ChiB/Sp ChiC/Sp ChiD) were incubated with 0.3 μM Sp CBP21 or Sp CBP50 and 1.0 mM reduced glutathione in 50 mM sodium phosphate buffer pH 7.0. After incubation at 37 °C for 7 days at 1000 rpm, after every 24 h, 100 μL of reaction mixture was transferred. To this 100 μL of 0.02N NaOH was added to stop the reaction and stored at −20 °C until products quantification by standard reducing end assay. Vertical bars represent standard deviation of triplicate experiments.

Figure 6. β-chitin hydrolysis enhancing effects of Sp CBP21 and Sp CBP50 with Sp ChiD. Reaction mixture (1 mL) containing 0.25 mg/mL of β-chitin, 0.25 μM/0.50 μM/0.75 μM/1.0 μM Sp ChiD incubated individually with 0.3 μM of Sp CBP21/Sp CBP50 or combining both Sp CBP21 and Sp CBP50 (0.15 μM +0.15 μM/0.30 μM +0.30 μM), in 50 mM sodium phosphate buffer pH 7.0. After incubation at 37 °C for 24 h at 1000 rpm, 100 μL of reaction mixture was transferred. To this 100 μL of 0.02N NaOH was added to stop the reaction and stored at −20 °C until products quantification by standard reducing end assay. Vertical bars represent standard deviation of triplicate experiments.

Sm CBP21 catalyzes cleavage of glycosidic bonds in crystalline chitin [10], opening up the inaccessible polysaccharide material for hydrolysis by normal glycoside hydrolases. Such unique enzymatic activity was discovered after detection of traces of previously unidentified chitooligosaccharides up on incubation of β-chitin nano whiskers with Sm CBP21. Vaaje-Kolstad et al., [7] reported that the CBPs bind to the insoluble crystalline substrate, leading to both structural changes and increased substrate accessibility to the Sm chitinases (Sm ChiA, Sm ChiB and Sm ChiC). The Sm CBP21 strongly promoted hydrolysis of crystalline β-chitin by Sm ChiA and Sm ChiC, while Sm ChiB it was essential for complete degradation, and Sm CBP21 activity was boosted by external electron donor [10]. Vaaje-Kolstad et al., [4,19] also showed that the LCBP33A and EJCBM33A increased the hydrolytic efficiency of LChi18A and EJChi18A, respectively to both α- and β-chitin. These results show the general importance of CBPs in chitin turnover.

Among the four Sp chitinases, Sp ChiA, Sp ChiB and Sp ChiC released chitobiose as major end product [20], while Sp ChiD released GlcNAc from chitin substrates (based on HPLC) [data not shown]. Hydrolysis of natural chitin variants by four Sp chitinases in the presence of Sp CBP21 and Sp CBP50 showed that efficiency of all the four Sp chitinases increased with both α- and β-chitin (Figure 5). The Sp chitinases were less active on α-chitin (data not shown) and Sp CBP21 and Sp CBP50 had only minor binding preference to α-chitin. Therefore, there was no significant...
increase in the product formation on α-chitin. The addition of \textit{Sp CBP21} and \textit{Sp CBP50} had only minor effect on hydrolysis of β-chitin by \textit{Sp ChiA} and \textit{Sp ChiC}, while efficiency of \textit{Sp ChiB} and \textit{Sp ChiD} increased significantly high. These results are in line with the earlier report on \textit{Sm CBP21} in β-chitin degradation. Interestingly, in both the organisms, the \textit{cbp21} gene is located 1.5 kb downstream to the \textit{cbp21} earlier report on \textit{ChiD} increased significantly high. These results are in line with the biology of \textit{S. proteamaculans} in agriculture, food, and pharmaceutical industries. The uniqueness of \textit{Sp CBP28} is being investigated in terms of its importance in biology of \textit{S. proteamaculans}.

**Materials and methods**

**Chemicals and enzymes**

Restriction enzymes, T4 DNA ligase, and Pfu DNA polymerase were from MBI Fermentas (Ontario, Canada). Primers were procured from Eurofins India (Bangalore, India). Isopropyl-β-D-thiogalactoside (IPTG), ampicillin, kanamycin, chloramphenicol and all other chemicals were purchased either from Sigma–Aldrich (Missouri, USA), or Merck (Darmstadt, Germany), or Hi-media labs (Mumbai, India). The polymeric substrates α- and β-chitin were kindly provided by Mahatni Chitosan (Veraval, India). Colloidal chitin (CC) was prepared according to Berger and Reynolds [21].

**Bacterial strains, plasmids, and media**

\textit{Serratia proteamaculans} 568 was grown in Luria-Bertani (LB) broth at 28°C for 16 h for the extraction of gDNA (QIAGen, Düsseldorf, Germany). Plasmid vectors pET 22a(+), pET 28a(+), and Escherichia coli Rosetta-gami 2(DE3) were used as vector and host (Novagen, Darmstadt, Germany) for expression, respectively. To express CBP genes, \textit{E. coli} Rosetta-gami 2(DE3) carrying pET 22b(+) or pET 28a(+) was grown in LB broth with ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL) or kanamycin (50 μg/mL) and chloramphenicol (25 μg/mL), respectively.

**Amplification and cloning of \textit{Sp CBPs}**

Three genes encoding CBPs (\textit{Sp cbp21}, \textit{Sp cbp28} and \textit{Sp cbp50}; GenBank accession no. ABV42576.1, ABV42205.1, and ABV43333.1, respectively) were amplified from the gDNA by referring to the annotated sequence of \textit{S. proteamaculans} 568 at 55°C annealing temperature using gene specific forward and reverse primers listed in Table 2. Expression vectors, and the amplicons were separately digested with \textit{NcoI} and \textit{XhoI} (pET 22b(+)), \textit{Sp cbp21} and \textit{Sp cbp28}, and \textit{Eco RI} and \textit{XhoI} (pET 28a(+) and \textit{Sp cbp50}), gel purified and ligated using T4 DNA ligase at 16°C for 16 h. The resultant plasmids were designated as pET 22b-\textit{Sp cbp21}, pET 22b-\textit{Sp cbp28} and pET 28a-\textit{Sp cbp50} to express \textit{Sp CBP21}, \textit{Sp CBP28} and \textit{Sp CBP50}, respectively in \textit{E. coli}.

**Expression and purification of \textit{Sp CBPs}**

Expression and purification of \textit{Sp CBP21}, \textit{Sp CBP50} and \textit{Sp CBP28} were done as described by Neeraja et al., [22], except that the \textit{Sp CBP50} was isolated from whole cell lysate by sonication the cell pellet. The cell pellet was suspended in Ni-NTA equilibration buffer (50 mM NaH₂PO₄, 100 mM NaCl and 10 mM imidazole pH 8.0). The cells were lysed by sonication at 20% amplitude with 30×15 s pulses (with 20 s delay between pulses) on ice, with a Vibra cell Ultrasonic Processor, converter model CV35, equipped with a 3 mm probe (Sonics, Newtown, CT, USA). To pellet the insoluble cell debris, sonicate was centrifuged at 15,200×g for 10 min at 4°C. The expressed protein was purified using Ni-NTA column as the expressed protein having C-terminal His-tag. After purification, the \textit{Sp CBPs} were buffer exchanged with 50 mM sodium phosphate buffer pH 7.0 using Macrosep Centrifugal Devices (Pall Corporation, USA), and stored at 4°C until use.

**Protein measurement**

Purified \textit{Sp CBPs} were quantified by BCA (bicinchonic acid) protein assay kit (Novagen, USA) using a standard calibration curve constructed from BSA (bovine serum albumin). For the chitin binding assay, protein concentration was measured from the absorption at 280 nm using the molar extinction coefficients (ε) calculated from the amino acid composition of the protein as described by Pace et al., [23].

**Insoluble substrate binding specificity**

Insoluble substrate binding of \textit{Sp CBPs} was done as described by Vaaje-Kolstad et al., [7] with slight modifications. The substrates, α-chitin, β-chitin, colloidal chitin and Avicel were used as insoluble substrates, and BSA was used as a background control for nonspecific adsorption. The binding mixture (1 mL) was incubated for 24 h at 37°C with vigorous shaking at 1300 rpm on thermostir (Thermostatir comfort; Eppendorf, Hamburg, Germany).

**Time course binding of \textit{Sp CBP21} and \textit{Sp CBP50} towards α- and β-chitin**

To study the time at which the binding of \textit{Sp CBP21} and \textit{Sp CBP50} (described above) was getting saturated with natural chitin variants (α- and β-chitin) was assessed at different time points up to 24 h.

**Adsorption isotherms of \textit{Sp CBP21} and \textit{Sp CBP50} towards α- and β-chitin**

Adsorption isotherms of \textit{Sp CBP21} and \textit{Sp CBP50}, towards α- and β-chitin, were obtained as described by Vaaje-Kolstad et al., [7] with minor modification. Varied concentration of an \textit{Sp CBP}, up to 10.0 μM, was incubated with α-/β-chitin for different saturation periods: 12 h for α-chitin and 6 h for β-chitin with \textit{Sp CBP21}, and 12 h for both α- and β-chitin with \textit{Sp CBP50}.

**Soluble substrate binding specificity**

Binding of \textit{Sp CBPs} to soluble polysaccharides (glycol chitin, laminarin and CM cellulose) was evaluated by affinity electrophoresis as described by Hardt and Laine [24] with slight modifications. Proteins (10 μg of \textit{Sp CBPs} and non-interacting BSA) were electrophoresed in 8.0% polyacrylamide gels impregnated with substrates (glycol chitin or laminarin or CM-cellulose) under non-denaturing conditions at 4°C. The gels were visualized by staining with Coomassie blue G-250.

**Sequence alignment for \textit{Sp CBPs} and homology modelling of \textit{Sp CBP21} and \textit{Sp CBP50}**

All three \textit{Sp CBPs} from \textit{S. proteamaculans} were aligned with \textit{Sm CBP21} using chustal2 (www.ncbi.nlm.nih.gov/Tools/msa/chustal2/). A 3D structure models of \textit{Sp CBP21} and \textit{Sp CBP50} were generated using the template structure of \textit{Sm CBP21} (PDB ID: 2BEM) by Modeller9v8 (http://www.salilab.org/modeller/). About 40 mod-
els and corresponding Ramachandran plots were generated for each protein to check the protein structure quality using PROCHECK. The figures were prepared using Pymol (http://www.pymol.org/).

Synergistic effect of Sp CBP21 and Sp CBP50 with Sp chitinases in chitin degradation.

Chitin degradation assay was performed as described by Vaaje-Kolstad et al., [19] with few modifications. A standard 1 mL reaction mixture containing 0.25 mg/mL of chitin substrates (α- or β-chitin), 1.0 μM of Sp chitinase (unless stated otherwise) [Sp chiA, Sp chiB, Sp chiC and Sp chiD (GenBank accession no. ABV39247.1, ABV40327.1, ABV42574.1 and ABV41826.1)] were amplified using gene specific primers (Table 2) and cloned, expressed, and purified similarly like Sp cbps (data not shown) and 1.0 mM reduced glutathione. Reaction mixtures were incubated in triplicates at 37°C for 7 days at 1000 rpm in a thermomixer. After every 24 h, 100 μL of reaction mixture was transferred and mixed with 100 μL of 0.02 N NaOH was added to stop the reaction and stored at −20°C until products quantification. Products were quantified by standard chitinase assay as described by Neeraja et al. (2010a, 22).

Supporting Information

Figure S1 Ni-NTA agarose purification of Sp CBPs. Recombinant Sp CBP21, Sp CBP28 and Sp CBP50 were purified using Ni-NTA agarose column chromatography. Elution buffer containing 250 mM imidazole was used to elute Sp CBPs from the column and loaded on 12% SDS-PAGE followed by staining with Coomassie brilliant blue G-250. Lane 1: Protein standards size in kDa indicated to the left, lane 2–4: Purified Sp CBP21, Sp CBP28 and Sp CBP50, respectively.

(DOCX)

Figure S2 Binding of Sp CBPs towards soluble polymeric substrates. Affinity non-denaturing gel electrophoresis was performed at 4°C by preparing 8% polyacrylamide gels. Ten micromolars of Sp CBP and BSA were electrophoresed without (A), or with 0.1% (w/v) substrates glycol chitin (B), laminarin (C), and CM-cellulose (D). Proteins were visualized by Coomassie blue G-250 staining after electrophoresis. Lane 1: BSA, lane 2–4: Sp CBP21, Sp CBP28 and Sp CBP50.

(DOCX)

Figure S3 Dose-response effects for Sp CBP21 and Sp CBP50 in degradation of β-chitin. Reaction mixture containing 0.25 mg/mL of chitin substrates (α- or β-chitin), 1.0 μM Sp ChiD incubated with different concentrations of Sp CBP21/Sp CBP50 (0.05–0.40 μM) as indicated in 50 mM sodium phosphate buffer pH 7.0. After incubation at 37°C for 24 h at 1000 rpm, 100 μL of reaction mixture was transferred. To this 100 μL of 0.02N NaOH was added to stop the reaction and stored at −20°C until products quantification by standard reducing end assay. Vertical bars represent standard deviation of triplicate experiments. (A and B) degradation of α- and β-chitin by Sp ChiD in the presence/absence of Sp CBP21 and reduced glutathione (RG), (C and D) degradation of α- and β-chitin by Sp ChiD in the presence/absence of Sp CBP50 and reduced glutathione (RG). Sp CBP21+RG or Sp CBP50+RG: Sp CBP21/Sp CBP50 with reduced glutathione, Sp CBP21+ or Sp CBP50+: only Sp CBP21/Sp CBP50 without reduced glutathione, Sp CBP21- or Sp CBP50 - : without Sp CBP21/ Sp CBP50 and reduced glutathione.

(DOCX)

Acknowledgments

The authors thank DST-FIST, UGC-SAP and DBT-CREBB for the support to the Department of Plant Sciences, University of Hyderabad. PP thanks the University of Hyderabad for research fellowship, Dr. Daniel van der Lelie, Brookhaven National Laboratory, USA for providing the S. proteamaculans 568, and Dr. Dominique Gillete, Mahatani Chitosan, Veraval, Gujarat, for chitinous substrates.

Author Contributions

Conceived and designed the experiments: JSSP ARP. Performed the experiments: PP PVPSA. Analyzed the data: PP PVPSA JSSP ARP. Contributed reagents/materials/analysis tools: PP PVPSA JSSP ARP. Wrote the paper: PP ARP.
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