Transglycosylation by chitinase D from *Serratia proteamaculans* improved through altered substrate interactions

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**Background:** *SpChiD*, family 18 glycosyl hydrolase that can transglycosylate chitooligosaccharides.

**Results:** Transglycosylation of *SpChiD* was improved in terms of quantity of TG products produced and the extended duration of TG.

**Conclusion:** The new findings unravel possibilities of modulating chitinases for improved transglycosylation.

**Significance:** Variants of *SpChiD* can be used to develop a bio-process for large scale production of longer chain chitooligosaccharides.

**SUMMARY**

We describe improvement of the transglycosylation (TG) by chitinase D from *Serratia proteamaculans* (*SpChiD*). The *SpChiD* produced less proportion of TG products up to 90 min, with 2 mM chitotetraose as the substrate and hydrolysed the products subsequently. Of the five residues targeted at the catalytic center, E159D resulted in substantial loss of both hydrolytic and TG activities. Y160A resulted in a product profile similar to *SpChiD* and a rapid turnover of substrate with slightly increased TG activity. Rest of the three mutants, M226A, Y228A and R284A displayed improved TG and decreased hydrolytic ability. Four of the five amino acid substitutions, F64W, F125A, G119S and S116G, at the catalytic groove increased TG activity, while W120A completely lost the TG activity with a concomitant increase in hydrolysis. Mutation of W247 at the solvent accessible region significantly reduced the hydrolytic activity with increased TG activity. The mutants M226A, Y228A, F125A, S116G, F64W, G119S, R284, and W247A accumulated approximately double the concentration of TG products like chitopentaose and chitohexaose, compared to *SpChiD*, respectively. The double mutant E159D/F64W regained the activity with accumulation of 6.0% of chitopentaose at 6 h, similar to *SpChiD* at 30 min. Loss of chitobiase activity was unique to Y228A. Substitution of amino acids at catalytic center and/or groove substantially improved the TG activity of *SpChiD*, both in terms of quantity of TG products produced and the extended duration of TG activity.

Chitin is the second most abundant natural polysaccharide consisting of β (1→4)-linked N-acetyl-D-glucosamine (GlcNAc) units in a linear form. Chitin is insoluble in water and mainly exists in two crystalline (α- and β-) polymorphic forms. The α-chitin consists of sheets of tightly packed alternating parallel and antiparallel chains (1) and is abundant in the exoskeleton of arthropods, insects, fungi and yeast cell walls. The chains are arranged in parallel orientation in β-chitin (2), which occurs less frequently in nature and is often extracted from squid pens. The insolubility of chitin is a major limitation in eliciting biological activities.

The polymeric chitin and the break down products like chitooligosaccharides (CHOS) are of increasing interest due to potential applications in agriculture and medicine. The CHOS are water-soluble, nontoxic, and biocompatible. Biological properties of CHOS include antimicrobial, antitumor activities, immuno-enhancing...
effects on animals (3) and disease protective responses in plants (4–6). Fully acetylated CHOS with degree of polymerization (DP) from 4 to 10 induced peroxidase activity in wheat, while the fully deacetylated CHOS of DP5 and DP7 induced neither peroxidase nor phenylalanine ammonia lyase activities (4). Oligomers of chitin, but not chitosan, were active elicitors of defense-related lignification in wounded (7) and intact wheat (Triticum aestivum L.) leaves (8) and in suspension-cultured wheat cells (9). Chitin octamer (DP8), by acting as a bivalent ligand, induced dimerization of chitin-elicitor receptor kinase 1 of Arabidopsis thaliana (AtCERK1) that was inhibited by shorter chitin oligomers (10). Dimerization of AtCERK1 was critical to activate immune responses.

In view of the wider biological applications for the CHOS, chemical methods for hydrolysis of chitin or chitosan were frequently used for industrial scale production (11). Conventional chemical approaches for synthesis proved in adequate to produce substantial quantities of CHOS owing to the complexities involved in the selective protection and subsequent manipulations of the various monosaccharide donors and acceptors (12). The chemical methods employ organic solvents in larger quantities followed by cumbersome purification methods. Therefore, enzymatic approach for CHOS production would be an alternative attractive. Chitinases (EC 3.2.1.14) are one of the glycosyl hydrolases (GH), best preferred biological tools for chitin degradation and production of CHOS with biological activity.

A few GHs show transglycosylation (TG), with which new glycosidic bonds are introduced between donor and acceptor sugar molecules. The regio- and stereo-selective synthesis of glycosidic bond can be achieved through retaining GHs showing TG, which follows double displacement reaction (13). The glycosyl-enzyme intermediate formed during the catalysis is a key step, deciding the direction of reaction towards hydrolysis or TG. Hydrolysis occurs when a water molecule attacks the glycosyl-enzyme intermediate leading to the release of a hydrolysed sugar. Alternatively, TG occurs when a carbohydrate molecule outcompetes with the water molecule (14).

The TG by chitinases could be improved for an efficient production of CHOS with higher DP by mutagenesis. Mutation of W167 at −3 subsite in Serratia marcescens chitinase A (15) and a chemical modification of hen egg white lysozyme at D101 and W62 at −4 and −2 subsites, respectively enhanced the TG activity (16). The variants of D140/311 and D142/313, analogous residues in ChiB and ChiA from S. marcescens, respectively, showed improved TG activity (14). Similarly the aromatic side chains of F166 and W197 in class V chitinase from Cycas revoluta were important for the TG acceptor binding than for the substrate binding (17). The mutational effects on TG activity of GH18 family chitinases were very less and also restricted to the changes in conserved catalytic residues or aromatic residues. Chitinase from Bacillus sp. and a family GH 85, β - N-acetyl-D-glucosaminidase from Streptococcus pneumoniae catalysed TG provided a potential useful tool for the chemo-enzymatic synthesis of glycoproteins (18, 19).

We have characterized a family 18 TG chitinase D from Serratia proteamaculans (SpChiD) that had a molecular mass of 44.4 kDa, producing TG products like DP7, DP10, DP11-12 and DP11-DP13 from DP3, DP4, DP5 and DP6 substrates, respectively (20). The SpChiD displayed TG activity only for 90 min with 2 mM of chitotetraose (DP4) as substrate, later only hydrolytic products with lower DP were produced. The TG products detectable during the initial stages of reaction become substrates for hydrolytic activity of SpChiD in a prolonged incubation. The availability of a TG GH18 chitinase like SpChiD provoked us to further improve the TG activity. We report here that the alteration of amino acid residues at catalytic center, catalytic groove and solvent accessible region substantially improve the TG activity both in terms of increasing the quantity of TG products and in extending the duration of TG activity.

**EXPERIMENTAL PROCEDURES**

**Fold Recognition and Homology Modeling**

Fold recognition by FUGUE (21) was done as described previously by Sashidhar et al. (22). The aminoacid sequence of SpChiD (NCBI_ID: YP_001478954) was obtained from NCBI database (http://www.ncbi.nlm.nih.gov/). A BLAST search of the aminoacid sequence against protein databank (PDB) was performed to
identify the 3D structures that could share high sequence homology. The 3D coordinates of the basic structure were obtained from the PDB (http://www.rcsb.org/pdb/home/home.do). We built the 3D structure of the SpChiD protein using the MODELLER (23) implemented in Discovery Studio 2.5 (Accelrys Inc, USA). The structure validation of the generated 3D models was performed using Verify_3D (24, 25) and Ramachandran plot (26).

Selection of Amino Acid Residues for Point Mutations

The aminoacid residues that might influence hydrolytic and TG activities of SpChiD were selected based on the sequence alignment and the crystal structures of the homologous proteins (1E15, 1ITX, 1E9L, 1HKM, 1EDQ and 1D2K). We considered the hydrophobic aromatic residues of chitinases that are mainly involved in catalytic interactions with chitin. Using the 3D model, a few aminoacid residues were selected for point mutations based on their probable involvement in hydrolytic and TG activities of SpChiD.

Bacterial Strains, Plasmids, Culture Conditions, Biochemicals and Enzymes

The plasmid pET-22b (+) and E. coli BL21 (DE3) (Novagen, Madison, USA) were used for heterologous expression. E. coli was grown in LB broth (1% peptone, 0.5% yeast extract, 1% NaCl) at 37°C. Ampicillin, 100 µg/ml, was added to the LB broth as required. Oligonucleotide primers were purchased from Eurofins India (Bangalore, India). Restriction enzymes, T4 DNA ligase and Pfu DNA polymerase were obtained from MBI Fermentas (Ontario, Canada). Isopropyl-β-D-thiogalactoside (IPTG), ampicillin and all other chemicals were purchased from Calbiochem or Merck (Darmstadt, Germany), or Hi-media labs (Mumbai, India). Ni-NTA His bind resin was procured from Novagen (Madison, USA) for protein purification. Different DP CHOS were obtained from Seikagaku Corporation (Tokyo, Japan), through Cape Cod (East Falmouth, USA).

Generation of SpChiD Mutants

SpChiD mutants were generated as described by Ke and Madison (27) with pET 22b-SpChiD as template (18). Mutagenic primers were designed and amplification was carried out in two different steps using Pfu DNA polymerase. In the first step of PCR, annealing temperature was 52°C and polymerization time was 90 sec. The product from the 1st step PCR was used as mega primer for the 2nd step PCR with annealing temperature of 60°C and polymerization time of 150 sec. Details of the primers used for the generation of SpChiD mutants were listed (supplemental Table S1). Agarose gel electrophoresis was performed and the ampiclon of desired size was gel eluted using Qiagen (Duesseldorf, Germany) Gel Extraction kit. Both the gel eluted ampiclons and expression vector pET-22b(+) were subjected to double-digestion with Eco I and Xho I at 37°C for 6 h, followed by PCR clean up using Qiagen Gel clean up kit. Double-digested ampiclons were ligated to Eco I and Xho I sites of the vector pET-22b (+), at 16°C for 16 h.

Protein Expression, Isolation and Purification

Highly efficient competent cells of E.coli BL21 (DE3) were transformed with the respective SpChiD mutants and positive clones were selected on LB-ampicillin plates. Expression of SpChiD and the mutant proteins was done as described by Neeraja et al. (28). Harvested culture pellet was processed for isolation of periplasmic fraction (PF) as described in pET manual (Novagen, Darmstadt, Germany), concentrated with buffer exchange using amicon filters (10 kDa cutoff, Millipore, Billerica, MA) and purified as described in Qiagen (Duesseldorf, Germany) manual using Ni-NTA affinity chromatography. The recombinant protein was eluted using buffers containing 50, 150 and 250 mM imidazole. The purified fractions were electrophoresed on 12% SDS-PAGE and visualised using coomassie brilliant blue. The purified protein in 20 mM sodium acetate, pH 5.6, was used for characterization.

Activity Determination and HPLC Analysis for TG

Zymogram analysis for activity determination was done according to Purushotham et al. (29). SpChiD mutants positive for zymogram analysis were considered for HPLC analysis to check the mutational effects on the activity of SpChiD. Two mM DP4 was incubated with 350 nM of the purified SpChiD and its variants. Alternatively, 35 nM SpChiD was incubated with 50 or 100 µM of DP4 to determine the substrate binding with SpChiD. Reaction was performed in 20 mM sodium acetate buffer,
pH 5.6, at 40°C. Fractions were collected up to 6 h, at regular intervals and the reaction was stopped using equal volume of 70% acetonitrile. Reaction mixture (20 µL) from each fraction was injected into HPLC (Shimadzu, Tokyo, Japan) using Hamilton (Hamilton Bonaduz, Switzerland) syringe. The products were analysed by using SHODEX Amino-P50 4E column (4.6 ID x 250 mm, Showa Denko K.K, USA) through isocratic elution with ACN: H2O in 70:30 (29). Flow rate of 0.7 ml/min was maintained and eluted CHOS were monitored at 210 nm. Quantification of CHOS was done by comparing peak areas of the products with peak areas obtained from known concentration of the CHOS. Mixture containing equal weight of CHOS ranging from DP1-DP6 was used for standard graph preparation. A linear correlation between peak area and concentration of CHOS in standard samples was established for quantification of reaction products up to DP6. Standard calibration curves for CHOS moieties were constructed separately. These data points yielded a linear curve for each standard sugar with the R^2 values of 0.997–1.0 allowing molar concentration of CHOS to be determined with confidence. The decrease in DP4 concentration was considered for specific activity measurements through a linear regression analysis using OriginPro 8 software.

RESULTS
Homology Modeling and Selection of the Mutant Sites –

The NCBI BLAST search of the SpChiD at PDB identified the crystal structure of putative chitinase II from Klebsiella pneumoniae (PDB_ID: 3QOK). The SpChiD had 91.1% of sequence similarity with the template crystal structure (supplemental Fig. S1). The modelled structure of the SpChiD (Fig. 1) was validated using PROCHECK (99.4% of the residues are in the allowed region) and Verify_3D (94.9% of residues had an average 3D-1D score > 0.2). Thus, the compatibility of SpChiD 3D model structure with its 1D amino acid sequence was confirmed. The coordinates of chitotetraose were built based on the crystal structure of PDB_ID: 1NH6.

To improve the TG activity of SpChiD, the aminoacid residues in the catalytic center, catalytic groove and in the surface exposed region were selected for mutagenesis. In the catalytic center, the residues M226, Y228, R284 and Y160 were closer to the catalytic triad DxDe (Fig. 2A). We have mutated E159 from the catalytic triad, F125 close to the catalytic triad and a few residues present in the catalytic groove including S116, G119, W120 and F64, where the substrate molecules show major interactions (Fig. 2B).

Further, W247 on the surface of the protein, which is expected to play a role in threading of chitin molecules to the catalytic center, was also selected for point mutation. Amplification and Cloning of SpChiD Mutants –

All the SpChiD mutants were amplified using gene-specific mutagenic primers with pET 22b-SpChiD as template. The amplicons of 1.2 kb were double-digested and cloned into Nco I & Xho I sites of pET 22b (+) expression vector. The clones were confirmed through double-digestion. The inserted mutation was confirmed by automated DNA sequencing. Expression, Purification and Dot Blot Assay for SpChiD and the Mutants –

E. coli BL21 (DE3) cells harbouring the plasmids of SpChiD and the mutants were used for protein expression. Induced cells were harvested and processed for isolation of PF. Soluble proteins of SpChiD and its variants in the PFs were passed through Ni-NTA affinity matrix to obtain pure proteins. SDS-PAGE analysis confirmed the purity of collected protein fractions and the mutant protein molecular mass was also around 44.4 kDa (supplemental Fig. S2A). The dark blue spots in the in gel assay confirmed the activity of SpChiD and the mutant proteins that had single/double aminoacid substitutions (supplemental Fig. S2B).

Hydrolytic and TG Activities of SpChiD –

The results with SpChiD showed a very sharp decrease in the initial concentration of DP4 substrate starting from 5-45 min (1.26% decreased to 0.2%), and completely depleted by the end of 120 min (supplemental Fig. S3A). The TG activity of SpChiD, although was detectable up to 90 min, the production of TG products (DP5 and DP6) was relatively more up to 30 min only (DP5
from 1.3% to 6.3%, and DP6 from 1.9% to 4%). Later, the TG activity decreased and completely extinguished after 90 min (DP5 and DP6 were 1.0%, 0.3%, respectively) (supplemental Fig. S3A). *Sp* ChiD produced chitobiose (DP2) as the major end product (56.4%) at the end of 120 min, along with the other two degradation products *N*-acetyl glucosamine (DP1) and chitotriose (DP3). The DP1 product (42.4%) was much higher than DP3 product (0.8%) at 120 min. Reaction of low enzyme (35 nM) with 50 or 100 µM DP4 substrate resulted in similar products profile that yielded both DP5 and DP6 as TG products (supplemental Fig. S4), suggesting that -2 to +2 and -1 to +3 are possible for productive binding.

**Effect of Mutations on Hydrolytic and TG Activities of *Sp*ChiD Mutants – Residues at the Catalytic Center – Glu159 to Asp Conversion –**

E159D mutation resulted in significant loss of both hydrolytic and TG activities as indicated by the presence of 92.5% of DP4 substrate at the end of 6 h (supplemental Fig. S3B and S5B). Only a trace amount of hydrolytic products DP1, DP2 and DP3 were detected even after 6 h. Among the TG products, only DP5 and DP6 were detected (no TG products >DP6), where in the former was detectable at 150th min and the latter at 60th min. The proportion of quantifiable oligomers, DP1 to DP6, generated by E159D mutant was 0.6%, 2.3%, 2.5%, 92.5%, 1.0% and 1.1% at the end of 6 h.

**Y160A, Met226, Tyr228 and Arg284 to Ala Conversion**

Y160A was unique from all other mutants generated, which retained TG activity even when hydrolytic activity increased. The concentration of DP4 substrate and DP3 product followed the same path from 60 min of reaction incubation with almost equal fractions of DP4 and DP3 (0.6% and 0.4%) (supplemental Fig. S3C and S5C). But, up to 45 min, a gradual reduction in DP4 (7.5%) substrate and simultaneous increase in DP3 (21.0%) product was observed. Reaction mixtures at 150 min showed DP1 (49.7%) and DP2 (49.6%) as the major end products. Y160A was the only mutant which produced nearly equal concentration of DP1 and DP2 products. Y160A also displayed TG activity up to 45 min. At 5 min, the concentration of DP5 and DP6 products was 8.8% and 4.6% that decreased to 3.0% and 0.9% by 45 min, respectively.

The product profile of the mutants M226A and Y228A was similar except that the latter lost chitobiase activity. A gradual decrease in the initial DP4 substrate, through time was detected (supplemental Fig. S3D&E and S5D&E) for M226A and Y228A. The relative proportion of the products produced by the mutant M226A was DP1-6.4%, DP2-13.5%, DP3-37.7%, DP5-12.0% and DP6-5.8% by 6 h. No DP1 product was detectable with Y228A, as the chitobiase activity of *Sp*ChiD was abolished. Fraction collected at 6 h showed that DP2 (14.4%), DP3 (40.7%), DP5 (13.6%) and DP6 (6.8%) were produced by Y228A. A rapid increase of DP5 and DP6 products up to 45 min was followed by a state of equilibrium, which continued till the end of 6 h. Both M226A and Y228A produced more of DP5 than DP6 product.

R284A substitution resulted in a gradual decrease of DP4 substrate, generating more of DP5 and DP6 products, although DP2 (40.8%) and DP3 (26.6%) were the major end products (supplemental Fig. S3F and S5F) at 6 h. The synthesis of DP5 and DP6 products started right from the beginning, but the concentration varied through time. Fraction collected at 30 min showed DP6 (9.0%) > DP5 (6.4%) whereas the fraction at 150th min had DP5 (9.8%) > DP6 (6.2%). Though there was an alteration in the proportion of TG products, both DP5 (8.2%) and DP6 (4.0%), were detectable till the end of 6 h.

**Residues at the Catalytic Groove – Phe64 to Trp Conversion and the Double Mutant E159D/F64W**

A decrease in the initial concentration of DP4 substrate was detected from 0-5min (62.9% to 48.5%) with F64W (supplemental Fig. S3G and S6A). There was no decrease in the hydrolytic ability of F64W up to 30 min, but produced more of TG products compared to *Sp*ChiD. There was an increase of quantifiable TG products with 12.9% of DP5 and 6.6% of DP6 after 60 min. Though there was a gradual decrease of TG products, more of DP5 was detectable than DP6 at 6 h. Hydrolytic products DP3 and DP2 remained as the major end products. When F64W was coupled with E159D, the double mutant E159D/F64W regained both hydrolytic and TG activities (Fig. 3A). Fraction collected at the end of 6 h revealed accumulation of 2.2%,
2.1%, 8.0%, 80.1%, 5.9% and 0.8% of oligomers from DP1-DP6 products, respectively.

**Trp120 to Ala Exchange** –

The mutation W120A led to a rapid increase in the hydrolysis of DP4 substrate, leaving only 0.5% at 30 min (supplemental Fig. S3H and S6B). The DP3 product also decreased to 2.4% at 30 min, while the concentration of DP1 and DP2 products was comparable to those produced by SpChiD. DP2 (56%) was the major end product. Substantial loss of TG activity, with a feeble activity up to 5 min (1.0% of DP5 and 0.5% of DP6), suggested that W120 is an important residue contributing to TG activity of SpChiD.

**Phe125, Gly119, Ser116 Exchange to Ala, Ser, and Gly, Respectively** –

The product profiles of F125A, G119S, and S116G showed decreased hydrolytic ability by the three mutants. F125A and G119S produced more or less equal quantities of DP2 with 24.0% and 22.2% and DP3 with 21.0% and 18.7%, respectively as the major end products at 6 h (supplemental Fig. S3I&J and S6C&D). These two mutants also produced more of DP5 and DP6 products but, the proportion was less in the early stage and increased gradually through time compared to SpChiD. Proportion of DP5 and DP6 products produced by F125A at 0 min was 1.3% and 1.5%, respectively, that increased to 10.4% of DP5 and 7.8% of DP6 by the end of 6 h. The product profile of G119S was also similar, where 1.5% of DP5 and 0.9% of DP6 products were detectable at the 0 min, increased up to 9.5% of DP5, and 8.4% of DP6 by the end of 6 h. S116G produced both DP5 and DP6 TG products right from 0 min with 2.2% and 4.1%, respectively (supplemental Fig. S3K and S6E). The concentration of TG products increased gradually up to 180 min with accumulation of 11.7% of DP5 and 8.8% of DP6 products. Nearly similar quantities of TG products were detectable up to 6 h with accumulation of 11.7% and 7.2% of DP5 and DP6, respectively.

**Residue at the Solvent Accessible Region (Trp247 to Ala)** –

Exchange of W247, at the solvent exposed region, to Ala resulted in the reduced hydrolytic ability (Fig. 4 and supplemental Fig. S7). The formation of TG products was slow at the early time points, which increased at later time points and persisted up to 6 h. W247A produced DP2 and DP3 as the major end products. The proportion of DP1 – DP6 products at the end of 6 h of reaction incubation were 4.3%, 18.2%, 18.1%, 42.3%, 9.8% and 7.2%, respectively.

The specific activity and the extent of TG activity for all the mutants were compared with SpChiD (supplemental Fig. S8) and listed in Table 1. The specific activity decreased for mutants E159D/F64W, E159D, F125A, G119S, S116G and W247A and significantly increased Y160A, F64W, Y228A, R284A, M226A, with a maximum of 8-fold increase for W120A. However, F125A, G119S, S116G, R284A and W247A produced more of DP5 and DP6 compared to GlcNAc. F64W in 60 min (12.9%) and other mutants M226A (12.0%), Y228A (13.6%), F125A (10.4%), and S116G (11.7%) in 6 h produced double the concentration of DP5 compared to SpChiD (6%) at 30 min (Fig. 5). Whereas, almost double the concentration of DP6 was generated by G119S (8.4%), F125A (7.8%) and W247A (7.2%) at 6 h compared to SpChiD which produced only 4% at 30 min (Fig. 5). The change in proportion of TG products (DP5 and DP6) produced by all the eleven single mutants at the respective time intervals, were quantified and compared against the native SpChiD (Fig. 6A&B).

**DISCUSSION**

*S. proteamaculans* 568, a member of family Enterobacteriaceae, was isolated as a root endophyte from *Populus trichocarpa* (30). The Carbohydrate Active enZYme data base (CAZyhttp://www.cazy.org/) (31) showed at least eight genes in the genome sequence of *S. proteamaculans* 568 could be potentially involved in chitin turnover. We have cloned and characterized *Sp ChiA, Sp ChiB, Sp ChiC*, and *SpChiD* from *S. proteamaculans* 568 (20, 29). *SpChiD* had very less sequence identity (<25% and only 23% identity with human chitotriosidase) with other well characterized chitinases, except with chitinase II from *Klebsiella pneumoniae* for which the enzyme characterization was not available. *SpChiD* was the first characterized bacterial, family 18 processive endo-chitinase with single catalytic domain. Among the characterized single-domain chitinases, *SpChiD* showed significant TG activity with a potential to synthesize CHOS with DP up to
SpChiD, therefore, attained importance as a unique hyper TG chitinase produced by an endophytic bacterium. The enormous potential of SpChiD to modulate chitin substrates may have implication in the plant-microbe interaction. The chitinase-catalysed TG activity provides a potentially useful tool for synthesis of CHOS and also the neoglycoproteins (18, 19).

TG could be improved by reducing the effective concentration of water, performing the TG reactions at high concentrations of substrate and the addition of large excesses of the acceptor molecule (12). The product association or product release were the rate determining steps for chitinase-catalyzed degradation of insoluble chitin or soluble chitosan, respectively (32). Moreover, deglycosylation step may be the rate determining step in hydrolysis/TG (14). Since the TG products are kinetically controlled, monitoring of the reaction is necessary to ensure maximum yields (12). But, in the present study there was no temporal difference between the hydrolytic and TG activities of SpChiD. As both hydrolytic and TG activities start simultaneously at 0 min (20) even at very low enzyme and substrate concentrations (supplemental Fig. S4), mutational approach could be most appropriate for improving the TG activity. Very few mutational studies were carried to improve the TG activity in terms of extended time of TG reaction and the quantity of TG products produced by a chitinase (14, 15, 17). The present study provides deeper insight into the three different possibilities of targeting SpChiD to improve TG activity as following:

Residues at the Catalytic Center

Among the five different amino acid substitutions made at the catalytic center, three showed increased TG activity. M226 and Y228, present on the β6 strand of the TIM barrel, with their side chains in proximity to the catalytic triad DxDeX, have the potential to interact with the sugar molecule during catalysis. The residue M226 is not directly involved in catalysis, but is an important supporting residue for catalytic activity of SpChiD. Substitution of M243 to Glu in family 18 chitinase from Aspergillus fumigatus, showed that the mutant M243E lost the TG activity towards PNP-(NAG)₂ but not with DP4 substrate (33). Docking studies revealed that M243E forms hydrogen bond with backbone oxygen of −1 sugar instead of a hydrophobic stack between M243 and +1 subsite sugar moiety. However, in the present study, substitution of M226 with Ala decreased the hydrophobic interactions needed for usual pace of reaction, which probably gives a chance to oxazolinium intermediate to stay longer at the catalytic center. Moreover it was evident that a chitin-chitosan hybrid polysaccharide could be synthesized via chitinase-catalyzed polymerization of an oxazoline derivative of a GlcN β (1→4) GlcNAc monomer. Monomer was designed as a transition-state analogue substrate (TSAS) for chitinase catalysis, which belongs to the glycoside hydrolase family 18 (34). Therefore, we speculate that the increased stay of substrate/intermediate resulted in increased TG activity both in terms of quantity of TG products formed, and the duration of TG activity of the mutant M226A. It was proposed that the Y214 in ChiB of S. marcescens is conserved and stabilizes the transition state (35). In Y228A, where similar kind of interactions were possible with the phenolic hydroxyl group of Y228, also improved the TG activity. The mutants M226A and Y228A accommodate smaller side chains on the β6 strands and therefore possess larger cavity revealed the possibility of increasing TG activity possibly by decreasing the rate of reaction. Quantifiable TG products DP5 and DP6 were detected in proportions 12.0% and 5.8% by M226A, 13.6% and 6.8% by Y228A where it was 6.3% and 4% only by SpChiD.

R284 located on the β7 strand of the TIM barrel, with its side chain protruding towards the active site and forms ion pair interactions with D229 in its free state. It was observed from the crystal structure of chitinase in complex with sugar (PDB_ID 1D2K, 1E15, and 1EDQ), that R284 also forms ion pairs with the substrate at the catalytic center. Based on these two observations, we mutated R284 to Ala, and caused perturbations in the ion pair forming ability of SpChiD with the incoming DP4 substrate. Such subtle changes at the catalytic center resulted in a gradual increase in the proportion of TG products DP5 (6.4%) and DP6 (9.0%) up to 30 min. But, the proportion of DP5 and DP6 products was reversed by the end of 150 min with 9.8% and 6.2%, respectively, and continued till the end of 6 h (8.2% of DP5 and 4.0% of DP6). Shifting towards increased synthesis of DP5
product by R284 could be due to the dynamic ion pair interactions with the substrate or within the protein remains to be examined.

The chitinase B from *S. marcescens* catalytic residue D142 is in the ‘down’ position, interacting with D140 for family 18 chitinases in their free state. Upon substrate-binding, D142 moves to the ‘up’ position and interacts with the substrate and E144 (35). Such dynamic changes in all the three catalytic residues play a crucial role during chitin degradation. As the carboxyl group of E204 could not be substituted by Asp in chitinase A1 of *Bacillus circulans* W12, it was concluded that the relative disposition of the carboxyl group of E204 to substrate was critical for the catalytic activity (36). In the present study, E159 located on the β4 strand of TIM barrel of *SpChiD* was mutated to Asp, which resulted in substantial loss of both hydrolytic and TG activities, suggesting that the length of the side chain in E159 was not only important for hydrolysis but also for TG activity. Due to a subtle change, with the removal of a single –CH₂ group, the mutant E159D had a significant difference in the activity. The adjacent residue Y160 substituted with Ala, reduced the time of TG to 45 min, against 90 min with *SpChiD*. The DP4 substrate was utilized rapidly within 60 min as against 120 min with *SpChiD*. The results indicated that the phenolic hydroxyl group in the Y160 was important for an extended TG activity up to 90 min. The mutant Y160A acquired subtle changes at the catalytic center that favoured hydrolysis but not TG.

**Residues at the Catalytic Groove**

We have selected five different amino acid residues in the catalytic groove for point mutations. F64 with Trp, G119 with Ser, S116 with Gly, and F125 and W120 with Ala were replaced. F64 was located on the β2 strand of the TIM barrel and its substitution with Trp resulted in increased synthesis of TG products up to 60 min (12.9% of DP5 and 6.6% of DP6), later channelled in the gradual decrease up to 6 h (8.0% of DP5 and 4.2% of DP6). Though the Phe and Trp residues confer aromaticity, the bulky side chain (increased surface area) of Trp facilitates more number of interactions with the incoming substrate (14). The Trp also might assist in correct positioning and guiding of the sugar molecule into the active site (37). The increased TG by F64W for 60 min and further gradual decrease may be because, the sugar molecules with higher DP will have more of possible interactions with the Trp residue. F64W when coupled with E159D, the double mutant E159D/F64W regained the TG activity by 3–folds. The hydrolysis decreased to a greater extent for E159D/F64W, but produced DP5 product as efficient as *SpChiD* at 30 min (Fig. 3B). In E159D, the relative positioning of the carboxylate group of Glu was perturbed by replacement with Asp. But, the increased aromatic surface area at the catalytic groove, in F64W conversion could overcome the loss of function of E159D to a greater extent, at least, on TG activity.

The residues G119 and W120 were present on the β3 strand of TIM barrel very close to the catalytic center. Both these residues were thought to maintain the architecture of the catalytic groove, to a major extent maintained by W120 with its bulky side chain. As these two residues are directly involved in threading of the chitin molecules towards the catalytic center, point mutations were planned to identify the key residue for TG in chitin path and also to increase the TG. To achieve this W120 was mutated to Ala and G119 was replaced with Ser. Exchange of Trp97 to Ala in ChiB of *S. marcescens* significantly increases the rate of hydrolysis (38), and removal of such a residue resulted in the loss of TG activity (17, 14). W120A had lost the ability of TG after 5 min, and almost completely degraded the DP4 substrate by 30 min. The very low TG activity at the early time intervals (0-5 min) could be due to the strong inherent TG activity of the *SpChiD*, but lost completely when the trafficking of water molecules along with CHOS increased through the groove, because of the lack of guiding aromatic residue at 120º position. This proved that the residue W120 was also involved in the direct interactions with the sugar molecules. However, increased TG activity was observed with the mutant G119S. There were pre-existing stacking interactions shown by W120 with the substrate which further increased with the adjacent Ser –OH group. Increased interactions at the positions 119 and 120 in turn increased the TG activity of G119S mutant.

For an efficient TG to occur, enzyme should have an active site architecture that disfavours correct positioning of the hydrolytic water molecule and/or favours binding of
incoming carbohydrate molecules, through strong interactions in the aglycon subsites (14). S93 in ChiB from S. marcescens stabilizes the first D (Asp140) in the DxDxE motif, when the second D (Asp142) turns away from Asp140 to Glu144 and the oxazoline intermediate (39, 35). The second D (Asp142) was shown to be important for TG activity (14). The QM/MM calculations (40) show that Asp142 is important in transition state and intermediate stabilization. The mutation D142N yielded an enzyme with high TG activity (14). It was proposed that active site electrostatics may change by this mutation and that this may affect the catalyzing water. In line with these observations, we have mutated S116 → Gly. The residue S116 is present on the β strand of the TIM barrel with its side chain traversing the void between the two Asp residues of the catalytic triad. Mutant S116G resulted in the decreased hydrolytic ability with concomitant increase in the TG activity. The residue F125 was present at the α3 helix of the TIM barrel with its side chain eclipsing the DxDxE region from the bottom-side of the active site. This was the conserved aromatic residue found in the family 18 chitinases, whose side chain forms π-π, CH-π interactions with W158 located between D157 and E159 of catalytic triad. The mutant F125A has shortage of the aromatic side chain because of which the ascribed interactions could be disturbed. These subtle changes in the interactions resulted in increased TG activity.

Residue at the Solvent Accessible Region –

W247 is on the turn region next to the β6 strand of the TIM barrel. Soluble substrates like CHOS were assumed to enter the substrate binding cleft from various directions, while a chitin chain from crystalline chitin enters the binding site only from the edge of the cleft (41). There was no significant effect on hydrolysis of DP5 substrate with the mutants Y56A and W53A, but the hydrolysing activity against β-chitin microfibrils decreased in chitinase A1 from B. circulans. But, in the present study decreased rate of hydrolysis followed by increased TG activity was observed for the mutant W247A. This aromatic residue with the bulky side chain could stack against the pyranoside rings and its replacement to Ala altered the inherent activity of the SpChiD. This clearly indicates that the residue W247 has an important role in the activity of SpChiD towards soluble CHOS substrates like DP4. There is no correlation between the $k_{cat}$ for hydrolysis and apparent TG activity. It is the other factors such as positioning and activation of the catalytic water and optimal aligning of the acceptor are likely to play a role (14).

TG received a considerable attention for the production of CHOS with longer DP. In order to synthesize adequate quantities of longer chain CHOS with biological activity there is a definite need for exploitation of TG activity. We conclude that the residues at catalytic center and groove are the better targets to improve the TG activity. The improved SpChiD variants are useful in developing a process for the production of longer chain CHOS.

REFERENCES


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FOOTNOTES

The abbreviations used are: Sp ChiD, chitinase D from *Serratia proteamaculans*; GH, glycosyl hydrolase; GlcNAc, *N*-acetyl-D-glucosamine; TG, transglycosylation; DP, degree of polymerization; CHOS, chitooligosaccharides; HPLC, high performance liquid chromatography; PF, periplasmic fraction.

FIGURE LEGENDS

**FIGURE 1.** The 3D model of *Sp*ChiD. Interactions of chitin tetramer with the catalytic residues (D155, D157 and E159) at the active site are highlighted. The side chains of catalytic residues were shown as sticks and subsites indicated. Pictures used for representation were made with PyMol (www.pymol.org).

**FIGURE 2.** Close view of 3D-model of *Sp*ChiD representing the residues targeted for mutation. A) The catalytic center (M226, Y228, R284, E159 and Y160), B) The catalytic groove (F64, F125, G119, S116 and W120) and one residue at the solvent accessible region W247. The side chains of residues mutated in this study were shown as sticks and subsites indicated. Pictures used for representation were made with PyMol (www.pymol.org).

**FIGURE 3.** Product profile of the double mutant E159D/F64W. Profiles were generated by *Sp*ChiD or its variants with 2 mM DP4 substrate in 20 mM sodium acetate buffer pH-5.6 at 40°C. A) Double mutant (E159D/F64W). Inset represents the differences between hydrolytic (DP1-DP3) and TG (DP5, DP6) products generated during the course of reaction. B) Comparison of quantifiable TG products DP5 (blue, filled symbols) and DP6 (red, open symbols) by *Sp*ChiD, E159D, E159D/F64W.

**FIGURE 4.** Product profile of the mutant W247A. HPLC quantification profile of both hydrolytic and TG products up to 360 min. The reaction mixture contained 2 mM of DP4 substrate, 350 nM of W247A, at pH-5.6, 40°C. The reaction products were analyzed by isocratic HPLC. Products were quantified from respective peak areas of products by using standard calibration curves of CHOS ranging from DP1 to DP6.

**FIGURE 5.** Quantifiable TG products (DP5 and DP6) accumulated by *Sp*ChiD and its mutants. Reaction mixture containing 2 mM DP4 and 350 nM of each of *Sp*ChiD and its indicated mutants were incubated separately for different time periods from 0 to 360 min at 40°C. Products were quantified from respective peak areas by using standard calibration curves of CHOS. Comparative profiles showing the quantity of DP5 (A) and DP6 (B) TG products produced at 30 min (C) and 6 h (D).

**FIGURE 6.** Comparison of quantifiable TG products. DP5 (A) and DP6 (B) accumulated at different time intervals up on incubation of 2 mM DP4 with *Sp*ChiD and its variants. Reaction was carried up to 6 h at pH-5.6, 40°C. Product quantification was done by a linear correlation between peak area and concentration of oligosaccharides in standard samples.
**TABLE 1:** Relative specific activity and TG activity of *Sp*ChiD and its mutants towards DP4 substrate.

<table>
<thead>
<tr>
<th>Name of the enzyme</th>
<th>Relative % of specific activity</th>
<th>Relative TG activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sp</em>ChiD</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>E159D</td>
<td>49.8</td>
<td>−</td>
</tr>
<tr>
<td>Y160A</td>
<td>151.3</td>
<td>+</td>
</tr>
<tr>
<td>M226A</td>
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<td>+++</td>
</tr>
<tr>
<td>Y228A</td>
<td>168.9</td>
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<td>R284A</td>
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<tr>
<td>W120A</td>
<td>830.6</td>
<td>−</td>
</tr>
<tr>
<td>F64W</td>
<td>160.7</td>
<td>+++</td>
</tr>
<tr>
<td>G119S</td>
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</tr>
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<td>S116G</td>
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<tr>
<td>W247A</td>
<td>75.8</td>
<td>+++</td>
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<tr>
<td>E159D/F64W</td>
<td>5.7</td>
<td>*</td>
</tr>
</tbody>
</table>

(+) minimum, (−) nil, (+++) high, (*) regained
Figure 1:
Figure 3:
Figure 4:
Figure 5:

A

B
Figure 6:
Transglycosylation by chitinase D from Serratia proteamaculans improved through altered substrate interactions
Jogi Madhuprakash, Karunakar Tanneeru, Pallinti Purushotham, Lalitha Guruprasad and Appa Rao Podile

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