

How nature can exploit nonspecific catalytic and carbohydrate binding modules to create enzymatic specificity

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Noncatalytic carbohydrate binding modules (CBMs) are components of glycoside hydrolases that attack generally inaccessible substrates. CBMs mediate a two- to fivefold elevation in the activity of endo-acting enzymes, likely through increasing the concentration of the appended enzymes in the vicinity of the substrate. The function of CBMs appended to exo-acting glycoside hydrolases is unclear because their typical endo-binding mode would not fulfill a targeting role. Here we show that the *Bacillus subtilis* exo-acting β -fructosidase SacC, which specifically hydrolyses levan, contains the founding member of CBM family 66 (CBM66). The SacC-derived CBM66 (*BsCBM66*) targets the terminal fructosides of the major fructans found in nature. The crystal structure of *BsCBM66* in complex with ligands reveals extensive interactions with the terminal fructose moiety (Fru-3) of levantriose but only limited hydrophobic contacts with Fru-2, explaining why the CBM displays broad specificity. Removal of *BsCBM66* from SacC results in a ~ 100 -fold reduction in activity against levan. The truncated enzyme functions as a non-specific β -fructosidase displaying similar activity against β -2,1- and β -2,6-linked fructans and their respective fructooligosaccharides. Conversely, appending *BsCBM66* to BT3082, a nonspecific β -fructosidase from *Bacteroides thetaiotaomicron*, confers exolevanase activity on the enzyme. We propose that *BsCBM66* confers specificity for levan, a branched fructan, through an "avidity" mechanism in which the CBM and the catalytic module target the termini of different branches of the same polysaccharide molecule. This report identifies a unique mechanism by which CBMs modulate enzyme function, and shows how specificity can be tailored by integrating nonspecific catalytic and binding modules into a single enzyme.

isothermal titration calorimetry | X-ray crystallography | prebiotics | biofuels | lectins

Complex carbohydrates represent a major nutrient for numerous microbial ecosystems, exemplified by bacterial and fungal communities established in the rumen and large bowel of mammals, where they play an important role in animal nutrition and human health, respectively (1, 2). It is also evident that these composite structures are of increasing industrial significance, particularly in the bioenergy and bioprocessing sectors (3). The enzymes that catalyze the degradation of these complex carbohydrates, primarily glycoside hydrolases but also polysaccharide lyases, are grouped into sequence-based families on the continuously updated CAZY database (4). Members of the same family display a common fold, and the catalytic mechanism and catalytic apparatus are also conserved (5). Substrate specificity within glycoside hydrolase families (GHs), however, can vary significantly (6). Glycanases that attack inaccessible substrates often contain noncatalytic carbohydrate binding modules (CBMs; see ref. 7 for review) that are also grouped into sequence-based families in the CAZY database. Against recalcitrant substrates,

CBMs enhance the activity of their cognate enzymes, and though the mechanism(s) by which this occurs remains uncertain, CBMs most likely fulfill a targeting function by increasing the effective concentration of the appended enzymes, in the vicinity of the substrate, thereby enhancing catalytic efficiency (8, 9).

Fructans, such as inulin and levan (polymers of predominantly β -2,1- or β -2,6-linked fructose units, respectively) are common dietary plant polysaccharides that are used extensively as prebiotics, to ensure that the human large-bowel microbiota maximizes human health by selecting for beneficial bacteria such as *Bifidobacter* (10). Fructans are metabolized by GH68 and, more frequently, by GH32 enzymes that include endo- and exo-acting levanases, inulinases, and nonspecific β -fructosidases, whereas transglycosylases catalyze the synthesis of these fructans (11). Understanding the mechanism(s) by which GH32 enzymes display such a range of different activities is important when designing fructan-based prebiotic strategies.

The catalytic modules of GH32 fructanases consist of a domain that adopts a five-bladed β -propeller fold and houses the active site, which is abutted onto a β -sandwich domain that likely plays a structural role (12). A cohort of GH32 enzymes, exemplified by SacC from *Bacillus subtilis*, an exo-acting levanase (13), contain, in addition to the catalytic module, a ~ 160 -residue sequence of unknown function. Here we show that the C-terminal 160-residue module of SacC (designated *BsCBM66*) is the founding member of CBM family 66 (CBM66) that binds to the nonreducing end of fructan polymers. Intriguingly, *BsCBM66*, despite displaying broad specificity, directs nonspecific β -fructosidases onto highly branched fructans. This study thus provides generic insights into how enzymatic specificity can be achieved through the recruitment of nonspecific catalytic and noncatalytic carbohydrate binding modules into a single protein. This report therefore provides unique strategies for engineering the specificity of carbohydrate modifying enzymes.

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Results

C-Terminal Domain of the Exolevanase SacC Is the Founding Member of a Large CBM Family. The *B. subtilis* exolevanase SacC comprises a GH32 catalytic module and a C-terminal region of unknown function (14). To test the hypothesis that the C-terminal domain of SacC is a CBM, the capacity of the protein module to bind to complex carbohydrates was evaluated. The data (Table S1) showed that the C-terminal module binds to levan, but not to any of the other polysaccharides evaluated, demonstrating that SacC contains a C-terminal CBM, designated hereafter as *BsCBM66*. Analysis of sequence-based relatives of *BsCBM66* using BLAST identified ~140 bacterial protein modules that display significant similarity to the *Bacillus* CBM (e < 10⁻⁶ and sequence identity > 30%), and therefore, *BsCBM66* is the founding member of CBM66. Notably, 106 of the CBM66 members are located in GH32 enzymes (Fig. 1; Fig. S1), strongly suggesting that the predominant role of CBM66 is in binding fructans. The remaining CBM66 members are linked to a range of CAZy enzymes, primarily glycoside hydrolases and lyases, associated with plant cell-wall degradation.

Influence of *BsCBM66* on SacC Function. Biochemical analysis revealed that SacC displays ~100-fold-higher activity for levan (β-2,6-glycosidic linkages) compared with inulin (β-2,1-glycosidic linkages) or oligosaccharides [with a degree of polymerization (DP) < 6] of either fructan (Table 1). The elevated activity against levan reflects a reduction in *K_m* and an increase in *k_{cat}* compared with the other substrates. The activity of truncated SacC comprising the GH32-containing catalytic module (defined hereafter as *BsLev32_{CM}*), but lacking *BsCBM66*, displayed similar activity as the full-length enzyme against sucrose and levan- and inulin-derived

oligosaccharides, exhibiting maximal activity against the disaccharides of the two fructans. Thus, *BsCBM66* does not contribute to the structural integrity of the active site of the enzyme, which appears to contain only two fructose-binding sites. Although the activities of *BsLev32_{CM}* and SacC were similar for inulin and several fructooligosaccharides, *BsLev32_{CM}* was ~100-fold less active than SacC against *B. subtilis* levan, reflecting a ~10-fold increase in *K_m* and a similar decrease in *k_{cat}* (Table 1). These data indicate that *BsCBM66* is able to target the nonspecific β-fructosidase, *BsLev32_{CM}*, to levan, a β-2,6-linked branched polysaccharide. To demonstrate that the truncation per se has not influenced enzyme specificity, the activity of a variant of SacC (W640A), in which *BsCBM66* was inactive, was evaluated. The W640A mutation did not influence the activity of SacC against oligosaccharides or inulin, but caused a substantial reduction in the capacity of the enzyme to hydrolyze levan (Table 1). This lower activity was similar to that seen for the isolated catalytic domain *BsLev32_{CM}*, confirming that a functional *BsCBM66* is integral to the elevated activity displayed by SacC against levan.

***BsCBM66* Targets the Nonreducing End of Fructans.** To explore the specificity of *BsCBM66* in more detail, isothermal titration calorimetry was used to determine the affinity of the protein for a range of fructose-containing oligosaccharides and polysaccharides (Fig. S2; Table 2). *BsCBM66* binds to all of the fructose-containing polymers evaluated, but displays extremely weak affinity for the monosaccharide (estimated *K_d* ~10 mM). With respect to oligosaccharides, *BsCBM66* displays ~twofold-higher affinity for the β-2,6-linked disaccharide levanbiose, compared with either β-2,6- or β-2,1-linked fructooligosaccharides with a DP > 2. These data indicate that the protein module contains two fructose-binding sites that can accommodate either a β-2,6- or β-2,1-linked disaccharide. The modest reduction in affinity for longer fructooligosaccharides may reflect an element of steric hindrance caused by these longer molecules. With respect to polysaccharides, *BsCBM66* displays a five- to sixfold preference for *B. subtilis* levan, which is heavily branched (12% of the fructose residues are at branch points) (15), compared with *Erwinia herbicola* levan (3% branched) (16) and chicory inulin, which is unbranched (17). The preference for the branched *Bacillus* levan likely reflects the disordered structure of the polysaccharide being more accessible to the protein.

To determine whether *BsCBM66* recognizes internal or terminal regions of levan, the stoichiometry of binding was compared with that of an inactive form (lacking the catalytic nucleophile) of the exo-acting β-fructosidase BT3082 (18). The data showed that both proteins bind to *Erwinia herbicola* levan at a frequency of ~1 in 30 fructose residues, which is entirely consistent with linkage analysis demonstrating that nonreducing terminal fructose residues comprise 3% of the polysaccharide (16). It would appear, therefore, that *BsCBM66* binds to the nonreducing end of levan chains, a view consistent with the crystal structure of the protein in complex with its target ligands, described below.

Mechanism by Which *BsCBM66* Recognizes Fructans. The crystal structure of *BsCBM66* was solved using the single-wavelength anomalous dispersion method using selenomethionine (SeMet)-labeled protein and refined using data extending to 1.7-Å resolution (Table S2). *BsCBM66* adopts a β-sandwich fold in which the two β-sheets contain seven and six antiparallel β-strands, respectively (Fig. 2). In contrast to the majority of CBMs, *BsCBM66* contains no obvious metal ions. The surface of the protein reveals a broad pocket centered on the concave β-sheet. Mutagenesis of the residues that comprise the pocket abolishes binding to levan, whereas mutations of other surface amino acids have no influence on ligand recognition (Table 2; Fig. S3). These data indicate that the pocket located on the concave β-sheet comprises the ligand-binding site. *BsCBM66* displays structural

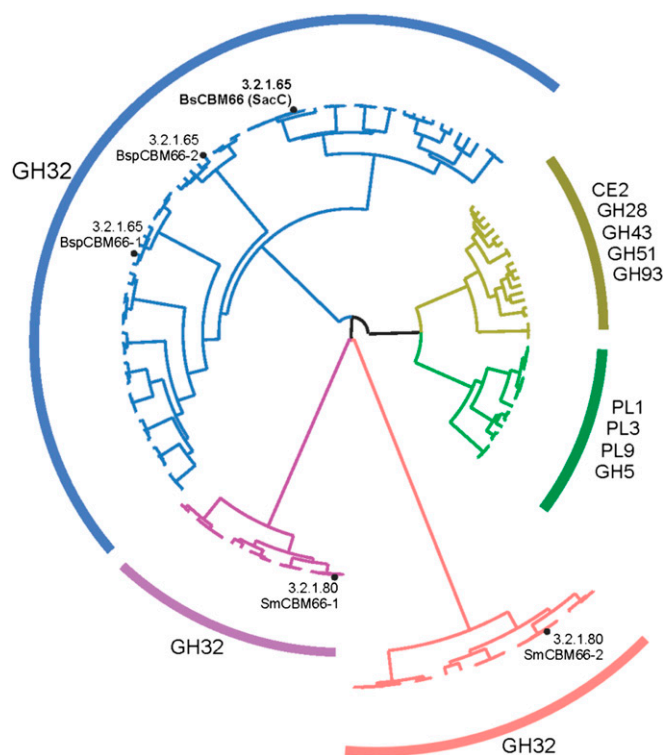


Fig. 1. Phylogenetic clustering of known CBM66 modules. The major clusters are shown with different branch colors. *BsCBM66* of SacC and other CBM66 modules appended to two other characterized enzymes are shown along with their enzyme commission (EC) number. In the outer region, the catalytic module families associated with the respective CBM66 modules are indicated. A more detailed figure, including all accession numbers to GenBank, is provided in Fig. S1.

Table 1. Influence of BsCBM66 on the catalytic activity of GH32 enzymes

Enzyme	Substrate	K_m , mM*	k_{cat} , min ⁻¹	k_{cat}/K_m , min ⁻¹ ·mM ⁻¹
SacC	Levan (<i>B. subtilis</i>)	0.087 ± 0.014	663 ± 35	7.6 × 10 ³
SacC W640A	Levan (<i>B. subtilis</i>)	1.1 ± 0.18	63 ± 6	5.7 × 10 ¹
BsLev32 _{CM}	Levan (<i>B. subtilis</i>)	1.2 ± 0.24	92 ± 7	7.5 × 10 ¹
BT3082-BsCBM66	Levan (<i>B. subtilis</i>)	0.026 ± 0.002	2,123 ± 183	8.2 × 10 ⁴
BT3082	Levan (<i>B. subtilis</i>)	0.94 ± 0.13	643 ± 56	6.8 × 10 ²
SacC	Levantriose	1.5 ± 0.26	56 ± 3	3.7 × 10 ¹
BsLev32 _{CM}	Levantriose	2.8 ± 0.56	74 ± 6	2.6 × 10 ¹
SacC	Levanbiose	3.4 ± 0.52	274 ± 18	8.6 × 10 ¹
BsLev32 _{CM}	Levanbiose	1.9 ± 0.41	114 ± 12	6.0 × 10 ¹
SacC	Inulin	0.62 ± 0.13	58 ± 4	9.4 × 10 ¹
BsLev32 _{CM}	Inulin	0.70 ± 0.14	45 ± 3	6.4 × 10 ¹
BT3082-BsCBM66	Inulin	0.36 ± 0.26	253 ± 26	7.0 × 10 ²
BT3082	Inulin	0.48 ± 0.051	423 ± 32	8.8 × 10 ²
SacC	Kestotriose	2.5 ± 0.28	152 ± 16	6.1 × 10 ¹
BsLev32 _{CM}	Kestotriose	3.7 ± 0.41	143 ± 11	4.0 × 10 ¹
SacC	Kestopentaose	0.92 ± 0.15	85 ± 5	9.2 × 10 ¹
BsLev32 _{CM}	Kestopentaose	1.3 ± 0.3	71 ± 6	5.5 × 10 ¹
SacC	Sucrose	34 ± 7.6	23 ± 3	6.7 × 10 ⁰
BsLev32 _{CM}	Sucrose	42 ± 8.8	21 ± 4	5.0 × 10 ⁰

*The molar substrate concentration of levan and inulin was determined by the stoichiometry of the binding of an inactive mutant of the exofructosidase and BsCBM66 to inulin and levan.

similarity to a range of proteins that interact with carbohydrates, including the mannan-binding lectin PAL (PDB ID code 2GMP; z-score of 14.9 and an rmsd of 2.5 Å over 151 Cα atoms), the galactose-binding peanut lectin (PDB code 1V6M; z-score 13.9 and an rmsd of 2.6 Å over 152 Cα atoms), and the β-sandwich domain of the GH32-containing catalytic module of the *Aspergillus japonicus* fructosyltransferase (PDB code 3LDK; z-score of 13.9 and an rmsd of 2.4 Å over 132 Cα atoms). These proteins all display <10% sequence identity with BsCBM66, and the ligand-

binding residues (see below) are not conserved in the three other proteins. It is unlikely, therefore, that these structural homologs of BsCBM66 will bind to fructans, although the two lectins are oligomeric and thus display enhanced affinity through avidity effects (19, 20).

To determine the mechanism of ligand recognition, the crystal structures of BsCBM66 in the presence of fructose or levantriose were solved to a resolution of 1.7 Å and 1.1 Å, respectively. Electron density for both ligands was evident (Fig. 2). As is typical

Table 2. Thermodynamics of the binding of BsCBM66 and a catalytically inactive variant of BT3082 to its target ligands determined by ITC

Protein	Ligand*	K_a (× 10 ³ M ⁻¹)	ΔG (kcal/mol)	ΔH (kcal/mol)	TΔS (kcal/mol)	n^{\dagger}
BsCBM66 wild type	Fructose	<1.0 (~0.1)	nd [‡]	nd	nd	nd
BsCBM66 wild type	Levanbiose	18.3 ± 0.6	-5.8	-13.2 ± 0.3	-7.4	1.1
BsCBM66 wild type	Levantriose	11.2 ± 0.3	-5.5	-11.2 ± 0.3	-5.7	1.1
BsCBM66 wild type	Levantetraose	8.2 ± 0.01	-5.3	-11.2 ± 0.2	-5.9	1.0
BsCBM66 wild type	Levanpentaose	7.8 ± 0.6	-5.3	-13.7 ± 0.1	-8.4	1.0
BsCBM66 wild type	Levan (<i>B. subtilis</i>)	18.4 ± 0.9	-5.8	-14.1 ± 0.8	-8.3	1.0
BsCBM66 wild type	Levan (<i>E. herbicola</i>)	3.5 ± 0.1	-4.8	-14.3 ± 0.9	-9.5	1.0
BsCBM66 wild type	Inulin (chicory)	3.1 ± 0.02	-4.7	-11.0 ± 0.4	-6.3	1.0
BsCBM66 wild type	Kestotriose	7.2 ± 0.06	-5.3	-10.8 ± 0.2	-5.5	1.0
BsCBM66 wild type	Kestotetraose	8.5 ± 0.2	-5.4	-11.3 ± 0.5	-5.9	1.0
BsCBM66 wild type	Kestopentaose	11.3 ± 0.1	-5.5	-12.0 ± 0.2	-6.5	1.0
BsCBM66N505A	Levan (<i>E. herbicola</i>)	1.4 ± 0.2	-4.3	-12.5 ± 0.4	-8.2	1.0
BsCBM66D522A	Levan (<i>E. herbicola</i>)	NB [§]	—	—	—	—
BsCBM66K562A	Levan (<i>E. herbicola</i>)	2.3 ± 0.08	-4.6	-15.0 ± 2.1	-10.4	1.1
BsCBM66N568A	Levan (<i>E. herbicola</i>)	NB	—	—	—	—
BsCBM66D570A	Levan (<i>E. herbicola</i>)	<1.0 (0.35)	nd	nd	nd	1.0
BsCBM66H573A	Levan (<i>E. herbicola</i>)	1.7 ± 0.06	-4.4	-12.8 ± 2.0	-8.4	1.0
BsCBM66K577A	Levan (<i>E. herbicola</i>)	NB	—	—	—	—
BsCBM66W640A	Levan (<i>E. herbicola</i>)	NB	—	—	—	—
BT3082 D131A	Levan (<i>E. herbicola</i>)	5.9 ± 0.2	-5.1	-22.6 ± 2.6	-17.5	1.1

*The structure of the various polysaccharides is as follows: *B. subtilis* levan, β-2,6-linked fructose polymer with 12% β-2,1-linked fructose branches (15); *E. herbicola* levan, β-2,6-linked fructose polymer with 3% β-2,1-linked fructose branches (16); chicory inulin, β-2,1-linked fructose polymer with no branches (17).

[†]ITC data were fitted to a single-site binding model for all ligands. For polysaccharide ligands, in which the molar concentration of binding sites is unknown, the n value (stoichiometry) was iteratively fitted as close as possible to 1 by adjusting the molar concentration of the ligand.

[‡]nd, not determined.

[§]NB, no binding detected.

for other CBMs, *BsCBM66* did not display significant conformational changes upon ligand binding; the rmsd between the apo- and ligand-bound forms of the protein is 0.1 Å. Fructose, which is in its furanose form, is sandwiched between two aromatic residues, Phe⁵⁷⁹ and Trp⁶⁴⁰, which make hydrophobic contacts with the ligand. The key polar residues that interact with the furanose sugar are Asp⁵²², in which Oδ1 and Oδ2 make polar contacts with O4 and O3 of fructose, respectively, whereas the Nε of Lys⁵⁷⁷ makes potential hydrogen bonds with O1, O6, and the endocyclic oxygen. The direct polar interactions between the protein and ligand are completed by Nδ2 of Asn⁵⁶⁸ and Oδ2 of Asp⁵⁷⁰, which both interact with O6. The biological significance of these interactions is illustrated by the observation that ligand binding is greatly compromised, or abolished, by alanine substitution of any of these amino acids (Table 2; Fig. S3). The O1 and O6 of the bound fructose are pointing at the surface of *BsCBM66*, preventing extension of the ligand in the nonreducing direction (levan and inulin are linked β-2,6 and β-2,1, respectively), whereas O2 is pointing into solvent, consistent with the protein binding to the

nonreducing termini of β-2,6- and β-2,1-linked fructans. The ligand-binding residues identified above are conserved in 37 CBM66 members (Fig. S4), and thus it is likely that these protein modules display specificity for the nonreducing end of levan and inulin.

In the complex with levantriose, only the nonreducing (Fru-3, equivalent to the fructose of the monosaccharide complex) and central fructose residues (Fru-2) have unambiguous electron density and could be modeled with certainty. As expected, Fru-3 binds essentially identically to fructose. In contrast, Fru-2 makes only a single polar contact with *BsCBM66*, between O4 and Nε2 of His⁵⁷³. This interaction, however, has little impact on ligand binding because the H573A mutation does not significantly affect affinity for levan (Table 2). The major contribution of Fru-2 to *BsCBM66* binding is likely through hydrophobic interactions between C4 and C5 of the sugar and the side chain of Trp⁶⁴⁰. Despite making extensive interactions with only Fru-3, *BsCBM66* binds extremely weakly to fructose. In solution, fructose has considerable conformational freedom, and thus the binding of *BsCBM66* to the monosaccharide in its β-furanose conformation will incur a significant entropic penalty. In contrast, because the fructose at the nonreducing end of fructan polymers adopts a β-furanose conformation in solution, there will be no significant loss in entropy when *BsCBM66* interacts with these polysaccharides, which may explain the elevated affinity for the polymer. The specificity for internal disaccharide sequences, although binding primarily to a single sugar moiety, resembles CBM36 and CBM60 proteins, where it was proposed that the presentation of a closed sugar ring to the CBMs may confer the enhanced affinity for polymeric ligands (21, 22).

Levan-Targeting Function of *BsCBM66* Is Generic to GH32 β-fructosidases.

It is possible that, in addition to modulating the specificity of SacC, CBM66s have a generic influence on the activity and specificity of GH32 β-fructosidases. To test this hypothesis, *BsCBM66* was linked, through a 15-residue Thr/Pro sequence, to the C terminus of a *Bacteroides thetaiotaomicron* nonspecific β-fructosidase, BT3082 (18). *BsCBM66*, indeed, mediated a substantial increase in the activity of the BT3082 β-fructosidase against levan, driven mainly by a reduction in K_m , but did not affect the activity of the enzyme against inulin or fructooligosaccharides from the two fructans (Table 1). It is apparent, therefore, that *BsCBM66* has a generic effect on GH32 β-fructosidases, by enhancing their activity against levan but not against inulin. CBM66, unlike the other CBM families described to date, clearly plays a central role in defining substrate specificity.

Significantly, BT3082 displays ~10-fold higher activity against levan and inulin than the catalytic module of SacC (*BsLev32_{CM}*), and this elevated activity is retained when the *Bacteroides* enzyme is coupled to *BsCBM66* (Table 1). Only 52 of the ~2,000 known GH32 enzymes contain a CBM66, and of the 180 characterized glycoside hydrolases from this family, 21 enzymes (which all lack a CBM66) display nonspecific β-fructosidase activity. It is likely that these general-acting β-fructosidases, similar to BT3082, may offer additional catalytic targets. Modifying the specificity of nonspecific β-fructosidases, *BsCBM66* (and by inference other members of the CBM66 family) provides a strategy for tailoring the function of these industrially significant catalysts.

Discussion

We have shown that nonspecific β-fructan-binding catalytic and noncatalytic protein modules can be recruited to generate an enzyme that specifically targets levan, a branched β-2,6-fructose polymer. The mechanism by which a nonspecific fructan-binding CBM can confer enzyme specificity is intriguing. Apart from the backbone linkage, the major difference between the two major fructans in nature is that levan contains a large number of β-2,6-linked branches attached β-2,1 to the polysaccharide backbone. Indeed, in levans, 3–12% of the fructose residues are at branch

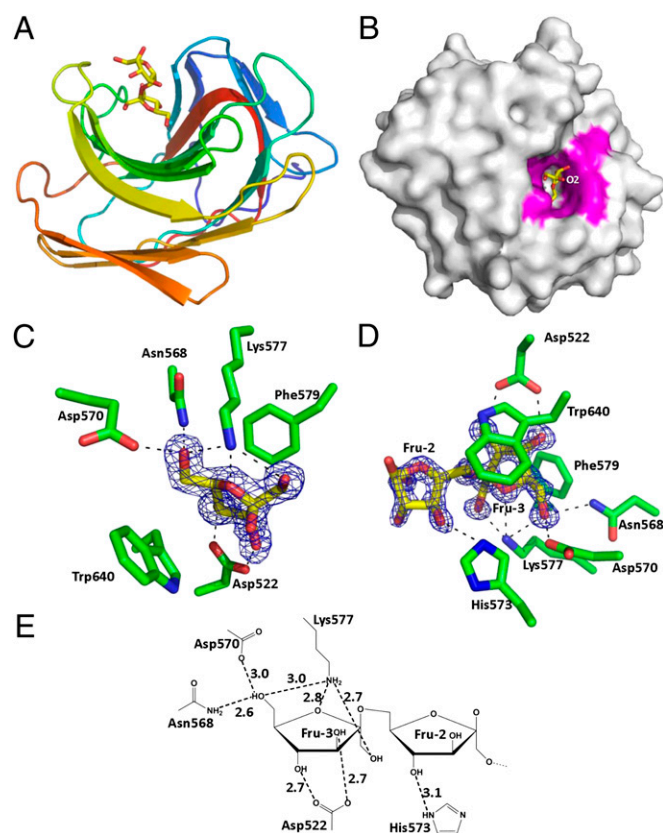


Fig. 2. The structure of *BsCBM66*. (A) Protein schematic of *BsCBM66* in complex with levanbiose (yellow), color ramped from N terminus (blue) to C terminus (red). (B) Surface representation of *BsCBM66* in complex with fructose demonstrating the solvent-accessible surface. Fructose is shown in yellow (carbon) stick representation. Amino acids whose side chains contribute to ligand recognition are colored magenta. (C and D) 3D position of amino acids of *BsCBM66* that contribute to fructose and levantriose recognition, respectively. The carbons of the amino acids are colored green and the carbohydrate ligands are yellow. The maximum-likelihood/σ-weighted $2F_{obs} - F_{calc}$ electron density map for the carbohydrate ligands is shown in blue mesh and contoured at 1.5σ ($0.65 \text{ e}^-/\text{\AA}^3$ for fructose and $0.86 \text{ e}^-/\text{\AA}^3$ for levantriose). In D, Fru-3 is the nonreducing fructose in levantriose and Fru-2 is the central sugar in the trisaccharide. There is no visible density for Fru-1. (E) Schematic representation of *BsCBM66* in complex with levantriose. The dotted lines show polar interactions with the distance in Å between the interacting atoms. A–D were drawn in PyMOL.

points (16, 17), whereas inulins contain very few, if any, branches. The branched structure of levan will result in polysaccharide molecules containing numerous nonreducing terminal fructose residues, and this presents a structure in which *BsCBM66* and *BsLev32_{CM}*, within *SacC*, will be able to bind to different terminal fructose residues of the same polysaccharide molecule. The ensuing avidity effect will result in much tighter binding of *SacC* to levan, compared with either the CBM or the catalytic module as discrete entities (Fig. 3). This model is entirely consistent with the lower K_m displayed by *SacC* compared with *BsLev32_{CM}*.

The proposed avidity mechanism for *BsCBM66* function may be a generic feature of CBMs that bind to the terminal residues of glycans, and are linked to exo-acting glycanases. Examples of such enzymes include GH84 exo-GlcNAcases, which often contain CBM32s that target terminal Gal-GlcNAc structures present on the surface of red blood cells (23). With respect to the 52 CBM66-containing GH32 enzymes, 40 are appended to ≥ 2 CBM66 modules, suggesting that the avidity effects may be more extensive in these proteins than observed in *SacC*. It would appear, therefore, that a generic role for CBM66 in GH32 enzymes is to target branched fructans conferring exolevanase activity on a cohort of nonspecific β -fructosidases. Inspection of CBM66 reveals, in addition to GH32 fructanases, a cohort of 12 enzymes containing GH43 or GH51 catalytic modules (Fig. 1), which are likely to be exo-acting arabinofuranosidases (6, 24). It is possible that the CBM66s in these enzymes will also bind to terminal arabinofuranose residues (the specificity of CBMs are often linked to the substrate hydrolyzed by the parent enzymes) (25), which may also lead to substantial increases in activity against branched substrates. Thus, it is possible that CBM66 is a family of protein modules that bind to the termini of branched carbohydrates and enhance enzyme activity against their substrates through avidity effects.

Because CBMs were first described ~25 years ago, initially as cellulose-binding domains reflecting their enzyme origin (26), there has been considerable debate over their role in enzyme action. Several reports indicate that CBMs potentiate the activity of plant cell-wall-degrading enzymes against insoluble substrates, likely through a targeting mechanism (refs. 8 and 9, and reviewed in ref. 27). In the proposed targeting mechanism, CBMs, by binding to components of the cell wall, increase the concentration of enzyme presented on the surface of the substrate. It is

not obvious why the more general targeting mechanism does not enhance activity greater than fivefold, particularly when one considers that the catalytic potential of the enzymes are several orders of magnitude greater than the observed activity against the plant cell wall (27). In contrast, it was initially believed that CBMs may disrupt the ordered structure of recalcitrant crystalline substrates such as cellulose, leading to increased enzyme access and hence catalytic activity (28, 29). Over the last quarter of a century, however, there have been only three reports of CBMs mediating modest (0.5- to fourfold) potentiation of enzyme activity *in trans*, pointing to a possible substrate disruption mechanism (30–32). However, the possibility of the formation of noncovalent CBM/enzyme interactions, which would argue in favor of a targeting function for the CBMs, was not explored. As shown in Fig. S5, *BsCBM66* mediates a substantial increase in the activity of *BsLev32_{CM}* and BT3082 *in trans*, thus suggesting a substrate disruption mechanism. Analytical ultracentrifugation of the catalytic modules in the presence of *BsCBM66* (described in *SI Materials and Methods*; Tables S3 and S4; and Fig. S6), however, revealed the presence of noncovalent heterodimers of *BsLev32_{CM}*-*BsCBM66* and BT3082-*BsCBM66*, and heterotetramers of BT3082-*BsCBM66*. Within these complexes it is likely that both the catalytic modules and the CBM can bind substrate, which would lead to enhanced affinity through avidity effects, similar to how enzyme activity is increased when *BsCBM66* is covalently attached to *BsLev32_{CM}* and BT3082. Indeed, if the CBM is increasing enzyme access, then the maximum activity afforded by *BsCBM66* would be equivalent to the rate of oligosaccharide hydrolysis (with a DP ≥ 2). The observation that *BsCBM66*, when covalently linked to *BsLev32_{CM}* in full-length *SacC*, mediates a 100-fold increase in exolevanase activity, compared with oligosaccharide hydrolysis, is consistent with the proposed targeting role of the CBM. The elevated activity against the polysaccharide precludes a mechanism in which *BsCBM66* mediates the disruption of levan, leading to increased enzyme access.

To summarize, the data presented demonstrate how specificity can be introduced into enzymes through the integration of catalytic modules and CBMs that, independently, display broad specificity. As such, this paper proposes a generic model for the biological significance of CBMs that target the terminal structures of complex glycans. The work described here provides a

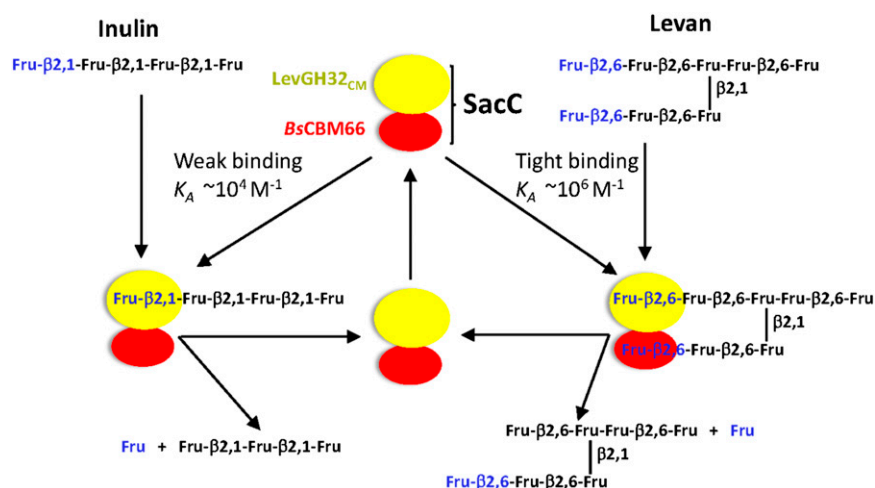


Fig. 3. Model for the role of *BsCBM66* in exo-acting β -fructosidases. The data described in Table 2 show that *BsCBM66*, which binds to terminal fructofuranose residues, enhances the activity of two GH32 broad-acting β -fructosidases against levan, but not against the other major fructan inulin. It is proposed that *BsCBM66* facilitates the enzymatic targeting of branched substrates, such as levan, by binding in synergy to the terminal fructose residues of a branch structure, leading to increased affinity through avidity effects. This avidity effect does not occur when the enzyme is attacking linear fructans such as inulin.

platform for engineering unique specificities into enzymes that could, potentially, increase their industrial utility. The proof of principle for this approach is provided by the introduction of exolevanase activity into a highly active nonspecific β -fructosidase. Such engineering strategies of fructan-degrading enzymes are particularly relevant to the pre- and probiotic industries, where fructans are widely deployed to promote the growth of beneficial bacteria.

Materials and Methods

Gene Cloning and Expression and Protein Purification. DNA encoding mature SacC (residues 23–677), the catalytic module of SacC (*BsLev32_{CM}*, residues 23–513), and the CBM of SacC (*BsCBM66*, residues 514–677) were amplified using appropriate primers. The amplified DNAs were cloned into *NheI/XhoI*-restricted pET21a, such that the encoded recombinant proteins contain a C-terminal His₆ tag. The DNA encoding *BsCBM66* was also cloned into pET16b, which supplies an N-terminal His₆-tag that can be removed with Factor X. The cloning of BT3082 into the *E. coli* expression vector pET22b was described previously (18). To generate *BsCBM66*-BT3082 His₆-tag, DNA encoding these two proteins were amplified by PCR and ligated into a pET22b variant, pET22jf, that contains a sequence to give two separate multicloning sites divided by a sequence encoding a 15-residue proline/threonine linker sequence (9). DNA encoding *BsCBM66* and BT3082 were cloned into the first (between *KpnI* and *HindIII*) and second (*EcoRI* and *XhoI*) multicloning sites, respectively. The expression and purification of the recombinant proteins used in this study are described in *SI Materials and Methods*.

Binding Studies. Affinity-gel electrophoresis was used to screen for the binding of *BsCBM66* to polysaccharides, following the method described in ref. 25, with the target polysaccharide at 100 μ g/mL. The binding of *BsCBM66* to its ligands was quantified by isothermal titration calorimetry (ITC), as described previously (25). Titrations were carried out in 50 mM Na-Hepes buffer (pH 7.5) at 25 °C. The reaction cell contained protein at 50 μ M,

and the syringe contained either the oligosaccharide at 10 mM or polysaccharide at 3–5 mg/mL. The titrations were analyzed using Microcal Origin version 7.0 software to derive n , K_a , and ΔH values, and ΔS was calculated using the standard thermodynamic equation, $RT \ln K_a = \Delta G = \Delta H - T \Delta S$.

Enzyme Assays. Fructosidase activity was determined by the continuous monitoring of fructose release using a linked enzyme assay system purchased from Megazyme International (sucrose/fructose/glucose detection kit). The reaction was carried out at 37 °C in 50 mM Na-Hepes buffer (pH 7.0) containing 2 mM MgCl₂, 1 mM ATP, and 1 mM NADP⁺; excess concentrations of linker enzymes (hexokinase, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase); and 1 mg/mL BSA. Through linker enzymes, glucose-6-phosphate, generated from the released fructose, is oxidized by glucose-6-phosphate dehydrogenase with concomitant reduction of NADP⁺ to NADPH, which was monitored at 340 nm using an extinction coefficient of 6,223 M^{−1}·cm^{−1}. For the *trans* experiments, fructose release was determined by high-performance anion exchange chromatographic measurement.

Phylogenetic Clustering. Methods used are described in *SI Materials and Methods*.

Analytical Ultracentrifugation. The use of AUC to determine protein mass is described in *SI Materials and Methods*.

Crystallization, Data Collection, Structure Solution, and Refinement. The methods used are described in *SI Materials and Methods*, and the crystal and data collection statistics are reported in Table S2.

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