

A single residue mutation abolishes attachment of the CBM26 starch-binding domain from *Lactobacillus amylovorus* α -amylase

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Abstract Starch is degraded by amylases that frequently have a modular structure composed of a catalytic domain and at least one non-catalytic domain that is involved in polysaccharide binding. The C-terminal domain from the *Lactobacillus amylovorus* α -amylase has an unusual architecture composed of five tandem starch-binding domains (SBDs). These domains belong to family 26 in the carbohydrate-binding modules (CBM) classification. It has been reported that members of this family have only one site for starch binding, where aromatic amino acids perform the binding function. In SBDs, fold similarities are better conserved than sequences; nevertheless, it is possible to identify in CBM26 members at least two aromatic residues highly conserved. We attempt to explain polysaccharide recognition for the *L. amylovorus* α -amylase SBD through site-directed mutagenesis of aromatic amino acids. Three amino acids were identified as essential for binding, two tyrosines and one tryptophan. Y18L and Y20L mutations were found to decrease the SBD binding capacity, but unexpectedly, the mutation at W32L led to a total loss of affinity, either with linear or ramified substrates. The critical role of Trp 32 in substrate binding confirms the presence of just one binding site in each α -amylase SBD.

Keywords Starch-binding domain · Alpha-amylase · Starch · Carbohydrate binding · CBM26

Introduction

The major vegetal reserve polysaccharide, starch, is hydrolyzed into soluble sugars by several enzymes distributed into all the life domains. Some of these enzymes contain carbohydrate-binding modules (CBMs) whose principal function is the attachment of the enzyme to the insoluble substrate, i.e., the starch granules, bringing the substrate to the catalytic domain of the enzyme, increasing the local concentration and facilitating hydrolysis [15]. It has been reported that enzymes that have lost their binding modules, specifically, their starch-binding domains (SBDs), diminish or even lose the capacity to hydrolyze insoluble starch but not the soluble substrate [11, 14, 19].

SBDs have been classified by its primary structure into eight families (CBM20, CBM21, CBM25, CBM26, CBM34, CBM41, CBM45 and CBM48). These families belong to a general classification developed for the carbohydrate-active enzymes (CAZY: <http://www.cazy.org/index.html>). In general, the architectures of the SBDs are structurally well conserved; they adopt a β -sandwich fold and show a conserved mode of α -glucan recognition, based on the hydrophobic interaction of aromatic residues such as tryptophan and tyrosine [2, 10]. Recently, the structure of the CBM26 from the maltohexaose-forming amylase from *Bacillus halodurans* (C-125) was solved. The SBD folds in a β -sandwich fashion with immunoglobulin-like topology. Its Trp 36 and Tyr 25 are responsible for stacking interaction against pyranose rings of maltose sugar residues. Tyr 23 contributes to a hydrogen bond formation with oxygen 6 of the sugar stacking against Trp 36 [3].

Only families CBM26 and 34 contain enzymes with SBDs of lactobacilli origin. CBM26 contains *Lactobacillus* α -amylases with unusual tandemly arranged SBDs, i.e., five identical modules in *L. amylovorus* and four in *L. plantarum*

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[6] and *L. manihotivorus* [12]. In a preceding report, it was shown that the *L. amylovorus* α -amylase with no tandem SBDs is incapable of binding and hydrolyzing starch granules even though it still hydrolyzed soluble starch [14]. The binding ability of just one *L. amylovorus* α -amylase SBD was established, suggesting that any of the CBMs might be able to bind starch with equal affinity [16]. The observed tandem organization allows the modules to display a significant increase in affinity for insoluble substrates acting in a synergistic way when they are together in the same polypeptide chain [8].

Although belonging to the same CBM26 family, the sequence similarity between the modules from the *L. amylovorus* SBD α -amylase (laSBD) and the *B. halodurans* maltohexaose-forming amylase SBD (bhCBM26) [3], the only CBM26 structure solved, is very low. However, some of the aromatic residues related to binding function are well conserved [16]. In this study, we have investigated the properties of the aromatic residues in one of the five identical modules of *L. amylovorus* α -amylase SBD. The finding suggests that in contrast to the *B. halodurans* maltohexaose-forming amylase SBD, the mutation of just one aromatic residue in laSBD can eliminate the starch binding ability. Considering the multi-interaction network between the carbohydrate and the binding domain, it is remarkable that a single residue mutation of Trp 32 results in an abolishment of this binding.

Materials and methods

Strains and plasmids

Escherichia coli XL10-Gold (Stratagene cloning system; Stratagene, La Jolla, CA) was used as host for general cloning work and for protein production. Recombinant cells were cultivated in Luria–Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with ampicil-

lin (100 μ g/ml). Plasmid pQ-1mod containing one SBD was constructed as previously described [8]. *E. coli* strain DH5 α [*supE44 lacU169* (Δ 80*lacZ* M15) *hsdR17 recA1 endA1 gyr96 thi-1 relA1*] was used as the initial host of pQ-1mod.

Construction of *L. amylovorus* α -amylase SBD mutants and DNA manipulations

The standard nucleic acid techniques used in this work, are those of Sambrook et al. [17]. Site-directed mutagenesis was used to construct mutants of the wild-type SBD. Point mutations were introduced to change tryptophan residues to leucine in two simple mutants named W11L and W32L and in a double mutant W11-32L. Conserved tyrosine residues were changed to leucine in mutants Y16L, Y18L, Y20L and Y85. Constructions were performed using a Quick Change Mutagenesis Kit (Stratagene, La Jolla, CA) with two antiparallel primers containing the desired mutation and pQ-1mod as template DNA (Table 1). PCRs were performed using *Pfu Turbo* DNA polymerase. Incorporation of the oligonucleotide primers generated a mutated plasmid containing staggered nicks. After PCR, the products were treated with *DpnI* to digest the parental DNA template and the newly synthesized DNA was selected. The nicked vector DNA which incorporated the desired mutations was then transformed into XL10-Gold cells. All constructs were validated by DNA sequencing (Laragen Inc).

Sequence alignment

SBD sequences were aligned using ClustalW [4]. Superimposition of binding residues between the CBM26 structure from *B. halodurans* amylase-bhCBM26 (PDB 2C3G) and the Rosetta model (<http://www.bioinfo.rpi.edu/~bystrc/hmmstr/server.php>) of one *L. amylovorus* α -amylase module (SBD Q48502) was done in a Swiss PDB Viewer (<http://www.expasy.org/spdbv>).

Table 1 Oligonucleotide primers used for site-directed mutagenesis

Mutant	Primers
W11L	P1f: 5' GAAAAGCCTTCAAGT TTGGG TAGTAGAGTTTATGCC 3' P2r: 5' GGCATAAACTCTACTACCC AAACT TGAAGGCTTTTC 3'
W32L	P1f: 5' GCTATAACTTCAGCT TTGCCT GGCAAAAAATGACC 3' P2r: 5' GGCATTTTTTTGCCAGGCA AAAGCT GAAAGTTATAGC 3'
Y16L	P1f: 5' GGGGTAGTAGAGTT TTAGCCT ATGTTTATAATAAAAAATACG 3' P2r: 5' CGTATTTTTATTATAAAACATAGGCT AAA ACTCTACTACCCC 5'
Y18L	P1f: 5' GGGGTAGTAGAGTTTATGC CTTAG TTTATAATAAAAAATACG 3' P2r: 5' CGTATTTTTATTATAAA CTA AGGCATAAACTCTACTACCCC 3'
Y20L	P1f: 5' GGGGTAGTAGAGTTTATGC CTATG TTTAAATAAAAAATACG 3' P2r: 5' CGTATTTTTATT TA AAACATAGGCATAAACTCTACTACCCC 3'
Y85L	P1f: 5' ACGGCTGATGCCACT TTAAT CAAAATGGTGTGC 3' P2r: 5' CGACACCATTTTGAT TAA AGTGGCATCAGCCGT 3'

Nucleotides in bold represent the mutations introduced by the substitutions

Protein purification

Cells containing the wild-type and the mutated plasmids were grown in LB medium with ampicillin at 100 µg/ml for 8 h at 37°C. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (0.4 mM), and incubation was continued for 8 h at 30°C. Cells were harvested by centrifugation (8,000 g for 10 min at 4°C) and washed in 20 mM Tris, pH 7.4 before lysis.

For protein purification, the *E. coli* pellet was resuspended to about 1/100 of the original culture volume in binding buffer (20 mM Tris pH 7.4, NaCl 0.5 M and imidazole 50 mM) with a protease inhibitor cocktail (Sigma-Aldrich, St Louis Mo). Cells were disrupted by sonication three times for 20 s at 60 Hz (Vibra-Cell; Sonics & Materials, Inc). Obtained polypeptides were purified from clarified extracts by immobilized metal affinity chromatography according to the manufacturer's protocols (Pharmacia Biotech).

Electrophoretic analyses

Sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis was performed according to the method of Laemmli [9]. Proteins were visualized by Coomassie brilliant blue staining as described by Blakesly and Boezi [1].

Starch-binding assay

The raw binding activity was determined as previously described [8]. Various amounts of peptides were added to a prewashed raw-cornstarch suspension (final concentration, 1%) in 0.1 M citrate-phosphate buffer, pH 5. The mixture was incubated at 4°C for 30 min with gentle shaking and centrifuged at 13,000g for 5 min. The protein concentration in the supernatant fluid was subtracted from the total protein to calculate the total amount of protein bound.

Affinity gel electrophoresis

Once the proteins were purified, the affinity of the mutants and wild-type laSBD's for a range of polysaccharides was determined by affinity gel electrophoresis (AE). The AE method was performed with the Bio-Rad Mini-Protean III system (Bio-Rad, Richmond, CA). Purified proteins were separated in native gels containing no carbohydrate or 1% of potato starch (Prolabo, Fontenay-sous-Bois, France), amylose, amylopectin, pullulan, glycogen, cellulose and birchwood xylan (all purchased from Sigma-Aldrich, St Louis Mo). Electrophoresis was run at room temperature at 100 V. Albumin was used as a negative, non-interacting control. Proteins were detected by staining with Coomassie brilliant blue.

Results

Sequence alignment and molecular modeling

Sequence comparison between the CBM26 members (CAZY: <http://www.cazy.org/index.html>) revealed no significant primary structure identity. Only two residues were fully conserved, one tryptophan and one tyrosine. However, when the alignment was made between one of the five modules forming the SBD of the *L. amylovorus* α-amylase (laSBD) and the single solved structure in this family (SBD from the *B. halodurans* maltohexaose-forming amylase—bhCBM26), the amino acid identity reached only 28% (Fig. 1).

The alignment showed that, even though the relevant binding amino acids were present in the laSBD and only a glycine was not conserved (Fig. 1a), structural alignments in web protein modeling servers did not recognize any similar structure. Superimposition of the Rosetta model of one module of the *L. amylovorus* α-amylase SBD (<http://www.bioinfo.rpi.edu/~bystrc/hmmstr/server.php>) with the PDB 2C3G SBD of the *B. halodurans* amylase and inspection of the amino acids related to the binding site showed that interactive residues display divergent orientations (Fig. 1b) (Deep View/Swiss PDB viewer- <http://www.expasy.org>).

Given the absence of closer solved structures, candidate residues were chosen to test the importance of hydrophobic stacking. Thus, in an initial approach, the aromatic residues (Trp11, Trp 32, Tyr 16, Tyr 18, Tyr 20 and Tyr 85) present in one module of the *L. amylovorus* α-amylase SBD were mutated to leucines. After verification of the sequences, the mutated plasmids were transformed into *E. coli* XL10-Gold. The mutated proteins were expressed in a similar way to the wild-type protein, indicating that the specified mutation did not change the global folding. The molecular size and purity of all proteins was confirmed by SDS-PAGE (Fig. 2).

Binding activity

The binding activities of the purified enzymes were initially evaluated in isotherm adsorption binding assays as previously described [8], but in the case of some mutants, the affinity was too low to be accurately quantified. Therefore, it was estimated by AE.

To explore the tyrosine and tryptophan contribution to the binding activity, four tyrosine residues were interchanged by leucines. They were Tyr 16, Tyr 18 and Tyr 20, located near Trp 32, and Tyr 85, in close proximity to the putative hydrogen bond-forming residues (Fig. 1). Two of those tyrosine residues showed a contribution to the raw starch binding (Fig. 3). The proteins with Y18L and Y20L mutations had a

Fig. 1 a SBD sequence alignment of *L. amylovorus* NRRLB-45 α -amylase SBD Q48502 (*L.a.*) and *B. halodurans* C-125 amylase PDB 2C3G (*B.h.*). Conserved residues are indicated by *; mutated residues are indicated by a closed circle and residues involved in binding in the *B. halodurans* α -amylase SBD are boxed [3]. **b** Superposition of the binding residues from *B. halodurans* C-125 amylase (PDB 2C3G; in red) and the Rosetta model of one module of the *L. amylovorus* α -amylase SBD (in black). Location of all residues corresponds to those of the three-dimensional structure of *B. halodurans* C-125 amylase

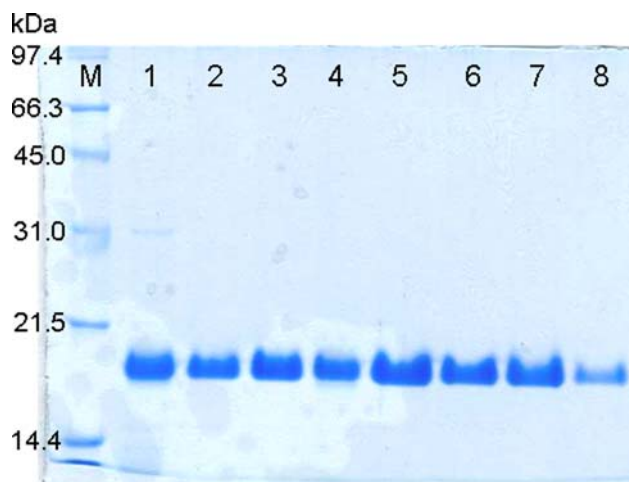
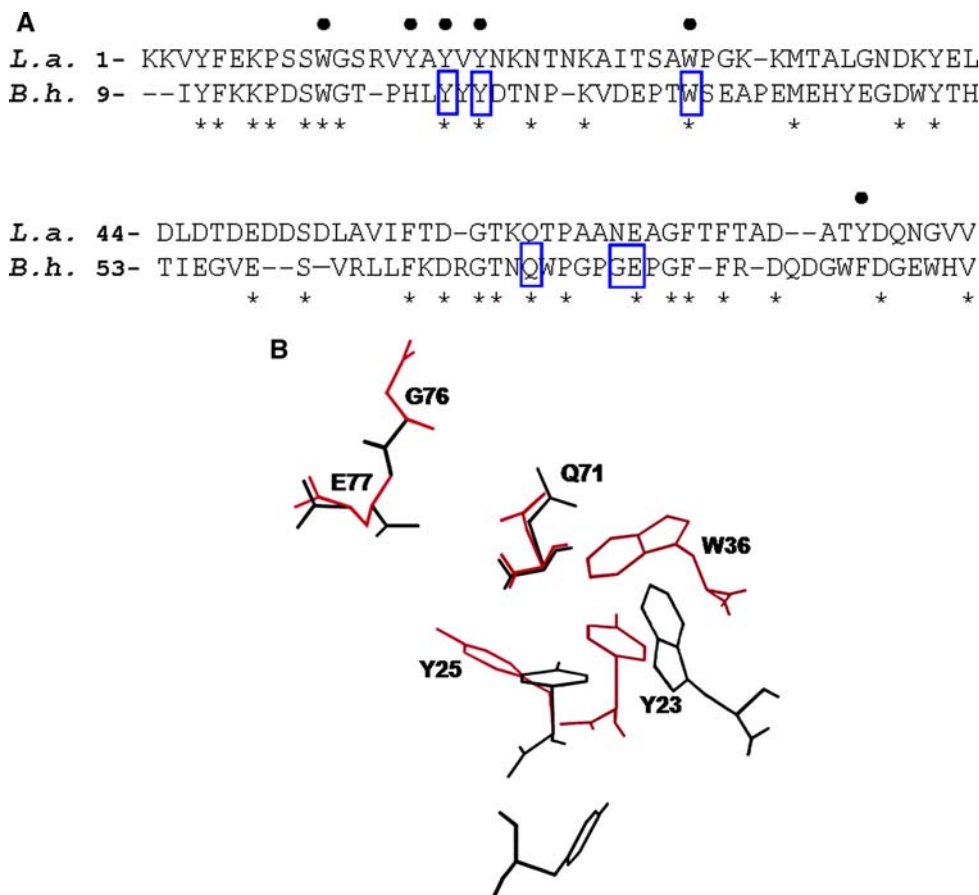


Fig. 2 SDS-PAGE analysis of the purified protein derived from wild-type and mutants. Lanes 1 WT *L. amylovorus* α -amylase SBD, 2 W11L, 3 W32L, 4 W11-32L, 5 Y16L, 6 Y18L, 7 Y20L, 8 Y85L

minor capacity of adsorption, in comparison to the wild-type protein. The different migration retardation observed between Y18L and Y20L (Fig. 3) suggested a different binding contribution of these two tyrosines. In contrast, the other tyrosine mutations (Y16L and Y85L) did not have any effect on the laSBD starch-binding function.

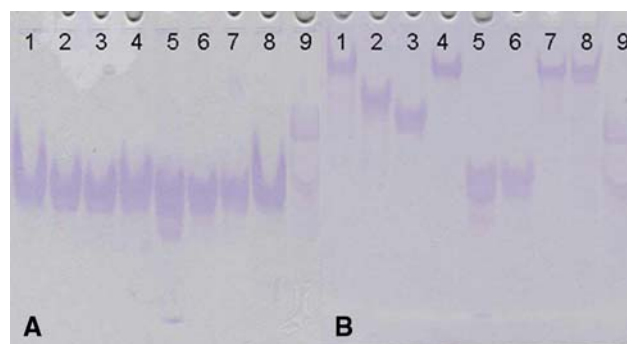
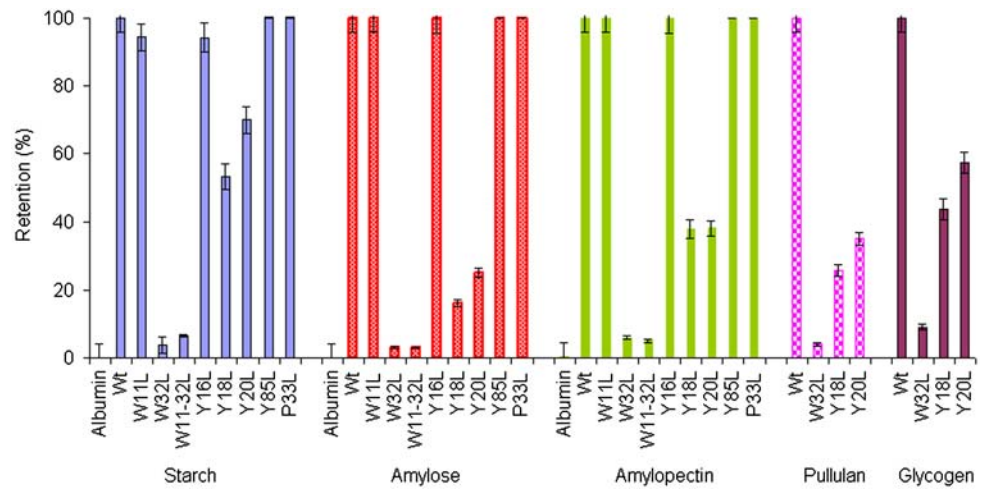


Fig. 3 Non-denaturing affinity electrophoresis in the absence (a) or presence (b) of potato starch. Lanes 1 Y85L, 2 Y20L, 3 Y18L, 4 Y16L, 5 W11-32L, 6 W32L, 7 W11L, 8 Wild-type *L. amylovorus* α -amylase SBD and 9 albumin

The same figure shows that W32L displayed no detectable affinity for starch suggesting that this residue plays the most important role in carbohydrate recognition. In order to verify that the observed effect was not the consequence of a conformational change in the protein, the proline residue, next to Trp 32, were also mutated. There was any effect of this mutation on binding ability. On the other side, Tyr 18 and Tyr 20 interactions, still present in the W32L mutant, were not enough to establish any binding to starch. The

Fig. 4 Binding of *L. amylovorus* α -amylase SBD and its mutants to different polysaccharides as assessed by affinity gel electrophoresis



mutation of Trp 11 to leucine had no apparent effect on the binding to starch.

SBD specificity for other polysaccharides

Starch is the natural substrate for amylases. The enzyme SBDs are designed for recognition of the glucose polymers, such as amylose and amylopectin, present in distinct proportions in different origin starches. Therefore, we tested the binding of one module and the mutated proteins to different polysaccharides. The data show (Fig. 4) that W32L and the double mutant W11-32L display no detectable affinity for all the tested ligands, whereas Y18L and Y20L exhibit significantly reduced binding to these polysaccharides. The similar proportional reduction in the affinities of the mutants for polysaccharides, compared to wild-type SBD, suggested that these three residues (Trp 32, Tyr 18 and Tyr 20) play an important role in the recognition of α , 1–4 and α , 1–6 glucans (e.g., amylose vs. amylopectin, pullulan and glycogen). In contrast, W11L, Y16L and Y85L mutant proteins display similar affinity to that of the wild-type SBD, which suggests that they do not contribute to ligand recognition. As expected, no retention was observed with cellulose or xylan.

Discussion

The *L. amylovorus* α -amylase SBD has a quite different primary sequence as compared to other described SBDs. The particular architecture composed of five tandem modules implies that this multiple SBD must bind starch with a different mechanism. However, it has been reported that each repeated unit is independent for binding to the starch granule [8, 16], and as a single unit, each module may conserve the general structure and mechanism of carbohydrate-binding reported for CBM [10].

In this mechanism, binding is facilitated by stacking interactions between the glucose residues and aromatic amino acids present in the carbohydrate-binding site. The obtained results indicate that residues Tyr 18, Tyr 20 and Trp 32 play a major role in the interaction of the *L. amylovorus* α -amylase SBD with its ligands, such as the nearest structure solved, the bhCBM26, where the base of the sugar binding is done by Trp 36, Tyr 23 and Tyr 25. Trp 36 and Tyr 25 stack against the pyranose rings of maltose [3]. Tyr 23 is flanked by Trp 36 and Tyr 25 and contributes a hydrogen bond with oxygen 6 of the sugar stacking against Tyr 25 [3]. Nevertheless, the binding abolishment caused by the Trp 32 mutation was not expected, since dramatic changes in binding activities were only observed when several mutations are produced in the same CBM. Thus, the binding nature of this specific tryptophan remains to be explained.

Even with the above-mentioned differences, the fact that a single tryptophan mutation eliminates the SBD interaction with all the tested polysaccharides supports the theory of “one binding site” in CBM26 family members. In the case of the two SBDs binding sites, abolishment of one site does not prevent the binding of the amylose chain to the other one [5, 13].

It has been suggested that tandem arrangement could be suited to the disruption of the starch structure [3]; analogous to the two binding sites described for *Aspergillus* glucoamylase CBM20 [18]. However, in the case of the five tandem SBD from lactobacilli, the disruption effect could not be identified [7]. Therefore, the presence of multiple SBDs, might be the result of the requirement of stronger binding to the substrate, given that, as it was reported, the tandem arrangement improved raw starch binding [8].

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