Review

Functional characteristics of the starch-binding domain of *Lactobacillus amylovorus* α-amylase

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Abstract: *Lactobacillus amylovorus* α-amylase (AmyA) presents an uncommon primary structure, constituted by two parts: the catalytic domain (amino acids 1 to 474) and the C-terminal domain (475-953). Structurally, the C-terminal domain is different from the other starch binding domains (SBD) of α-amylases since it is composed of five identical repeated units (RU) of 91 amino acids each one. A truncated *AmyA*, encoding the N-terminal region (first 474 aa) comprising the catalytic domain and excluding the putative SBD was expressed in a non-amylolytic *Lactobacillus plantarum* strain. This protein was unable to bind or hydrolyze raw starch, showing that repeated units constitute an SBD. The importance of the repeated units on the starch binding ability was analyzed by expressing either one or five RUs of the C-terminal region in *Escherichia coli*, as independent domains. Starch binding assays showed that the five RUs alone bind to insoluble starch, suggesting that the catalytic domain is not required for adsorption. Moreover, one RU was also bound to the starch grain. These observations suggest that each RU is acting as an independent fixation module, as observed in some glycoside hydrolases, but not in amylases.

Key words: carbohydrate-binding domain, CBM family 26, starch-binding domain, Lactobacilli amylases, raw starch.

Abbreviations: CD, catalytic domain; GH, glycoside hydrolase; RU, repeated unit; SBD, starch-binding domain.

Introduction

The starch-binding domain (SBD) is a functional domain, which can bind granular starch increasing thus the local concentration of substrate at the enzyme active site, and may also disrupt the structure of starch surface thereby enhancing the amylolytic rate (Sorimachi et al., 1997; Southall et al., 1999). In the primary structure classification of glycoside hydrolases (GHs) (Coutinho & Henrissat, 1999), the carbohydrate-binding modules (CBMs) are organized into 42 families, which include several specificities, such as cellulose, xylan, chitin and starch binding. The most generalized and studied family of starch-binding modules is the CBM20; this family contains ~120 different proteins and the granular starch-binding function has been demonstrated in several cases. These modules are present in approximately 10% of amylolytic enzymes from GH13 (almost all CGTases, in a few α-amylases and in maltotetraohydrolases, maltopentaohydrolases, maltogenic α-amylases and acarviose transferases), GH14 (some β-amylases), and GH15 (most fungal glucoamylases) (Janecek & Sevcik, 1999). The SBD is usually positioned at the C-terminal end of proteins, except for the glucoamylase from *Rhizopus oryzae* (Ashikari et al., 1986) and the *Thermoactinomyces vulgaricus* α-amylase (Abe et al., 2004), which contain the SBD at their N-termini. These SBDs belong, however, to CBM21 and CBM34, respectively (for a review, see Rodriguez-Sanoja et al., 2005). In particular, the yeast α-amylase from *Lipomyces kononenkoae* possessing the N-terminal CBM21 has been described by Ramachandran et al. (2005). The SBD is usually composed of about one hundred amino acid residues, which produce several β-strand segments forming an open-sided, distorted β-barrel structure (Mikami et al., 1999; Penninga et al., 1996).

The α-amylases of lactobacilli

Recently the *Lactobacillus amylovorus*, *Lactobacillus plantarum* and *Lactobacillus manihotivorans* α-amylases were described. The three enzymes are able to hydrolyze raw starch on acidic pH (Imam et al., 1991; Giraud et al., 1994; Guyot & Morlon-Guyot, 2001). The gene sequences of these enzymes share an iden-

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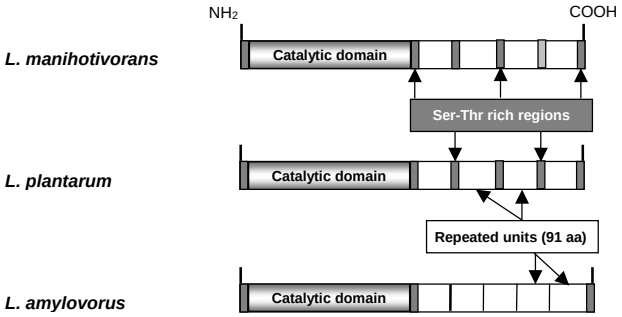

Fig. 1. The structure of the α-amylase genes from lactobacilli. The tandem repeats are shown in white, whereas the serine-threonine rich regions are in dark grey.

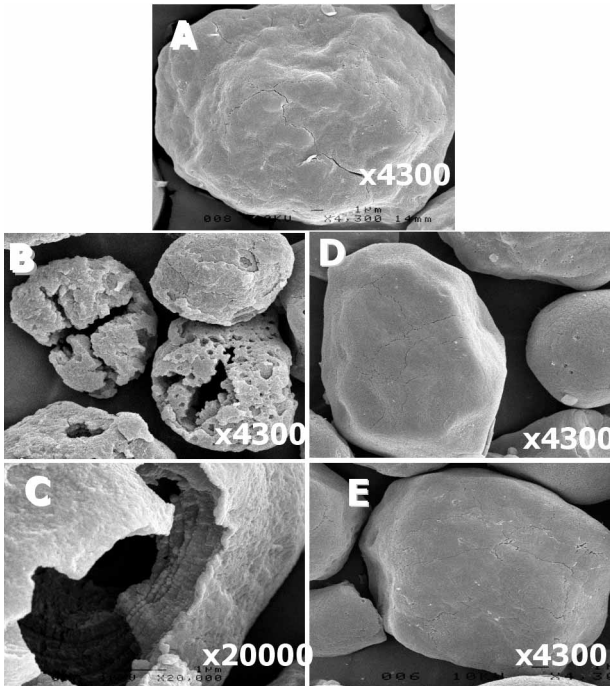

Fig. 2. Scanning electron micrographs showing the initial aspect of the corn-starch granules (A) and the aspect of the granules after their fermentation with the*Lactobacillus amylovorus* α-amylase for 24 (B) and 48 h (C), or with the α-amylase having the repeated units deleted after 24 (D) and 48 h (E). For experimental details, see Rodríguez-Sanoja et al. (2000).

tity of 98% (Morlon-Guyot et al., 2001). The three enzymes are organized into two functional domains: the catalytic domain (CD) (amino acids 1 to 474) and the SBD (amino acids 475–953 in *L. amylovorus* α-amylase).

The catalytic domain belongs to the family GH13 (Coutinho & Henrissat, 1999) and contains the conserved sequence regions as described by Vihinen & Mäntsälä (1989), Rumbak et al. (1991) and Janecek et al. (1999). The three lactobacilli CDs share 99.2% sequence identity, and 65.5% and 61.5% with *Bacillus subtilis* and *Streptococcus bovis* α-amylases, respectively (Morlon-Guyot et al., 2001).

In contrast, the SBD of the lactobacilli, classified by Coutinho & Henrissat (1999) into the family CBM26, has a structure that is completely different from the common SBD (CBM20). The lactobacilli α-amylases present an SBD formed by almost 500 amino acids organized in tandem repeated units (RUs) of 91 amino acids each (Fig. 1) – four identical repeats for *L. manihotivorans* (Morlon-Guyot et al., 2001) and *L. plantarum* and five identical repeats for *L. amylovorus* (Giraud & Cuny, 1997). Similar modular organization has also been found in the family CBM25: in the α-amylases from *Bacillus* sp. no. 195 (Sumitami et al., 2000) and *Clostridium acetobutylicum* ATCC 824 (Noelling et al., 2001) both with two repeats forming a putative SBD, and in the maltopentaose-producing amylase from an alkaliphilic Gram-positive bacterium with three C-terminal repeats of unknown function (Candussio et al., 1990). A similar situation is in some pullulanases from the family CBM41 with two N- or C-terminal modules (Coutinho & Henrissat, 1999).

The RUs are flanked by two serine-threonine rich regions and, in the *L. plantarum* and *L. manihotivorans* α-amylases, these sequences are also present between the RUs (Morlon-Guyot et al., 2001) (Fig. 1). The presence of these regions may increase the random coil regions and perhaps the mobility of the RUs in the *L. plantarum* and *L. manihotivorans* α-amylases in contrast with the SBD from *L. amylovorus*. The mentioned regions have a "consensus" sequence (TTSDSSSSSSTTTET) that resembles the serine-threonine rich *O*-glycosylated Gp-I domain of glucoamylase I from *Aspergillus niger,* involved in the maintenance of protein structure against stress, adsorption onto raw starch granules, and secretion (Libby et al., 1994; Semimaru et al., 1995; Goto et al., 1999; 2004; Juge et al., 2002). It is worth mentioning, however, that in the case of the lactobacilli α-amylases the sequences are not glycosylated (Rodríguez-Sanoja et al., 2005).

Nevertheless, the presence of RUs is very usual in other substrate-binding domains, as in the glucosyltransferases and dextran-saccharases from *Leuconostoc* and *Streptococcus* (Monchois et al., 1999, Shah & Russell, 2000), or in the xylanolytic and cellulolytic enzymes from *Cellulomonas*, *Aeromonas* and *Clostridium*, where the linkers favor correct conformations and independent actions of joined functional domains (Linder & Teeri, 1997; Bolam et al., 2001; Boraston et al., 2002).

Starch binding domain in lactobacilli α-amylase

To identify the role of tandem repeats in the *L. amylovorus* α-amylase, the whole gene and the gene resulted from the deletion of the tandem repeats were expressed in a non-amylolytic *L. plantarum* strain. The proteins were purified until electrophoretic homogeneity and their biochemical characteristics were compared (Rodríguez-Sanoja et al., 2000).

Both enzymes exhibit similar α-amylase activity

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L. amylovorus (X5)          TKVYFEKPSSWGS-RVYAYVYNKNTNKAITSAWPGK-KMTALGNDKYELDLDTDEDDSDLAVIFTD-GTKQTPAANEAGFTFTADATYD
L. manihotivorans(X4)       TKVYFEKPSSWGS-TVYAYVYNKNTNKAITSAWPGK-EMTALGNDEYELDLDTDEDDSDLAVIFTD-GTNQTPAANEAGFTFTADATYD
L. plantarum (X4)           TKVYFEKPSSWGS-TVYAYVYNKNTNKAITSAWPGK-EMTALGHDEYELDLDTDEDDSDLAVIFTD-GTNQTPAANEAGFTFTADATYD
B. fibrisolvens                YFYNTEGWDKVCAYTW------GATALGDWPGK-ELTQDEDGWYSVVLPA-GPSEDLNIIFNNGNN
S. bovis 148 RU2             VTFDNPGGWDSANAYLYY--GNPVQYPLGVWPGT-QMTKDDAGNFYLDLPEEYADVNAKIIFNQ-PGTSNQYPYSEGFNLVKSGNYNKDGL
S. bovis 148 RU1             IYFQNPDNW-SE-VYAYMYSAKDNKL_LGAWPGTK-MTKEASGRYSITVPASYAEEGVKVIFTN-NQGSQYPQNE-GFDFKAEGLYSKAGLMPDVP
B. subtilis                   IGYQNPDHWGNVNAYIYKHDGGGAIELTGSWPGK-AMTKNADGIYTLTLPANADTADAKVIFNN-GSAQVPGQNHPGFD
B. subtilis SUH4-2            IGYQNPNHW-SVNAYIYKHDGGRAIELTGSWPGK-PMTKNADGIYTLTLPADTDTTNAKVIFNN-GSAQVPGQNQPGFDYVQNGLYNDSGLSGSLPH
B. subtilis subtilis          IGYQNPNHW-SVNAYIYKHDGSRVIELTGSWPGK-PMTKNADGIYTLTLPADTDTTNAKVIFNN-GSAQVPGQNQPGFDYVLNGLYNDSGLSGSLPH
C. acetobutylicum 1 RU1       VHFKDPNGWSAPNIYYY---DPAGKLTGPGWPGV-KMNSDGNGWYSYTI---QNWTSAKVLFDD-GTNQIPGVNQPGIDVTGEEWYENGKLYQANPD
C. acetobutylicum 1 RU2       VHYKNPTNWSEPSVYYD---NTAGGVKGPDWPGV-KMNNDGNGWYSYII---KDTTAAKATFND-ETNK-----SSVIDVTGEEWYENGTLYQYNPD
C. acetobutylicum 2 RU2       VHYKNPTNWSEPSVYYD---NTAGGVKGPDWPGV-KMNNDGNGWYSYII---KDTTAAKATFND-ETNK-----SSVIDVTGEEWYENGTLYQYNPD
C. acetobutylicum 2 RU1       VHFKNISTWAAPNIYFY---DATGGVTGPEWPGA-KMKDDGNGWYSYTI---DNCTSAKVLFND-GVNQIPGHNEPGFDVSGEEWYKDGNWYKSNPN
B. halodurans C-125           IYFKKPDSWGTPHLYYY---DTNPKVDEPTWSEAPEMEHYEGDWYTHTI---EGVESVRLLFKDRGTNQWPGPGEPGFFRDQDGWFDG-EWHVDRP
Bacillus sp. H167              YFKNPDTWGTPHIYYY---ETEPKVEEPSWGSAPEMELVENGWYKYTI---EGAESARVLFKDAGANQWPGPGEEGFYRDQDGWFDG-EWHSEKP
Bacterium DSM5853            VYFKKPADWGTPHIYYY---DTFPEEPEVTWTTAPEMTLVEDDWYVYVF---ENAESANIIFKDSSGKQIPGPNEPGFFIDQIGWYDGVKWLDSDP
Consensus                     VYFKNPDNWGSPNVYYYYHDDNTGKVPTGSWPGKPEMTKDGNGWYSYTLPADEDTENAKVIFNDGGTNQTPGPNEPGFDFTGDGWYDNGGWYQSNPD
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Fig. 3. Sequence alignment of the family CBM26 members. Line 1: *Lactobacillus amylovorus* α-amylase (U62096); line 2: *Lactobacillus manihotivorans* α-amylase (AF126051); line 3: *Lactobacillus plantarum* α-amylase (U62095); line 4: *Butyrivibrio fibrisolvens* α-amylase (M62507); lines 5, 6:, *Streptococcus bovis* 148 α-amylase (AB000829); line 7: *Bacillus subtilis* α-amylase (AB015592); line 8: *B. subtilis* SUH4-2 α-amylase (AF116581); line 9: *Bacillus subtilis subtilis* 168 α-amylase (D50453); lines 10, 11: *Clostridium acetobutylicum* 1 α-glucosidase (AE007725); lines 12, 13: *Clostridium acetobutylicum* 2 fusion of α-glucosidase and glycosidase (AE007786); line 14: *Bacillus halodurans* C-125 α-amylase (AP001508); line 15: *Bacillus* sp. H167 maltohexaose-forming amylase (X55452); line 16: alkalophilic Gram-positive bacterium DSM5853 maltopentaose-forming amylase (X53373). The residues highlighted in gray boxes have been reported in some members of CBM20 as part of the carbohydrate binding sites.

on a range of soluble substrates. However, the truncated α-amylase showed lower activity for soluble starch with one-third-fold decrease in the catalytic constant (K_{cat}) and one-third increase in K_M compared to the whole enzyme. Moreover, the direct RU region contributes to the stability of the enzyme against pH and temperature by maintaining the intact conformation of the protein. But the most important difference between these α-amylases is the inability of the truncated one to hydrolyze raw starch. Figure 2 illustrate the initial aspect of the starch granules and after their fermentation with the *L. amylovorus* α-amylase; past 24 h it can be seen that the starch granules become rougher and perforated and after 48 hours many starch granules displayed large cavities and their lamellar organization could be observed. However the granules treated with the truncated α-amylase did not show degradation after either 24 h or 48 h. In subsequent studies adsorption of both enzymes to raw-starch granules was assayed at various protein concentrations: the whole α-amylase was adsorbed to raw starch, whereas the α-amylase without tandem repeats was not able to bind raw starch, the binding being the possible reason for the incapacity of the truncated enzyme to degrade the granules (Rodríguez-Sanoja et al., 2000).

Once the role of tandem repeated units as an SBD was established, the sequence of one of the *L. amylovorus* SBD repeats was aligned with the other members of the CBM26 (this family comprises 18 proteins, but the starch-binding ability has been demonstrated only in two cases). The alignment (Fig. 3) enabled us to identify, in each repeat, the amino acid residues that appear in other CBM family as part of the carbohydrate-binding sites (Sorimachi et al., 1997). Further analysis was carried out to determine if the tandem repeats were acting as a unit or as separate modules, whose functions may be added in order to improve the adsorption.

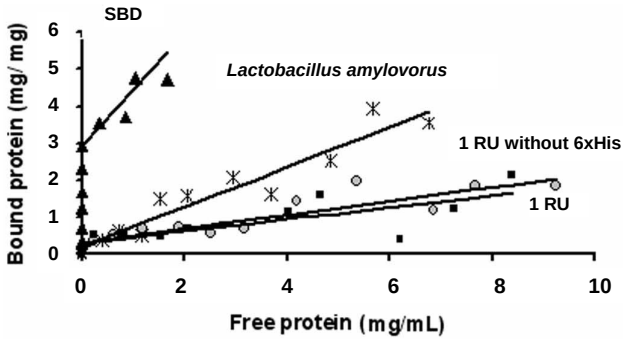

Fig. 4. Binding plots of the *Lactobacillus amylovorus* α-amylase and its derivatives against raw cornstarch. Several protein concentrations were mixed with pre-washed granular cornstarch. Reaction tubes were shaken at 4 °C for 30 min. The starch was pelleted by centrifugation and the supernatant was assayed for protein.

To answer these questions, a His-tagged version of one and five repeats, was produced. The peptides produced and also the whole native α-amylase were purified by affinity chromatography to electrophoretic homogeneity and their ability for adsorption was performed by the method described previously (Rodríguez-Sanoja et al., 2000). Figure 4 shows the resulting binding plots of mg of bound protein per gram of starch versus mg of unbound protein. Notice that one RU is able to bind raw starch without an observable effect of the His-tag. This adsorption was specific because the *L. amylovorus* α-amylase without tandem repeats is not capable of fixing to starch granules (Rodríguez-Sanoja et al., 2000). However, the whole α-amylase binds to insoluble starch stronger than the unit alone. This may suggest that higher the number of repeats, higher the adsorption is. Furthermore, when the binding of the five tandem RUs was compared, at low protein concentrations all the protein was bound to starch. The binding was evidently stronger than that of a single repeat but it was also higher than that of the whole

α-amylase. These observations suggest that each repeat may bind the starch granule acting as an independent fixing module with an additive or synergic effect between the units. This phenomenon, known as cooperativity, has never been described for amylases, nevertheless, it has been observed in some cellulases and chitinases with multiple CBMs (Linder et al., 1996; Bolam et al., 2001; Boraston et al., 2002).

The presented information describes an SBD, different from the best known CBM20, constituted by modules that may act as individual functional units. However, there is not yet enough information to establish if a single RU is sufficient to allow the binding and the hydrolysis of the insoluble starch by the α-amylase, neither to ascertain if the origin of the tandem repeats in the lactobacilli α-amylases is the result of the requirement of better adsorption.

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