Glucoamylase starch-binding domain of *Aspergillus niger* B1: molecular cloning and functional characterization

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Carbohydrate-binding modules (CBMs) are protein domains located within a carbohydrate-active enzyme, with a discrete fold that can be separated from the catalytic domain. Starch-binding domains (SBDs) are CBMs that are usually found at the C-terminus in many amylolytic enzymes. The SBD from *Aspergillus niger* B1 (CMI CC 324262) was cloned and expressed in *Escherichia coli* as an independent domain and the recombinant protein was purified on starch. The *A. niger* B1 SBD was found to be similar to SBD from *A. kawachii*, *A. niger* var. *awamori* and *A. shirusami* (95–96 % identity) and was classified as a member of the CBM family 20. Characterization of SBD binding to starch

indicated that it is essentially irreversible and that its affinity to cationic or anionic starch, as well as to potato or corn starch, does not differ significantly. These observations indicate that the fundamental binding area on these starches is essentially the same. Natural and chemically modified starches are among the most useful biopolymers employed in the industry. Our study demonstrates that SBD binds effectively to both anionic and cationic starch.

Key words: binding affinity, binding reversibility, modified starch.

INTRODUCTION

Starch-degrading enzymes are widely distributed throughout many species of animals, plants and micro-organisms. These enzymes have been classified as α -amylases, β -amylases and glucoamylases belonging to glycoside hydrolase families 13, 14 or 15 [1]. Similar to other polysaccharide-degrading enzymes, some of these enzymes have a modular structure consisting of a catalytic domain, a starch-binding domain (SBD) and a highly O-glycosylated linker that connects the two domains [1,2]. The fungal SBDs belong to the carbohydrate-binding module (CBM) associated with families 20 and 21 (for detailed classification, see <http://afmb.cnrs-mrs.fr/CAZY/>) [3–5].

Binding of the starch-degrading enzyme to its substrate is a critical step in starch hydrolysis since it involves the phase transfer of a soluble enzyme to the insoluble form of the substrate. It is believed today that similar to other CBMs, the SBDs play a pivotal role in this phase transfer [6]. The three-dimensional structure of SBD revealed anti-parallel β -sheet structures forming a bifacial protein that contains two starch-binding sites [7,8]. The enzyme to substrate interaction is enthalpy-driven and the two binding sites in the SBD appear to bind independently [6,9]. Studies on the binding of SBD to β -cyclodextrin (amylose helix analogue) have shown that the two starch strands are bound approximately perpendicularly to each other. It was suggested that this orientation of the binding sites might force the starch strands apart, thus increasing the available hydrolysable surface [8]. Atomic force microscopy studies supported this hypothesis and demonstrated that the amylose chains undergo a conformational change on binding to the SBD. Mutation of a single binding site eliminates this conformational change, indicating that both sites are needed for efficient starch deformation [6].

Several *Aspergillus* glucoamylase species have been cloned and characterized [10–13]. These glucoamylases contain conserved amino acid sequences within SBDs, which have been found also in bacteria and fungi [4,5]. The *Aspergillus niger* SBD was cloned, expressed and purified as a fully functional domain in *A. niger* [14] and in *Saccharomyces cerevisiae* [15]. Natural and chemically modified starches are among the most useful biopolymers in the industry. Utilization of SBD to link functional groups to starch is very attractive; however, very little is known about the interaction of SBD and chemically modified starches. In the present study, we cloned the *A. niger* B1 (CMI CC 324262) SBD. We chose this organism, since its glucoamylase is known to be an efficient starch-degrading enzyme. The recombinant SBD, expressed in *Escherichia coli*, revealed two equilibrium/metastable binding states when tested with granular starch. Kinetic studies on its binding to different types of starch indicate that the fundamental binding area on the starches examined are essentially the same.

EXPERIMENTAL

Enzymes and chemicals

Chemicals (including potato and corn starch) were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise stated. Enzymes were purchased from Fermentas AB (Vilnius, Lithuania). *Taq* polymerase was purchased from Promega (Madison, WI, U.S.A.). The TB medium contained 1.2 % (w/v) bactotryptone, 2.4 % (w/v) bacto yeast extract, 0.4 % (v/v) glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4 . The Luria–Bertani (LB) medium contained 1 % (w/v) tryptone, 0.5 % (w/v)

Abbreviations used: BCA, bicinechonic acid; CBD, cellulose-binding domain; CBM, carbohydrate-binding module; LB, Luria–Bertani; SBD, starch-binding domain.

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yeast extract and 1 % (w/v) NaCl. Cationic (degree of substitution is equal to 0.042) and anionic corn starch derivatives were purchased from Galam (Israel).

Preparation of vectors

Standard DNA manipulations and PCR protocols were conducted by the method of Ausubel et al. [16]. DNA products were analysed on a 1 % agarose gel and extracted using Qiagen (Hilden, Germany) extraction kit. Transformation was performed using a Gene pulser II electroporator (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and selection accomplished on LB-agar plates supplemented with 50 µg/ml kanamycin.

Construction of pET29-SBD

The glucoamylase SBD gene, residues 498–616 [18], was PCR-amplified from *A. niger* B1 (CMI CC 324626) chromosomal DNA, which was prepared using cetyltrimethylammonium bromide (CTAB method) as described by Dan et al. [19]. Two primers, the 5'-SBD (5'-AAACCATGGAATTCAGCAAGACC-AGCACCAGTAC-3') and the 3'-SBD (5'-TTTTGAGCTCTCAGATATCCCGCCAGGTGTCAGTAAC-3'), were designed based on sequence similarities found in *Aspergillus* glucoamylases. The resultant *sbd* gene fragment was cloned into pET29a vector (Novagen, Madison, WI, U.S.A.) yielding pET29-SBD.

Expression and purification of SBD

Small-scale protein expression was performed in *E. coli* BL21(DE3) (Novagen) harbouring the pET29-SBD plasmid. Inoculum was prepared by subculturing a single bacterial colony into 10 ml of LB medium containing 50 µg/ml kanamycin and incubating the culture at 37 °C to an absorbance A_{600} 4.0. Inoculum (5 ml) was transferred to 500 ml of fresh TB medium containing 50 µg/ml kanamycin and the cells were grown in an orbital shaker at 250 rev./min at 37 °C to an A_{600} 0.9. At this point, isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM, followed by overnight incubation at 28 °C. Bacterial cells were harvested by centrifugation at 5000 g for 10 min and washed twice with 50 ml of 100 mM Tris (pH 8.0). The bacterial pellet was suspended in 50 ml of lysis buffer [100 mM sodium acetate (pH 6), 1 mM EDTA, 0.1 % Triton X-100, 10 mM MgCl₂, 20 µg/ml lysozyme, 50 µg/ml DNase I and 0.5 mM PMSF] and incubated at 37 °C for 1 h. The insoluble bacterial debris was removed by centrifugation at 15 000 g for 10 min. For large-scale protein expression, a 10 litre batchwise fermentation procedure was used (BioFlo IV; New Brunswick Scientific, Edison, NJ, U.S.A.) with similar conditions that resulted in a final dry weight yield of 130 g of bacterial pellet. The pellet was then homogenized (APV Rannie homogenizer; APV Rannie, Copenhagen, Denmark) in 1.2 litres of lysis buffer to a final total protein concentration of 10 mg/ml. For protein purification, 500 g of granular corn starch (prewashed with 2 litres of 100 mM sodium acetate buffer, pH 6) was mixed with the bacterial lysate and stirred for 2 h at room temperature (25 °C). The starch was separated by centrifugation at 5000 g for 10 min followed by three washes with 1 litre of washing buffer (100 mM sodium acetate, pH 6). SBD was eluted from the starch using 3 litres of 500 mM maltose. The solution was dialysed against washing buffer using Filtron tangential ultrafiltration (Filtron, Northborough, MA, U.S.A.), equipped with a 5 kDa cut-off membrane. The protein was concentrated to 1 mg/ml (60 mM)

and to a final volume of 360 ml. The purified protein was then analysed by SDS/PAGE [16].

Association rate of SBD to granular corn starch

SBD (1 ml), diluted in sodium acetate buffer (100 mM, pH 6), in the concentration range 15.2–60 µM was mixed with 20 mg of prewashed granular corn starch and incubated at 25 °C for 5 h. The binding was terminated at different time intervals by sedimentation of the starch after centrifugation (13 000 g, 10 min). To remove proteins that were bound non-specifically, granular starch was washed once with a washing buffer containing 1 M NaCl and twice with washing buffer alone. The adsorbed SBD was assayed directly on the beads using a Micro bicinchoninic acid (BCA) reagent kit (Pierce, Rockford, IL, U.S.A.).

Binding reversibility assay

A binding reversibility assay was performed by the method of Norde and Haynes [20]. Briefly, SBD concentrations ranging from 7.2 to 37.5 µM were adsorbed to prewashed granular corn starch for 1 h at 25 °C. Desorption from the starch was performed, while the most concentrated SBD mixture (37.5 µM) was diluted in individual test tubes to final SBD concentrations ranging from 7.2 to 30.2 µM, followed by mixing for an additional hour. After centrifugation at 13 000 g for 10 min, the non-bound protein concentration was assayed by the Micro BCA protein-assay method and the amount of adsorbed protein was calculated from the difference between the initial and final protein concentrations in the supernatant.

Modelling of adsorption and analysis of adsorption data

The maximum amount of bound protein $B_{\max}$ and K_d values were determined by non-linear regression of the binding isotherms using a modified model for saturation binding, comprising one binding site as developed by Swillens [21]. The following equation describes the well-known binding model derived from the law of mass action:

$$B = B_{\max} F / K_d + F \quad (1)$$

where B (µmol) represents the bound protein, $B_{\max}$ (µmol) the maximum amount of bound protein, F (µmol) the free protein in the system and K_d (µmol) the equilibrium dissociation constant. F is calculated by subtracting B from the total protein added (T) as follows:

$$F = T - B \quad (2)$$

Combining eqns (1) and (2) yields

$$B = B_{\max}(T - B) / K_d + (T - B) \quad (3)$$

Eqn (3) was used to calculate the non-linear regression curves. The units of the $B_{\max}$ and K_d values calculated were converted into µmol/g and µM respectively.

Binding assays

The starch-binding isotherm (saturation binding assay) was generated as follows: 1 ml of purified SBD (12–72 µM) was added to 0.02 g of prewashed granular starch and mixed for 1 h. The bound SBD was determined by using the Micro BCA assay directly on the beads.

Figure 1 Amino acid sequence alignment of *Aspergillus* SBDs

Amino acid sequence alignment to other *Aspergillus* SBDs was performed using DNAMAN 4.11 software (Lynnon BioSoft, Quebec, Canada). (A) Multiple-sequence alignments of six C-terminal SBDs from glucoamylase genes. Abbreviations of the sources of individual SBD genes are as follows: A.N. B1, *A. niger* B1 (accession no. AF495813); A.N. awa, *A. niger* var. *awamori* (GenBank® accession no. K02465); A. kaw, *A. kawachii* (GenBank® accession no. D00427); A. shi, *A. shirousami* (GenBank® accession no. D10460); A. nid, *A. nidulans* (GenBank® accession no. AF208224); A. ory, *A. oryzae* (GenBank® accession no. D10698). Residues belonging to the consensus sequences are highlighted in black. (B) Phylogenetic rooted tree of *Aspergillus* SBDs. The tree is based on the multiple sequence alignment of the same SBD genes. The branch lengths are proportional to the sequence divergence.

Data analysis

Kinetic and affinity parameter data analyses were performed using GraphPad Prism 3.02 for Windows (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Cloning and expression

Based on conserved amino acid sequences shared by SBDs, two primers were designed that enabled the cloning of *A. niger* B1 SBD (accession no. AF495813). Figure 1(A) illustrates the deduced amino acid sequence and the multiple sequence alignment of the *Aspergillus* SBDs. The *A. niger* B1 SBD sequence was found to possess a high degree of similarity to SBDs from *A. kawachii*, *A. niger* var. *awamori* and *A. shirousami*. The *A. niger* B1 SBD can be classified, along with other *Aspergillus* SBDs, as a member of the CBM family 20. According to the SBD phylogenetic tree (Figure 1B), *A. niger* B1 emerged from a common ancestral source with *A. niger* var. *awamori* and *A. oryzae*. The calculated molecular mass of the putative protein is 16.5 kDa (including an S-tag sequence at the N-terminus of SBD [22]). This is in agreement with its apparent molecular mass as estimated by SDS/PAGE analysis (Figure 2).

Figure 2 SDS/PAGE analysis of SBD production and purification

SBD expression and purification steps were subjected to SDS/PAGE (12.5% gel) followed by Coomassie Brilliant Blue R-250 staining for protein detection. Lane 1, 50 µg of protein from the bacterial lysate soluble fraction; lane 2, 2 µg of SBD that was purified on corn starch and eluted with 0.5 M maltose.

Table 1 Steps in expression and purification of SBD

Large-scale production of SBD was performed in a 10-litre batchwise fermentation process. The SBD was then purified from the bacterial lysate on granulated corn starch to high purity, as described in the Experimental section.

Fraction	Total protein (mg)*	Purity (%)†	Yield (%)
Soluble	1200	15	100
Purified‡	360	95	30

*Determined by Micro BCA.

†Estimated by densitometry analysis.

‡Purified on corn starch.

Overexpression of SBD in *E. coli* was performed to enable biochemical characterization of its non-glycosylated form [14]. Protein production was accomplished using the pET system [23]. A yield of approx. 120 mg/l recombinant protein was obtained after induction with isopropyl β-D-thiogalactoside at optimized growth conditions of 28 °C for 14 h. The protein was located predominantly in the soluble fraction of the bacterial lysate (Figure 2). SBD was affinity-purified in a single step on granular corn starch, and eluted with 0.5 M maltose. This procedure yielded an SBD solution that was 95% pure (Figure 2 and Table 1). The final yield of pure protein was 30% (Table 1).

SBD association rate and reversibility

The kinetic study of SBD adsorption on granular corn starch is shown in Figure 3. After 1 min of incubation, 60–70% of the binding (relative to the equilibrium state) was observed. From that point onwards, adsorption of SBD on starch was biphasic; a fast initial phase (up to 60 min) with a linear relationship between incubation time and bound protein, followed by a slower phase (after 60 min) in which no significant increase in bound protein was obtained with prolonged incubation time. More than 95% of the SBD was bound to the starch within the first 60 min. When different SBD concentrations were tested, no significant differences were observed in the binding rate ($3.1 \pm 0.2 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$), as indicated by the initial slope of the binding curves. The level of bound protein at equilibrium was dependent on the SBD concentration.

Figure 3 Association rate assay
 The association rate of SBD was tested by applying SBD at different concentrations (◆, 15.3 μM ; ●, 30.5 μM ; ▼, 45.8 μM ; ■, 61 μM) to corn starch for 5 h. Bound protein was tested at different time points to determine the association rate.

Figure 4 Adsorption/desorption isotherm
 SBD, at different concentrations, was allowed to adsorb to corn starch to the point of equilibrium (■). After equilibrium was reached, the highest SBD-containing mixture was diluted to allow desorption (□) as described in the Experimental section.

Adsorption/desorption experiments (Figure 4) are critical tests to study the reversible nature of adsorption. The desorption experiments revealed that a new equilibrium was established after dilution, which was not on the same isotherm (desorption exhibits hysteresis). These results prove that the ascending and descending isotherms do not overlap, which is a prerequisite for irreversible binding [20].

Figure 5 Binding of purified SBD to starch
 Binding was tested with cationic starch (■), corn starch (▲), potato starch (◆) and anionic starch (▼). SBD, at different concentrations, was bound to various starch granules until equilibrium, as described in the Experimental section. Protein concentrations were determined by direct measurement of bound SBD on the starch granules.

Table 2 Binding parameters of SBD for different types of granular starch
 Binding characteristics of SBD for different types of granular starch were calculated from the binding isotherms as described in the Experimental section. Kinetic and affinity parameter data analyses were performed using GraphPad Prism 3.02 software.

Starch type	K_d (μM)	B_{max} ($\mu\text{mol/g}$)
Corn starch	3.247 ± 0.949	0.563 ± 0.001
Potato starch	3.303 ± 0.727	0.084 ± 0.004
Cationic corn starch	3.513 ± 0.594	0.999 ± 0.032
Anionic corn starch	4.605 ± 1.259	0.269 ± 0.017

SBD-binding affinities and capacities

Equilibrium adsorption isotherms were used to study the affinity of purified SBD for starch originating from distinct sources (corn or potato) or possessing dissimilar ionic characteristics (anionic and cationic corn starch). In general, at low protein concentrations, the amount of bound protein demonstrated a linear dependence on the free protein concentration. In all starch granules tested, it almost approached the SBD saturation, but not attained even at the highest level of protein concentration tested (Figure 5).

The binding isotherms were used to calculate binding parameters. The K_d and B_{max} values were calculated from a non-linear regression of the adsorption isotherms (Figure 5). The binding parameters are summarized in Table 2. The affinities of SBD to different types of starch are similar (mean K_d is 3.6 μM). On the other hand, B_{max} values are markedly different. Cationic starch demonstrated the highest capacity levels (0.999 $\mu\text{mol/g}$) followed by corn starch (0.563 $\mu\text{mol/g}$) and anionic starch (0.269 $\mu\text{mol/g}$), the lowest capacity being achieved with potato starch (0.084 $\mu\text{mol/g}$).

DISCUSSION

Cloning and expression

The results presented in Figure 1 demonstrate a high level of identity among SBDs from other *Aspergillus* species. The tryptophan residues are highly conserved and are set within the SBDs of different starch-degrading enzymes found in bacteria and fungi [4,5,24]. SBD cloned in the present study contains these two tryptophan residues in addition to other conserved residues that are known to participate in starch binding (Trp⁵⁴³, Lys⁵⁷⁸, Glu⁵⁹¹ and Asn⁵⁹⁵ in site 1, and Thr⁵²⁶, Tyr⁵²⁷, Gly⁵²⁸, Glu⁵²⁹, Asn⁵³⁰ and Asp⁵⁵⁴ in site 2) [4,7]. The phylogenetic tree (Figure 1B) further establishes the identification of this clone as an *A. niger* variant that differs from its closest relatives.

E. coli BL21, harbouring the *sbd* expression vector, was grown under conditions that favoured protein solubility over inclusion-body formation. The recombinant SBD was affinity-purified on starch directly from the crude bacterial lysate. SDS/PAGE analysis (Figure 2) indicated that the protein was purified to near homogeneity in a single step. Belshaw and Williamson [25] addressed the apparent low efficiency of maltose in releasing SBD from starch. They suggested that the distance between the two glucose residues that are situated on different chains of the double-helix amylose fit the two binding subsites of SBD optimally. Therefore it is not surprising to find that maltose is not the optimal elution agent. More recent studies by Giardina et al. [6] further substantiate their observation.

Association rate and reversibility

In this section, we address the question as to whether the adsorption of SBD to starch (the transfer of the protein from solution to the solid state) is a reversible process. We initially determined the time necessary to reach binding equilibrium. Studies performed on cellulose-binding domains (CBDs) revealed that CBDs bind to cellulose very rapidly and reach equilibrium within a few minutes [17,26]. The time elapse for SBD to reach binding equilibrium is longer (Figure 3). The experiments conclude that with all protein concentrations tested, equilibrium was obtained after approx. 60 min.

A reversible adsorption process is defined if the departure from adsorption equilibrium is infinitesimally small, so that in the reverse process (desorption) the variables characterizing the state of the system return to the same values in the reverse order. Therefore in a reversible adsorption process, the ascending branch (increasing concentration in the solution) and the descending branch (decreasing concentration in the solution) of the isotherm must overlap. If the ascending and descending branches of the isotherm do not overlap, the process is defined as irreversible and the deviation between the ascending and descending branches is defined as hysteresis [20].

Our results demonstrated that under the conditions tested, SBD binds almost irreversibly to starch (Figure 4). From three-dimensional structure analysis of the free [7] and bound forms of SBD [8], it has been established that the SBD undergoes conformational changes on binding. When the hysteresis is small, it can be explained by protein-protein interaction such as those seen with *Trichoderma* Cel7A [27]. However, in SBD, we consider the observed hysteresis as one that originates from the nature of SBD binding to starch. SBD possesses two binding sites that are almost independent, and abolishing either site does not prevent binding to starch [4,7–9]. Nevertheless, both sites must be functional to induce conformational changes in the amylose molecule [6], which leads to starch granule structure disruption

[6,28]. According to the model developed by Giardina et al. [6], binding of SBD to parallel amylose chains results in a novel two-turn helical amylose complex structure. The resultant starch conformational changes may be responsible for the irreversible binding and for the relatively low on-rate binding compared with CBDs.

Affinity and capacity

Binding affinity can be quantified by calculating the partition coefficient K from the equilibrium adsorption isotherms, provided that the number of available ligand-binding sites does not differ and that the surface of the substrate is uniform and offers only a single class of binding sites. The latter assumption is not valid when insoluble starch is used; therefore the dissociation constant K_d calculated from a binding assay of intact SBD to granular starch displays an apparent constant. Dalmia and Nikolov [29] used a three-parameter model to analyse the wide range of adsorption isotherms, which included an estimation of the non-specific protein-protein interaction that was inferred from the binding isotherms. This non-specific interaction was attributed to the intact glucoamylase that was employed in the study. In the present study, the binding of SBD to starch was determined directly on starch granules and non-specific binding was decreased by washing the starch granules with a washing buffer containing 1 M NaCl. We employed a two-parameter model to analyse the binding affinities of the isolated SBD for different types of starches. Analysis of our experimental data by employing the three-parameter model proposed by Dalmia and Nikolov [29] failed to explain the results. The results obtained in the study agree with the previously reported partition coefficient K_d obtained for glucoamylase G1 and for its isolated SBD [9,14,32].

Based on the results of the present study, we propose that the similar binding affinities to different types of starch (natural and chemical derivatives) could result from the fact that the fundamental binding area of each starch species is essentially the same. According to the model (eqn 3), B_{max} represents the total amount of potential binding sites on the ligand surface area. The deviating B_{max} can be rationalized based on the differences between the starches. Maize starch granules are relatively small (diameter range 3–26 μm , mean diameter 15 μm) and contain 25–28% amylose. Potato starch granules are relatively large (diameter range 5–100 μm , mean diameter 30 μm) and contain 18–21% amylose [30,31]. We propose that parameters, such as the surface area derived from the size and shape of granules or the varying ratios of amylopectin to amylose, may affect B_{max} values. The chemical modification of starch seen in ionic starches may result in additional physical changes to the starch granule surfaces in addition to charge density; therefore we could not conclude the exact reason for the apparent differences in B_{max} values. Natural and chemically modified starches are among the most useful biopolymers in the industry. Our study demonstrates that SBD binds effectively to both anionic and cationic starch.

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