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Expression, Purification, and Synergistic Effects in Starch Binding of Starch-Binding Domains (SBD-SBD4) Derived from *Aspergillus niger* 2316 Glucoamylase

Yunfeng Zhang[1,2], Qing Yang[1]*, Qin Ji[2], Denzhan Wang[2] and Chun Xu[2]

[1]College of Life Sciences, Nanjing Agricultural University, Nanjing, Jiangsu Province, China.
[2]Jiangsu Key Laboratory for Eco-Agricultural Biotechnology Around Hongze Lake, Huaiyin Normal University, Huai'an, Jiangsu Province, China.

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A starch-binding domain encoding region of glucoamylase (GASBD) was amplified by PCR from *Aspergillus niger* 2316 and sequenced (GenBank JQ814697). It consists of 324bp nucleotides and encodes a peptide of 108 amino acids. To investigate the affinities of artificial multiple-repeat GASBDs for insoluble starch *in vitro*, the pET28a plasmids harbouring, respectively, single GASBD and multiple-repeat GASBDs from two to four were constructed and expressed in *Escherichia coli* BL21(DE3). The recombinant GASBD(s) proteins were purified to homogeneity by Ni^{2+}–NTA affinity chromatography and the affinities of the purified recombinant proteins for insoluble starches were determined. GASBD2, GASBD3, and GASBD4 had, respectively, approximately 5, 8 and 10-fold higher affinities for starch than single GASBD, indicating that the multiple-repeat GASBDs act in a synergistic way when binding to starches.

Key words: Glucoamylase, Starch-binding domain, Protein expression, Starch affinity, Synergistic effect.

Glucoamylase (GA) (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) catalyses the release of β-D-glucose from the non-reducing ends of starch and related substrates by hydrolysing α-1,4 bonds using a single active site[1,2]. They are typically microbial enzymes[3], present in archaea, bacteria[4,5] and fungi. Glucoamylase of *Aspergillus niger* type has a modular structure, in which a catalytic domain with an $(\alpha/\alpha)_6$-barrel conformation[6], classified as a member of the glycoside hydrolase family 15[7], is connected to a starch-binding domain (SBD) by a highly O-glycosylated linker[2].

SBD is a functional domain that can attach the amylolytic enzyme to the insoluble granular starch, thereby locally increasing the enzyme concentration at the substrate surface. Another proposed role is to disrupt the structure of the starch surface resulting in a higher amylolytic rate[8,9]. The SBDs are usually relatively small and their structures are very well conserved among different enzymes, as well as among different microbial species. According to amino acid sequence similarity, various SBDs have been grouped into 10 of the 64 carbohydrate-binding module (CBM) families. SBDs belong to families CBM20, CBM21, CBM25, CBM26, CBM34,

* To whom all correspondence should be addressed.
Tel: +86-25-84395221; Fax: +86-25-84395221;
E-mail: qyang19@njau.edu.cn

CBM41, CBM45 , CBM48, CBM53and CBM58 (the CAZy database, http://www.cazy.org/ ; last update on 20 March 2012) [10]. Among CBM families, the CBM20 is the most generalized and studied family. Crystallographic studies revealed that most CBM20s possess two separate carbohydrate-binding sites. Each site contains two or three exposed aromatic amino acid (Trp and Tyr), which play a pivotal role in binding carbohydrate structures[11–13]. Several studies show that SBDs retain their affinity for insoluble starch, even when they are separated from the other constituent domains of the natural proteins. When Saccharomyces glucoamylase lacking a starch-binding domain was fused with a additional SBD, the hybrid glucoamylase, in contrast to the native enzyme, had a significant starch binding capacity and higher activity on insoluble starch[14].

Several starch-degrading enzymes contain multiple SBDs organized in tandem repeat modules. For example, two SBD modules, both from CBM25, were found in the α-amylases from *Bacillus* sp. no. 195[15] and *Clostridium acetobutylicum* ATCC 824[16]; Three SBD modules were found in the maltopentaose-forming amylase from an alkaliphilic Gram-positive bacteria[17]. In CBM26 family, four SBD modules have been found in the α-amylases from *Lactobacillus manihotivorans*[18] and *Lactobacillus plantarum* and five modules in the α-amylase from *Lactobacillus amylovorus*[19]. The multiple repeats SBDs in natural enzymes can be considered with the potential to bind starch with very high affinities. The tandem CBM26-CBM25 of the maltohexaose-forming amylase from *Bacillus halodurans* had roughly 50-fold affinity enhancement for insoluble starch than the single CBMs and the enhanced affinity is attributed to the simultaneous interaction of the two tandem SBDs present in the enzyme through an avidity effect[20]. Synergistic effects was also found in the SBD of family CBM26 from the *Lactobacillus amylovorus*, which displays a significant increase in affinity for insoluble substrates acting in a synergistic way[21]. Similar performances were also reported for some natural cellulases and xylanases[22,23].

Although glucoamylase starch-binding domain (GASBD) and multiple-repeat SBD modules in natural enzymes have been extensively discussed, synergistic effects of the artificial multiple-repeat GASBDs derived from *Aspergillus niger* has never been described. Therefore, in this study, a starch-binding domain-encoding region of glucoamylase (GASBD) from *A. niger* 2316 was amplified and the pET28a plasmids harbouring single GASBD and multiple-repeat GASBDs were constructed and expressed in *Escherichia coli* BL21(DE3). GASBD(s) proteins were purified by Ni^{2+}–NTA affinity chromatography and the binding properties of the GASBD(s) for insoluble starch *in vitro* were qualitatively and quantitatively characterized.

MATERIALS AND METHODS

Corn starch and potato starches were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Enzymes were purchased from Fermentas AB (Vilnius, Lithuania). *Taq* polymerase was purchased from Promega Life sciences (Madison, WI, U.S.A.). Ni^{2+}-nitrilotriacetate (Ni^{2+}-NTA) resin was acquired from Qiagen Inc. (Valencia, CA, USA). All other chemicals were analytical grade or molecular biological grade, from Shanghai Chemical Industries (Shanghai, China).

Strains, Plasmids, and Media

Aspergillus niger strain 2316 provided by China Center of Industrial Culture Collection (CICC) was used to isolate the GASBD gene. The SBD from *A. niger* has been classified as a member of the CBM20 family. *E. coli* BL21 (DE3) was used for expression of recombinant proteins. pET28a (Novagen, Madison, WI, USA) plasmid was used for making *E. coli* expression plasmid. The Terrific-Broth (TB) medium contains 1.2% (w/v) Difco Bacto-tryptone, 2.4% (w/v) Difco 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 contains 1% (w/v) Difco Bacto-tryptone, 0.5% (w/v) Difco Bacto-yeast extract and 1% (w/v) NaCl.

Cloning of the GASBD Gene

The genomic DNA of *A. niger* 2316 was extracted with cetyltrimethyl ammonium bromide (CTAB) method as described by Dan *et al*[24]. A SBD-encoding fragment was amplified by polymerase chain reaction (PCR) (95°C for 30 s, 60°C for 30 s, and 72°C for 1 min and 30 cycles) with the primers: SBD-F (5' -AAATATTAGC GGATCCCTGCACCACTCCCACC-3') and SBD-R

(5' -TTCGAGCAAGCTTCTACCGCCAGG TGTCAGTAAC-3') with genomic DNA of *A. niger* 2316 as a template. Two primers contain *BamH*I and *Hind*III restriction sites (underlines), respectively. The amplified fragment was cloned into *BamH*I and *Hind*III sites of the pUC19 (Takara Bio Inc., Shiga, Japan), giving the pUC19-GASBD plasmid. The construct was used to analyse the GASBD sequence.

Construction of *E.coli* Expression Plasmids

Four expression plasmids were constructed in this study. The pET28a-GASBD, pET28a-GASBD2, pET28a-GASBD3, and pET28a-GASBD4 plasmids were used for the expression of GASBD to GASBD4 fusion proteins in *E. coli*, respectively.

The pET28a-GASBD was assembled from the pUC19-GASBD plasmid. After digesting pUC19-GASBD with *BamH*I and *Hind*III, the *BamH*I-*Hind*III GASBD fragment was inserted in pET28a to obtain a pET28a-GASBD plasmid.

The pET28a-GASBD2, pET28a-GASBD3 and pET28a-GASBD4 plasmids were constructed from a pTrcHisB vector. For making pTrcHisB-GASBD2, the *EcoR*I-*Hind*III GASBD fragment was amplified by PCR with the primers SBD2-F1 and SBD2-R1(see Table 1) using pUC19-GASBD as a template. The amplified fragment was inserted into pTrcHisB plasmid to give the pTrcHisB-GASBD plasmid. In a similar way, the *BamH*I-*Bgl*II GASBD fragment was generated by PCR with the primers SBD2-F2 and SBD2-R2 (see Table 1) using pUC19-GASBD as a templet. The resultant fragment was inserted into the pTrcHisB-GASBD to obtain a pTrcHisB-GASBD-GASBD plasmid. Then an artificial linker (*Bgl*II- *EcoR*I) derived from pBIN19/ SBD2[25] was inserted between two GASBDs to give the pTrcHisB-GASBD2 plasmid.

Table 1. Oligonucleotide primers used in this study

Primers	Sequences
SBD2-F1	5' -TAATACGCGAATTCTGCACCACTCCCACCG-3'
SBD2-R1	5' -TTCGAGCAAGCTTCTACCGCCAGGTGTCAGTAAC-3'
SBD2-F2	5' -AAATATTAGCGGATCCCTGCACCACTCCCACC-3'
SBD2-R2	5' -TTAGGTACGCAGATCTCCGCCAGGTGTCAGT-3'
SBD3-F	5' -TAATACGCAGATCTCCGACGCCGACGCCCACC-3'
SBD3-R	5' -TATATAGCCGGATCCGCGCCAGGTGTCAGT-3'

A *Bgl*II-*BamH*I fragment, containing the linker and GASBD, was amplified with the primers SBD3-F and SBD3-R (see Table 1) using the pTrcHisB-GASBD2 plasmid as a templet. The amplified fragment was cloned into the pTrcHisB-GASBD2 at *Bgl*II site to give the pTrcHisB-GASBD3 plasmid. In the same way, the *Bgl*II-*BamH*I fragment was inserted into the *Bgl*II sites of the pTrcHisB-GASBD3 to obtain the pTrcHisB-GASBD4 plasmid.

The pTrcHisB-GASBD2, pTrcHisB-GASBD3, and pTrcHisB-GASBD4 plasmids were digested with *BamH*I and *Hind*III, respectively. The *BamH*I-*Hind*III GASBDs fragments were, respectively, inserted into the corresponding sites of the pET28a plasmid to generate the pET28a-GASBD2, pET28a-GASBD3 and pET28a-GASBD4. All plasmids were sequenced to verify their correctness.

The four plasmids were, respectively, transformed into *E. coli* BL21(DE3) for expression of recombinant GASBD(s) proteins.

Expression of Recombinant Proteins

The recombinant *E. coli* BL21(DE3) cells, containing the pET28a-GASBD(s), were grown in 10 mL LB medium supplemented with 50 µg/mL kanamycin at 37°C for 12 h. Five mL of the resultant bacterial culture was transferred to 200 mL fresh TB medium containing 50 µg/mL kanamycin and the cells were grown at 37°C to an OD_{600} 0.8. At this point, expression of proteins was induced by adding of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.4 mM, and incubation was continued at 24°C for 12 h. Uninduced cells

were also grown without adding IPTG as control.

Cells were harvested by centrifugation (5,000 g, 10 min, 4°C) from 200 mL culture and washed twice with 50 mL of 20mM Tris-HCl (pH 8.0). The pellets were resuspended in 20mL lysis buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8.0) and disrupted by sonication (VCX750, SONICS, CT, USA) on ice at 300W for 20 min (sonication for 3 s and intermission for 6 s). Total cell lysates of the induced and uninduced cell were analyzed by 15% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [26], and the proteins were stained with Coomassie Brilliant Blue R-250.

Protein Purification

The cells were harvested from 1 L bacterial culture by centrifugation. Cell pellets were resuspended in 50 mL lysis buffer and lysed by sonication. The soluble and the insoluble fractions were separated by centrifugation at 13,000g for 10 min at 4 °C. The supernatant was collected and filtered through a 0.22μm filter.

The purification of the recombinant proteins was based on the His-tag protocol. The 2 mL of Ni^{2+}–NTA resin (Valencia, CA, USA) was settled in the column and equilibrated with 2 column volumes of lysis buffer.The filtered supernatant was loaded onto the Ni^{2+}–NTA column at a flow rate of 1 mL/min. The column was then washed with 15 column volumes lysis buffer containing 20 mM imidazole. Subsequently, the target protein was eluted with 5 column volumes lysis buffer containing 300 mM imidazole at a flow rate of 1 mL/min. The fraction containing target protein was pooled, dialyzed against 100 mM sodium acetate buffer (pH6.0) at 4 °C and concentrated by Amicon Ultra centrifugal filter devices 5000 MWCO (Millipore, Billerica, MA, USA). Purified GASBD(s) proteins were lyophilized and stored at –20°C. The purity of the protein was assessed by 15% SDS–PAGE.

Qualitative Binding Assay

Qualitative binding assays were carried out as described by Abou *et al*[27]. with several modifications. The 50, 100 and 200 μg purified GASBD(s) proteins were respectively mixed with 10 mg of insoluble corn starch in a 1mL of 100 mM sodium acetate buffer (pH 6.0) solution. The mixtures were incubated at 25°C with gentle stirring for 2 h, and then centrifuged at 13,000 g for 5 min at 4°C. Bovine serum albumin (BSA, 200 μg) was used as a negative control. The proteins in the supernatant (the unbound fraction) and the precipitated starch (the bound fraction) were analysed by SDS-PAGE.

Association Rate of GASBD(s) to Corn Starch

The purified GASBD(s) protein at the concentration of 15.4, 20.6 and 27.8 μM were, respectively, mixed with 10 mg of prewashed corn starch in a 1mL of 100 mM sodium acetate buffer (pH 6.0) solution. The reaction mixtures were incubated at 25°C with gentle stirring. The binding reaction was terminated at 1, 5, 10, 20, 30, 60, 90, 120, 180, and 240 min, respectively, by sedimentation of the starch after centrifugation (13,000 g, 5 min, 4°C). The protein concentration in the supernatant (unbound protein) was determined by bicinchoninic acid (BCA) assay[28], and the amount of bound protein was calculated from the difference between the total and unbound protein concentrations. The amount of bound protein was expressed as micromole of protein per gram of starch (μM/g).

Adsorption Isotherms

The purified GASBD, GASBD2, GASBD3 and GASBD4 proteins at the concentration of 1, 2, 4, 6, 8, 10, 15, 20, and 25 μM were, respectively, mixed with 10 mg of prewashed corn starch or potato starch in 1 mL of 100 mM sodium acetate buffer (pH 6.0) and incubated at 25°C for 2 h. After centrifugation at 13,000 g for 5 min at 4 °C, the unbound protein was determined by BCA assay. The amount of bound protein was calculated from the difference between the total and unbound protein concentrations.

The binding parameters were determined by nonlinear regression of the adsorption isotherms using a modified model for saturation binding, comprising one binding site as developed by Swillens[29]. The following equation describes the well known binding model derived from the law of mass action: $B = B_{max} F/(K_d + F)$, where B (μM/g) represents the bound protein per gram of starch, B_{max} (μM/g) the maximum amount of bound protein per gram of starch, F (μM) the free protein in the system and K_d (μM) the equilibrium dissociation constant.

Nucleotide Sequence Accession Number

The nucleotide sequence of the GASBD fragment discussed in this study has been submitted to the GenBank nucleotide sequence

database, and can be accessed under accession no. JQ814697.

RESULTS AND DISCUSSION

Cloning and Homology Analysis of the GASBD Gene

The DNA fragment encoding the GASBD (GenBank JQ814697) was amplified by PCR using genomic DNA of *A. niger* 2316 as a templet. Two primers SBD-F and SBD-R were designed based on sequence similarities found in *Aspergillus* glucoamylase SBDs. The amplified fragment was subcloned into pUC19 plasmid and sequenced. The data shown that the SBD in its natural setting is present at C-terminus of GA, and it consists of 324 bp nucleotides encoding a peptide of 108 amino acids.

The nucleotide sequence of the GASBD gene from *A. niger* 2316 was compared with other known *Aspergillus* glucoamylase SBDs in the GenBank database. The GASBD gene revealed the following degrees of homology: 99.07% with SBDs from *A. niger* T21, *A. awamori* NRRL3112 and *A. ficuum*, 92.90% with SBD from *A. awamori* KT11, 92.28% with SBD from *A. niger* B1, 91.05% with SBDs from *A. awamori* X100 and *A. kawachii*, 90.74% with SBD from *A. shirousami*.

Construction of *E.coli* Expression Plasmids

To study the starch binding capacity and their possible synergy of the artificial multiple appended GASBD modules, the pET28a-GASBD, pET28a-GASBD2, pET28a-GASBD3, and pET28a-GASBD4 plasmids were constructed for expression of GASBD, GASBD2, GASBD3, and GASBD4 (Figure 1) in *E. coli* BL21(DE3) . The multiple

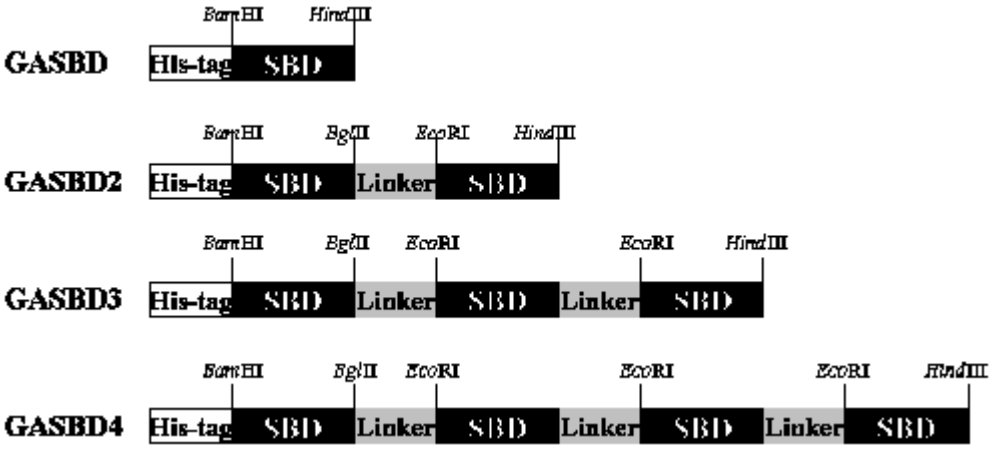

Fig. 1. Schematic structures of recombinant GASBD(s) in pET28a plasmids

appended GASBDs were covalently joined via an artificial Pro-Thr-rich linker (The amino acid sequence of the linker: 5' -PTPTPTTPTPT PTTPTPTPST-3'). The function of linker is to provide sufficient flexibility for each GASBD to bind to starch independently[30]. The *E. coli*-produced GASBD, GASBD2, GASBD3, and GASBD4 proteins have the predicted molecular masses of 15.62kDa, 29.81kDa, 44.00kDa, and 58.19kDa, respectively, including an His-tag sequence at the N-terminus of recombinant proteins.

Expression of Recombinant GASBD(s)

The expression of recombinant GASBD(s) was investigated in *E. coli* BL21 (DE3). The SDS–PAGE assay showed that recombinant GASBD(s) proteins were expressed and the molecular mass of four recombinant proteins were consistent to predicted ones (Figure 2).

Purification of Recombinant GASBD(s)

The recombinant proteins were purified from the soluble fractions by Ni^{2+}–NTA affinity chromatography, as described in the method section. Large amounts of GASBD2 and GASBD3 proteins was efficiently bound to the Ni^{2+}–NTA resin, while only a small amount was lost in the flow-through (Figure 3A, lanes 4, 8). The 20 mM imidazole wash was successful in the removal of

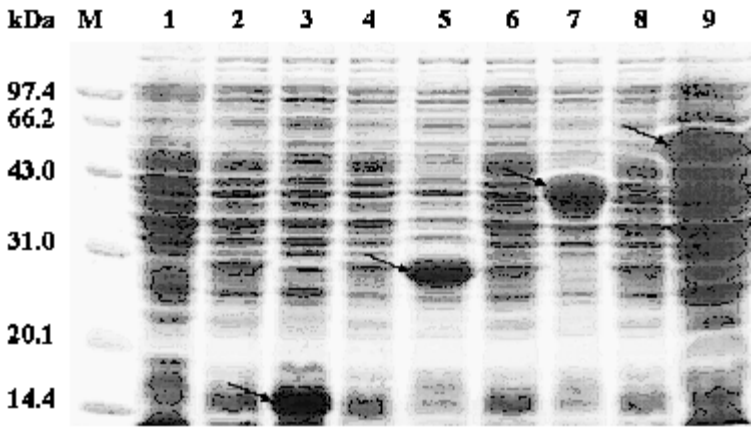

Fig. 2. SDS-PAGE analysis of GASBD(s) expression. Lane M, Protein marker (kDa); Lane 1, Total proteins of *E. coli* BL21 (DE3) without recombinant plasmid as control; Lanes 2, 4, 6, and 8, Total proteins of recombinant GASBD, GASBD2, GASBD3 and GASBD4 bacterial lysates without IPTG induction; Lanes 3, 5, 7, and 9, Total proteins containing GASBD, GASBD2, GASBD3 and GASBD4 after IPTG induction. Arrows indicate the position of the expressed GASBD, GASBD2, GASBD3 and GASBD4 proteins.

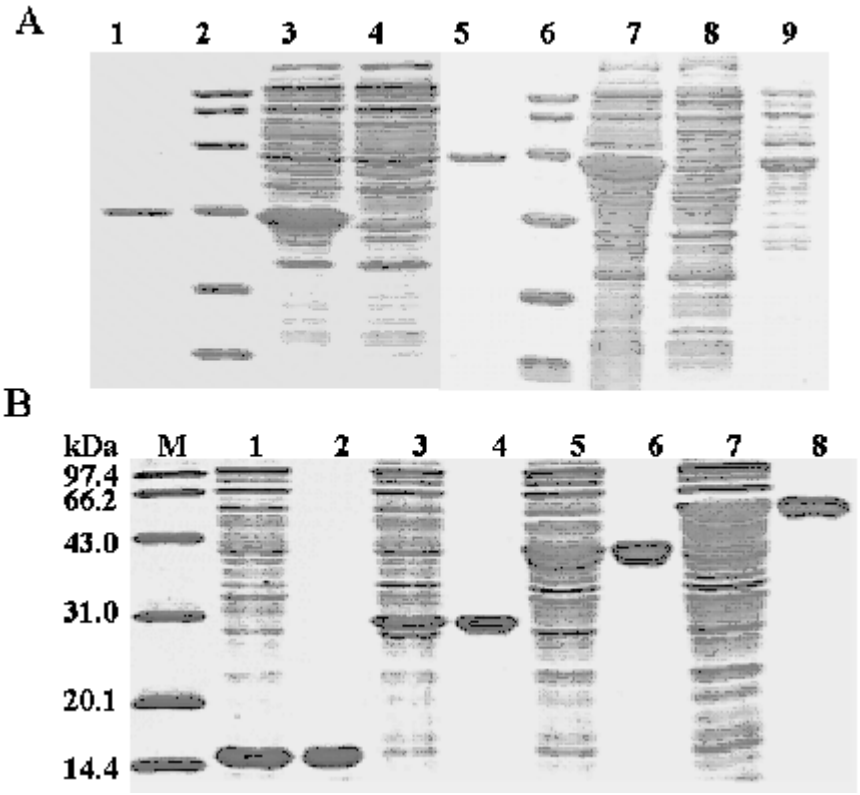

Fig. 3. Purification of recombinant GASBD(s)**.** (A) SDS-PAGE analysis of GASBD2 and GASBD3 affinity purification. Lanes 1 and 5, the eluted fractions with 300 mM imidazole; Lanes 2 and 6, protein marker; Lanes 3 and 7, soluble fractions from induced cells; Lanes 4 and 8, column flow through; Lane 9, the eluted fractions with 20 mM imidazole. (B) SDS-PAGE Analysis of the purity of recombinant GASBD(s). Lane M, Protein marker; Lanes 1, 3, 5, and 7: soluble fractions of recombinant GASBD, GASBD2, GASBD3 and GASBD4 bacterial lysate; Lanes 2, 4, 6, and 8: The purified GASBD, GASBD2, GASBD3 and GASBD4 proteins.

contaminating proteins (Figure 3A, lane 9). Increasing the imidazole concentration to 300 mM, majority of GASBD2 and GASBD3 proteins was then eluted. As shown in Figure 3A and 3B, the purity of the GASBD(s) was over 95% as analyzed by a computer-assisted scanner. These results indicated that solubly expressed GASBD(s) were rapidly and efficiently purified using the one-step Ni^{2+}–NTA affinity chromatography.

Qualitative Binding Assays of GASBD(s)

The binding ability of GASBD(s) to insoluble corn starch was qualitatively examined by incubating the protein with a starch suspension, and then the amounts of protein in the supernatant (unbound protein) and in the precipitate fraction (bound protein) were analyzed by SDS-PAGE. BSA incubated with corn starch was conducted as a nonspecific binding control. The results, which are shown in Figure 4, clearly demonstrated that the GASBD(s) proteins displayed significant affinities for corn starch, whereas BSA did not. The affinity of GASBD(s) for corn starch was enhanced with increasing numbers of GASBD in the fusion proteins.

To determine the time to reach the maximal capacity of the purified proteins to bind to insoluble

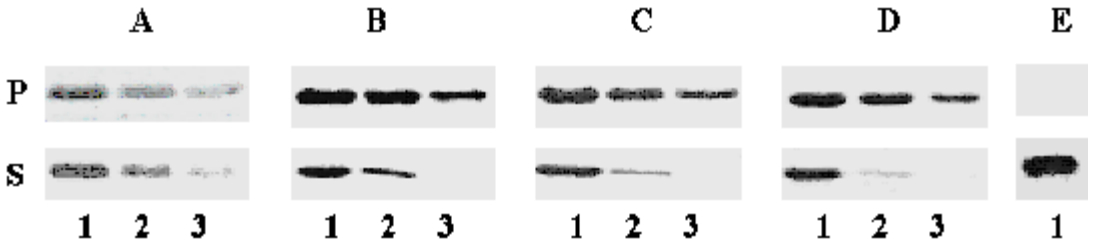

Fig. 4. Binding of the GASBD(s) proteins to insoluble corn starch. The purified GASBD(A), GASBD2(B), GASBD3(C), GASBD4(D) protein solutions were incubated with insoluble corn starch. After centrifugation, the proteins in the supernatant and the precipitate were analyzed by SDS-PAGE. The protein amounts are 200 µg (Lane1), 100 µg (Lane2), and 50 µg (Lane3). P: Proteins in precipitates; S: Proteins in supernatants. BSA (E) served as a negative control.

starch, a time-course experiment was performed. The results shown in Figure 5 demonstrated that the adsorption exhibited a linear phase relationship between incubation time and bound protein in the initial period (up to 60 min). The maximal binding was achieved after 2 h. Moreover, no significant differences in the binding equilibrium were observed in different concentrations of GASBD. The time to reach the maximal binding to corn starch of GASBD2, GASBD3 and GASBD4 proteins were also 2 h, which was consistent with that of GASBD (data not shown).

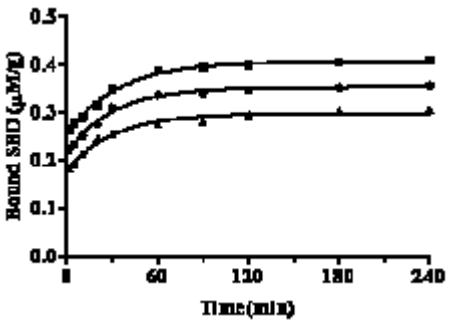

Fig. 5. Association rate of GASBD. Time course of adsorption of GASBD to corn starch. Purified GASBD protein at different concentrations (▲, 15.4 µM; ●, 20.6 µM; ■, 27.8 µM) were added to 10 mg of prewashed corn starch and incubated at 25°C with gentle stirring for 4 h. Bound protein was tested at different time points to determine the association rate.

Adsorption Isotherms of GASBD(s)

Adsorption isotherm measurements were carried out at 25°C for 2 h to determine the binding affinities of GASBD(s) proteins for insoluble corn starch and potato starch. Figure 6 shows adsorption isotherms of GASBD(s) on starches. As can be seen from the Figure 6A and 6B, at low protein concentrations, the amount of micromole of protein binding per gram of starch demonstrates a linear increase. The reason is that there were more free binding sites on the granule surface available for proteins. However, with a rise in protein concentrations, the most of binding sites on the granule surface in contact with proteins were immediately covered by proteins, consequently leading to the amount of bound proteins slowly increased.

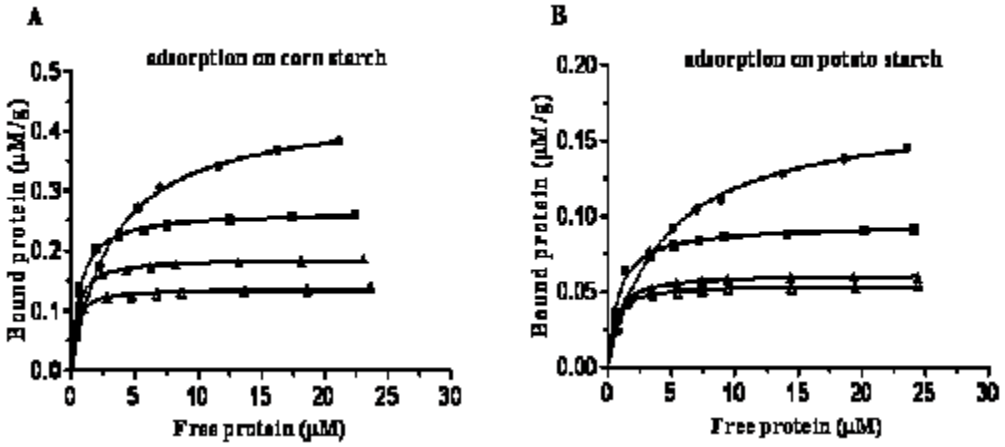

Fig. 6. Adsorption isotherms of four GASBD(s) onto insoluble starch. Adsorption isotherms of GASBD(●), GASBD2(■), GASBD3(▲) and GASBD4(Δ) onto insoluble corn starch (A) and potato starch(B) were performed at 25°C as described in the Materials and Methods. The datas reported are the means for three independent experiments.

Binding parameters of GASBD(s) for insoluble corn starch and potato starch were calculated from a nonlinear regression of the adsorption isotherms (Figure 6). Table 2 summarizes the results on equilibrium association constant (K_a) and the maximum amount of bound protein per gram of starch (B_{max}). K_a(association content, 1/K_d) represents the binding affinity of a protein for starch granules indicating the greater a K_a value, the greater the binding affinity of protein for starch granules. A comparison of the K_a values in the Table 2 showed that the GASBD2, GASBD3 and GASBD4 have approximately 5, 8, and 10-fold higher binding affinity than GASBD for both corn starch and potato starch. The greater than two-, three-, and four-fold increase in the binding affinity cannot be explained only by duplication, triplication, and quadruplication of the binding sites in GASBD2, GASBD3 and GASBD4, indicating that the repeated SBDs in GASBDs proteins act in synergy to bind the insoluble starches. However, the B_{max} values of GASBD(s) proteins for corn starch are 2.5~3.0-fold higher than that for potato starch. It is known that granule size can be different between corn starch and potato starch. The diameter of starch granules range from 3 to 26 μm (mean diameter 15 μm) in corn, and 5 to 100 μm (mean diameter 30 μm) in potato[31,32]. Therefore, corn starch granules have larger specific surface area than potato ones in the same unit mass. For this reason, there are more binding sites on the corn granule surface available for proteins.

To our knowledge, this paper provides the first report of synergy in the artificial multiple

Table 2. Binding parameters of four GASBD(s) to insoluble starches

Proteins	Corn starch		Potato starch	
	K_a ($\times 10^6$ M^{-1})	B_{max} (μM/g)	K_a ($\times 10^6$ M^{-1})	B_{max} (μM/g)
GASBD	0.305± 0.019	0.441 ± 0.008	0.227 ± 0.020	0.170 ± 0.003
GASBD2	1.546± 0.057	0.265 ± 0.004	1.196 ± 0.048	0.094 ± 0.002
GASBD3	2.381 ± 0.073	0.187 ± 0.003	1.712 ± 0.062	0.061 ± 0.001
GASBD4	3.184 ± 0.123	0.136 ± 0.001	2.193 ± 0.083	0.054 ± 0.001

K_d and B_{max} values were calculated by nonlinear regression using a one-site binding model (GraphPad Prism 5.0 software, San Diego, CA, U.S.A.). Values are the means ± SD for three independent measurements.

tandem repeat SBD2 to SBD4 derived from *A. niger* glucoamylase *in vitro*. However, there is also a similar report, in which an artificial tandem repeat of a family 20 starch-binding domain (SBD2) derived from *Bacillus circulans* cyclodextrin glycosyltransferase via the same linker peptide as used in our pET28a-GASBD2 plasmid displayed approximately 10-fold higher binding affinity than single SBD for soluble starch[25]. Whereas the GASBD2 only has approximately 5-fold higher affinity than single GASBD for starch. Although, in both cases, the starch binding domains were used for making recombinant proteins belong to the same CBM family, as well as two SBDs were separated by the same linker, but they derived from different microorganisms. Moreover, affinity analysis methods, as well as ligands using for affinity analysis were different. These could be reasons to lead to affinity differences. Similar performances was also reported in the five tandem SBD modules of family CBM26 from *Lactobacillus amylovorus* α-amylase[21]. Although the results in both cases were similar, the SBDs used for making recombinant proteins belong to different CBM family, one from family CBM20 and the other from family CBM26, which have multiple tandem SBD modules in the natural enzyme.

CONCLUSIONS

In conclusion, the glucoamylase starch binding domain (GASBD) from *Aspergillus niger* 2316 consists of 324 bp nucleotides and encodes a peptide of 108 amino acids. The engineered GASBD2, GASBD3 and GASBD4 proteins have approximately 5, 8, and 10-fold higher binding affinity than single GASBD for both corn starch and potato starch, showing that the multiple-repeat SBD in GASBDs proteins may act in a synergistic way when binding to their ligands.

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