New type of starch-binding domain: the direct repeat motif in the C-terminal region of *Bacillus* sp. no. 195 α-amylase contributes to starch binding and raw starch degrading

Jun-ichi SUMITANI, Tadashi TOTTORI, Takashi KAWAGUCHI and Motoo ARAI[1]

Department of Applied Biological Chemistry, College of Agriculture, and Research Institute of Applied Science and Technology, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, 599-8531 Osaka, Japan

The α-amylase from *Bacillus* sp. no. 195 (BAA) consists of two domains: one is the catalytic domain similar to α-amylases from animals and *Streptomyces* in the N-terminal region; the other is the functionally unknown domain composed of an approx. 90-residue direct repeat in the C-terminal region. The gene coding for BAA was expressed in *Streptomyces lividans* TK24. Three active forms of the gene products were found. The pH and thermal profiles of BAAs, and their catalytic activities for *p*-nitrophenyl maltopentaoside and soluble starch, showed almost the same behaviours. The largest, 69 kDa, form (BAA-α) was of the same molecular mass as that of the mature protein estimated from the nucleotide sequence, and had raw-starch-binding and -degrading abilities. The second largest, 60 kDa, form (BAA-β), whose molecular mass was the same as that of the natural enzyme from *Bacillus* sp. no. 195, was generated by proteolytic processing between the two repeat sequences in the C-terminal region, and had lower activities for raw starch binding and degrading than those of BAA-α. The smallest, 50 kDa, form (BAA-γ) contained only the N-terminal catalytic domain as a result of removal of the C-terminal repeat sequence, which led to loss of binding and degradation of insoluble starches. Thus the starch adsorption capacity and raw-starch-degrading activity of BAAs depends on the existence of the repeat sequence in the C-terminal region. BAA-α was specifically adsorbed on starch or dextran (α-1,4 or α-1,6 glucan), and specifically desorbed with maltose or β-cyclodextrin. These observations indicated that the repeat sequence of the enzyme was functional in the starch-binding domain (SBD). We propose the designation of the homologues to the SBD of glucoamylase from *Aspergillus niger* as family I SBDs, the homologues to that of glucoamylase from *Rhizopus oryzae* as family II, and the homologues of this repeat sequence of BAA as family III.

Key words: glucoamylase, polysaccharide, post-translational processing.

INTRODUCTION

α-Amylases (α-1,4 glucan-4-glucanohydrolase, EC 3.2.1.1) are widely distributed in animals, plants and micro-organisms and catalyse the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and related polysaccharides to produce the α-anomer. Various proteinaceous α-amylase inhibitors have been isolated from plants and microbes [1–3]. They specifically inhibit animal and some *Streptomyces* α-amylases but have no effect on plant and most microbial α-amylases. *Bacillus* sp. no. 195 has been isolated as a producer of an α-amylase sensitive to these inhibitors. Purification and characterization [4] and gene cloning [5] of the enzyme have been reported. In fact, the deduced amino acid sequence showed that the primary structure of the enzyme was more similar to those of animals (34–37% identity) and *Streptomyces* α-amylases (45–51%) than bacterial α-amylases (approx. 23%). We therefore designated the enzyme as *Bacillus* 'animal'-type α-amylase (BAA). In the C-terminal region of BAA, a repeat sequence containing two blocks of 90 amino acid residues each, conserved in several amylases from Gram-positive bacteria, mainly from the genus *Bacillus*, has been found [5]. It was suspected that the natural BAA was processed proteolytically in the C-terminal region because of the lower molecular mass of the purified natural BAA determined by SDS/PAGE than that of the mature BAA estimated from the nucleotide sequence. Moreover, it was expected that the repeat sequence in the C-terminal region of BAA was a cleavage signal for proteinases in the culture supernatant such as α/β-amylase from *Bacillus polymyxa* [6]. To clarify the occurrence of the processing and the role of the repeat sequence in the C-terminal region of BAA, we tried to express the *baa* gene in *Streptomyces lividans* because of the high GC content of the gene. In this paper we describe the characterization of three active forms of recombinant BAAs expressed in *S. lividans*. We found that the starch-adsorption ability and the raw-starch-degrading activity of BAAs depend on the existence of the repeat sequence. This repeat sequence is identified as a putative new type of starch-binding domain (SBD).

EXPERIMENTAL

Materials

Soluble starch, used as substrate for the assay of amylase activity, was purchased from Merck. Corn starch, paramiron, and chitin were from Wako Pure Chemical Industries. Xylan from birch wood was from Sigma, chitosan was from Funakoshi Co., and

Abbreviations used: BAA, 'animal'-type α-amylase from *Bacillus* sp. no. 195; BAA-SBD, starch-binding domain of α-amylase from *Bacillus* sp. no. 195; CBD, cellulose-binding domain; G1-SBD, starch-binding domain of glucoamylase 1 from *Aspergillus niger*; PPA, α-amylase from pig pancreas; SBD, starch-binding domain; TAA, α-amylase (Taka-amylase) from *Aspergillus oryzae*.

[1] To whom correspondence should be addressed (e-mail motoo@biochem.osakafu-u.ac.jp).

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pustulan was from Calbiochem. Yeast extract and tryptone as medium components were products of Difco Laboratories. Restriction endonucleases and modification enzymes were obtained from Nippon Gene Co. and New England Biolabs and were used in accordance with the procedures recommended by the suppliers. Sephacryl S-200 and Sephadex G-75 were purchased from Pharmacia LKB Biotechnology. Butyl-Toyopearl 650M was a product of Tosoh Corp. All other chemicals were commercial products of reagent grade or molecular biological grade.

Bacterial strains, media and culture conditioning

Bacillus sp. no. 195 was grown at 30 °C in the medium described previously [4]. *Escherichia coli* JM109 was used as the host strain for the manipulation of the *baa* gene. *E. coli* was grown at 37 °C with shaking in a Luria–Bertani medium [1 % (w/v) peptone/0.5 % yeast extract/0.5 % NaCl (pH 7.2)] supplemented with 100 μg/ml ampicillin when appropriate. *S. lividans* TK24 was grown at 30 °C in a maltose-Bennett medium [1 % (w/v) maltose/0.2 % peptone/0.1 % meat extract/0.1 % yeast extract (pH 7.2)], containing 10 μg/ml thiostrepton when appropriate.

General DNA manipulations

General molecular biological experiments involving plasmid purification, enzyme digestion and modification, and *E. coli* transformation, were performed in accordance with the methods described in Molecular Cloning [6a] or Current Protocols in Molecular Biology [6b].

Amino acid sequence analysis

The amino acid sequence of the N-terminus was determined by automated Edman degradation with a protein sequencer system (PSQ-1; Shimadzu Co.). For purified recombinant BAAs, approx. 100 pmol was spotted on a glass fibre disk treated with Polybrene and sequenced directly. A C-terminal fragment generated by excision of BAA-α *in vitro* (see below) was subjected to SDS/PAGE and electroblotted to an Immobilon membrane (Millipore) with 0.025 M Tris/HCl/0.192 M glycine/10 % (v/v) methanol. The membrane was washed thoroughly with water and stained with Coomassie Brilliant Blue R-250; the blot was then air-dried. The 10 kDa protein band of the C-terminal fragment of BAA-α was cut out and sequenced directly.

Expression of *baa* gene in *S. lividans*

The expression plasmid was constructed by inserting the 2.5 kbp *Sac*I–*Pst*I fragment from pBAA104 [5] between the same restriction sites of *Streptomyces* expression vector pSEV1 [7]; the resultant plasmid was designated pBAA2. The plasmid was introduced into *S. lividans* TK24 by the method of Hopwood et al. [8]. The transformant was grown at 30 °C in a maltose-Bennett medium supplemented with thiostrepton (final concentration 10 μg/ml).

Enzymic assays

Amylase activity was assayed as follows. The reaction mixture, consisting of 0.1 ml of enzyme solution and 0.1 ml of 1.5 % (w/v) soluble starch in 10 mM Tris/HCl buffer, pH 7.0, was incubated at 37 °C for 10 min and the amount of reducing sugar liberated was measured by the Somogyi–Nelson method [9] with maltose as a standard. One unit of activity was defined as the amount of the enzyme that liberated reducing sugar equivalent to 1 μmol of maltose/min under these conditions. Amylase activity was also measured by the blue-value method of Fuwa [10], modified for the fractions from the column chromatography and desorption assays. The reaction mixture, 0.4 ml of 1.5 % (w/v) soluble starch dissolved in 100 mM Tris/HCl buffer, pH 7.0, and 0.2 ml of enzyme solution, was incubated at 37 °C for 10 min; the reaction was stopped by the addition of 1.0 ml of stop solution [0.5 M acetic acid/0.5 M HCl (5:1, v/v)]. A 50 μl aliquot was removed from the reaction mixture and added to 2.5 ml of 0.01 % I_2/0.1 % KI solution and left at room temperature for 20 min; the A_{660} of the resulting solution was measured. One unit of amylase activity was defined as the amount of enzyme that decreased A_{660} to one-half of that obtained in the same period when buffer was used in place of the enzyme solution.

Measurement of protein

In the purification steps, the protein elution profile was monitored spectrophotometrically as A_{280}. The protein was measured with a Protein Assay Kit (Bio-Rad) by using the method of Bradford [11], with bovine plasma γ-globulin as a standard.

Purification of recombinant BAAs

Culture supernatant from a 4.0-litre culture was added to $(NH_4)_2SO_4$ at 80 % satn. Precipitate was recovered by centrifugation (10000 ***g***, 20 min, 4 °C) and re-dissolved in a small volume of 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$, then dialysed extensively against the same buffer. After removal of the precipitate by centrifugation (10000 ***g***, 20 min, 4 °C), the dialysed sample was applied to a column of Sephacryl S-200 (3.0 cm × 100 cm) equilibrated with 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$ and eluted at a flow rate of 50 ml/h with the same buffer. Amylase activities were divided into three peaks, designated BAA-γ, BAA-β and BAA-α on the basis of the elution order. At this step BAA-α and BAA-β were purified to homogeneity as judged by SDS/PAGE. BAA-γ was purified further as follows. The fractions after Sephacryl S-200 chromatography were collected and applied to a column of Toyopearl HW55 (2.6 cm × 10 cm) equilibrated with 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$ and 1.5 M $(NH_4)_2SO_4$. The enzyme was eluted with a linear gradient of 1.5–0 M $(NH_4)_2SO_4$ in 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$. The active fractions were collected and the enzyme was precipitated with $(NH_4)_2SO_4$ at 80 % satn. The precipitate was recovered, re-dissolved and dialysed as described above. The dialysed sample was applied to a column of Mono Q HR5/5 equilibrated with 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$ on an FPLC system. The enzyme was eluted with a linear gradient of 0–0.2 M NaCl in 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$, at a flow rate of 60 ml/h. The active fractions were collected and added to 1.5 M $(NH_4)_2SO_4$. The enzyme was applied to a column of Toyopearl HW55 (2.6 cm × 10 cm) as described above. A single protein peak was obtained and BAA-γ was found to be homogeneous on SDS/PAGE.

SDS/PAGE and far-Western blot analysis

SDS/PAGE was performed by the method of Laemmli [12]. Far-Western blot analysis was done as follows. After SDS/PAGE and electroblotting as described above, the membrane was incubated overnight in 5 % (w/v) skimmed milk in PBS. After being washed five times with PBS, the membrane was incubated for 1 h with PBS containing purified T-76 (a proteinaceous α-amylase inhibitor from *S. nitrosporeus*) (1 mg/ml) and 1 % (w/v)

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BSA. After being washed five times with PBS, the membrane was incubated for 1 h with PBS containing T-76 antiserum (1:2000 dilution) and 1% (w/v) BSA. After being washed, the blot was incubated with goat anti-rabbit IgG (Sigma) at 1:5000 dilution for 1 h at room temperature. For the detection of bound peroxidase-conjugated antiserum, 3,3′-diaminobenzidine was used as the chromogenic substrate.

Physicochemical and enzymic properties of recombinant BAAs

The effects of pH and temperature on the recombinant BAAs were examined as described previously [4]. For the kinetic analysis of the hydrolysis of starch, initial rates were determined by linear regression analysis and kinetic parameters were calculated from Hanes–Woolf plots. A TLC analysis of the final products of BAAs with amylose as a substrate was performed as follows. The reaction mixtures were spotted on a silica-gel plate (Kieselgel $60F_{254}$; Merck) and developed twice with the following solvent system: butan-1-ol/ethanol/chloroform/25% (v/v) NH_4OH (4:5:2:8, by vol.). After being sprayed with H_2SO_4 containing 1% vanillin, followed by heating for several minutes at 110 °C, the products were identified with the use of malto-oligosaccharides as standards.

Adsorption and desorption assay

The adsorption of BAAs to various polysaccharides was performed as follows. Each of the BAAs (1.4 mM) was mixed with 20 mg each of the polysaccharides in a final volume of 1 ml of 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$. After incubation at 4 °C for 1 h, polysaccharides were removed by centrifugation (18500 ***g***, 10 min, 4 °C) and the amount of unbound protein remaining in the supernatant was measured with a protein assay kit. Estimation of the association constant (K_a) of the enzymes to corn starch was performed by measuring the amounts of free and total protein as above, except that the concentration of protein was varied between 0.13 and 0.33 μM and an equilibration time of 30 min was used. Each tube contained 20 mg of corn starch in 1 ml of 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$. A Langmuir type of isotherm was chosen. By plotting $1/B$ (bound enzyme) against $1/F$ (free enzyme), association constants of the enzymes to corn starch were estimated from the slopes of the line by using the following equation:

$$1/B = 1/(B_{max}K_aF) + 1/B_{max}$$

All experiments were performed in triplicate.

Desorption of BAA-α with various monosaccharides and disaccharides was done as follows. BAA-α adsorbed on corn starch was prepared under the same conditions as the adsorption assay. The samples were then washed with 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$, mixed with 1 ml of 100 mM monosaccharide or disaccharide, incubated at 4 °C for 1 h, and removed by centrifugation (18500 ***g***, 10 min, 4 °C). The enzymes desorbed from the starch in the supernatants were detected by the blue-value method [15].

Determination of digestion rate of raw starch

Wheat, corn, rice, potato and sweet-potato raw starches were washed with water, centrifuged and freeze-dried. Reaction mixtures (3 ml) containing 0.15% raw starch and 0.8 unit/ml enzyme in an appropriate buffer containing 5 mM $CaCl_2$ were incubated at 30 °C for 12 h with shaking. The buffers used were 20 mM Tris/HCl buffer, pH 7.0, for BAAs and α-amylase from pig pancreas (PPA), and 20 mM acetate buffer, pH 5.0, for α-amylase (Taka-amylase) from *Aspergillus oryzae* (TAA). After the reaction the mixtures were centrifuged and the quantities of reducing sugars in the supernatant were measured with the Somogyi–Nelson method as described above, with maltose as a standard. The rate of raw starch digestion, r_d, was defined as:

$$r_d(\%) = 100(A_1/A_2)$$

where A_1 is the molar concentration of the reducing sugars in the supernatant after the reaction and A_2 is the molar concentration of glucose units of whole raw starch before the reaction.

Excision of the C-terminal region of BAA-α *in vitro* in *Bacillus* sp. no. 195 culture supernatant

Bacillus sp. no. 195 was grown in the medium described above, with 1% (w/v) glucose instead of soluble starch. After removal of the cells by centrifugation (10000 ***g***, 5 min, 4 °C), the culture supernatant was passed through a membrane filter (0.20 μm) (Advantec) to remove the cells completely. The culture fluid (50 μl) was added to 50 μg of BAA-α in 100 μl of 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$. The reaction mixture was incubated at 30 °C and a 15 μl aliquot from the reaction mixture was transferred after various durations (0–24 h) to a fresh tube containing 15 μl of 2 × SDS/PAGE sample buffer on ice. The samples were stored at −20 °C until all were finished, then applied to SDS/PAGE by the method of Schägger and von Jagow [13]. After electroblotting, the N-terminal amino acid sequence of the 10 kDa fragment generated from BAA-α was analysed as described above.

RESULTS

Expression of *baa* gene in *S. lividans* TK24 and purification of recombinant BAAs

Amylase activity sensitive to a proteinaceous inhibitor was detected in the culture supernatant of *S. lividans* TK24 harbouring the expression plasmid pBAA2, but not in that of the transformant with the original plasmid pSEV1. A previous study

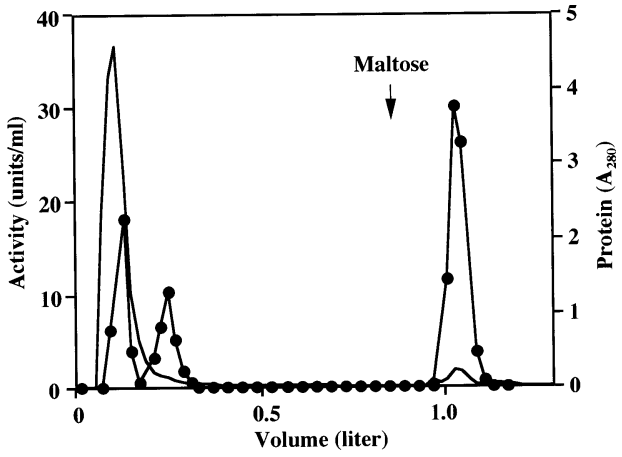

Figure 1 Gel filtration of recombinant BAA on Sephacryl S-200

Crude enzyme after dialysis was applied to a column (4 cm × 80 cm) of Sephacryl S-200 equilibrated with 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$. BAA-α was eluted with a buffer containing 10 mM maltose at the point indicated. Symbols: ●, amylase activity; line without symbols, A_{280}.

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Table 1 Purification of recombinant BAAs

Enzyme	Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Fold purification
BAA-α,β,γ	Culture fluid	9360	16000	1.71	100	1.00
	$(NH_4)_2SO_4$ precipitate	2750	13500	4.91	84.7	2.87
BAA-α	Sephacryl S-200	295	3830	12.9	24.0	7.54
BAA-β	Sephacryl S-200	70.8	972	13.7	6.10	8.01
BAA-γ	Sephacryl S-200	2400	2200	0.917	13.8	0.536
	Toyopearl HW-55	469	2020	4.30	12.7	2.51
	Mono Q	78.5	984	12.5	6.17	7.31
	Toyopearl HW-55	39.9	624	15.6	3.91	9.12

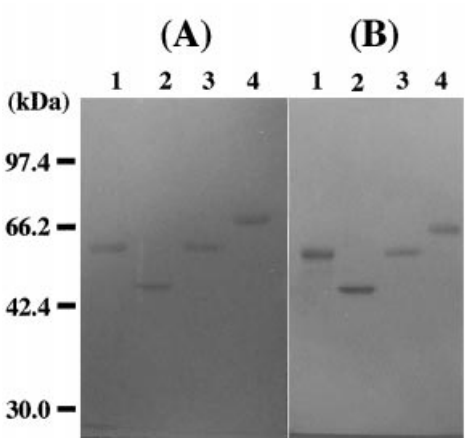

Figure 2 SDS/PAGE and far-Western analysis of recombinant BAAs

Purified enzymes were subjected to an SDS/PAGE [12% (w/v) gel]; the gels were analysed by Coomassie Brilliant Blue R-250 staining for protein detection (**A**) and far-Western blot for immunological detection of proteinaceous α-amylase inhibitor bound to amylase (**B**). Lanes 1, natural BAA purified from *Bacillus* sp. no. 195; lanes 2, BAA-γ; lanes 3, BAA-β; lanes 4, BAA-α. The positions of molecular mass markers are indicated at the left.

showed that BAA from *Bacillus* sp. no. 195 was efficiently purified by Sephacryl S-200 column chromatography. A concentrated culture supernatant from the transformant of pBAA2 was therefore applied to a column of Sephacryl S-200 (Figure 1). However, the amylase activity appeared in three peaks, in contradiction of previous results [4]. The first peak (designated BAA-γ) was eluted at almost the same volume as that predicted from its molecular mass. The second peak (BAA-β), in the same manner as the natural enzyme, was eluted in later fractions than predicted from its molecular mass, because of an interaction between the protein and the resin. The third peak (BAA-α) was not eluted under these conditions until buffer containing 10 mM maltose was added. BAA-α and BAA-β were purified to homogeneity as judged by SDS/PAGE by this step. The purification of the three recombinant BAAs is summarized in Table 1.

Physicochemical properties of recombinant BAAs

The purified enzymes were subjected to SDS/PAGE and far-Western blot analysis with proteinaceous amylase inhibitor T-76 and anti-(T-76) antibody (Figure 2). It was estimated that the molecular masses of BAA-α, BAA-β and BAA-γ were 69, 60 and 50 kDa respectively; that of the natural BAA was estimated to be 60 kDa. The molecular mass of BAA-α agreed with that of the mature protein estimated from the nucleotide sequence, suggesting that BAA-β and BAA-γ could be truncated forms of BAA-α. We therefore analysed the N-terminal amino acid sequences of recombinant BAAs. As a result, all of the enzymes including natural BAA were found to have the same N-terminal sequence (results not shown). This indicates that the signal sequence of BAA was functional in *S. lividans* and also that BAA-β and BAA-γ as well as natural BAA were truncated in the C-terminal region. The temperature and pH profiles of recombinant BAAs and the natural BAA were almost the same (results not shown).

Enzymic properties of recombinant BAAs

The kinetic constants of recombinant BAAs were determined (Table 2). All of the recombinant BAAs showed almost the same affinities and reaction velocities for *p*-nitrophenyl maltopentaoside and soluble starch. The hydrolysis products of amylose with the recombinant BAAs with amylose as a substrate were analysed by TLC (results not shown). Maltose, maltotriose and maltotetraose were detected from the early stage of the reaction with all of the BAAs, although maltotetraose disappeared gradually. Glucose was not detected by TLC. It was therefore suggested that maltotetraose was a substrate for all of the BAAs, yielding two maltose molecules. It was also found that all the recombinant BAAs were bound to a proteinaceous amylase inhibitor, T-76 (Figure 2B). The sensitivities of recombinant BAAs to inhibition by T-76 were also the same in spite of the diversities in the C-terminal repeat sequence, suggesting that the repeat sequence is not involved in the sensitivity to T-76 (results not shown).

Adsorption and desorption assays

Natural BAA adsorbs starch and interacts with Sephacryl S-200 resin containing allyl dextran; these properties have been applied to the purification of the enzyme [4]. Recombinant BAAs were separated into three peaks with the Sephacryl S-200 column (Figure 1); in particular, BAA-α required the presence of 10 mM maltose for elution from the resin. These results indicate that each of the BAAs has its own strength of interaction with certain polysaccharides. We therefore investigated the abilities of recombinant BAAs to adsorb on various insoluble polysaccharides (results not shown). BAA-α was tightly adsorbed, BAA-β was partly adsorbed and BAA-γ was hardly adsorbed on corn starch, Sephacryl S-200 and Sephadex G-75. In contrast, the BAAs did

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Table 2 Kinetic parameters of BAAs

Enzyme	*p*-Nitrophenyl maltopentaoside			Soluble starch		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	K_m (mg/l)	k_{cat} (s^{-1})	k_{cat}/K_m ($mg^{-1} \cdot s^{-1} \cdot l$)
BAA-α	1.6	18	11	69	200	2.9
BAA-β	1.9	17	8.9	93	230	2.5
BAA-γ	1.5	14	9.3	61	230	3.8

Table 3 Ability of amylases to digest raw starches

The reaction mixture contained 0.15% various raw starches and 0.8 unit/ml of enzyme. Tris/HCl buffer (20 mM, pH 7.0) was used for BAAs and PPA, and 20 mM acetate buffer, pH 5.0, for TAA. After incubation for 12 h the derived reducing sugars in the supernatant were measured by the Somogyi–Nelson method. The digestion rate was calculated as described in the text.

Amylase	Digestion rate (%)				
	Maize	Wheat	Rice	Potato	Sweet potato
BAA-α	13.1	11.6	13.5	1.13	7.38
BAA-β	3.00	4.50	4.88	0.563	1.50
BAA-γ	0.375	1.00	1.50	0.275	0.225
PPA	3.5	7.75	6.38	0.75	2.13
TAA	0.188	0.400	0.875	0.125	0.15

not interact with paramyron, β-1,6-glucan, xylan, chitin or chitosan under the conditions described in the Experimental section. Moreover, the association constants (K_a) of BAA-α and BAA-β for corn starch were estimated from the plot of $1/F$ against $1/B$ (where F and B are free and bound enzyme concentrations respectively), with an equilibration time of 30 min. From these plots, K_a values of 1.0×10^7 M^{-1} for BAA-α and 1.2×10^6 M^{-1} for BAA-β, and B_{max} values of 2.9×10^{-8} mol/mg for BAA-α and 2.7×10^{-8} mol/mg for BAA-β binding to corn starch were obtained. Because BAA-α strongly adsorbed corn starch in the recombinant BAAs, we examined the desorption specificity with various monosaccharides and oligosaccharides from corn starch (results not shown). BAA-α was specifically desorbed with maltose and β-cyclodextrin; however, cellobiose, isomaltose, sucrose, trehalose, gentiobiose and glucose had no effect on the desorption of corn starch under the conditions described in the Experimental section.

Degradation of raw starch by recombinant BAAs

Each recombinant BAA expressed in *S. lividans* had a characteristic strength of adsorption for starch. Consequently, the relation between the adsorption ability and the raw-starch-degrading activity was examined (Table 3). Thus BAA-α and BAA-β showed activities of 653 unit/μmol and 140 unit/μmol for raw corn starch respectively, whereas BAA-γ showed no activity. Moreover, the raw-starch-digesting abilities for BAAs were compared with those of PPA, which has a strong raw-starch-digesting ability, and TAA, which does not. The digesting abilities of BAA-α for all of the raw starches tested were stronger than those of PPA. BAA-β had weaker abilities than PPA. In contrast, BAA-γ and TAA showed little activity towards these raw starches.

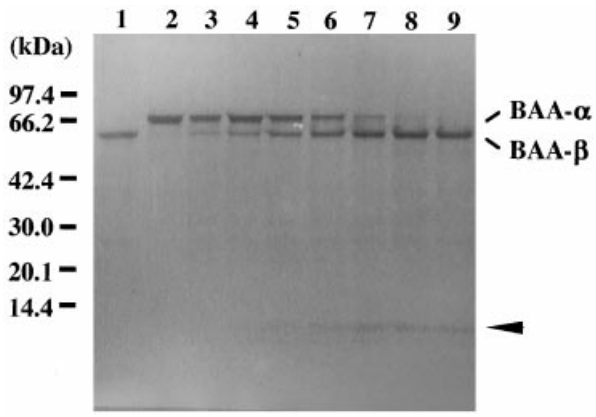

Figure 3 SDS/PAGE analysis of the excision of BAA-α in the culture supernatant of *Bacillus* sp. no. 195

BAA-α was incubated in the culture supernatant of *Bacillus* sp. no. 195 at 30 °C for the following durations: lane 2, 0 min; lane 3, 2 min; lane 4, 4 min; lane 5, 8 min; lane 6, 16 min; lane 7, 32 min; lane 8, 64 min; lane 9, 16 h. Lane 1, natural BAA. The positions of molecular mass markers are indicated at the left. The arrowhead indicates the 10 kDa peptide generated by the excision of BAA-α.

Excision of the C-terminal region of BAA-α *in vitro* in culture supernatant of *Bacillus* sp. no. 195

It has been supposed that natural BAA is processed in the spacer region between the two repeat sequences in the C-terminal region after secretion into the extracellular culture supernatant. To confirm this hypothesis, the purified BAA-α corresponding to an intact product of the *baa* gene was incubated in the culture supernatant of *Bacillus* sp. no. 195 for various durations. As shown in Figure 3, the concentration of BAA-α gradually decreased and that of a new protein whose molecular mass was the same as that of natural BAA increased. Moreover, a 10 kDa peptide was generated in parallel with the excision of BAA-α. The processed BAA-α corresponding to natural BAA and the 10 kDa peptide remained stable. To determine the site of the excision, amino acid sequencing of the N-terminal region of the 10 kDa peptide was performed. As shown in Figure 4, the N-terminal amino acid sequence of the 10 kDa peptide was SAPAPADT (single-letter amino acid codes), corresponding to the sequence of BAA from Ser-554 to Thr-561. Therefore the cleavage site of the C-terminal region of BAA was the N-terminal side of Ser-554, 10 residues upstream of the second repeat sequence. As a result, the molecular masses of the processed BAA-α and 10 kDa peptide calculated from the nucleo-

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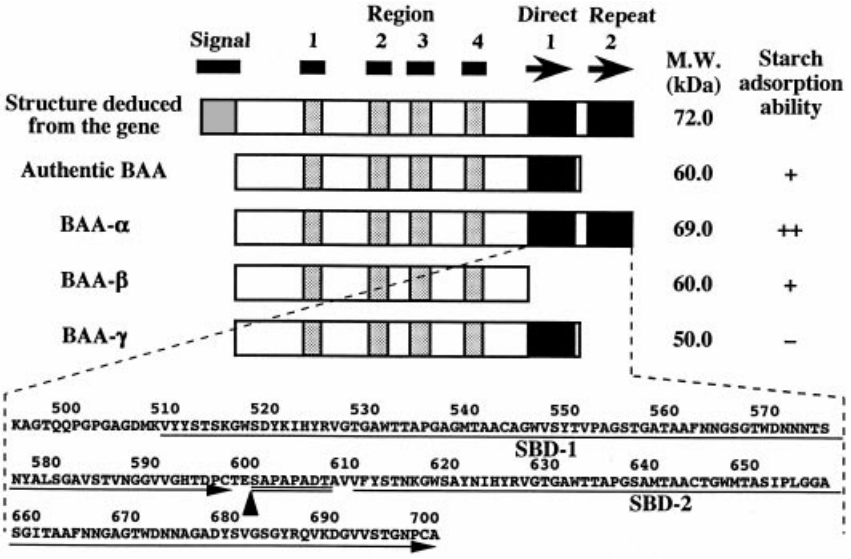

Figure 4 Schematic representation of the structures of BAAs produced from *Bacillus* sp. no. 195 and *S. lividans* TK24, and the cleavage site of the C-terminal fragment of BAA in the culture supernatant of *Bacillus* sp. no. 195

Regions 1, 2, 3 and 4 indicate the conserved regions in an amylolytic enzyme. A starch adsorption assay was performed as described in the text. The amino acid sequence around the SBD is shown. The repeat sequence is shown by direct arrows. The sequence corresponding to the N-terminal sequence of the 10 kDa peptide is doubly underlined. The vertical arrow indicates the cleavage site of the proteinase in the culture supernatant of *Bacillus* sp. no. 195.

tide sequence were 58696 and 10098 Da respectively, in good agreement with the estimate by SDS/PAGE (60 and 10 kDa respectively).

DISCUSSION

S. lividans harbouring the expression plasmid of the *baa* gene produced three forms of BAA, the only difference being the repeat sequence number in the C-terminal region arising from post-translational processing. The structures of the three BAA derivatives produced in *S. lividans* are summarized in Figure 4. We showed that the precursor of BAA (BAA-α) was immediately processed at the region sandwiched between the two repeat sequences in the C-terminal region after secretion into the medium (Figure 3). We also confirmed that this processing occurred at a specific point, by a clear determination of the N-terminal sequence of the excision product in the excision experiment *in vitro*. However, the specificity of processing might depend on the domain structure of BAA-SBD, which is extremely sensitive to extracellular proteinases, rather than on the specificity of the proteinases, which recognize certain amino acid sequences. Indeed, BAA-SBD was processed not only in the culture supernatant of *Bacillus* sp. no. 195 but also in that of *S. lividans*, in spite of incomplete digestion. Therefore the repeat sequence in the C-terminal region of BAA can be regarded as the processing signal, as in α/β-amylase from *B. polymyxa* [6]. However, the presence of the repeat sequence in the C-terminal region also conferred binding to and activity towards raw starch (Figure 4 and Table 3) on the N-terminal catalytic domain of BAA. Thus it is apparent that the repeat sequence in the C-terminal region of BAA functions as an SBD. So far there have been reports of many polysaccharide-binding domains separated from their catalytic domains in polysaccharide-degrading enzymes. In these studies the cellulose-binding domains (CBDs) have been characterized in great detail. There have been many reports on CBDs, which have been grouped into several families on the basis of amino acid sequence similarity [14,15]; there have been fewer reports on SBDs. The SBD of glucoamylase 1 from *Aspergillus niger* (G1-SBD) has been well characterized [16–20]. To our knowledge, the amino acid sequences of most of the SBDs reported are similar to that of G1-SBD, with the exception of the SBDs lying at the N-terminal region of glucoamylases from *Rhizopus oryzae* and *Arxula adeninivolans* [21]. The present study shows that BAA-SBD is a new type of SBD because the amino acid sequence of BAA-SBD has no similarity to others reported previously. Several internal or C-terminal repeat ssequence of some bacterial amylases share amino acid similarity with BAA-SBD [5], suggesting that the repeat sequences of these enzymes might also be functional SBDs and that these SBDs form a new SBD family. We propose the designation of the homologues to G1-SBD as family I SBDs, the homologues to SBD of *R. oryzae* glucoamylase as family II, and the homologues to the BAA-SBD as family III. The domain structures of these three families of SBD are shown diagrammatically in Figure 5. Although the family I SBDs have already been classified into two groups (a bacterial type and a fungal type) on the basis of amino acid sequence similarities [22], it can be regarded as a 'subgroup' because of similarities in their entire sequences and structures. The family I SBDs are widely distributed in micro-organisms, whereas the family III SBDs are found exclusively in Gram-positive bacteria, mainly the genus *Bacillus*. The distribution of family II SBDs cannot yet be deduced, because of a lack of data.

Temperature and pH profiles of the catalytic domain of BAA-SBD are not affected by the existence of an SBD, although the SBD of α-amylase from *Cryptococcus* sp. [23] and many CBDs of cellulases and xylanases from various origins stabilize the function of the catalytic domain against denaturation at high temperature [24]. It is evident that the affinity and activity of BAA-α towards raw starch are higher than those of BAA-β. However, it is not yet clear whether the difference between the properties of BAA-α and BAA-β towards raw starch depends on the number of repeat units in the C-terminal region or whether

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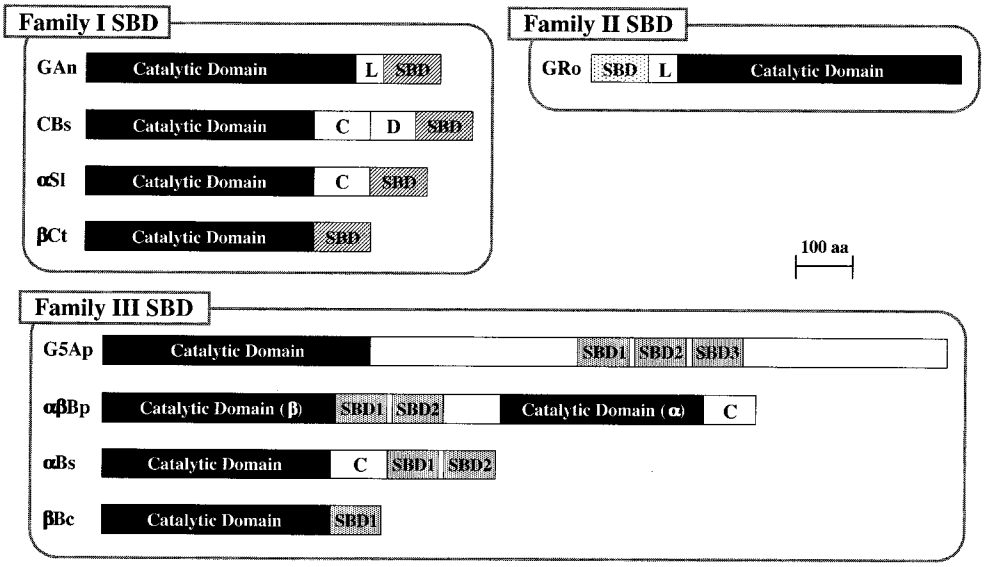

Figure 5 Schematic representation of the domain structure of amylolytic enzymes possessing SBDs

The domain organizations of amylolytic enzymes proposed by Jespersen et al. [28] are represented and classified into three families on the basis of their SBD structure. The black, hatched/grey and white areas indicate the catalytic, starch-binding and other domains respectively. L, heavily glycosylated linker regions. C-domains are functionally unknown but are clearly identified by sequence comparison and hydrophobic-cluster analysis; D-domains follow the C-domain with an immunoglobulin fold as described elsewhere [28]. Enzyme abbreviations: GAn, glucoamylase, *A. niger* [29]; CBs, cyclodextrin glucanotransferase, *Bacillus* sp. strain 1011 [30]; αSl, α-amylase, *S. limosus* [31]; βCt, β-amylase, *C. thermosulfurogenes* [32]; GRo, glucoamylase, *R. oryzae* [33], G5Ap, maltopentaose-producing amylase, alkaliphilic Gram-positive bacterium [34]; αβBp, α/β-amylase, *B. polymyxa* [35]; αBs, α-amylase, *Bacillus*. sp. no. 195 [5]; βBc, β-amylase, *B. circulans* [36].

the role of the repeat unit positioned at the N-terminal side is to function as a linker region to keep an appropriate distance between the catalytic domain and the SBD. Glucoamylase 1 from *A. niger* and many cellulases, xylanases and chitinases from various origins contain a linker sequence between the catalytic domain and the polysaccharide-binding domain [14,25,26]. In addition, it is as yet unknown why the C-terminal repeat unit was immediately trimmed from BAA. Although there seems to be no improvement in the enzymic properties of BAA by the conversion from BAA-α to BAA-β, a growth advantage might be achieved.

In studies on G1-SBD, the importance of conserved tryptophan and tyrosine residues in binding to starch was demonstrated by chemical modification [27], site-directed mutagenesis of conserved residues [20] and the three-dimensional structure of the SBD–β-cyclodextrin complex [19]. Similarly, two tryptophan and three tyrosine residues are highly conserved in the repeat sequences in the C-terminal region of some bacterial amylases, including BAA [5]. It is expected that some of these would be connected with ligand binding.

It will be of interest to investigate the relationship between structure and function in BAA, to elucidate how the repeat sequence of BAA-SBD is involved in binding to starch. Moreover, it is possible that the SBD could be used as an affinity tag to purify recombinant proteins in molecular biology. It might also be possible to confer the raw starch-degrading ability on an amylase without activity against insoluble starches by connecting it to the SBD. At present we are attempting to identify the amino acid residues in BAA-SBD that participate in the binding to polysaccharides.

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