Properties of Branching Enzyme from Hyperthermophilic Bacterium, *Aquifex aeolicus*, and Its Potential for Production of Highly-branched Cyclic Dextrin

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Abstract: By sequencing the whole genome of the hyperthermophilic bacterium, *Aquifex aeolicus*, a gene (Aq722) encoding a protein with high similarity to the branching enzyme (BE, EC 2.4.1.18) has been found. Based on the putative amino acid sequence, a polynucleotide encoding the BE gene was chemically synthesized and the gene was expressed in *Escherichia coli*. More than 95% of the recombinant enzyme was present within the cells as insoluble but catalytically active aggregates. Heat treatment of the aggregate suspension at 70°C resulted in about 30% solubilization of the BE activity. Enzymatic properties of both soluble and insoluble forms of the BE were analyzed. Both forms exhibited maximum activities at 75°C and pH 7.5–8.0, and were stable at temperatures up to 85°C. The insoluble-form BE was less stable than the soluble form in neutral and acidic pH regions; after 30-min incubation at 70°C and pH 7.0, 90 and 50% activities of soluble and insoluble forms remained, respectively. When amylopectin was used as a substrate, the *A. aeolicus* BE cyclized the B-chains, which connect the cluster structures of amylopectin. The product (highly-branched cyclic dextrin), with weight-average degree of polymerization (DP_w) 1200, has a ring structure with DP_w 50 and non-cyclic chains with an average unit chain length of 16 connected to the ring.

Key words: branching enzyme, hyperthermophilic bacterium, cyclization, thermostable enzyme

Starch, the major reserve polysaccharide of plants, consists of two components: amylose and amylopectin. Amylose is an essentially linear glucan linked with α -1,4-glucosidic linkages, while amylopectin is highly branched. Partially degraded starch, dextrin, is widely used in food, paper and other industries. To produce dextrans with various sizes, starch is treated with α -amylase (EC 3.2.1.1) or acid under appropriate conditions.

The branching enzyme (BE, 1,4- α -D-glucan: 1,4- α -D-glucan 6- α -D-(1,4- α -D-glucano)-transferase, EC 2.4.1.18) catalyzes transglycosylation to form α -1,6-glucosidic linkages of amylopectin or glycogen. It has been demonstrated that, in addition to the branching reaction, BE catalyzes the cyclization reaction of amylose and amylopectin.^{1–3} When amylopectin was used as a substrate, the thermostable BE from *Bacillus stearothermophilus* TRBE14 mainly acted on the inner B-chain of amylopectin and degraded amylopectin by cyclization reaction to produce large cyclic glucans with a limited molecular size (highly-branched cyclic dextrin, HBCD; Fig. 1). HBCD has a narrow size distribution, high solubility in water and a low propensity for retrogradation compared to other commercially available dextrin.⁴ HBCD is also useful to improve the physical properties of starch: it has been demonstrated that the addition of HBCD to other starches reduces the viscosity of the starch pastes and increases their transparency.⁵ Although the *B. stearothermophilus* BE is a thermostable enzyme exhibiting maximum activity at 50°C,⁶ a higher reaction temperature is desirable for starch processing because the gelatinization temperature of

starch is 60–75°C. Recently, Shinohara *et al.*⁷ reported *Rodothermus obamensis* BE. It has optimum activity at 65°C and is stable up to 85°C.

Aquifex aeolicus is a hyperthermophilic bacterium with a growth-temperature maximum near 95°C. From the whole genome-sequencing project, a gene (Aq722) encoding a protein with a high similarity (56.5%) to BE has been found.⁸ The aim of this paper is to test the activity of the putative BE, and to evaluate the potential of the enzyme for starch processing.

MATERIALS AND METHODS

Chemicals. Enzymatically synthesized amylose (AS-1000; average molecular mass, 1000 kDa) was purchased from Nakano Vinegar, Co., Ltd. (Aichi, Japan). Type III amylose was obtained from Sigma. Waxy corn starch was purchased from Sanwa Denpun Kogyo Co., Ltd. (Nara, Japan). Isoamylase was purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Glucoamylase of *Rhizopus* sp. was obtained from Toyobo (Osaka, Japan).

Construction of a plasmid for the expression of *A. aeolicus* BE gene and the production of BE. A gene (1935 bp) encoding *A. aeolicus* BE was chemically synthesized. A *Bam*HI site (underlined) and ribosome binding sequence (doubly underlined) were placed in the upstream region to the start codon (italic), and an *Eco*RI site (underlined) was placed in the downstream region to the stop codon (italic), as follows;

CTTGGATCCTGATTAAAGGAGGTAATAACATATG... (coding sequence)...TAATGAATTCCTC.

The ribosome binding sequence was designed based on the consensus sequence described by Hershey.⁹ The syn-

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Fig. 1. Schematic representation of cyclization of amylopectin catalyzed by BE.

Open and closed triangles indicate the α -1,4 linkages cleaved by BE and glucosyl acceptors, respectively. Solid lines indicate a 1,4- α -D-glucan chain. Arrows are α -1,6-glucosidic linkages. The reducing end of a glucan chain is shown by "R."

thetic gene was digested with *Bam*HI and *Eco*RI, and then inserted to pUC19 to construct a recombinant plasmid, pAQBE1.

E. coli XL-2 Blue (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*[F', *proAB*⁺, *lacI*^q, *lacZ* Δ M15, Tn10 (Tet^r), Amy, Cam^r]) carrying pAQBE1 was cultured in 200 mL of L broth (1% Bacto-Tryptone (Difco Laboratories, Detroit, MI, USA), 0.5% Bacto-yeast extract (Difco), 0.5% NaCl, pH 7.3) containing 100 μ g/mL ampicillin at 37°C with a shaking rate of 120 rpm. At the mid-log phase, isopropyl- β -D-thiogalactopyranoside was added at a final concentration of 0.1 mM. After 20 h, cells were harvested and washed twice with 10 mM sodium phosphate buffer (pH 7.5). The cells were suspended in 20 mL of the same buffer, and broken by sonication at 4°C to obtain a cell-free extract.

Assay of BE activity. The BE activity was assayed as described previously²⁾ with slight modification. The enzyme solution (50 μ L) was mixed with the substrate solution (0.12% (w/v) amylose, 50 μ L) and incubated at 70°C for 10 min. The enzyme was diluted to an appropriate concentration with 10 mM sodium phosphate (pH 7.5) including 0.05% (w/v) Triton X-100. The substrate solution was prepared by mixing 100 μ L of 1.2% (w/v) type-III amylose (Sigma) dissolved in dimethyl sulfoxide, 200 μ L of 50 mM potassium phosphate buffer (pH 7.5) and 700 μ L of distilled water. Reactions were terminated by adding 1 mL of 0.4 M HCl, and then 1 mL of iodine reagent was added to the solution. The iodine reagent was made daily from 0.125 mL of stock solution (0.26 g of I₂ and 2.6 g of KI in 10 mL of water) mixed with 0.5 mL of 1 N HCl and diluted to 65 mL. One unit of enzyme activity was defined as the amount of BE that can decrease the absorbance of the amylose-iodine complex by 1%/min at 660 nm.

Preparation of *A. aeolicus* BE product (*P*_{Aae}) from waxy corn amylopectin. Waxy corn starch (20 g) was suspended in 100 mL of 10 mM sodium phosphate buffer (pH 7.5) and gelatinized by heating in a boiling water bath for 1 h. After cooling to 70°C, 6000 U of *A. aeolicus*

BE were added to the paste. The mixture was incubated at 70°C for 16 h and then heated at 100°C for 1 h. After the removal of insoluble materials by filtration and centrifugation, the dextrin was precipitated by adding an equal volume of ethanol and then lyophilized. No sugar could be detected in the supernatant by the phenol-sulfuric acid method.¹⁰⁾

Preparation of *B. stearothermophilus* BE product (*P*_{Bst}) from waxy corn amylopectin. The *B. stearothermophilus* BE product was prepared from 20% (w/v) waxy corn starch as described previously.⁴⁾

Measurement of molecular weight of glucan. Glucan was subjected to high-performance liquid chromatography (HPLC) with a multi-angle laser-light-scattering photometer (MALLS; DAWN DSP, Wyatt Technology Co., Ltd., CA, USA) and a differential refractive index detector (RI; RI-71, Showa Denko, Tokyo, Japan). Columns were Shodex OHpak SB-806M HQ (8.0 $\times$ 300 mm, Showa Denko) with a precolumn (OHpak SB-G). Elution was carried out at 40°C with 0.1 M NaNO₃ at a flow rate of 1.0 mL/min. Samples (2–4 mg/mL, 50 μ L) were injected into the column after filtration through a 0.22 μ m membrane (Millex GS, Millipore). The molecular weight was determined according to the instruction manual of DAWN-DSP on the basis of the following equation.

$$R_{\theta \rightarrow 0}/K^*c = M - 2A_2cM^2$$

where $R_{\theta \rightarrow 0}$ is the Rayleigh ratio extrapolated using the individual scattering angle θ to 0, K^* is an optical constant, c is the concentration of solute molecules calculated from the RI response using the refractive index increment (dn/dc) value of 0.145 mL/g for α -glucan, M is the weight-average molecular weight and A_2 is the second virial coefficient.

By neglecting the second term, we determined M with a $R_{\theta \rightarrow 0}/K^*c$ versus $\sin^2(\theta/2)$ plot (Debye plot) for θ approaching 0. M was calculated at each 0.5 s slice of the elution period and then used to determine weight- and number-average molecular weights of the glucan.

Other procedures. The chain-length distribution of the glucan was analyzed using high-performance anion exchange chromatography (HPAEC) with a pulsed amperometric detector (Dionex, CA, USA) as described previously.²⁾ The reducing ends of the glucan were quantitated using a modified Park-Johnson method.¹¹⁾ The non-reducing end of the glucan was quantitated using F-kit Glycerol (Behringer Mannheim, Tokyo, Japan) after rapid Smith-degradation¹²⁾ of the glucan. The total sugar of the glucan was measured by the phenol-sulfuric acid method.¹⁰⁾ The glucoamylase treatment to isolate the cyclic portion of the glucan was performed as described previously.²⁾ Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a gradient gel (10–15%) with PhastSystem (Pharmacia), and the gel was stained with PhastGel Blue R (Pharmacia). Unless otherwise specified, all chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

RESULTS

Expression of the gene of *A. aeolicus* BE in *E. coli*.

The cell-free extract of *E. coli* XL2-Blue carrying

pAQBE1 was prepared as described in the MATERIALS AND METHODS section. SDS-PAGE analysis showed that a 66-kDa protein was produced in the extract (data not shown). Based on the amino acid sequence, the molecular mass of *A. aeolicus* BE was calculated to be 74,166 Da.⁸⁾ The differences between the calculated mass and estimated mass from SDS-PAGE remains to be explained. BE activity of the extract assayed at 70°C was 9850 U/mL. After 10-min centrifugation at low speed ($3000 \times g$), most of the 66-kDa protein and 86% of the BE activity were recovered in the insoluble fraction. By using a densitometer, the specific activity of BE was estimated to be approximately 10,000 U/mg protein. To confirm the branching activity (α -1,6-glucosidic-linkage synthesizing activity) of the BE, amylose was incubated with the BE at 70°C. The ability of amylose to form an iodine complex (absorbance at 660 nm) decreased rapidly (Fig. 2). Then, the 10-min and 60-min reaction products were subjected

Fig. 2. Activity of the *A. aeolicus* BE with amylose.

A reaction mixture (1 mL) including 0.5% (w/v) amylose AS-1000 and 470 U of the *A. aeolicus* BE in 10 mM sodium phosphate buffer (pH 7.5) was incubated at 70°C. At the indicated reaction time, 5 μ L of the mixture was removed and mixed with 1 mL iodine reagent (0.5 mg of I_2 and 5 mg of KI in 0.4 mM HCl), and then absorbance at 660 nm was measured.

to HPAEC analysis before and after treatment with isoamylase (Fig. 3). α -1,4-Glucan chains with DP 5–40 were detected after isoamylase treatment (Fig. 3B). The peak eluted at 40–45 min should be glucans with DP larger than 60, because in this HPAEC system, such large-sized glucans cannot be separated and are eluted together in this area (40–45 min). Before isoamylase treatment, oligosaccharides were also detected (Fig. 3A). These oligosaccharides are assumed to be the result of BE action on amylose, because BE maintained the reducing-terminal-side moiety of the donor chain after the cleavage of α -1,4 linkage followed by transfer to the acceptor chain.³⁾ These results indicate that the BE catalyzes the transfer of α -1,4-glucan chains with various sizes to form α -1,6 branch points, and confirm that the gene Aq722 encodes a functional branching enzyme.

Characterization of *A. aeolicus* BE prepared from *E. coli* carrying pAQBE1.

The insoluble BE could be partially solubilized by heat treatment as follows. The suspension of BE was incubated at 70°C for 30 min and then centrifuged at $10,000 \times g$ for 10 min. About 30% of the activity was recovered in the supernatant. The effect of pH and temperature on the stabilities and activities of the soluble and insoluble forms of BE were analyzed. Both forms were stable up to 85°C and the activities were maximal at 75°C (Fig. 4). In a temperature range of 50 to 70°C, the soluble form has 10–20% higher activity than the insoluble form. Both forms were stable in the range of pH 8 to 10 (Fig. 5). In the acidic and neutral pH ranges, the soluble form again had higher stability. The optimum pH for both forms was around 8.0 (Fig. 5).

Fig. 3. HPAEC analysis of products formed from the activity of *A. aeolicus* BE with amylose.

The products of *A. aeolicus* BE from the experiment shown in Fig. 2 (10 or 60 min) were subjected to HPAEC before (A) and after (B) treatment with isoamylase. Each elution profile is shown. The numbers on peaks indicate chain lengths.

Fig. 4. Effect of temperature on the stabilities (A) and activities (B) of soluble (●) and insoluble (○) forms of the *A. aeolicus* BE.

(A) The enzyme activity was assayed at each temperature in 10 mM sodium phosphate (pH 7.5) and 0.025% Triton X-100. (B) To examine the thermal stability, the enzyme in 10 mM sodium phosphate (pH 7.5) and 0.05% Triton X-100 was heated at the indicated temperature for 10 min and then immediately cooled on ice. The remaining activity was assayed at 70°C.

Action of *A. aeolicus* BE on amylopectin.

The action of both insoluble and soluble forms of BE was analyzed by HPLC-MALLS-RI and isoamylase treatment followed by HPAEC. Although Figs. 6 and 7 show the results using the insoluble form of BE, no differences in the action of amylopectin were observed between the soluble and insoluble forms of BE under the conditions tested. The BE efficiently degraded amylopectin to a limited molecular size (Fig. 6) with no increase in reducing power (data not shown). This result suggests that amylopectin was degraded by a cyclization reaction. Analyses of chain-length distributions revealed that the long chains (CL 42–57) were decreased and that middle-sized chains (CL 25–38) and short chains (CL 8–11) were increased by the BE action (Fig. 7). On the other hand, overall chain-length distribution was not significantly changed. The results suggest that the BE cyclized the long chains of amylopectin to form highly-branched cyclic dextrin (Fig. 1), as *B. stearothermophilus* BE did.

For further comparison of the activities of *A. aeolicus* BE and *B. stearothermophilus* BE with amylopectin, products P_{Aae} and P_{Bst} were prepared and subjected to structural analyses (Table 1). No significant differences between the two products were observed. The elution curves of gel-filtration chromatography monitored by the RI of P_{Aae} and P_{Bst} also overlapped each other (data not shown). The glucoamylase-resistant glucans derived from P_{Aae} and P_{Bst} were relatively small molecules with DP_w

Fig. 5. Effect of pH on the stabilities (A) and activities (B) of soluble (●) and insoluble (○) forms of the *A. aeolicus* BE.

(A) The enzyme activity was assayed at 70°C in Britton-Robinson buffer and 0.025% Triton X-100 adjusted to the indicated pH. (B) To examine the pH stability, the enzyme was incubated at 70°C for 30 min in Britton-Robinson buffer adjusted to the indicated pH. After the enzyme solution was diluted with 10 mM sodium phosphate buffer (pH 7.5) and 0.05% Triton X-100, the remaining activity was assayed at 70°C.

Fig. 6. Activity of *A. aeolicus* BE with amylopectin.

A reaction mixture (1 mL) including 0.4% (w/v) waxy corn starch gelatinized by heating and 1.6 U of *A. aeolicus* BE in 10 mM sodium phosphate buffer (pH 7.5) was incubated at 70°C. At the indicated reaction time, 0.2 mL of the mixture was removed, mixed with 7 μ L 1 N HCl to adjust the pH to around 5 and heated at 100°C for 10 min. Then, the mixture was filtrated using a 0.22 μ m membrane and subjected to HPLC-MALLS-RI. The change in weight-average molecular mass of the product compared to reaction time is shown. The molecular weight of the 0-h product was probably underestimated because the products could only be partially passed through the membrane, and only filtrate was loaded onto the column.

Fig. 7. Chain-length distribution of products formed from the activity of *A. aeolicus* BE with amylopectin.

The products of *A. aeolicus* BE from the experiment shown in Fig. 6 (0 or 20 h) were subjected to HPAEC after debranching with iso-amylase. The ratio of each peak area to the sum of the peak areas (DP 6–57) is shown. Symbols: black bar, 0 h; striped bar, 4 h; white bar, 20 h.

Table 1. Structural parameters of the product by *A. aeolicus* BE (P_{Aae}) and *B. stearothermophilus* BE (P_{Bst}) from amylopectin.

	P_{Aae}	P_{Bst}
CL_n^{*1}	16.2	15.5
DP_n^{*2}	940	1020
DP_w^{*3}	1200	1530
DP_w/DP_n	1.3	1.5
GA-resistant(%) ^{*4}	5.7	6.3
DP_w of GA-resistant	53	60

^{*1}Average chain length; CL_n =amount of total sugar residues/amount of non-reducing residues. ^{*2}Number-average degree of polymerization. ^{*3}Weight-average degree of polymerization. ^{*4}Content of glucoamylase-resistant component (% of total sugar residues as glucose).

50–60, while their reducing ends could not be detected (less than 1/1000 glucosyl residues), demonstrating that both P_{Aae} and P_{Bst} have a cyclic portion in their structures. Both P_{Aae} and P_{Bst} were highly soluble in water and their solutions were quite stable: no increase in turbidity was observed after 3-week storage at 4°C of 30% (w/v) solutions of P_{Aae} and P_{Bst} .

DISCUSSION

It has been demonstrated that the *B. stearothermophilus* BE can catalyze not only branching reaction but also cyclization reaction.^{1–3} When amylopectin is used as a substrate, the *B. stearothermophilus* BE mainly cyclizes the B2, B3 and B4-chains of amylopectin.^{2,4} This paper showed that the *A. aeolicus* BE also cyclized the B-chains of amylopectin. The structure of P_{Aae} was essentially the same as that of P_{Bst} . It has been considered that BE generally catalyzes cyclization reaction, but the ratio of cyclization reaction to branching reaction might vary depending on the sources of BE. Boyer *et al.*¹³ analyzed the action of maize BE isozyme I by gel-filtration analysis and

chain-length analysis. They observed that the enzyme action retarded the retention time of amylopectin although the change of molecular weight was not measured. In this reaction, the average chain-length of amylopectin was decreased from 25.2 to 9.8. Most recently, Kawabata *et al.*¹⁴ demonstrated that *Neurospora crassa* BE produced a highly-branched macromolecule from amylopectin. It is not clear whether the macromolecule has a cyclic portion or not. These results may suggest that the ratios of branching to cyclization by maize BE and fungus BE are higher than the ratios by the thermostable BEs. Detailed structural analyses and comparisons of products using various BEs are needed for further understanding the reaction specificity of BE.

The *A. aeolicus* BE was a highly thermostable enzyme with an optimum temperature of 75–80°C, and more than 90% of the activity was retained after 10-min incubation at 90°C. The enzyme is the most thermostable BE reported to date. Some enzymes (*e.g.*, α -amylase from *Bacillus licheniformis*) are known to be stabilized in the presence of a high concentration of substrates. Preliminary experiments indicated that the *A. aeolicus* BE efficiently works at 90°C and is active even at 100°C in the presence of 20% waxy corn starch. In addition to the thermostability, the higher expression level (0.1–0.2-g BE protein/L of culture broth) of the *A. aeolicus* BE gene in *E. coli* compared to that of the *B. stearothermophilus* BE gene (0.02–0.1-g BE protein/L) is also beneficial for applications. These properties suggest that the *A. aeolicus* BE is useful for the production of highly-branched cyclic dextrin.

It has been reported that conformations of some thermophilic enzymes expressed in mesophiles are different from those of the native enzymes prepared from original sources.^{15–19} It has also been demonstrated that heat treatment of the recombinant enzymes results in conformational changes to the same or close forms of the native

enzymes from original sources.¹⁵⁻¹⁹⁾ In such cases, specific activities and/or stability of the recombinant proteins were increased by the heat treatment. The *A. aeolicus* BE, produced as an insoluble form, was partially solubilized by incubation at 70°C for 30 min, and stability in the neutral and low pH regions was increased. No change in the molecular weight of BE was observed by SDS-PAGE after heat treatment (data not shown). The results suggest that the conformation of BE was changed by heat treatment as the cases described in references Nos. 15-19.

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超好熱性細菌 *Aquifex aeolicus* 由来の

ブランチングエンザイム：その性質と高度分岐環状デキストリン合成への応用

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超好熱性細菌 *Aquifex aeolicus* のゲノム全塩基配列が決定され、ブランチングエンザイム(BE, EC 2.4.1.18)と相同性を有するタンパクをコードする遺伝子が見つかった。その推定アミノ酸配列をもとにして遺伝子を化学合成し、大腸菌内で発現させた。その結果、組換えタンパク質のうち 95% 以上が活性のある不溶性凝集体として菌体内に蓄積された。この凝集体を 70°C で加熱することによって、30% 程度が可溶化された。不溶型および可溶型 BE の反応至適条件は双方とも 75°C, pH 7.5-8.0 であったが、安定性には違いがあり、不溶型は中性-酸性領域でやや不安定であった。本 BE はアミロペクチンのクラスター構造を連結する B 鎖を主に環状化し、高度分岐環状デキストリンを生産した。その重量平均重合度(DP_w)は 1200, 平均鎖長は 16 であり、環状構造部分の DP_w は約 50 であった。