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ORIGINAL PAPER

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A novel thermostable branching enzyme from an extremely thermophilic bacterial species, *Rhodothermus obamensis*

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Abstract A branching enzyme (EC 2.4.1.18) gene was isolated from an extremely thermophilic bacterium, *Rhodothermus obamensis*. The predicted protein encodes a polypeptide of 621 amino acids with a predicted molecular mass of 72 kDa. The deduced amino acid sequence shares 42–50% similarity to known bacterial branching enzyme sequences. Similar to the *Bacillus* branching enzymes, the predicted protein has a shorter N-terminal amino acid extension than that of the *Escherichia coli* branching enzyme. The deduced amino acid sequence does not appear to contain a signal sequence, suggesting that it is an intracellular enzyme. The *R. obamensis* branching enzyme was successfully expressed both in *E. coli* and a filamentous fungus, *Aspergillus oryzae*. The enzyme showed optimum catalytic activity at pH 6.0–6.5 and 65 °C. The enzyme was stable after 30 min at 80 °C and retained 50% of activity at 80 °C after 16 h. Branching activity of the enzyme was higher toward amylose than toward amylopectin. This is the first thermostable branching enzyme isolated from an extreme thermophile.

Introduction

Branching enzyme (BE; α-1,4-glucan: α-1,4-glucan 6-α-glucosyltransferase; EC 2.4.1.18) is one of the key enzymes in the biosynthesis of starch or glycogen (Okita et al. 1981). The enzyme produces α-1,6 branching linkages by a transglycosylation reaction of α-1,4-glucan. BE-encoding genes have been isolated and their enzymatic properties have been characterized in various organisms, from bacteria to mammals.

BE belongs either to the α-amylase superfamily (Jepersen et al. 1993) or to family 13 of the glycosyl hydrolase groups (Herissat 1991). Both families include such enzymes as α-amylase, α-glucosidase, cyclodextrin glucanotransferase, pullulanase, isoamylase and oligo α-1,6- glucosidase. These enzymes share four conserved catalytic regions (I, II, III, IV), which were also found in BE (Baba et al. 1991, Takata et al. 1992, Kuriki et al. 1996). The importance of several invariant residues has been analyzed. For example, a catalytic triad consisting of Asp405, Asp526 and Glu458 (*Escherichia coli* BE numbering) was confirmed by site-directed mutagenesis using *Bacillus stearothermophilus* BE and maize SBE II (starch branching enzyme II; Takata et al. 1994; Kuriki et al. 1996).

Intermolecular reaction by BE has been demonstrated using potato BE (Borovsky et al. 1979). Thus, the enzyme first cleaves α-1,4-glucosidic linkages of α-glucan and then transfers the chain fragments at non-reducing ends to other α-1,4-glucans to create α-1.6 branching linkages. Intramolecular reaction was reported using *Bacillus* BE (Takata et al. 1996a, b). Such intramolecular branching reactions produced cyclic glucans from amylose and amylopectin. Cyclic glucan products from waxy rice amylopectin by BE were uniform in their size, with the degree of polymerization being 900 (146,000 kDa; Takata et al. 1996b). They were highly soluble and gave a stable solution with a low viscosity, which is advantageous for food industries (Takata et al. 1997).

Due to the unique α-1,6-transglycosylation activity, BE might have the potential to create new applications in starch-related industry, where thermostable enzymes are highly desirable. To obtain a thermostable BE, we screened thermophilic bacteria and archaea for the homologue of the bacterial BE gene, *glgB*. In this report, we describe a novel thermostable BE from *Rhodothermus obamensis*, an extremely thermophilic bacterium isolated from a shallow hydrothermal vent, with an optimum growth temperature of 80 °C (Sako et al. 1996).

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We also report that heterologous expression of *R. obamensis* BE was achieved both in *E. coli* and a filamentous fungus *Aspergillus oryzae*.

Materials and methods

Bacterial strains

Thermophilic bacterial strains used for our PCR screening were *R. obamensis* JCM9785, *Sulfolobus solfataricus* JCM8930, *Thermotoga neopolitana* DSM4359, *Thermus filiformis* DSM4687 and *Bacillus thermodenitrificans* DSM465. *E. coli* strain K12 was used as the positive control for PCR screening. *E. coli* DH12S (Life Technologies) and TOP10 (Invitrogen) were used as cloning and BE expression strains, respectively.

PCR amplification of *glgB* genes

Bacterial genomic DNA was prepared as described by Murray and Thompson (1980). PCR amplification of *glgB* genes was carried out in a 50-µl PCR reaction mixture [0.1 ng genomic DNA/µl, 0.125 mM each dNTP, 2.5 mM $MgCl_2$, 2 pM each primer, 0.025 units *Taq* polymerase (Boehringer Roche Diagnostics)/µl with 1× reaction buffer]. Degenerate primers used here were designed using conserved amino acid sequences of known bacterial BEs (A–C in Fig. 1). The codons used were based on the *E. coli* codon usage. The reaction profile included one cycle of denaturation at 94 °C for 5 min, annealing at 50 °C for 45 s and extension at 72 °C for 1 min, followed by 30 cycles with denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 1 min. An amplified fragment with the expected size was gel-purified using the QIAquick gel extraction kit (Qiagen) and cloned into a pT7Blue (Novagen) vector. Inserted DNA fragments were sequenced by the dideoxy chain-terminating method, analyzed with the ABI Prism 310 genetic analyzer (PE Applied Biosystems). Deduced amino acid sequences of amplified fragments were aligned with previously reported BE amino acid sequences to examine whether those fragments encoded BE. Protein sequence alignment was performed with AlignX in the Vector NTI Suite (InfoMax), using the ClustalW method (Thompson et al. 1994).

Cloning of *R. obamensis glgB*

R. obamensis genomic libraries were constructed, using the pBluescript SK(–)(Stratagene) vector, from either single- or double-digested genomic DNA with various restriction enzymes. *E. coli* strain DH12S (Life Technologies) was used as a host strain of the libraries. Colony lift was performed using Hybond-N^+ (Amersham Pharmacia Biotech) membranes and then hybridized to a digoxigenin (DIG)-labeled pMSra8-insert probe prepared with a DIG-labeling kit (Boehringer Roche Diagnostics). Detection was performed with a DNA detection kit (Boehringer Roche Diagnostics), as described by the supplier. Two plasmids, pMSra10 and pMSra29, obtained from this screening covered the whole *R. obamensis glgB*. The sequence was used to design primers to generate BE expression vectors.

R. obamensis BE expression in *E. coli*

R. obamensis glgB was amplified by PCR from genomic DNA, using the following primers to introduce restriction enzyme sites at both ends: 5′-TTCC*TCATGA*GCTGGCTCACGGAAGAAGACA-3′ (italics: *Bsp*HI), and 5′-GTTTA*AAGCTT*TTCAGGACGGCTACC-3′ (italics: *Hin*dIII). After digesting the amplified fragment with the restriction enzymes, *Bsp*HI and *Hin*dIII, the fragment was ligated into a pBAD/*myc*-HisA vector (Invitrogen) linearized with *Nco*I and *Hin*dIII (pMSra33). The product was devoid of a *myc*-His tag, because the stop codon was placed upstream of the tag sequence. Plasmid pMSra33 was used for the transformation of *E. coli* expression strain, TOP10. BE production by the *E. coli* strain was carried out overnight in SM medium (containing, per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 5 g glucose, pH 7) with 100 µg ampicillin/ml at 28 °C. BE expression was induced with 0.02% arabinose for enzyme production. Cells were harvested by centrifugation and re-suspended in 20 mM sodium phosphate buffer (pH 6.0). Cell suspensions were sonicated on ice and debris was removed by centrifugation to obtain crude cell extracts.

R. obamensis BE expression in a filamentous fungus

A mutant strain derived from *A. oryzae* IFO4177, deficient in α-amylase and major protease activities, was used as a BE expression host. We constructed two expression vectors, either with or without the *c-myc* epitope (EQKLISEEDL) at the C-terminal of the *R. obamensis* BE. The plasmids possessed the *A. nidulans amdS* gene as a selection marker; and the *R. obamensis glgB* gene was fused to the *A. oryzae* α-amylase gene promoter (Christensen et al. 1988) of the modified version. Transformation of *A. oryzae* using the *amdS* selection marker was carried out as described by Christensen et al. 1988). For protein production, transformants were grown in shaking flasks at 34 °C for 3 days, using a medium containing maltose (per litter: 45 g maltose, 7 g yeast extract, 12 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 2 g K_2SO_4, 5 g urea, 1 g NaCl, 0.5 ml metal solution, pH 5.0). Cell extracts were prepared with the extraction buffer (50 mM potassium phosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mg pepstatin A/l, pH 6.0) by vigorous mixing with glass beads, followed by removal of debris by centrifugation.

Enzyme assays

Amylolytic activity of cell extracts was first evaluated by an amylopectin plate assay, where the plates included 0.2% amylopectin and 2% agar in 0.1 M potassium phosphate (pH 7.0). Samples (20 µl) were applied into wells on the plates and incubated at the indicated temperatures overnight. The plates were then stained with iodine solution freshly diluted from a 50× solution consisting of 20 g KI/l, 2 g I_2/l and 0.03 M HCl in water.

BE activity was measured either by an iodine-staining assay or by a branching linkage assay as described by Takeda et al. (1993) and Takata et al. (1994), with some modifications.

Iodine-staining assay

This assay monitors the decrease in the color of glucan-iodine complex detected by absorbence at 660 nm (A_{660}). A cell extract (50 µl) was mixed with the same volume of substrate solution consisting of 0.1% amylose type III (Sigma), dissolved in 100 mM Tris-HCl buffer (pH 7.0), unless otherwise noted. After 30 min incubation at the designated temperature, each reaction was terminated by adding 2 ml of freshly prepared iodine solution (0.01% I_2, 0.1% KI, 0.38% 1 N HCl in water). After 15 min incubation at room temperature for color stabilization, the A_{660} was measured. One unit of enzyme activity was defined as the activity that decreased A_{660} by 1%/min.

Branching linkage assay

The branching linkage assay estimates the formation of α-1,6-branching linkages by measuring liberated reducing ends after isoamylase treatment. Samples (90 µl) of 0.5% substrate solution (either amylose [type III from Sigma], amylopectin [waxy rice starch, Mothyl B from Shimada Chemicals, Japan], or glycogen [type II from Sigma] in 50 mM Tris-HCl, pH 7.2) and enzyme solution were mixed to make a 100-µl total mixture and were incubated at 60 °C for 15 min. The reaction was terminated by boiling

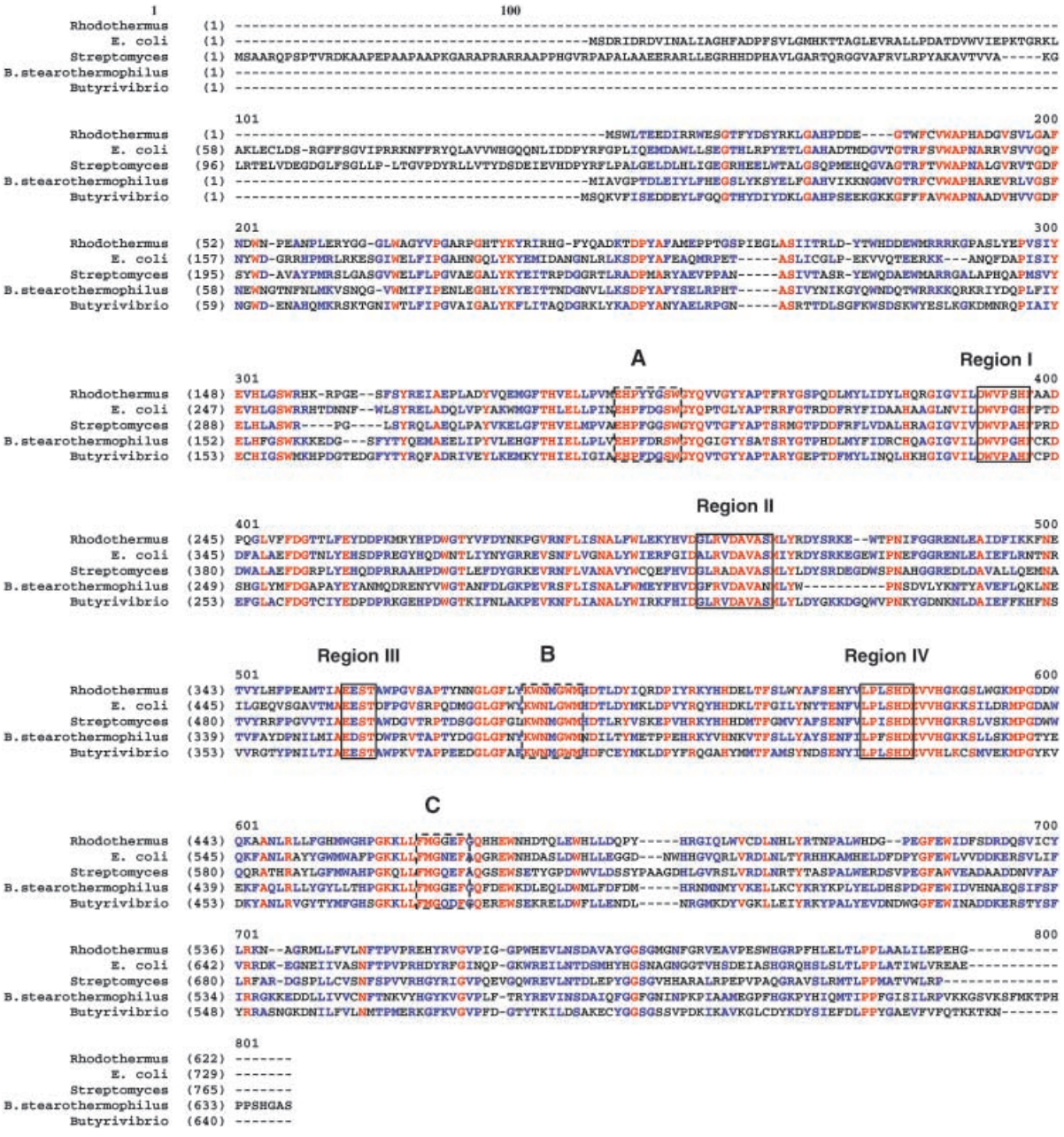

Fig. 1 Multiple alignment of bacterial branching enzymes (BEs). BE amino acid sequences from *Rhodothermus obamensis* (*Rhodothermus*), *Escherichia coli* (*E. coli*) *Streptomyces aureofaciens* (*Streptomyces*), *Bacillus stearothermophilus* (*B. stearothermophilus*), and *Butyrivibrio fibrisolvens* (*Butyrivibrio*) are aligned. Non-homologous and identical residues are indicated with *black and red letters*, respectively. Similar amino acid residues are shown with *blue letters*. *Four solid boxes* (*Regions I–IV*) indicate the conserved four regions of α-amylase family. Three other *boxes with broken lines* designate regions (*A–C*) of conserved amino acid sequences from which degenerate primers were designed

the mixture for 4 min. Subsequently, 10 µl of 1 M sodium acetate buffer (pH 4) and 3 µl of *Pseudomonas* isoamylase (1 mg/ml, Hayashibara Co.) were added to the reaction mixture, which was then incubated at 45 °C for 45 min. Reducing ends in the solution were measured by the modified 3,5-dinitrosalicylic acid method described by Luchsinger and Cornesky (1962). The sample solution was mixed with 0.4 ml of DNS solution (0.1 g 3,5-dinitrosalicylic acid, 20 ml 2 N NaOH, 6 g Rochelle salt, 10 ml of deionized water) and boiled for 5 min. After cooling the sample tubes with running water, 1.8 ml of water were added to the reaction mixture and the A_{525} was measured. To calculate BE activity, the amount of branches originally existing in the substrate was measured by the same procedure with water instead of BE solution and was subtracted from the amount of branches when BE was added. The amount of branch linkages was equivalent to the amount of reducing ends produced. One unit of enzyme activity was defined as the activity that formed 1 µmol branching linkage/min.

R. obamensis BE purification and electrophoresis

Endogenous *E. coli* enzymes were inactivated at 60 °C for 30 min and removed by centrifugation. The heat-treated lysates were dialyzed and concentrated by ultra-filtration. After passing the concentrated sample through a Macro-Prep ceramic hydroxyapatite (type I) column (Bio-Rad), the active fractions were further puri-

Table 1 List of amino acid sequences in bacterial branching enzymes (BEs)

Species	Accession number	Similarity (%) to *Rhodothermus obamensis* BE
Escherichia coli	P07762	46.7
Bacillus caldolyticus	P30537	44.6
Bacillus stearothermophilus	P30538	44.3
Bacillus subtilis	P39118	41.7
Synechococcus sp.	P16954	50.7
Synechocystis sp.	P52981	48.0
Butyrivibrio fibrisolvens	P30539	43.2
Agrobacterium tumefaciens	P52979	44.6
Streptomyces aureofaciens	P52980	46.4
Haemophilus influenzae	P45177	43.6
Mycobacterium tuberculosis	Q10625	49.0

fied in a Super-Q Toyopearl column (Tosoh, Japan) with a linear gradient of 0–0.6 M NaCl in 50 mM potassium phosphate buffer (pH 7.4). The purity of recovered BE was approximately 80% of the total protein remaining in the final product, but it was free from amylolytic activities other than that from *R. obamensis* BE. SDS-PAGE analysis was carried out with 7.5% acrylamide gels, stained with Coomassie brilliant blue. Isoelectric focusing (IEF) analysis was performed using Ampholine polyacrylamide gel plates (pH 3.5–9.5) with Multiphor II (Amersham Pharmacia Biotech). Active staining was performed by overlaying 2% agar solution containing 0.1% amylose type III in 50 mM Tris-HCl buffer (pH 7), followed by incubation at 40 °C overnight. Enzyme activity was visualized by exposure to iodine vapor.

Nucleotide sequence accession number

The nucleotide sequence of *R. obamensis glgB* has been registered in GenBank/EMBL/DDBJ under accession number AB060080.

Results

Cloning of *R. obamensis glgB* gene

To design degenerate primers to screen novel *glgB* genes, we used conserved regions found in the alignment of BE amino acid sequences from bacterial species listed in Table 1. Eleven primers each for forward and reverse sequences were used for PCR screening with various primer combinations. Among them, two combinations: primer A-I (5′-GAGCACCCCYTCGACGGCAGTTGG-3′) with B-I (5′-CATCCAICCWAKRTTCCA-3′), and primer B-II (5′-AARTGGAAYATGGGTTGGATG-3′) with C-II (5′-RAAYTCTTTTCCCATR AA-3′) successfully amplified DNA fragments from *R. obamensis* of the expected sizes, as produced by the control strain of *E. coli*. However, other thermophiles described in Materials and methods did not show any expected bands in this PCR screening. The 0.6-kb amplicon from *R. obamensis* genomic DNA obtained using primers A-I and B-I was cloned into a plasmid, producing pMSra8. Since the deduced amino acid sequence of the pMSra8 insert shared a high similarity with other BEs in Table 1 with a range of 55–64%, we concluded that this DNA fragment most likely encoded partial *R. obamensis glgB* and we used it as a probe to screen several *R. obamensis* genomic libraries by colony hybridization. The whole *R. obamensis glgB* encoding sequence was obtained in two plasmids, pMSra 10 and pMSra29, that carried a 3.5-kb *Bam*HI fragment and a 1.3-kb *Kpn*I–*Sal*I fragment, respectively.

Analysis of *R. obamensis glgB*

The *R. obamensis glgB* (GenBank accession no. AB060080) included an ORF consisting of 1,866 bp, corresponding to 621 amino acids and producing a protein with a predicted size of 72 kDa. The inferred amino acid sequence lacked a signal peptide, suggesting this enzyme is intracellular. The size of *R. obamensis* BE was similar to BEs from *Bacillus* strains and *Butyrivibrio fibrisolvens*, because there were approximately 100 amino acids of N-terminal truncation, compared with the other BEs from bacterial species in Table 1, such as *E. coli* (Fig. 1). The whole *R. obamensis* BE amino acid sequence had high levels of similarity to reported bacterial BEs, in the range of 41–51% sequence identity (Table 1). Figure 1 shows multiple alignments of amino acid sequences of BEs from *R. obamensis* and other bacterial species. The BE shares four highly conserved sequences not only with other prokaryotes but also with eukaryotes, although the degree of similarity to eukaryotes is lower than that to prokaryotes (Table 2). These include residues reported to play important roles in the catalytic activity and substrate-binding activity of BE (Arg303 in Region II, and His240, His423 in regions I and IV, respectively, using *R. obamensis* BE numbering; Table 2; Funane et al. 1998; Libessart et al. 1998). *R. obamensis* BE also possesses perfectly conserved catalytic triad amino acid sequences (Takata et al. 1994; Kuriki et al. 1996) at Asp305, Asp424 and Glu357 (Table 2). These data suggest that *R. obamensis glgB* encodes functional BE.

R. obamensis glgB expression in *E. coli*

Expression plasmid pMSra33 was constructed under an arabinose-inducible *ara*BAD promoter. SDS-PAGE observation indicated that a concentration of more than 0.0002% arabinose was necessary to produce a recogniz-

Table 2 Sequences of various BEs around the conserved four regions of the α-amylase family. Amino acid residues consisting of a catalytic triad are indicated with italics. Accession numbers of eukaryotic BE sequences are as follows: *Saccharomyces cerevisiae* P32775, Human Q04446, maize SBEII Q08047, *Aspergillus nidulans* AB026630

Category	Origin	Region I	Region II	Region III	Region IV
Bacterial BE	*R. obamensis*	235 DWVPSH	301 GLRV*D*AVAS	357 *E*EST	419 LPLSH*D*
	E. coli	335 DWVPGH	401 ALRV*D*AVAS	458 *E*EST	521 LPLSH*D*
	B. stearothermophilus	239 DWVPGH	305 GFRV*D*AVAN	352 *E*DST	415 LPFSH*D*
	Synechococcus sp.	369 DWVPGH	435 GIRV*D*AVAS	492 *E*EST	555 LALSH*D*
	M. tuberculosis	341 DWVPAH	407 GLRV*D*AVAS	464 *E*EST	527 LPLSH*D*
Eukaryotic BE	*Saccharomyces cerevisiae*	283 DVVHSH	352 GFRF*D*GVTS	417 *E*DVS	481 YCESH*D*
	A. nidulans	271 DVVHSH	338 GFRF*D*GVTS	397 *E*DVS	461 YAESH*D*
	Human	286 DVVHSH	353 GFRF*D*GVTS	412 *E*DVS	476 YAESH*D*
	Maize SBEII	376 DVVHSH	443 GFRF*D*GVTS	502 *E*DVS	565 YAESH*D*

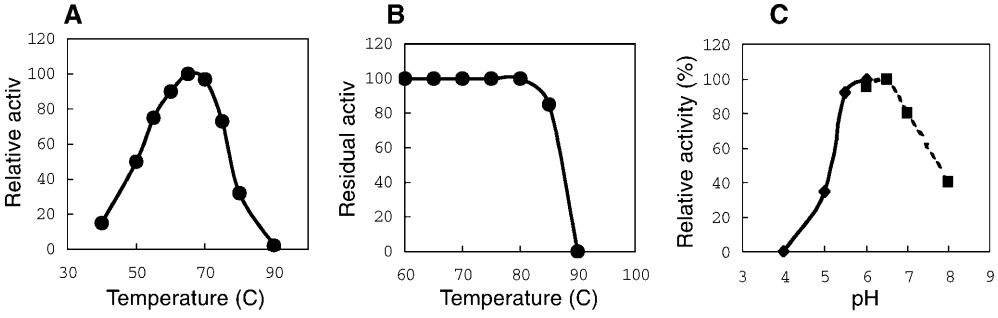

Fig. 2A–C Characterization of *R. obamensis* BE. The iodine-staining assay was employed for all the following experiments. **A** To assess the optimum temperature, samples were incubated with a substrate at the indicated temperature at pH 7.0. **B** Thermal stability was evaluated by incubating samples for 30 min at the indicated temperature; and the BE reaction was then carried out at 60 °C, pH 7.0. **C** Optimum pH was assessed by incubating samples with a substrate at 60 °C at the indicated pH. A *solid line* and a *broken line* indicate pH ranges where 0.5 M sodium citrate buffer and 0.5 M potassium phosphate buffer were used, respectively

able amount of BE (data not shown). Since *E. coli* crude extracts included *E. coli*-derived amylolytic activities that could mask *R. obamensis* BE activity, we needed to remove all endogenous amylolytic activity including *E. coli* BE. First, we used SDS-PAGE to check how much the endogenous protein bands were reduced by treating *E. coli* lysates at various temperatures for 20 min, with subsequent centrifuging to remove debris. This observation suggested that heating at 60 °C or higher for 20 min significantly reduced endogenous proteins, while *R. obamensis* BE remained in the supernatant (data not shown). Second, we tried to visualize the remaining endogenous amylolytic activity of *E. coli* by an iodine plate assay and an iodine-staining assay. Both assays indicated the complete loss of *E. coli*-derived amylolytic activity after 20 min of pre-treatment at 60 °C (data not shown). This suggested that the 60 °C/20 min pre-treatment was sufficient to inactivate all endogenous amylolytic activity of *E. coli*.

Characterization of *R. obamensis* BE expressed in *E. coli*

Heat-treated *E. coli* lysate was subjected to further purification as described in Materials and methods. Resulting active fractions did not give a single band in SDS-PAGE analysis but showed a couple more extra bands (lane 2 in Fig. 3). However, IEF analysis showed a single band by iodine vapor-staining. This confirmed that the remaining extra protein in the sample did not possess amylolytic activity. Given this, the final sample was used for BE characterization.

According to the iodine-staining assay, the optimum temperature of *R. obamensis* BE activity was 65 °C (Fig. 2A), which was also confirmed by the branching linkage assay (data not shown). The enzyme was fully stable after 30 min incubation at 80 °C and 80% of its activity was preserved when incubation was at 85 °C (Fig. 2B). After longer incubation (16 h at 80 °C), half of the enzyme activity remained (data not shown). The optimum pH of the enzyme was around pH 6 (Fig. 2C). Concentration of *R. obamensis* BE in the sample was estimated as 0.21 mg/ml, based on the intensity of the protein band on SDS-PAGE analysis (Fig. 3). Therefore, the specific activity of *R. obamensis* BE by iodine-staining assay at 60 °C and pH 7.0 was calculated as approximately 50,000 units/mg enzyme protein.

The branching linkage assay verified the formation of α-1,6-branch linkages by confirming the reduced power of BE products appearing after isoamylase treatment. The branching linkage of *R. obamensis* BE which gave 10,000 units by the iodine-staining assay were 7.0 units and 1.2 units, respectively, toward amylose and amylo-

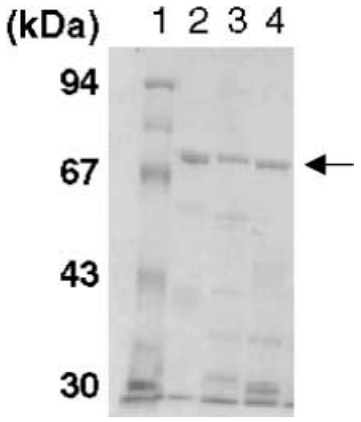

Fig. 3 SDS-PAGE gel analysis of *R. obamensis* expression. All lysates were incubated at 60 °C for 30 min. *Lane 1* Size marker, *lane 2 E. coli* lysates after column purification (as described in Materials and methods), *lane 3 Aspergillus oryzae* lysates (BE with the *c-myc* tag), *lane 4 A. oryzae* lysate (BE without the *c-myc* tag). An *arrow* indicates bands of *R. obamensis* BE

pectin. These results indicated that *R. obamensis* BE had a 6-fold higher specific activity toward amylose than toward amylopectin. Glycogen was also tested as a substrate, but the enzyme produced hardly any extra branching linkages.

R. obamensis BE expression in *A. oryzae*

R. obamensis BE was expressed in *A. oryzae* with or without the *c-myc* epitope tag. The proportion of expressed BE in the lysates after heat treatment was more than half of the total soluble proteins (Fig. 3). *R. obamensis* BE production in *A. oryzae* turned out to be 100 times more (for the same amount of medium) than that in *E. coli* as a host. Those samples were evaluated by iodine-staining assay. The enzyme expressed in *A. oryzae* showed the same temperature optimum, thermostability and pH optimum as the one expressed in *E. coli*. The presence of the *c-myc* tag did not alter those characters (data not shown).

Discussion

In this report, we describe the cloning, heterologous expression and characterization of a gene, *glgB*, from an extremely thermophilic bacterium, *R. obamensis*. The gene encodes a BE with the optimum temperature of 65 °C and with more than 80% stability after 30 min incubation at 85 °C. Since *Bacillus stearothermophilus* BE, the most thermostable BE previously reported (Kiel et al. 1991; Takata et al. 1994), has its optimum at 53 °C and 30 min incubation at 60 °C leaves around 80% stability, the *R. obamensis* BE is one of the most thermostable BE ever reported. *R. obamensis* BE was successfully expressed in both *E. coli* and *A. oryzae*. Differences in the host strains did not change the BE activity profiles.

Thermostable enzymes are often desirable in the starch industry, because the gelatinization temperature of starch is 60–75 °C. Since thermophilic microorganisms are usually good sources of thermostable enzymes, we had tried to obtain BEs from extremophiles such as *Sulfolobus* and *Thermotoga*. Our degenerate primers did not amplify their *glgB* homologues, probably due to their codon usage being different from that of *E. coli*, which has a preference for G and C at the third position of each codon. In contrast, codons of *Sulfolobus* and *Thermotoga* have a bias towards A and T at the third position. Considering that the whole *Sulfolobus* genome has already been sequenced, it is also suggested that the archaea may not even possess a *glgB* gene homologue.

During *R. obamensis glgB* amplification to construct an *E. coli* expression plasmid, we accidentally obtained a *glgB* variant resulting from two amino acid replacements, at positions 397 (Tyr to Cys) and 419 (Leu to Pro) due to PCR errors. Interestingly, these amino acid substitutions reduced the BE thermostability. The variant was stable up to only 70 °C after 30 min incubation, whereas the wild-type *R. obamensis* BE was fully stable at 80 °C after 30 min incubation. The observation indicated that either or both Try397 and Leu419 contributed to the thermostability of this enzyme. Recent work suggested that the protein thermal stability was accompanied by an increase in hydrogen bonds or salt bridges, or by better packing in buried regions (Scandurra et al. 1998). Decreased thermostability in the BE variant might be caused, for example, by a potential loss of hydrogen bonding with Tyr397, or a large conformational change caused by these amino acid substitutions. Substitution of Leu419 might also have altered protein structures around an adjacent putative catalytic residue Asp424.

Substrate specificity of BEs varies among different BEs. For example, one of the maize branching enzymes (SBEI) prefers amylose, while its isozyme SBEII showed much higher activity towards amylopectin than amylose (Guan et al. 1997). *R. obamensis* BE showed six times higher specificity towards amylose than amylopectin, which is similar to *E. coli* BE, which also prefers amylose to amylopectin (4-fold increase in activity; Guan et al. 1997). It may be interesting to examine whether other bacterial BEs also have a substrate preference similar to that of *R. obamensis* BE and *E. coli* BE.

Hilden et al. (2000) showed that bacterial BEs were divided into two groups in their phylogenetic tree, based on amino acid sequences. Group 1 includes BEs from *Agrobacterium tumefaciens*, *E. coli*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, *Streptomyces aureofaciens*, *Synechococcus* sp. and *Synechocystis* sp., while group 2 includes three *Bacillus* strains and *Butyrivibrio fibrisolvens*. The authors found that all the BE amino acid sequences in group 1 turned out to include a N-terminal extension, while all those in group 2 did not. We also generated a phylogenetic tree of bacterial BEs including *R. obamensis* BE, based on their amino acid sequences. Interestingly, *R. obamensis* BE shares higher sequence similarity to group 1 BEs, even though it lacks the N-ter-

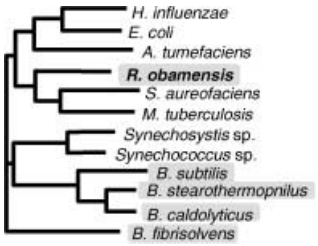

Fig. 4 A phylogenetic tree of bacterial BEs. BE amino acid sequences were obtained from the NCBI protein database (http://www.ncbi.nlm.nih.gov) and aligned using ClustalW (Thompson et al. 1994). The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987), using the AlignX program in Vector NTI. Bacterial species possessing BE without the N-terminal extension are indicated in *shadowed boxes*. The accession numbers of the BE sequences are indicated in Table 1

minal extension (Fig. 4). Considering that a 16S rRNA gene tree showed similar topology to this BE tree (data not shown), the evolutionary origins of those bacterial strains might not necessarily reflect the presence/absence of the N-terminal extension. The function of the N-terminal extension is not known yet.

It is known that some bacterial enzymes, such as *E. coli* β-glucuronidase (Roberts et al. 1989) or *Erwinia* pectate lyase (Bartling et al. 1996) are expressed in filamentous fungi. However, expression levels are not high enough for use in industrial applications (2 mg/l for *Erwinia* pectate lyase). We achieved high levels of bacterial BE expression using *A. oryzae*, which were significantly higher than using *E. coli*. Together with good thermostability, which is beneficial for simple recovery by heat treatment, high expression using a fungal host would make *R. obamensis* BE feasible for industrial production and other applications.

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