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Properties of the Glucan Branching Enzyme of the Hyperthermophilic Bacterium *Aquifex aeolicus*

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Glucan branching enzymes are responsible for the synthesis of α(1→6) glycosidic bonds in glycogen and amylopectin. The glucan branching enzyme of the hyperthermophile *Aquifex aeolicus* is the most thermo-active and thermostable glucan branching enzyme described. The gene encoding this glucan branching enzyme was overexpressed in *E. coli* and purified using γ-cyclodextrin affinity chromatography. Subsequently, the enzyme was subjected to a biochemical characterization. The optimum temperature for activity was 80°C, and the enzyme was stable up to 90°C. Its thermostability may be explained by the relatively high number of aromatic amino acid residues present, in combination with a relatively low number of glutamine/asparagine residues. The Km for amylose was 4 μM and the Vmax was 4.9 U/mg of protein (at optimal pH and temperature). The side-chain distribution of the branched glucan formed from amylose was determined.

Keywords: Glucan branching enzyme; Starch; *Aquifex aeolicus*; Thermostability; Glycoside hydrolase; α-amylase superfamily

INTRODUCTION

A large variety of microorganisms use glycogen, a polysaccharide composed of an α(1→4) linked glucan polymer with branches via α(1→6)glycosidic linkages, as a storage compound (François and Parrou, 2001; Preiss, 1984). It structurally resembles plant amylopectin, one of two glucan polymers found in starch (Myers *et al*., 2000). The major difference between glycogen (8–9%) and amylopectin (3.5%) is the degree of α(1→6) branching. Also the average length of the side-chains differs, usually 10–12 glucose residues for glycogen versus 20–23 glucose residues for amylopectin (Marshall, 1974; Robyt, 1998; Sandhyarani *et al*., 1992). Glycogen is synthesized from ADP-glucose by the action of an ADP-glucose specific glycogen synthase forming a linear α(1→4) glucan, and by branching enzyme (1,4α-D-glucan:1,4-α-D-glucan 6α-D(1,4-α-D-glucano)-transferase; E.C. 2.4.1.18) introducing α(1→6) linkages (Preiss, 1984). Similar enzymes also act in starch biosynthesis in plants, in which multiple forms of the enzymes are usually present (Myers *et al.*, 2000).

Currently, four microbial glucan branching enzymes active at higher temperatures have been described. The *Bacillus stearothermophilus* glucan branching enzyme, active at 55°C, has been studied in most detail (Kiel *et al*., 1991; Takata *et al*., 1994). The *Rhodothermus obamensis* glucan branching enzyme is optimally active at 65°C, and stable up to 80°C (Shinohara *et al*., 2001). Gruyer *et al*. (2002) reported evidence for the presence of a branching enzyme active at 80°C in the hyperthermophilic archaeon *Thermococcus hydrothermalis*. However, no characteristics of this enzyme have been reported. Recently, Takata *et al*. (2003) overexpressed the *A. aeolicus* glucan branching enzyme in *E. coli* and reported some of its basic properties. They found that most of the enzyme was present in inclusion bodies. The enzyme was partially purified by isolating these inclusion bodies using centrifugation, followed by a partial solubilization using a heat treatment. It was shown that the enzyme is active at 80°C, making it the most thermo-active glucan branching enzyme known.

We have cloned and overexpressed the Aq722 gene in *E. coli* and subsequently purified the enzyme to homogeneity from heat-treated cell extracts using γ-cyclodextrin affinity chromatography.

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In this paper we describe a number of biochemical characteristics of the pure enzyme. Also the side-chain composition of the branched glucan formed from amylose is described.

MATERIALS AND METHODS

The Aq722 gene of *A. aeolicus* was cloned in the expression vector pET3b (Novagen) resulting in pVOA01. *E. coli* strains TOP10 (Invitrogen), which was used for cloning purposes, and BL21-CodonPlus(DE3)-RP (Stratagene), which was used for enzyme production, were grown in Luria Bertani medium (Sambrook *et al*., 1989) supplemented with ampicillin (100 μg ml^{-1}) and/or chloramphenicol (30 μg ml^{-1}), when appropriate. Arabinose (0.2% w/v final concentration) was added to the Luria Bertani medium to induce gene expression. Genomic DNA of *A. aeolicus* was a generous gift of Dr. R. Huber (University of Regensburg, Regensburg, Germany). Restriction endonucleases, *Pwo* DNA polymerase, and T4 ligase were purchased from Boehringer Mannheim and used as recommended by the manufacturer. General DNA manipulations were done as described by Sambrook *et al*. (1989).

PCR and Cloning

PCR to amplify Aq722 was done as follows: *A. aeolicus* chromosomal DNA (200 ng) was used in a 25 μl reaction in the presence of 1 μM forward primer with an *Nde*I restriction recognition site (5′-GGGATTCCATATGA AG AAGTTCAGTCTCATCAGTGATTAC-3; the *Nde*I site is underlined), 1 μM reverse primer with a *Bgl*II restriction recognition site (5′-GGAAGATCTTCATCCTTCGTGCTTTAA ATAGATCACGG-3′; the *Bgl*II site is underlined), 400 μM dNTP, and 1.25 units of *Pwo* DNA polymerase. The first step in the PCR reaction was denaturation at 95°C for 5 min; this was followed by 35 cycles of 30 s at 95°C, 15 s at 55°C, and 150 s at 72°C. The final step was incubation at 72°C for 7 min. After ethanol precipitation, the PCR product was digested with *Nde*I and *Bgl*II, purified from a 1% agarose gel using the Qiagen gel extraction kit according to the manufacturer's instructions, and ligated into an *Nde*I/*Bgl*II digested pET3b vector with T4 DNA ligase. This ligation product was transformed to *E. coli* TOP10 cells. A number of transformants were subjected to restriction analysis to identify a clone with an insert of the correct size (pVOA01). Subsequently, pVOA01 was transformed to *E. coli* BL21-CodonPlus(DE3)-RP cells (Stratagene) for overexpression. Nucleotide sequencing was done by GATC (Germany). Site-directed mutants were made using the Quickchange XL site-directed mutagenesis kit of Stratagene.

Enzyme Purification

Recombinant *A. aeolicus* glucan branching enzyme was produced during overnight growth of *E. coli* BL21-CodonPlus(DE3)-RP cells with plasmid pVOA01 at 37°C. Cells were harvested by centrifugation (10,000 × *g* for 15 min), resuspended in lysis buffer consisting of 20 mM triethanolamine (pH 8.5), 100 mM KCl, 5 mM DTT, and 1% Triton X-100 and subsequently subjected to a heat treatment by incubation at 85°C for 45 min. This cell suspension was cooled down to 37°C, a few crystals of bovine *DNase* I (Roche) were added, and the mixture was incubated for 10 min at 37°C. Cell debris and denatured proteins were removed by centrifugation (25,000 × *g* for 30 min) and the supernatant was used for enzyme purification.

Epoxy-activated Sepharose (Amersham-Pharmacia) (15 g) was resuspended in 200 ml ultra-pure water. The swollen Sepharose (approximately 50 ml) was washed with 3 L ultra-pure water, subsequently with 600 ml NaOH (0.1 N), and collected by filtration. The Sepharose was resuspended in 100 ml NaOH (0.1 N) containing 20 g of γ-cyclodextrin. Covalent coupling of the γ-cyclodextrin to the activated Sepharose took place during gentle shaking at 45°C for 20 h. Subsequently, the γ-cyclodextrin Sepharose was washed according to the manufacturer's protocol. Approximately 20 ml Sepharose was poured into a column and used for enzyme purification.

Lysate of heat-treated *E. coli* cells expressing pVOA01 was applied to the γ-cyclodextrin Sepharose column and washed with 25 mM potassium phosphate (pH 7.6) containing 1 mM DTT (buffer A). The enzyme was eluted with buffer A containing 50 mM γ-CD. The estimated size of the protein produced from Aq722 is 74 kDa. Fractions containing a prominent 70–75 kDa protein band as determined by SDS-PAGE, were pooled and concentrated using a centriprep column (Amicon) according to the manufacturer's protocol. The concentrated enzyme was dialyzed five times (for 8–16 h) against fresh buffer A and once against buffer A containing 20% glycerol to remove γ-CD which might inhibit the enzyme activity. Purified enzyme was frozen in liquid nitrogen and stored at −80°C until needed.

SDS-PAGE was carried out as described by Sambrook *et al*. (1989). N-terminal amino acid sequencing was done as described previously (Kralj *et al*., 2002).

Enzyme Activity Assays

Two methods were used for measuring branching enzyme activity.

Iodine staining method

As described by Takata *et al*. (1994), using amylopectin as a substrate, which is a more qualitative estimation of the enzyme's activity. An iodine–iodide solution forms a complex with the hydrophobic interior of $\alpha(1 \rightarrow 4)$ linked glucan chains, as present in amylose or in amylopectin. On average the side-chains of amylopectin are 20–23 glucose residues in length giving a red colour when stained with an iodine–iodide solution. Due to the action of glucan branching enzyme, the amylopectin side-chains become shorter and the colour gradually disappears. By following the change in absorbance at 660 nm of an assay mixture containing amylopectin, a iodine–iodide solution, and an enzyme sample the branching enzyme activity can be estimated.

Incubation of glucan branching enzyme with amylose

And the subsequent debranching of the produced, branched glucan with isoamylase followed by a modified Park-Johnson method to quantitatively measure the number of reducing ends formed (Takata *et al*., 1994). For this activity assay, amylose V (AVEBE, Foxhol, The Netherlands) was used as a substrate. Removal of amylopectin still present in amylose V, and reduction of the amylose to obtain a zero background, were done as described by Takeda *et al*. (1993). The average degree of polymerization of the amylose was 1395, with less than 1% amylopectin, as determined by gel-permeation chromatography coupled to combined multi-angle laser light scattering/refractometric index detectors.

Substrate for activity assays was prepared by dissolving treated amylose (15 mg) in 1 N NaOH (200 µl), followed by the addition of 500 µl demineralized water and 100 µl potassium phosphate buffer (250 mM, pH 7.5). The pH was adjusted to 7.5 with approximately 200 µl HCl (1 N). The substrate solution was pre-incubated for 10 min at 80°C and the reaction was started by the addition of 10 µl of an appropriate dilution of the purified *A. aeolicus* enzyme. Every 2 min (up to 12 min) a sample of 30 µl was taken and put directly in boiling water for 2 min to inactivate the enzyme. Subsequently, 3 µl acetate buffer (1 M, pH 3.9) and 0.175 U isoamylase (Megazyme) were added to 20 µl of the sample. The mixture was incubated for 30 min at 40°C, resulting in the removal of all $\alpha(1 \rightarrow 6)$ branches introduced by the branching enzyme. Debranched glucans were precipitated with 100 µl ice-cold ethanol and recovered by centrifugation for 5 min at 16,100 × *g*. The pellet was heated briefly at 80°C and subsequently it was dissolved in 2.5 µl NaOH (1 N). Finally, 77 µl ultra-pure water was added and this solution was analysed.

The reducing power of the saccharides present was measured by the modified Park-Johnson method (Takeda *et al.*, 1993). 40 µl potassium ferricyanide (0.1% w/v) and 40 µl Na_2CO_3 (0.48% w/v)/$NaHCO_3$ (0.065% w/v)/KCN (0.065% w/v) were added to the solution. The mixture was heated at 100°C for 15 min, cooled to room temperature, and 0.3% (w/v) ferric ammonium sulfate in 50 mM H_2SO_4 (200 µl) was added. After 20 min incubation at room temperature the absorbance at 715 nm was measured. Maltose was used as a standard. One unit of enzyme activity is defined as the amount of branching enzyme that produces 1 µmol of $\alpha(1 \rightarrow 6)$ linkages per min.

For activity determination after SDS-PAGE, 0.2% (w/v) amylopectin was added as a substrate to the running gel. After electrophoresis the amylopectin containing gel was washed three times for 5 min with 30 ml of ultra-pure water to remove the SDS and to allow renaturation of the branching enzyme protein. The gel was then washed once with 30 ml potassium phosphate buffer (25 mM; pH 7.5). The buffer was removed by washing with ultra-pure water and the gel was incubated for 1 h at 80°C. The gel was stained with an iodine–iodide solution, which was made freshly from 0.2 ml of stock solution (0.26 g of I_2 and 2.6 g KI in 10 ml of water) mixed with 0.2 ml of 1 N HCl and diluted to 50 ml (Takata *et al*., 1994). As stated above, the staining with iodine is based on formation of a complex between the iodine with the $\alpha(1 \rightarrow 4)$ linked glucan chains present in the amylopectin giving the SDS-PAGE gel a dark red colour. Due to branching enzyme activity the length of these chains is reduced resulting in a reduced staining intensity.

For determination of the temperature optimum, purified enzyme was incubated with reduced amylose V in a phosphate buffer (25 mM potassium phosphate, pH 7.6) at temperatures between 50ºC and 100ºC. To determine the pH at which the enzyme is most active, purified enzyme was incubated with reduced amylose V in a phosphate buffer (25 mM, pH 6.5–8.5) at 80ºC. The enzyme activity was measured by sampling the incubation mixture and determining the number of $\alpha(1 \rightarrow 6)$ glycosidic linkages produced as described above. Temperature stability was determined by pre-incubating purified enzyme in a potassium phosphate buffer (25 mM, pH 7.5) at the appropriate temperature for 2 h and subsequently determining the residual enzyme activity as described above.

Determination of the Side-Chain Composition

To determine the side-chain distribution of the products made, treated amylose V was incubated with enzyme as follows: 25 mg of amylose (80% dry

weight) was dissolved in 600 µl NaOH (1N); then 2.6 ml demineralized water and 200 µl MOPS (0.5 M; pH 7.5) were added. The pH was adjusted to 7.5 with approximately 600 µl HCl (1N). This gave an amylose solution with a final concentration of 5 mg/ml. Amylose (190 µl) was pre-incubated for 10 min at 80°C. The reaction was started by the addition of 62.5 µg enzyme solution (4.8 U/mg of protein). The assay mixtures were incubated up to 320 min and the reaction was stopped by boiling for 2 min. After cooling to room temperature, 20 µl acetic acid (1M; pH 3.5) and 5 µl buffer or 0.875 U isoamylase (Megazyme) was added followed by incubation for 1 h at 40°C. The isoamylase treatment was stopped by boiling for 2 min. Subsequently, the side-chain composition was determined by analysis of a sample of the isoamylase treated material on a high performance anion exchange chromatography system with a pulsed amperometric detection system (HPAEC-PAD). To calculate the mass percentage of a specific oligosaccharide up to DP32, the area of each oligosaccharide was multiplied by the corresponding detector response factor.

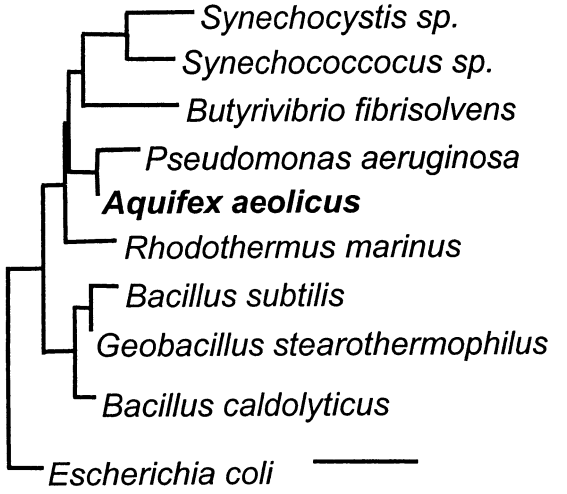

FIGURE 1 Dendrogram showing the phylogenetic relationships between various bacterial glucan branching enzymes. The tree is based on an amino acid alignment, excluding the N-terminal stretch of 100 amino acids (when present). The horizontal bar indicates 10% sequence distance. Sequences were retrieved from the NCBI database and had the following accession numbers: *R. obamensis*, D247652; *E. coli*, M13751; *P. aeruginosa*, AE004642; *B. fibrisolvens*, M64980; *B. stearothermophilus*, D87026; *B. caldolyticus*, Z14057; *B. subtilis*, Z99119. Alignment and dendrogram constructions were according to van Hijum *et al*. (2002).

RESULTS AND DISCUSSION

Overexpression of Aq722 in *E. coli* and Purification of the Glucan Branching Enzyme

The *A. aeolicus* genome contains an open reading frame, Aq722 of 1890 nucleotides, encoding a protein of 630 amino acids with an estimated molecular weight of 74,167 Da and an isoelectric point of 6.18. Takata *et al*. (2003) confirmed that Aq722 encodes a glucan branching enzyme. Aq722 is part of a glycogen biosynthesis operon, located between the *glgA* and *malM* genes, encoding respectively a putative glycogen synthase and an amylomaltase enzyme. Glucan branching enzymes are built up of a number of domains (Jespersen *et al*., 1991): the A-domain containing the catalytic residues, a C-terminal domain, and in most cases one or two N-terminal domains. Bacterial branching enzymes can be divided into two groups. The first group includes only bacterial enzymes, e.g. from *E. coli*, *Haemophilus influenzae*, *Agrobacterium tumefaciens*, *Streptomyces coelicolor*, *S. aureofaciens*, *Synechococcus* sp. and *Synechocystis* sp. (Hilden *et al*., 2000). These all have an additional stretch of amino acids at the N-terminus. A number of plant branching enzymes also have this N-terminal stretch. However, phylogenetically the plant branching enzymes form a separate cluster together with the enzymes from other eukaryotes such as *Saccharomyces cerevisiae* and humans. The second group is composed of branching enzymes lacking such an N-terminal stretch and includes enzymes from *Butyrivibrio fibrisolvens, Bacillus stearothermophilus*, *B. subtilis* and *B. caldolyticus*. The glucan branching enzyme from *A. aeolicus* also lacks this N-terminal amino acid stretch and thus resembles the *Bacillus* enzymes. However, phylogenetically it is most closely related to the glucan branching enzyme from *P. aeruginosa*, which has an N-terminal stretch (Fig. 1).

We amplified Aq722 directly from genomic DNA of *A. aeolicus* using PCR and the proofreading *Pwo* DNA polymerase. The PCR product was cloned into the *Nde*I/*Bgl*II site of the expression vector pET3b, yielding plasmid pVOA01. The nucleotide sequence of the cloned gene was identical to the gene sequence that was published by Deckert *et al*. (1998), indicating that no mistakes had been introduced by the PCR step. When studying the Aq722 gene sequence it turned out that there is a very strong bias for AGA or AGG as codons for arginine. Of the 29 arginine codons, 28 are AGA or AGG. There is also a bias in the proline codon usage: 12 out of the 30 codons present are CCC. Therefore *E. coli* BL21-CodonPlus (DE3)-RP strain was used for overproduction of the cloned Aq722 PCR product. This *E. coli* strain provides extra copies of the *argU* and *proL* genes encoding tRNA molecules that recognize the arginine codons AGA and AGG and the proline codon CCC, respectively (Instruction manual Stratagene).

SDS-PAGE of *E. coli* cells expressing the Aq722 gene showed that an extra protein of 71 kDa was produced, compared to *E. coli* cells carrying only the vector (data not shown). The size of 71 kDa of this band is close to the predicted size of 74,167 Da of the Aq722 product. A cell extract and a cell debris

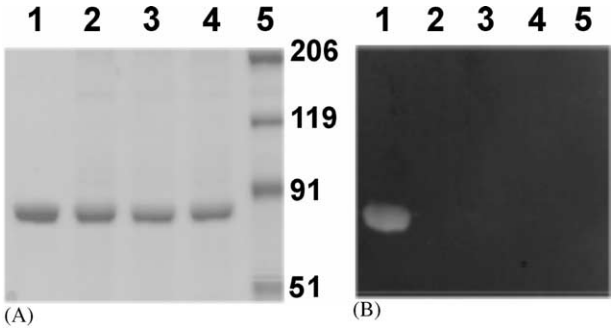

FIGURE 2 A. SDS-PAGE of purified wild type Aq722 glucan branching enzyme (lane 1), and of purified mutant enzymes with modified catalytic residues: D311N (lane 2), E362Q (lane 3) and D430N (lane 4). Lane 5 contains a protein marker. B. The same enzyme samples were also run on SDS-polyacrylamide gels containing amylopectin, subsequently washed to allow renaturation of the proteins, followed by incubation at 70°C and staining with iodine–iodide solution. The data show that the wild type enzyme was active towards amylopectin (lane 1), but not the mutant proteins (lanes 2–4).

fraction was prepared from *E. coli* cells containing the pVOA01 plasmid by sonication, heat treatment (30 min at 85°C), and centrifugation. When these two fractions were subjected to SDS-PAGE analysis it appeared that most of the 71 kDa protein was present in the cell debris fraction as inclusion bodies, as reported by Takata *et al*. (2003). The formation of inclusions bodies or protein aggregates is very common when overproducing enzymes from (hyper)thermophilic microoganisms in *E. coli* (Lilie *et al*., 1998). With sufficient 71 kDa protein present in the cell extracts, this fraction was used for further enzyme purification.

Viksø-Nielsen and Blenow (1998) reported purification of branching enzyme I of potato by γ-cyclodextrin sepharose affinity chromatography. We used the same method to purify the 71 kDa Aq722 protein from heat-treated cell extracts of *E. coli* BL21(DE3) containing the pVOA01 (Fig. 2). Following SDS-PAGE, purified branching enzyme could be reactivated in the gels and showed a clear white band when the amylopectin containing gel was stained with a iodine-iodide solution (Fig. 2). The N-terminal amino acid sequence of the purified enzyme (MKKFSL) was identical to the one expected based on the translated Aq722 gene sequence.

Mutagenesis of the Catalytic Residues

Alignment of the deduced amino acid sequence of the Aq722 glucan branching enzyme with those of other glucan branching enzymes allowed identification of the four conserved regions and the conserved amino acids typical for members of family 13 of glycoside hydrolases (Fig. 3; Baba *et al*., 1991; Henrissat, 1991; Leemhuis *et al*., 2003; Takata *et al*., 1994). The Aq722 catalytic residues were identified as Asp 311, Glu 362 and Asp 430, corresponding to the amino acids Asp 308, Glu 351, and Asp 419 of the *B. stearothermophilus* enzyme, previously shown to constitute the catalytic triad (Takata *et al*., 1994). When we changed the amino acids of the putative catalytic triad of the *A. aeolicus* enzyme into their amido counterparts (Asp311Asn, Glu362Gln, Asp-430Asn) by site-directed mutagenesis, no branching enzyme activity was found with purified mutant enzymes. Protein bands of the correct size were visible on SDS-PAGE (Fig. 2), indicating that proper expression of the mutant proteins occurred. Also four other conserved amino acids involved in substrate binding (Funane *et al*., 1998; Libessart and Preiss, 1998) could be identified easily based on the amino acid sequence alignment: Asp 240, His 245, Arg 309, and His 429 (Fig. 3).

Biochemical Properties

A number of basic properties of the purified enzyme were determined. The optimal temperature and pH for activity were respectively 80°C and 7.5 (Fig. 4). These values coincide well with those reported by Takata *et al*. (2003). With respect to thermostability, the branching enzyme of *A. aeolicus* showed 70% residual activity when incubated for 2 h at 90°C. Upon incubation at 96°C the activity was lost rapidly, while at 100°C the enzyme was immediately inactivated (Fig. 4). To analyse whether the high temperature stability can be deduced from the primary sequence information, the amino acid sequence was examined and compared to the sequences of other branching enzymes characterized thus far. Two major differences were found: the *A. aeolicus* sequence showed a higher number of aromatic amino acid residues (17.4%) in combination with a lower number of glutamine/asparagine residues (4.8%) (Table I). Also the number of cysteine residues in the *A. aeolicus* enzyme was lower when compared to other branching enzymes. Similar observations were made for the *R. obamensis* enzyme, which is active at 65°C and is stable up to 80°C (Shinohara *et al*., 2001). Aromatic stacking caused by hydrophobic residues is believed to increase the thermostability of a protein (Maves and Sligar, 2001). For xylose isomerase it has been postulated that deamination of the thermo-labile amino acid residues asparagine and glutamine and the oxidation of cysteine as a result of a temperature increase contribute to inactivation (Vieille *et al*., 1995; Vieille *et al*., 2001; Volkin and Klibanov, 1989). Decreasing the number of these amino acid residues thus lessens the chance of inactivation and may contribute to thermostability.

Glucan branching enzyme activity was quantified using amylose as a substrate. Because this is virtually free of $\alpha(1 \rightarrow 6)$ branching points, the exact

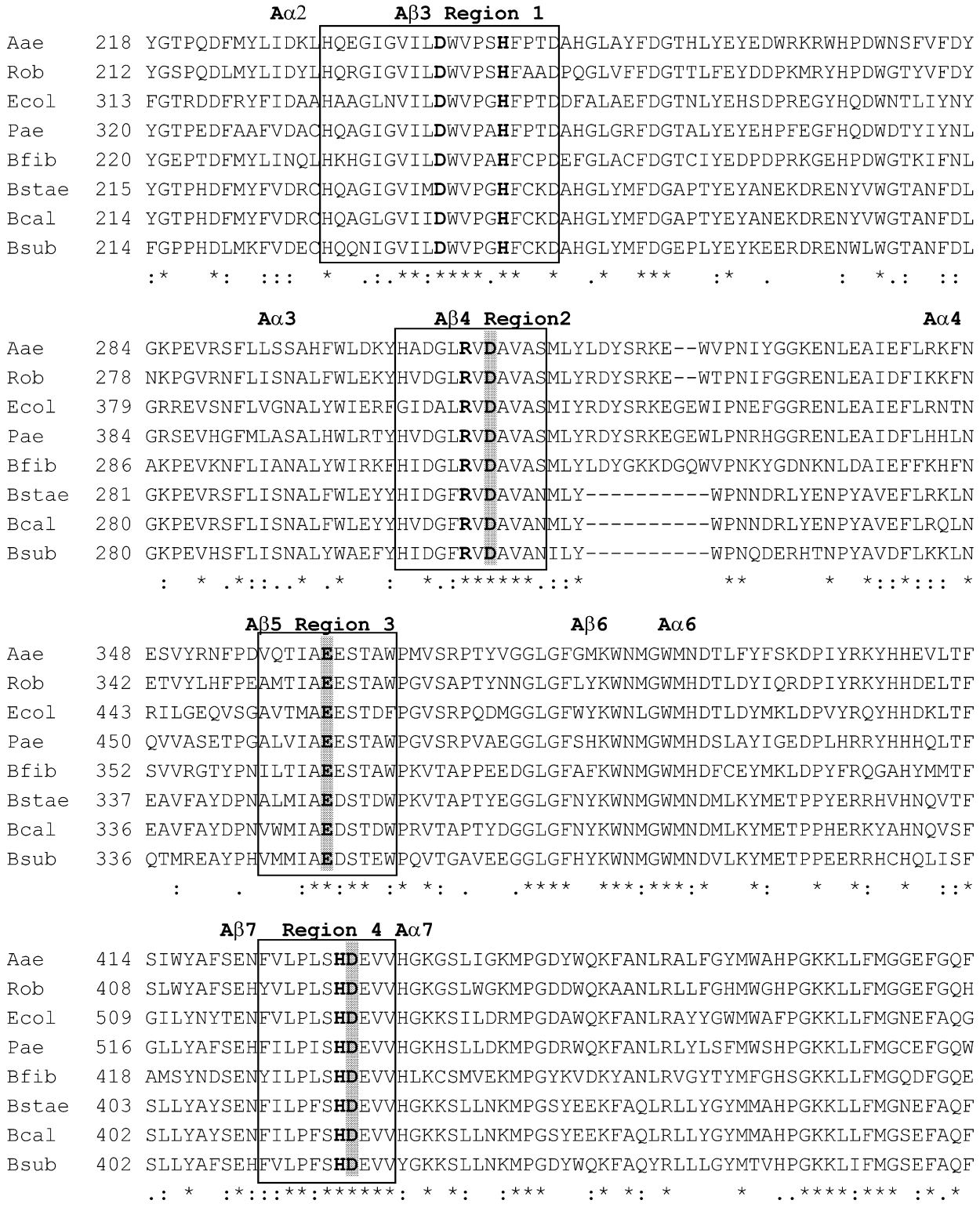

FIGURE 3 Partial amino acid sequence alignment of various bacterial glucan branching enzymes showing the four conserved regions known to be present in members of the α-amylase family. The fully conserved amino acid residues are highlighted in bold; the three catalytic residues are highlighted with a grey background. Aae, *Aquifex aeolicus*; Rob, *Rhodothermus obamensis* (BD247652); Ecol, *Escherichia coli* (M13751); Pae, *Pseudomonas aeruginosa* (AE004642); Bfib, *Butyrivibrio fibrisolvens* (M64980); Bstae, *Bacillus stearothermophilus* (D87026); Bcal, *Bacillus caldolyticus* (Z14057); Bsub, *Bacillus subtilis* (Z99119); between parentheses the NCBI accession numbers are given. The sequences were retrieved from the NCBI database. Symbols: (*) fully conserved residues; (:) fully conserved strong group; (.) fully conserved weaker group. The four conserved regions are shown in boxes. Also indicated are the approximate positions of a number of α-helices and β-sheets that are part of the A-domain of branching enzymes.

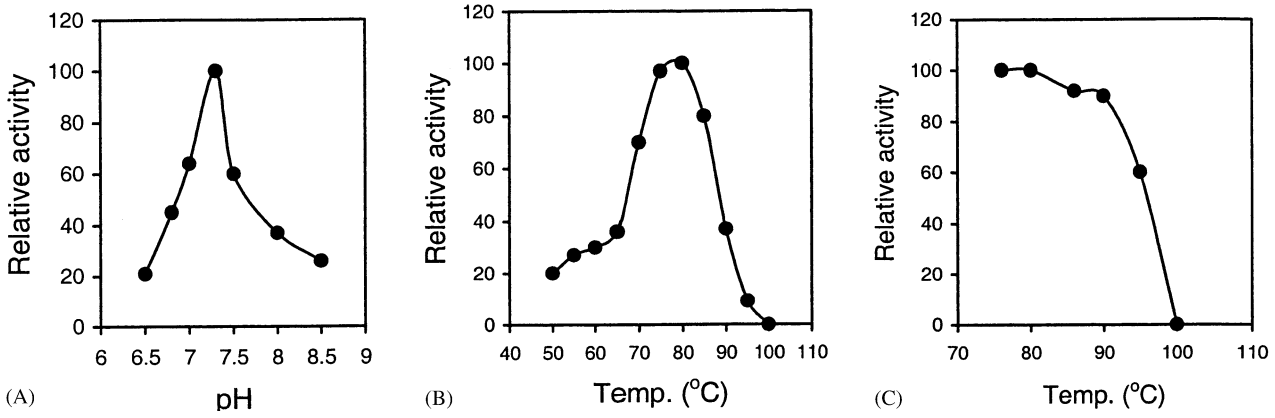

FIGURE 4 Activity profiles of the purified *A. aeolicus* branching enzyme in relation to pH (A) and temperature (B). The temperature stability is shown in panel C.

TABLE I Percentage of aromatic amino acids, glutamine and asparagine in relation to the temperature optimum for activity and stability of three bacterial and two maize branching enzymes

Source	%FWY[1]	%QN[2]	Temperature optimum (°C)	Temperature stability (°C)
A. aeolicus	17.4	4.8	80	90
R. obamensis	16.8	5.2	65	80
B. stearothermophilus	16.3	7.7	55	60
E. coli	13.2	7.6	30	nd
Maize I	12.2	6.6	30	nd
Maize II	13.4	6.8	20	nd

[1]FWY, respectively phenylalanine, tryptophan, tyrosine QN[2], respectively glutamine and asparaginend, not determined

number of newly introduced branching points can be determined by incubating amylose with the branching enzyme, subsequently debranching the product formed using isoamylase and then determine the number of reducing ends produced. The enzyme followed Michaelis-Menten kinetics. The Vmax on amylose was 4.9 U mg^{-1} and the Km 4.0 μM when determined under optimal conditions. With respect to these catalytic properties, the *A. aeolicus* enzyme is similar to the *B. stearothermophilus* glucan branching enzyme (Takata *et al*., 1994).

Side-Chain Composition of the Branched Glucan Formed from Amylose

The length of the side-chains of the branched glucan produced in time was determined by incubating purified branching enzymes with amylose and subsequent analysis of the side-chain distribution after debranching with isoamylase and analysis by HPAEC (Fig. 5). Halfway through the incubation time, the branched glucan produced had relatively long side-chains. At the end of the incubation, the

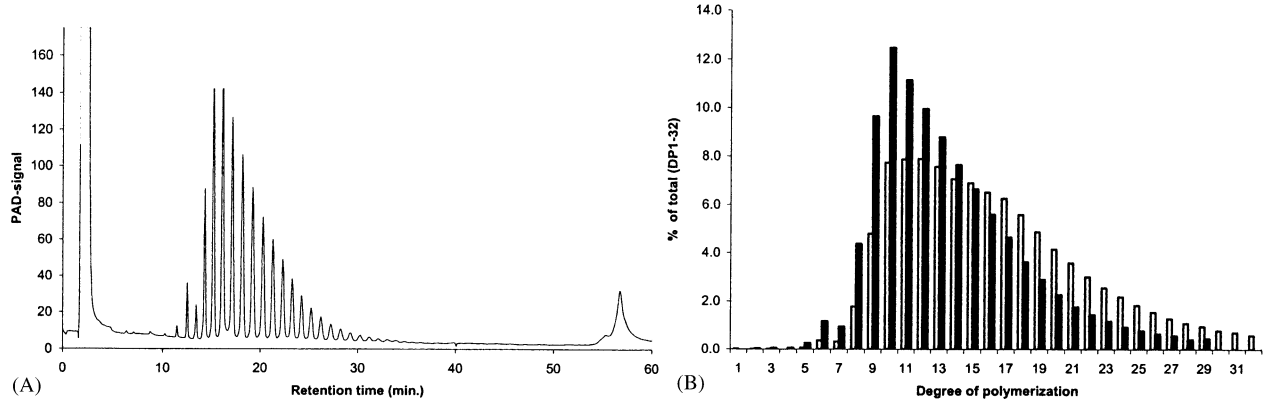

FIGURE 5 Side-chain distribution of the branched glucan produced when the *A. aeolicus* glucan branching enzyme was incubated with amylose. Panel A shows the HPLC profile; panel B shows the side-chain distribution of the products expressed as percentage of the total mass of all oligosaccharides up to DP32. The mass of each oligosaccharides was calculated by converting the PAD signal (Panel A) using a detector response factor, which was different for each oligosaccharide. The white bars represent the distribution found after 160 min of incubation; the black bars are the results obtained after 320 min of incubation. Longer incubation times did not result in different profiles. Incubations without enzyme did not result in a branched glucan.

side-chains had become shorter. Similar observations were made for the maize I and II branching enzymes (Kuriki *et al*., 1997), and for the *A. aeolicus* enzyme incubated with amylopectin (Takata *et al*., 2003). Thus, glucan branching enzymes initially transfer longer oligosaccharide chains to the acceptor molecule. When the reaction proceeds in time these newly synthesized longer chains are apparently used as donor substrate, and are thus shortened. The final side-chain profile of the branched glucan made by the *A. aeolicus* enzyme had an optimum at 10 glucose residues. The products made by the *B. stearothermophilus* and *E. coli* enzymes showed different, bimodal profiles, with optima at side-chains containing 7 or 11 glucose residues (Guan *et al*., 1997; Takata *et al*., 2003). The final branched glucans made by maize branching enzyme type I and type II did not show this bimodal distribution, with optima at 11 and 7 glucose residues, respectively (Kuriki *et al*., 1997). The branched glucan produced with Q-enzyme of potato even had a maximum at 16 glucose residues (Praznik *et al*., 1992). These differences in side-chain distribution of branched glucan products made by the various enzymes are very likely caused by subtle differences in the architecture of their active sites. Recently, the first 3D structure of a glucan branching enzyme was published (Abad *et al*., 2002). Future studies may provide detailed insights into structural factors of the different enzymes determining differences in the side-chain distribution.

CONCLUSIONS

The glucan branching enzyme of the hyperthermophile *Aquifex aeolicus* was successfully overexpressed in *E. coli*. The enzyme was purified to homogeneity from heat-treated *E. coli* cell extracts using γ-cyclodextrin affinity chromatography. The enzyme was optimally active at 80°C and pH 7.5 making it the most thermoactive glucan branching enzyme described. The thermostability of the enzyme might be explained by the relatively high number of aromatic amino acid residues in combination with a relatively low number of glutamine/asparagine residues. During its action on amylose, the enzyme initially transfers longer oligosaccharides resulting in a branched glucan with long side-chains. The final branched product has relatively small side-chains, indicating that during the later stage of action, the enzymes used the longer side-chains as donor substrate. The final product thus is a branched glucan that structurally resembles glycogen.

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