Molecular and Biochemical Characterization of an Endo-β-1,3-glucanase of the Hyperthermophilic Archaeon Pyrococcus furiosus*

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We report here the first molecular characterization of an endo-β-1,3-glucanase from an archaeon. Pyrococcus furiosus is a hyperthermophilic archaeon that is capable of saccharolytic growth. The isolated lamA gene encodes an extracellular enzyme that shares homology with both endo-β-1,3- and endo-β-1,3,1,4-glucanases of the glycosyl hydrolase family 16. After deletion of the N-terminal leader sequence, a lamA fragment encoding an active endo-β,1,3-glucanase was overexpressed in Escherichia coli using the T7-expression system. The purified P. furiosus endoglucanase has highest hydrolytic activity on the β-1,3-glucose polymer laminarin and has some hydrolytic activity on the β-1,3,1,4 glucose polymers lichenan and barley β-glucan. The enzyme is the most thermostable endo-β,1,3-glucanase described up to now; it has optimal activity at 100–105 °C. In the predicted active site of glycosyl hydrolases of family 16 that show predominantly endo-β,1,3-glucanase activity, an additional methionine residue is present. Deletion of this methionine did not change the substrate specificity of the endoglucanase, but it did cause a severe reduction in its catalytic activity, suggesting a structural role of this residue in constituting the active site. High performance liquid chromatography analysis showed in vitro hydrolysis of laminarin by the endo-β,1,3-glucanase proceeds more efficiently in combination with an exo-β-glycosidase from P. furiosus (CelB). This most probably reflects the physiological role of these enzymes: cooperation during growth of P. furiosus on β-1,3-glucans.

β-1,3-glucanases are widely distributed among bacteria, fungi, and higher plants. They are classified as exo-β-1,3-glucanases (β-1,3-glucan glucohydrolase (EC 3.2.1.58)) and endo-β-1,3-glucanases (β-1,3-glucan glucanohydrolase (EC 3.2.1.6 and EC 3.2.1.39)). Distinct physiological roles of β-1,3-glucanases have been proposed. In plants, involvement in cell differentiation and defense against fungal pathogens has been suggested (1). In fungi, β-1,3-glucanases seem to have different functions in morphogenetic processes, β-glucan mobilization, and fungal pathogen-plant interactions (2). Recently, the first metazoan β-1,3-glucanase, which may be involved in the early embryogenesis, has been described (3). In bacteria, a metabolic function has been reported for endo-β,1,3-glucanases and endo-β-1,3,1,4-glucanases (4). Both types of bacterial enzymes are polysaccharide endohydrolases with closely related specificities (5, 6). β-1,3-glucanases hydrolyze 1,3-β-glucosyl linkages, but they usually require a region of unsubstituted, contiguous 1,3-β-linked glucosyl residues. In contrast, β-1,3,1,4-glucanases catalyze the hydrolysis of 1,4-β-glucosyl linkages only when the glucosyl residue itself is linked at the O-3 position (7).

Genes encoding bacterial β-1,3- and β-1,3,1,4-glucanases have been cloned and sequenced from different Bacillus species (8–16), Fibrobacter succinogenes (17, 18), Cellvibrio mixtus (19), Thermotoga neapolitana (20), Ruminococcus flavefaciens (21, 22), Oerskovia xanthineolytica (23), Clostridium thermocellum (24, 25, 26, 27), and Rhodothermus marinus (28). All bacterial endo-β-1,3-glucanases (laminaran) known to date share sequence similarity with endo-β-1,3,1,4-glucanases (lichenan) and have been classified in the same family 16 of glycosyl hydrolases (29, 30, 31). On the other hand, eukaryal endo-β,1,3,1,4-glucanases and endo-β,1,3-glucanases have been classified in family 17 of glycosyl hydrolases. However, the first metazoan β-1,3-glucanase, obtained from a sea urchin, shares homology with both β-1,3- and β-1,3,1,4-glucanases of glycosyl hydrolase family 16 (3).

Presently, no β-specific endoglucanases have been reported in the Archaea, the third domain of life (32, 37). In this study, we report the characterization of an endo-β,1,3-glucanase from Pyrococcus furiosus, a heterotrophic hyperthermophilic archaeon that is able to grow optimally at 100 °C on a wide range of carbohydrate substrates, including starch, maltose, and cellobiose (32, 33). P. furiosus was found to contain several hydrolytic enzyme activities related to sugar degradation (34) and utilizes a modified Embden-Meyerhof pathway, which involves at least three unique enzymes: two ADP-dependent kinases (35) and a glyceraldehyde-3-phosphate:ferredoxin oxidoreductase (36). However, P. furiosus does not grow on cellulose or carboxymethylcellulose, and nothing has been described concerning the growth on other β-linked carbohydrates substrates, such as laminarin, β-glucan, and lichenan (32, 37).

The P. furiosus lamA gene was characterized and found to encode a secreted endo-β,1,3-glucanase belonging to glycosyl hydrolase family 16. Further characterization of the overproduced catalytic domain of this enzyme revealed that it was the most thermostable endo-β,1,3-glucanase detected so far. A mutant form of the catalytic domain was obtained after site-directed mutagenesis of the lamA gene and revealed that a conserved methionine residue is required for full activity. Based on the specificity of the endo-β,1,3-glucanase and the capacity of P. furiosus to grow on laminarin (described herein), a role for this extracellular enzyme during the fermentation of β-1,3-linked glucosyl polymer is proposed.

**EXPERIMENTAL PROCEDURES**

**Organisms and Growth Conditions—**P. furiosus (DSM 3638) was used in this study (32). Escherichia coli BL21 (DE3) harboring pLySE

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Endo-β-1,3-glucanase from Pyrococcus furiosus

![Diagram of genetic organization of the lamA region.](image)

**Fig. 1. Genetic organization of lamA region.** This DNA fragment was originally cloned as a fragment from chromosomal DNA of *P. furiosus* and inserted into the EcoRI/PstI sites of pTZ19R (41). Primers BG194 and BG195, used in the cloning of the lamA gene, and primers BG226 and BG227, used in site-directed mutagenesis of LamA, are indicated by arrows. Relevant restriction sites are indicated. H, HindIII; E, EcoRI; N, Ncol; B, BamHI; P, PstI.

(39) was used as the host strain for the recombinant plasmid of pGEF+ (a pET3d derivative; Ref. 38). *E. coli* TG1 was used as the host strain for the cloning vectors pLUW530 and pLUW531 (40). *E. coli* was grown in LB medium in a rotary shaker at 37 °C. Ampicillin was added to LB medium in a final concentration of 100 μg/ml. Isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 0.4 mM for the induction of gene expression.

**Cloning of the Endo-β-1,3-glucanase Gene—** A 4.8-kilobase pair fragment containing the lamA gene coding for *P. furiosus* endo-β-1,3-glucanase was cloned into pTZ19R (41). Based on its sequence, primers were designed to amplify the lamA gene by the polymerase chain reaction on a DNA Thermal Cycler (Perkin-Elmer Corp., Norwalk, CT) (Fig. 1). The two primers (with Ncol and BamHI restriction sites in boldface), were as follows: BG 194, 5′-GGCGCGCATGTTGCTCCGAAG-TGATAGAATTAGT, sense; and BG 195, 5′-GGCGCGGATCCTCAAC-AAGATGCTTGCAGTA, antisense. In addition to the template and the primers, the PCR reaction mixture contained 0.2 mM dNTPs, Taq DNA polymerase buffer, 5 mM MgCl2, and 5 units of DNA polymerase (Life Technologies Inc.) and was subjected to 30 cycles of amplification (30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C). The putative signal peptide of the endoglucanase was excluded to produce the mature enzyme in the cytoplasm of *P. furiosus* (42).

**Overexpression of the lamA Gene and Purification of the Endo-β,1,3-glucanase—** An overnight culture of *E. coli* BL21 (DE3), pLysE harboring pLUW530 was diluted 1:20 and grown until the A600 reached 0.6. The culture was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 4 h. Cells were harvested by centrifugation, resuspended in 100 mM sodium citrate buffer (pH 5.5), and sonicated using a Branson sonifier. Cell debris was removed by centrifugation (10,000 × g for 10 min). The resulting supernatant was heated for 30 min at 70 °C, and precipitated proteins were removed by centrifugation. The supernatant was subsequently dialyzed against Tris-HCl buffer (20 mM, pH 8.0) and loaded on a Q-Sepharose Fast Flow column (Pharmacia) (5 × 25 cm) that was equilibrated with the same buffer. Bound proteins were eluted by a linear gradient of NaCl (0–1 M in Tris-HCl buffer). Active fractions eluted around 0.5 M NaCl.

Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE)1 using the method of Laemmli (43). Proteins samples for SDS-PAGE were prepared by heating for 10 min at 110 °C in an equal volume of sample buffer (0.1 M citrate-phosphate buffer, 5% SDS, 0.9% 2-mercaptoethanol, 20% glycerol, pH 6.8).

**β-Glucanase Assay, Protein Determination, and Western Blot—** The standard assay for β-glucanase activity was carried out at 80 °C, for 10 min, using 0.5% (w/v) laminarin (Sigma) as substrate in 0.1 M phosphate (NaH2PO4, K2HPO4) buffer (pH 6.5). The reducing sugars released were detected by the dinitrosalicylic acid method, with glucose as a standard (44). Enzyme and substrate blanks were also included. 1 unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugars/min. Protein concentrations were determined by the method of Bradford, with bovine serum albumin as the standard (45).

Antibodies were raised against LamA by injecting enzyme purified from *E. coli* into rabbits. The proteins were separated by SDS-PAGE before Western blotting onto nitrocellulose filters. The blots were incubated with serum containing antibodies raised against LamA (dilution, 1:1000). The reaction of proteins with the antibodies was detected using the alkaline phosphatase system (Promega).

**Temperature and pH Optima, Thermal Stability, and Kinetic Parameters—** The optimal temperature was determined by running the standard assay at temperatures from 80 °C to 115 °C. The optimal pH of the enzyme was determined by running the standard assay at 80 °C using citrate-phosphate (citric acid, NaH2PO4) buffer (0.1 M) and phosphate (NaH2PO4, K2HPO4) buffer (0.1 M) for pH ranges 4–7 and 6–8, respectively. Thermostability was determined using diluted enzyme (0.14 mg/ml pure enzyme was diluted 10 times in 200 mM Tris-HCl buffer, pH 6.5), incubated at 80, 90, and 100 °C. Samples were taken at time intervals, and residual activity was determined by the standard assay at 80 °C. Michaelis-Menten constants were determined from Lineweaver-Burk representations of data obtained by determining the initial rate of laminarin and lichenan hydrolysis under the assay conditions described above and using a range of 0.25–10 mg of substrate/ml.

**Mutagenesis of the lamA Gene—** The plasmid pLUW500, carrying the lamA gene, was used as a template for mutagenesis following the splicing by overlap extension polymerase chain reaction (46). The first step comprised the use of primers introducing the mutation and the two flanking primers (BG 194 and BG 195), described above. The two mutagenic primers were as follows: BG 227, 5′-GGCAAGAATTCAT-TGTATTTCATCCACAAATGG, antisense; and BG 228, 5′-GAATGA-TAGACGACAGCCACGAA. The second step used the overlapping products of the first two polymerase chain reactions as a template and the flanking primers (BG 194 and BG 195) to yield the lamA gene with the mutation. To simplify detection of mutants, an EcoRI site (in boldface in the primers, above) was created. The amplified DNA was cloned as a HindIII/BamHI fragment into the pLUW500 gene. The mutation was checked by dideoxynucleotide chain termination method (47) with a Li-Cor automatic sequencing system (model 4000L). Computer analysis of sequencing data was performed by using the PC/GENE program, version 5.01 (IntelliGenetics Inc., Mountain View, CA) and the GCG package, version 7.0, at the CAOS/CAMM Center of the University of Nijmegen (Nijmegen, The Netherlands).

**RESULTS**

Cloning and Sequencing of the lamA Gene Encoding an Endo-β-1,3-glucanase from *P. furiosus*—The nucleotide sequence of a part of the 4.8-kilobase pair DNA insert from pLUW500 (41) revealed the presence of an 894-bp open reading frame (Fig. 1). The encoded 297-amino acid protein was found to be homologous to a family of bacterial β-1,3- and β-1,3,1,4-endoglucanases (see below) and contains a putative signal peptide of 20 residues (49). The molecular mass of the predicted mature protein was found to be 31,550 Da, and the estimated isoelectric point was 5.15. To overproduce this β-glucanase, the lamA gene was amplified by the polymerase chain reaction using primers and cloned into pGEF+ (38) under control of the T7 promoter (39). The resulting plasmid, containing the catalytic domain of the endo-β-1,3-glucanase (starting at residue 35 and called LamA), was named pLUW530. The plasmid pLUW530, harboring lamA, was checked by DNA sequence analysis.

**Primary Structure Comparison—** Comparison of the deduced primary structure of lamA with enzymes present in the GenBank Data Base indicated that the highest similarity was with representatives of family 16 of glycosyl hydrolases, which includes bacterial endo-β-1,3- and endo-1,3,1,4-glucanases, Streptomyces coelicolor agarase, and Alteromonas carrageenovora α-carrageenase. Scores of 52.4 and 46.9% identity were found with the β-glucanases of *T. neapolitana* and *O. xanthinolytica*, respectively. Multiple alignments of the deduced amino acid sequence of *P. furiosus* lamA and other β-glucanases showed many conserved amino acids (Fig. 2), including glutamate 170 and glutamate 175 *(P. furiosus* amino acid numbering is used throughout the paper).

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1. The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.

2. W. G. B. Voorhorst, manuscript in preparation.
The results of structural analysis and mutagenesis (50–53), combined with inhibitor attachment studies of Bacillus β-1,3-glucanases (52, 54), showed the direct involvement of these two carboxylic acids in catalysis.

![Image](url)

**Fig. 3.** SDS-PAGE of E. coli BL21 (DE3), pLyse containing the plasmid pLUW530, producing the catalytic domain of *P. furiosus* LamA. Lane 1, crude cell extract; lane 2, soluble fraction; lane 3, supernatant after heat incubation (30 min at 70 °C); lane 4, purified LamA; lane 5, purified fraction after anion exchange chromatography. lane 6, E. coli crude extract; lane 7, supernatant of a heat incubation (30 min at 70 °C) that resulted in the denaturation and loss of activity was obtained, and the isolated enzyme was estimated by SDS-PAGE to be at least 95% pure (Fig. 3).
LamA has the highest specificity for the soluble β-1,3-glucan laminarin. A 10-fold lower activity was observed on β-1,3,1,4-linked glucans, such as lichenan and barley β-glucan. The β-1,4-linked substrates (carboxymethyl cellulose, cellulose, and xylan) were not hydrolyzed. Based on the substrate specificity, the LamA enzyme produced in *E. coli* was characterized as a β-1,3-glucanase (laminaranase; EC 3.2.1.39). The mode of action of LamA was examined by analyzing the corresponding hydrolysis products by high performance liquid chromatography (Fig. 4). The enzyme cleaved laminarin randomly, yielding glucose, laminaribiose, and higher laminari-oligosaccharides. These results confirm that LamA is an endo-β-1,3-glucanase.

The combined action of LamA and CelB (an exo-β-glycosidase from *P. furiosus*) resulted in the almost complete hydrolysis of laminarin (96%) to glucose. The incubation of laminarin under the same conditions with CelB only resulted in the degradation of 5% of the laminarin, with only glucose as the end product.

The laminarase enzyme activity was investigated at different pH values and temperatures (Fig. 5). The optimal pH was found to be 6–6.5. The enzyme exhibited at least 80% of its optimal activity over a rather broad pH range, from 5 to 7 (Fig. 5A). The temperature for maximum activity was 100–105 °C. The enzyme was almost inactive at 40 °C and began to show significant activity above 60 °C (Fig. 5B). Thermal stability was investigated by incubating the pure enzyme for up to 110 h at different temperatures (Fig. 6). The enzyme retained 100% of its laminarase activity after 110 h of incubation at 80 °C. At higher temperatures, thermostability decreased: at 90 °C and 100 °C, the enzyme showed a half-life of 64 and 19 h, respectively. At 110 °C, the enzyme was rapidly inactivated, with a half-life of only 15 min (data not shown).

**Site-directed Mutagenesis of the *P. furiosus* Endo-β-1,3-glucanase**—To investigate whether the deletion of methionine at position 174 in LamA would affect the β-1,3 substrate specificity of the enzyme, a mutated lamA gene was constructed, resulting in plasmid pLUW530. Relative activities and kinetic parameters of mutant and wild-type enzymes were determined with laminarin and lichenan as substrates (Table II). The specific activities of the mutant LamA, on both laminarin and lichenan, are about 10-fold lower than the respective wild-type values. Catalytic parameters were determined at 80 °C and pH 6.5 (optimal conditions for the wild-type enzyme). The methionine deletion led to an enzyme with 13.1% of wild-type *V* max and a slightly higher *Km* on laminarin as a substrate. With lichenan as substrate, the mutant LamA had 7.9% of the wild-type *V* max and the same *Km* value.

**Detection of the Endo-β-1,3-glucanase in *P. furiosus***—The unexpected discovery of the endo-β-1,3-glucanase in *P. furiosus* prompted us to study the capacity of this hyperthermophilic archaeon to grow on β-1,3-linked glucose polymers. Remarkably, *P. furiosus* was found to grow on laminarin (0.3% w/v) and lichenan (0.3% w/v) as substrates (data not shown), and endo-β-1,3-glucanase activity was detected in the culture medium. Western blot analysis with immune serum directed against LamA gave an immunoreactive band with the culture supernatant of *P. furiosus* grown on laminarin as a substrate (Fig. 3). The product from *P. furiosus* appeared to be slightly bigger (by approximately 1 kDa) than the one expressed in *E. coli*, which is in agreement with the fact that we deleted, during the overexpression step, a N-terminal fragment of 34 amino acids, which is larger than the predicted signal peptide of 20 amino acids.

**FIG. 4.** High performance liquid chromatography analysis of *P. furiosus* LamA and CelB action on laminarin (0.5% w/v). A, laminarin control, incubation at 80 °C for 4 h; B, incubation with LamA (5 µg/ml) at 80 °C for 4 h; C, incubation with CelB (5 µg/ml) at 80 °C for 4 h; D, incubation with LamA (5 µg/ml) and CelB (5 µg/ml) at 80 °C for 4 h. Samples were analyzed on an Aminex HPX-87-H column (Bio-Rad). Identified products are laminarin (1), laminoli oligomers (2), laminaribiose (3), and glucose (4).

**DISCUSSION**

In this paper, we describe the overexpression of the lamA gene, which encodes an extremely thermostable endo-β-1,3-glucanase from the hyperthermophilic archaeon *P. furiosus*. The overproduction and purification of the endo-β-1,3-glucanase LamA was carried out by cloning a *lamA* gene fragment into the T7 expression system after deletion of the hydrophobic N-terminal sequence (34 amino acids). This is the first description of an endo-β-1,3-glucanase from an archaeon, and it is the most thermostable endo-β-1,3-glucanase known up to now. The second most thermostable endoglucanase of the family 16 of glycosyl hydrolases characterized is that of the thermophilic bacterium *R. marinus*, which has an optimal temperature of 85 °C and has 85% residual activity after 16 h incubation at 80 °C. The optimal temperature of the *P. furiosus* β-glucanase is 100–105 °C, and the enzyme retained 100 and 50% of its activity after incubation for 85 h at 80 °C and 16 h at 100 °C, respectively. The optimal temperature of the enzyme is near the optimal growth temperature (100 °C) of the *P. furiosus* strain (35). The enzyme activity has a broad pH optimum around pH 6, which is comparable to other endo-β-1,3-glucanases and endo-β-1,3,1,4-glucanases, with the exception of the *Bacillus brevis* enzyme, which has optimal activity at pH 9.0. The *P. furiosus* β-glucanase was able to hydrolyze both β-1,3-glucan and β-1,3,1,4-glucan but not the β-1,4-linked substrates like cellulose. Bacterial laminarases and lichenases often yield trisaccharides and tetrasaccharides as the main degradation products (36). On the other hand, like other bacterial and fungal endo-β-1,3-glucanases (2, 28, 29), the endo-β-1,3-glucanase of *P. furiosus* hydrolyzes β-1,3-glucan with glucose, laminaribiose, and laminaritriose as end products.

All of the bacterial laminarases and lichenases sequenced so far have been classified in the same family 16 of glycosyl hydrolases. The mechanism for β-glucanase action must take into account two general considerations (55, 59). The hydrolysis of the glycosyl bond can proceed via either retention or inver-
acid. Several experiments with electrostatic stabilization, and the second acts as a general setting. The first acts either as a nucleophile or by providing groups of the enzyme to be present in the appropriate spatial orientation of the configuration at the anomeric carbon atom. Because NMR analysis has indicated retention of configuration in case of the Bacillus licheniformis β-glucanase (60), the same stereochemical course is assumed to hold true for all other members of the family (61). This mechanism requires two functional

molecule (Fig. 2). From glutamate 170 to phenylalanine 176, there is a strict alternation of polar and nonpolar side chains in the catalytic sites of the endo-β-1,3,1–4-glucanases. This organization is different in the endo-β-1,3-glucanases because of the insertion of an extra methionine residue between isoleucine 173 and glutamate 175. Assuming that both the active site structure and the catalytic mechanism are similar within a family, this residue has been proposed to form a kink in the β-strand and thereby allowing the methionine side chain to point toward the hydrophobic core and the glutamate 175 to participate in catalysis (50, 63). This structural rearrangement of the active site most likely would affect catalysis. Moreover, substrate specificity analysis within glycosyl hydrolyze family 16 shows that these enzymes can be classified in two subfamilies: (i) a group of β-glucanases able to hydrolyze β-1,3-glucan or both β-1,3- and β-1,3–1,4-glucan (such as in the case of P. furiosus and R. marinus); and (ii) a group of β-1,3–1,4-glucanases. The structural change brought about by the inserted methionine has been correlated with the altered specificity, from β-1,3-1,4 linked substrates to β-1,3 linkages ones (28, 50). The role of this extra methionine in P. furiosus β-glucanase was investigated by site-directed mutagenesis. Removal of methionine 174 in the P. furiosus β-glucanase resulted in enzyme activity that was decreased by a factor of 10 for both laminarin and lichenan. However, we did not observe a shift of relative affinity of the mutant enzyme from β-1,3-glucan toward β-1,3-1,4-glucan substrates. Hence, deletion of methio-

| Substrate | Enzyme | Specific activity | Activity | $K_m$ | $V_{max}$ |
|-----------|--------|------------------|----------|------|----------|
| Laminarin (β-1,3(Glc)) | Wild type | 1073 | 100 | 2.8 | 100 |
| | mutant | 111.6 | 10.4 | 3.5 | 13.1 |
| Lichenan (β-1,3–1,4(Glc)) | Wild type | 85 | 100 | 4.7 | 100 |
| | mutant | 7.1 | 8.3 | 4.9 | 7.9 |

$^a$ Enzyme activity was measured in 100 mM phosphate buffer, pH 6.5, at 80 °C for 10 min.

$^b$ Percentages of residual activity and $V_{max}$ are expressed relative to the wild type for each substrate.

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Fig. 5. Influence of pH and temperature on the activity of the purified endo-β-1,3-glucanase of P. furiosus. A, enzyme activity was measured at 80 °C for 5 min, using laminarin as substrate, in 100 mM citrate-phosphate buffer (●) at pH 6–8. 100% activity corresponds to 935 units/mg of protein. B, enzyme activity was measured in 100 mM phosphate buffer at pH 6.5 for 5 min, using laminarin as substrate. 100% activity corresponds to 1842 units/mg of protein. Each assay was performed with 0.68 μg of protein/ml.

Fig. 6. Thermal stability of the P. furiosus endo-β-1,3-glucanase. The enzyme was preincubated at 80 °C (●), 90 °C (●), and 100 °C (●) in 200 mM Tris-HCl buffer, pH 6.5. Residual activity was measured in 100 mM phosphate buffer at pH 6.5 for 5 min, using laminarin as substrate. 100% activity corresponds to 945 units/mg of protein.
nine 174 affected mostly $V_{\text{max}}$, not $K_m$. The results suggest that the methionine 174 residue may assist in catalysis as a neighboring group to the essential glutamate 175 and suggest a structural role of this residue in the constitution of the active site. However, it does not significantly contribute to specific substrate binding, as seen by the unchanged $K_m$ upon deletion. Moreover, a mutant $B. macerans$ $\beta$-glucanase in which a methionine has been inserted in the active site region has been reported to be enzymatically inactive (50). These results suggest that further modifications are required to extend the substrate specificity of $\beta$-1,3-glucanases (for example, amino acids responsible for the binding of the different polysaccharide substrates). Fferrer et al. (23) noted that conserved regions of the $\beta$-glucanases family are rich in aromatic residues, tryptophan in particular. This residue was demonstrated to play a role in substrate binding of some cellulases (64). Furthermore, a limited number of tryptophan residues are invariant either in $\beta$-1,3-glucanases or in $\beta$-1,3,1,4-glucanases (in positions 46, 50, 89, 150, 154, 257, and 270), suggesting a possible function in $\beta$-glucanase substrate specificity of these residues. Moreover, Planas and co-workers (51) proposed, after site-directed mutagenesis on $B. licheniformis$ $\beta$-1,3,1,4-glucanase, a possible role in substrate binding of two glutamates (in position 122 and 203), which are conserved only among $\beta$-1,3,1,4-glucanases. A better understanding of the basis of the $\beta$-1,3- and $\beta$-1,3,1,4-glucanase substrate specificity awaits a detailed comparison of the three-dimensional structures of these two classes of enzymes and experimental verifications of the derived conclusions by protein engineering.

Previously, it was observed that $P. furiosus$ is capable to degrade $\alpha$-linked glucose polymers such as starch and glycogen by the concerted action of $\alpha$-amylase, pullulanase, and $\alpha$-glucosidase (65). Here, we have demonstrated that, in addition, $P. furiosus$ is able to grow on $\beta$-linked glucose polymers. Two enzymes are involved in the hydrolysis of laminarin to glucose: the LamA-encoded extracellular endo-$\beta$-1,3-glucanase and $\beta$-glucosidase (CelB). The physiological substrate of the latter endo-$\beta$-1,3-glucanase may be a $\beta$-1,3-linked carbohydrate component of the cell wall of a variety of marine organisms, such as eukaryotic algae (laminarin) or methanogenic archaea (pseudopeptidoglycan) (48). As it has been demonstrated in vitro, the LamA and CelB enzymes are antagonistic to catalyze the hydrolysis of sugars polymers to glucose in a cooperative manner. The extracellular endo-$\beta$-1,3-glucanase with high-level thermostability could function around the living area of $P. furiosus$, degrading the $\beta$-1,3-glucose polymer to smaller oligomers that are imported into the cell and hydrolyzed to glucose by the exo-$\beta$-glucosidase (CelB). Thus, these two enzymes could start the catabolic pathway of $P. furiosus$ growing on $\beta$-1,3-glucose polymer that could resemble the natural substrate of the strain in vitro conditions. Moreover, the lamA gene is located near the $\beta$-glucanase celB gene and two alcohol dehydrogenases (Fig. 1).\(^\text{2}\) Clustering of the lamA and celB genes may be of functional significance. The regulation of the celB and lamA genes in $P. furiosus$ is currently under investigation.\(^\text{2}\)

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REFERENCES
1. Grenier, J., Potvin, C., and Asselin, A. (1993) Plant Physiol. 103, 1277–1283
2. de la Cruz, J., Pinter-Toro, J. A., Bentzen, T., Libbey, A., and Romero, L. C. (1995) J. Bacteriol. 177, 6937–6945
3. Batchman, E. S., and McClay, D. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6888–6893
4. Watatanabe, T., Kasahara, N., Aida, K., and Tanaka, H. (1992) J. Bacteriol. 174, 186–190
5. Chen, L., Fincher, G. B., and Hoj, P. B. (1993) J. Biol. Chem. 268, 13318–13326
6. Varghese, J. N., Garrett, T. P., Colman, P. M., Chen, L., Hoj, P. B., and Fincher, G. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2785–2789
7. Parrish, F. W., Perlin, A. S., and Reese, E. T. (1960) Can. J. Chem. 38, 2094–2104
8. Borris, R., Buettner, K., and Maentseelaue, P. (1990) Mol. Gen. Genet. 222, 278–283
9. Bueno, A., Vazquez de Aldana, C. R., Correa, J., Villa, T. G., and del Rey, F. (1996) J. Bacteriol. 172, 2160–2167
10. Fiske, M. J., Tobey-Fincher, K. L., and Fuchs, R. L. (1990) J. Gen. Microbiol. 136, 2377–2383
11. Gosalbes, M. J., Perez-Gonzalez, J. A., Gonzalez, R., and Navarro, A. (1991) J. Bacteriol. 173, 7705–7710
12. Hofmeister, J., Kurtz, A., Borris, R., and Knowles, J. (1986) Gene 49, 177–187
13. Lloberas, J., Perez-Pons, J. A., and Querol, E. (1991) Eur. J. Biochem. 197, 337–343
14. Louw, M. E., Reid, S. J., and Watson, T. G. (1993) Appl. Microbiol. Biotechnol. 38, 507–533
15. Murphy, N., McConnell, D. J., and Cantwell, B. A. (1994) Nucleic Acids Res. 12, 5355–5367
16. Varghese, J. N., Garrett, T. P., Colman, P. M., Chen, L., Hoj, P. B., and Fincher, G. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2785–2789
Endo-β-1,3-glucanase from Pyrococcus furiosus

57. Welfle, K., Misselwitz, R., Welfle, H., Politz, O., and Borris, R. (1995) Eur. J. Biochem. 229, 726–735
58. Bacon, J. C., Gordon, A. H., Jones, D., Taylor, I. F., and Webley, D. M. (1970) Biochem. J. 120, 67–71
59. Svensson, B., and Soggard, M. (1993) J. Biotechnol. 29, 1–37
60. Malet, C., Gimenez-barbero, J., Bernabe, M., Brossa, C., and Planas, A. (1993) Biochem. J. 296, 753–758
61. Gebler, J., Gilkes, N. R., Claeyssens, M., Wilson, D. B., Beguin, P., Wakarchuk, W. W., Kilburn, D. G., Miller Jr, R. C., Warren, R. A., and Withers, S. G. (1992) J. Biol. Chem. 267, 12559–12561
62. Hahn, M., Keitel, T., and Heinemann, U. (1995) Eur. J. Biochem. 232, 849–858
63. Richardson, J. S., Getzoff, E. D., and Richardson, D. C. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2574–2578
64. Din, N., Forsythe, I. J., Burtnick, L. D., Gilkes, N. R., Miller, R. C., Warren, R. A. J., and Kilburn, D. G. (1994) Mol. Microbiol. 11, 747–755
65. Sunna, A., Moracci, M., and Antranikian, G. (1997) Extremophiles 1, 2–13