***Thermotoga neapolitana* bglB gene, upstream of lamA, encodes a highly thermostable β -glucosidase that is a laminaribiase**

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The gene for thermostable 1,3- β -glucosidase BglB was cloned from the chromosome of *Thermotoga neapolitana* and its primary sequence was determined. The purified recombinant β -glucosidase B had a monomer molecular mass of 81 kDa in accordance with the amino acid sequence predicted from the nucleotide sequence of clone pTT51. It was a member of glycosylhydrolase family 3 and belonged to enzyme class EC 3.2.1.21. β -Glucosidase B had a specific activity of 255 U mg⁻¹ on 4-nitrophenyl(PNP)- β -glucoside at the optima of pH (5.5) and temperature (90 °C), and K_m values of 0.1, 10 and 50 mM for PNP- β -glucoside, laminaribiose and cellobiose, respectively. The gene *bglB* was located immediately upstream of the laminarinase gene *lamA*. Both genes were transcribed from the same DNA strand and were not separated by a palindromic transcription terminator. The two purified enzymes 1,3- β -glucosidase BglB (laminaribiase) and 1,3- β -glucanase LamA (laminarinase) were together capable of completely degrading laminarin to glucose.

Keywords: *Thermotoga neapolitana*, nucleotide sequence, thermostability, β -glucosidase, BglB

INTRODUCTION

Thermotoga neapolitana is a saccharolytic, extremely thermophilic, organotrophic Gram-negative bacterium which grows up to a maximum temperature of 90 °C. It is able to ferment polysaccharides such as starch, xylan and cellulose (Huber & Stetter, 1992). Due to their physico-chemical stability the depolymerases produced by this micro-organism are an attractive source of catalysts for biotechnological applications. Clones expressing cellulase, xylanase, 1,3- β - and 1,3-1,4- β -glucanase and β -glucosidase activities have been isolated by Dakhova *et al.* (1993). The sequences of the genes for endo-xylanases XynA and XynB (Zverlov *et al.*, 1996; accession no. Z49961), β -1,3-glucanase LamA (Zverlov

et al., 1997; accession no. Z47974), and endoglucanases CelA and CelB (accession no. Z86103) have recently been reported.

A prerequisite for commercial 1,3- β -glucan hydrolysis, e.g. for yeast extract production or for conversion of algal biomass to fermentable sugars, is the availability of a thermostable endo-1,3- β -glucanase (a laminarinase like LamA) and in addition a 1,3- β -glucosidase, degrading the resulting 1,3- β -oligosaccharides (laminari-dextrins) by splitting off monomeric glucose residues from the non-reducing end. The clone pTT26, isolated from the genomic library of *T. neapolitana* (Dakhova *et al.*, 1993), was shown to contain upstream of the laminarinase gene *lamA* an ORF of 298 aa with high similarity to the C-terminus of β -glucosidases (Zverlov *et al.*, 1997). However, this potential β -glucosidase reading frame was incompletely cloned.

In this paper we describe the cloning and sequencing of the complete β -glucosidase B gene upstream of *lamA*, and the characterization of the recombinant BglB protein produced in *Escherichia coli* as an extremely

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Abbreviation: PNP- β -glucoside, 4-nitrophenyl- β -D-glucoside.

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thermostable β -glucosidase. BglB is shown to be a laminaribiase, being able to hydrolyse, on co-incubation with laminarinase LamA, laminarin completely to glucose.

METHODS

Bacterial strains, plasmids and growth conditions. *E. coli* DH5 α (*supE44*, Δ *lacU169* (ϕ 80 *lacZ* Δ 15M), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*) was used as host (Hanahan, 1983). Plasmid pTT26, containing the laminarinase gene of *T. neapolitana* Z2706-MC24, was described in Dakhova *et al.* (1993). Recombinant *E. coli* cells were grown at 37 °C in Luria-Bertani broth (LB) containing ampicillin (100 μ g ml⁻¹). The pGEM-T vector system (Promega) was used for cloning the PCR products and vector pUC18 for cloning DNA fragments from chromosomal DNA.

Recombinant DNA techniques and sequence analysis. Preparation of chromosomal and plasmid DNA, endonuclease digestion, ligation and transformation were carried out using standard procedures (Sambrook *et al.*, 1989). Enzymes for DNA modification were purchased from Boehringer Mannheim and used according to the manufacturer's recommendations.

The DNA sequence was determined from supercoiled double-stranded plasmid DNA from both strands (Thermosequencing Cycle Sequencing Kit; Amersham) with biotinylated primers. DNA fragments were detected with a GATC 1500 Direct-Blotting Electrophoresis apparatus using streptavidin-conjugated alkaline phosphatase and the chromogenic substrate NBT-BCIP (Promega). Sequence data were analysed and compared with DNASIS/PROSIS for Windows software (Hitachi Software Engineering). Nucleotide and protein sequence databases were screened using the FASTA and BLAST software at the NCBI-server (<http://www.ncbi.nlm.nih.gov>).

Cloning of *bglB*. The *Pst*I–*Hind*III DNA fragment of clone pTT26 (0.8 kb) was labelled with digoxigenin (DIG DNA Labelling Kit; Boehringer Mannheim) and used as DNA probe to select clone pTT50 from a genomic library of cloned chromosomal *Bam*HI–*Hind*III DNA fragments (Fig. 1). The 18 bp oligonucleotide primer P1 (5'-agtgcctttatcacgcac-3') was used for asymmetric PCR with the Expand High Fidelity PCR System (Boehringer Mannheim) to amplify the upstream region of clone pTT50 from chromosomal DNA (Fig. 1). In asymmetric PCR only one primer is used, which binds specifically to one DNA strand; the complementary strand is synthesized by unspecific binding, analogous to the random primer method (L. Richter, unpublished).

Enzyme purification. Frozen recombinant *E. coli* cells from a 250 ml culture were resuspended in 0.1 vol. 50 mM phosphate buffer (pH 6.1 + 1 mM PMSF), disintegrated by ultrasonic treatment and heated for 25 min at 77 °C. The protein precipitate was separated by centrifugation (4500 r.p.m.) and the supernatant ultrafiltered through a 10 kDa filter, changing the buffer to 30 mM Tris/HCl (pH 8.0). The suspension was applied to a Toyo-DEAE 650M column (2 $\times$ 1.5 cm) equilibrated with the same Tris/HCl buffer, followed by washing with 5 vols buffer. Adsorbed proteins were eluted by a 0–1 M NaCl gradient (10 column volumes). Fractions containing activity on 4-nitrophenyl(PNP)- β -glucoside were ultrafiltered (30 kDa polysulfon filter) with a buffer change to 30 mM imidazole-HCl buffer (1M/HCl; pH 6.5), followed by heating to 75 °C for 15 min and centrifugation. The supernatant containing 79% of the activity was applied to a phenyl-Sepharose column (CL-4B, 2.5 $\times$ 1.5 cm) in 30 mM 1M/HCl

buffer (pH 6.5 + 1 M NaCl) and eluted with a 1–0 M NaCl gradient. The hydrophobic protein adsorbed to the column was eluted by applying a 0–50% ethylene glycol gradient in 50 mM 1M/HCl, pH 6.5. Homogeneity of the protein contained in fractions active on PNP- β -glucoside was checked by SDS-PAGE.

Enzyme assays and detection of hydrolysis products. Enzyme aliquots in standard assays were incubated in phosphate-citrate buffer (50 mM, pH 6.2) with 0.7% (w/v) substrates at 85 °C. Reducing sugars released from polymeric substrates were detected by the 3,5-dinitrosalicylic acid method (Wood & Bhat, 1988). One unit of activity is defined by the release of 1 μ mol glucose-equivalent min⁻¹. Digests of laminarin were analysed by HPLC (System Gold; Beckman Instruments): 0.5% (w/v) laminarin was digested in MES buffer (25 mM, pH 6.0) at 90 °C. Hydrolysis products were separated at 85 °C in H₂O using an Aminex HPX-42A column (300 $\times$ 7.8 mm; Bio-Rad) fitted with a Microguard precolumn (Deash; Bio-Rad), and detected by refractometry. The pH of all buffers was adjusted at 20 °C.

Oat spelt xylan, CM-cellulose, lichenan, laminarin, PNP- β -D-glycosides, laminaribiose and cellobiose were obtained from Sigma-Aldrich. Laminaridextrins as size standards for HPLC were obtained from Seikagaku.

RESULTS

Cloning and sequencing of *bglB*

In a search for a clone containing the complete β -glucosidase gene of *T. neapolitana*, six clones expressing β -glucosidase, isolated previously from a genomic library (Dakhova *et al.*, 1993), were investigated by DNA–DNA hybridization analysis. However, none of the existing clones contained the β -glucosidase gene upstream of *lamA*. To clone the complete β -glucosidase gene located upstream of *lamA*, *Hind*III–*Bam*HI-digested chromosomal DNA of *T. neapolitana* Z2706-MC24 was ligated to pUC18 DNA. Clone pTT50 was selected (Fig. 1) due to its hybridization to the *Pst*I–*Hind*III fragment of pTT26. Its primary structure was

Fig. 1. Genetic map of the *bglB*–*lamA* genomic region. Clone pTT26 was described earlier (Dakhova *et al.*, 1993), pTT50 was selected from a partial library and pTTP1 was a product of asymmetrical (single primer) PCR with primer P1 (indicated). Clone pTT51 was used for expression of BglB. The palindromic structure resembling a putative transcription terminator is designated 'ter'. The GenBank accession number for the complete sequence of clone pTT51 (3786 bp) is Z77856, and for the sequence of clone pTT26 it is Z47974.

Table 1. Purification table for recombinant BglB

Activity was determined by degradation of PNP- β -glucoside in 50 mM phosphate buffer, pH 6.1, at 90 °C.

Step	Vol. (ml)	Total activity (U)	Protein (mg)	Protein concn (mg ml ⁻¹)	Yield (%)	Sp. act. (U ml ⁻¹)	Purification (-fold)
Cleared lysate	24	268	178	7.4	100	1.5	1.0
Heated lysate	22	262	29.7	1.3	98	8.8	5.9
Ultrafiltrate	10	215	26.0	2.6	80	8.3	5.5
DEAE column	7.6	166	9.7	1.3	62	17.1	11.4
Ultrafiltrate	2.0	160	8.8	4.5	60	18.2	12
2nd heating	1.9	141	2.5	1.3	53	57	38
Phenyl-Sepharose	6.6	84	0.33	0.05	31	255	169

determined and shown to contain only one part each of the *lamA* and the β -glucosidase ORF. The upstream DNA of the β -glucosidase ORF was amplified from chromosomal DNA by asymmetric PCR using a single oligonucleotide primer, P1. The 1 kb PCR product was ligated to pGEM-T vector DNA (pTTP1; Fig. 1). To combine the two overlapping DNA fragments containing the presumable β -glucosidase gene, the 2.7 kb insert DNA fragment of *Pst*I–*Bam*HI restricted pTT50 DNA was ligated to plasmid pTTP1 DNA digested with restriction endonucleases *Pst*I (polylinker site of the vector near the P1 primer site) and *Bam*HI, to yield clone pTT51. Its physical map corresponded to the chromosomal region as evaluated by DNA–DNA hybridization of the insert fragment with several restriction digests of chromosomal DNA. Clone pTT51 expressed high β -glucosidase activity in the recombinant *E. coli* host.

The nucleotide sequence (2696 bases) of clone pTT51 (accession no. Z77856) overlapped with the previously published *lamA* DNA sequence (accession no. Z47974; Zverlov *et al.*, 1997) and revealed one long ORF from base 428–2626 ending with an opal termination codon (TGA). A potential ribosome-binding site (Shine–Dalgarno sequence) was found, 7 bp from an ATG start codon at base 467, which would result in a coding region of 720 aa with a calculated molecular mass of 81 625 Da. Upstream of the β -glucosidase gene neither a palindromic structure reminiscent of a transcription terminator nor a similarity to bacterial promoter sequences was apparent. Despite the lack of an obvious promoter structure, and despite cloning of the insert fragment in opposite direction to the *lacZ* reading frame of the pGEM-T vector, about 4 mg BglB protein (1 culture)⁻¹ was produced by the recombinant *E. coli* (pTT51) cells.

Analysis of the deduced amino acid sequence revealed the presence of the glycosylhydrolase family 3 consensus pattern (PROSITE PS00775) between aa 233–257. Since a member of glycosylhydrolase family 1 (β -glucosidase subgroup A) has already been identified and named *blgA*

in *Thermotoga maritima* (Liebl *et al.*, 1994), the β -glucosidase gene of *T. neapolitana* (β -glucosidase subgroup B) was designated *bglB*. Upstream of *bglB* an incomplete ORF *orf1* was detected, with an intergenic distance of 170 bp between its stop codon and the potential Shine–Dalgarno sequence of *bglB*. The deduced amino acid sequence of this incomplete ORF showed 35–42% sequence identity to the C-terminus of the ATP-binding proteins OppD and OppF of the oligopeptide transport complexes e.g. of *E. coli* (accession no. D90763), *Bacillus subtilis* (accession no. X56347) or *Streptococcus pyogenes* (accession no. X89237).

Purification and characterization of the recombinant β -glucosidase B

BglB was purified to electrophoretic homogeneity by heat precipitation of host proteins, ion exchange and hydrophobic interaction chromatography (Table 1). Analysis of the purified enzyme on SDS-PAGE revealed one sharp protein band with a molecular mass of 81 kDa. The purified protein showed maximum activity at a temperature of 95 °C and at a pH of 5.5. Half of its activity was inactivated in 1, 3 and > 18 h at 95, 90 and 85 °C, respectively. The enzyme was most active on the substrates PNP- β -D-glucopyranoside with a specific activity of 255 U mg⁻¹ on PNP- β -glucoside under optimal conditions (100%) and on PNP- β -D-xylopyranoside (43%). It had almost no activity on α -glucoside and other aryl- β -glycosides of hexoses and pentoses (Table 2). BglB was also active on the alkyl- β -glucosides laminaribiose (1,3- β -) and cellobiose (1,4- β -) with an almost 15-fold higher reaction velocity on laminaribiose (Table 2). To evaluate the substrate preferences, the Michaelis constant was deduced from a graph of the activity of purified BglB [in phosphate-citrate (PC) buffer, pH 5.5, 90 °C, 10 min] with different concentrations of the substrates PNP- β -glucoside (K_m = 0.1 mM), cellobiose (50 mM) and laminaribiose (10 mM).

Table 2. Substrate specificity of BglB

The purified protein was incubated at pH 6.2 in 50 mM PC buffer at 90 °C for 10 min; 100% activity on PNP- β -glucoside corresponds to 255 U (mg protein)⁻¹. ND, Not determined.

Substrate	Relative activity (%)	K _m (mM)
PNP- β -glucoside	100	0.1
PNP- β -xyloside	43	ND
PNP- β -cellobioside	15	ND
PNP- β -fucoside	10	ND
PNP- β -lactoside	0	ND
PNP- β -galactoside	2.3	ND
PNP- α -glucoside	0	ND
Cellobiose	2.3	50
Laminaribiose	31.8	10

T.n.	M-EKVNEILSQTLEEKSETCSGGWTSVGVKSHSGWRCGETHPVPRVGLPAFVLADGGFAGLRINP	66
C.t.	MAVDIKKIITKQMLEKAGLCSG-----LDPWHTKFPVERLGIPTSIMQDGGSLAR--K	51
T.n.	TRENDE-----NTYTTAFPEIMLASTWNRELLEEVGKMGEEVREYGVVLLGPAFNIHRNPLCG	128
C.t.	QREDAETIADINNSVPATCFPSAAGLACSWDELVERVGAALGEECAENVSILLGEGANIKKSLQ	118
T.n.	RNFYYSDEPVLISGEMASSFVKGVSQGVGACIKHPANNQETWRVVDITVIERALREIYLRGFEI	195
C.t.	RNFYTFEPDYLISSELAASHIKGVQSQGVGACIKHPANNQEHRRVTVDITVDERTLREIYFASFEN	180
T.n.	AVKSKFMSVMSAYNKLNGKYCSQNEWLLKKVLEEWGFEFGFVMSWAGDNFVEOLKAGNDLIMP	262
C.t.	AVKKARFVVMCAYNKLNGEYCSNRYLLTEVLKNEMHDDGFVVDGAVNDRVSGLDGLLEMP	251
T.n.	KAYQVMTERRDEIEEIMALKKGLSEEVLDCEVNIILKVLVNAFSPQNYRYSNKFDELEHAKVAYE	329
C.t.	-TSHGITDKK-----IVEAVKSGKLSNTILNRAVERILKVII--MALENKKENAYEQDAHRLARQ	310
T.n.	AGAEGVLLKME-EALPLSENSKIALFQTGQIETIKGTSGSDTHPRYAISILEGIKERGNFDEEL	395
C.t.	AAAESVLLKNEDDVLPLKSGTIALIGAFVKPKRYQSGSSSHITPTRLDDIYEELKKAGA--DK-V	374
T.n.	AKIY-EDY-IKKMRETEEYKPRDSWGTIIKPKLSENFSEKVEVHLAKKNDVAVIVISRISGEGYD	460
C.t.	NLVYSEGRLNDGIDEELI-----NEAKKAASSSDVAV--VEAGLPDE--YE	418
T.n.	RKPV-KGDFYLSDDTDLINTVSREFHEQKKVILVNLIGSPVEVSWRDLVDGILLVWQAQGETR	526
C.t.	SEGDFRTMSIPENQNRLEAVA---EVQSNIVVLLN-GSPVE-MPWIDKVSLEAYLGQALGG	480
T.n.	IVADVLTGRINPSPGKLETTFFPRDSDVPSW-TFPGEPKDNPKVYVEEDIVGYRYDTFGVEPAYE	592
C.t.	RWRMCYSVK-SIVGKLAETFPVKLSHNSYSYLMFGE---DDRVEYKGLFVGIRYDTKGIEFLFP	542
T.n.	FGYGLSYITFEYSDLAV---SFDGETLRVQYRIENTGGAGKEVSQVYIKAPKGIKDPFQELKAF	655
C.t.	FGHGLSYITFEYSDISVDKKDVSNSIINVSVKVGKMGAKETVQLVYKDVKSVRERPEKELKGF	609
T.n.	HNTRLNPFGESEEVVLEIPVDLASFNGE--EMVVEAGEYEVVRGASSRNKLGKTFSVSGEERRFKP	720
C.t.	EKV-FLNFGEEKTFTTLDKRAFAYNTQIKDNRVESGEFLILIGRSSRDIVLKSERVNSTVKIRK	675

Fig. 2. Sequence alignment of the β -glucosidase genes (*bglB*) of *T. neapolitana* and *C. thermocellum*. T.n., *T. neapolitana* gene *bglB* (accession no. Z77856); C.t., *C. thermocellum* gene *bglB* (accession no. X15644). Identical amino acids are emphasized by a colon, patterns conserved throughout the glycosylhydrolase family 3 β -glucosidases by boxes. The putative active site amino acid residue is indicated by the arrowhead.

The enzyme seemed therefore to be ideally suited for hydrolysis of laminaridextrins. A mixture of laminaribiose, -triose, -tetraose, etc., derived from the degradation of laminarin by purified recombinant 1,3- β -glucanase LamA of the same organism (Zverlov *et al.*, 1997), was incubated with BglB. The reaction products were analysed by HPLC. After 2 h incubation, only glucose could be detected as the end product, indicating complete hydrolysis of the oligosaccharides by the β -glucosidase B (Fig. 3).

DISCUSSION

The deduced amino acid sequence of *T. neapolitana* gene *bglB* was up to 48% identical (out of 645 aa residues) to the β -glucosidases of *Clostridium thermocellum* (BglB, accession no. X15644), *C. stercorarium* (BglZ, accession no. Z94045), *Butyrivibrio fibrisolvens* (BglA, accession no. M32210), and to other bacterial and eukaryotic β -glucosidases belonging to glycosylhydrolase family 3 (Henrissat & Bairoch, 1993). The homology was especially high in two regions (Fig. 2), the N-terminal aa 42–390 (71.8% sequence similarity to BglB of *C. thermocellum*) and the C-terminal aa 437–722 (78.6% similarity). These homologous regions corresponded to the well-conserved N- and C-terminal parts of β -glucosidase subclass B (Gräbnitz *et al.*, 1989; Rojas & Romeu, 1996), which are block-inverted in the β -glucosidases of *B. fibrisolvens* and *Ruminococcus albus* (accession no. X15415). It should be mentioned that the point of permutation is situated around aa 400 in a non-conserved region (Fig. 2).

Almost all conserved residues derived from nine bacterial and eukaryotic β -glucosidases (Rojas & Romeu, 1996), especially the blocks of well-conserved residues, were also present in BglB. This also holds true for the SDW motif containing the Asp-243 residue, which was observed to bind the conduritol B-epoxi inhibitor in the *Aspergillus wentii* β -glucosidase (Bause *et al.*, 1980). This presumptive active site lies at the end of an extraordinarily well-conserved region (aa 127–246) having 71.4% sequence identity and 92.4% similarity with BglB of *C. thermocellum*.

The *bglB* gene of *T. neapolitana* is the first β -glucosidase

Fig. 3. HPLC analysis of undigested laminarin, and of the hydrolysis products after digestion of laminarin with β -glucanase LamA (73 kDa) and β -glucosidase LamA + β -glucosidase BglB. G1, glucose; G2, laminaribiose; G3, laminaritriose; G4, laminaritetraose.

gene of glycosylhydrolase family 3 to be cloned and sequenced in *Thermotogales*. A β -glucosidase with a pH optimum, K_m and substrate specificity similar to the purified recombinant β -glucosidase BglB has been purified from *Thermotoga* sp. FjSS3-B.1 (Ruttersmith & Daniel, 1993). β -Glucosidases corresponding to BglB in pH optimum and substrate specificity have also been isolated from *T. neapolitana* and *T. maritima* cultures (Bok *et al.*, 1994).

The N-terminus of BglB does not resemble a typical bacterial leader peptide, thus making secretion through the membrane unlikely. This is consistent with the observation that 80% of the β -glucosidases could be isolated from the cytoplasm of *T. neapolitana* cells (Bok *et al.*, 1994). The deduced 16 N-terminal aa of BglB were identical to the N-terminus of the purified native FjSS3-B.1 β -glucosidase (MEKVNEILSELTLLEEK) except for a conservative exchange ($Q_{10} \rightarrow E_{10}$), confirming the identity of both proteins and the translation start point deduced from the *bglB* nucleotide sequence (Ruttersmith & Daniel, 1993).

The *Orf1* gene product, encoded upstream of BglB, is possibly an ATP-binding protein component of a complex ABC transporter machinery, and might also have a function in laminarin utilization. Since the ATP-binding genes *oppD-oppF* and their homologous genes (PIR accession nos P37313, P45094, P08007, P36637) are the last proteins transcribed in the corresponding ABC transporter operons (Podbielski *et al.*, 1996), the presence of an *opp* operon upstream of *bglB* is likely and awaits further investigation. The lack of palindromic nucleotide sequences between *bglB* and *lamA* or *orf1*, and the absence of sequences resembling a typical promoter in both intergenic regions, support the hypothesis that the genes are transcribed from a promoter located upstream of *orf1*. Preliminary Northern blot experiments with cloned DNA fragments from clone pTT26 resulted in the detection of a large mRNA in *T. neapolitana* (> 7 kb, data not shown) suggesting a read through of transcription in that genomic region which could end at the presumptive terminator structure downstream of *lamA* (Zverlov *et al.*, 1997).

β -Glucosidases of glycosylhydrolase family 3 can be distinguished from family 1 enzymes by their activity on β -xyloside and β -galactoside: BglB of *T. neapolitana* behaved as a typical glycosylhydrolase of family 3, being active on β -xyloside, but having only a very limited activity on β -galactoside (Table 2); it was classified as a β -glucosidase (EC 3.2.1.21). Due to its activity on 1,3- β -oligosaccharides and its possible involvement in the *in vivo* hydrolysis of 1,3- β -glucans, BglB may be designated as laminaribiase.

β -Glucosidase BglB has, besides its activity on 1,4- β -glucosides, high activity on laminaribiose and could provide *T. neapolitana* cells with the ability to use 1,3- β -glucans as carbon source. The clustering of both genes, endoglucanase LamA and the laminaribiase BglB, in tandem and their possible cotranscription suggests a common *in vivo* function in the 1,3- β -glucan hydrolysis.

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