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Characterization, cloning and sequencing of a thermostable endo-(1,3-1,4) β-glucanase-encoding gene from an alkalophilic *Bacillus brevis*

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Abstract. A *Bacillus brevis* gene coding for an endo-(1,3-1,4)-β-glucanase was cloned in *Escherichia coli* and sequenced. The open reading frame contains a sequence of 759 nucleotides encoding a polypeptide of 252 amino acid residues. The amino acid sequence of the β-glucanase gene showed only a 50% similarity to previously published data for *Bacillus* endo-(1,3-1,4)-β-glucanases. The optimum temperature and pH for enzyme activity were 65–70° C and 8–10, respectively. When held at 75° C for 1 h, 75% residual activity was measured. The molecular mass was estimated to be about 29 kDa on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and the enzyme was found to be resistant to SDS.

Introduction

The enzyme endo-(1,3-1,4)-β-glucanase (EC 3.2.1.73) is able to hydrolyse the mixed linked (1,3-1,4)-β-glucans that constitute the major part of the endosperm cell walls of cereals such as oats and barley. Several species of the genus *Bacillus* secrete β-glucanases. Many of the β-glucanase-encoding genes have been cloned and sequenced: from *B. subtilis,* (Murphy et al. 1984, Tezuka et al. 1989), *B. amyloliquefaciens,* (Hofemeister et al. 1986); *B. macerans,* (Borriss et al. 1990) and *B. licheniformis* (Lloberas et al. 1991).

The major application of endo-(1,3-1,4)-β-glucanases are to be found within the brewing industry, where they are used to substitute and supplement malt enzymes (Cantwell and McConnell 1983). Thermostable β-glucanases are particularly suited for this, as they are not inactivated as rapidly during the kilning and mashing processes.

In this paper we describe the isolation of an alkalophilic *B. brevis* producing a thermostable β-glucanase, the subsequent cloning and sequencing of the gene and the characterization of certain novel biochemical properties of the enzyme.

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Materials and methods

Isolation of alkalophilic bacteria producing endo-(1,3-1,4)-β-glucanase. Approximately 3 g soil was pasteurized (Norris et al. 1981) before being used to inoculate 100 ml of alkalophilic enrichment broth, pH 10.0 (0.5% yeast extract, 0.5% polypeptone, 0.1% dipotassium hydrogen phosphate, 0.02% magnesium sulphate and 1% sodium carbonate as modified from Takeuchi et al. (1989) and incubated for 4–5 days at 37° C. Aliquots from the turbid broth cultures were then inoculated onto the same medium solidified with agar (15 g/l) and incubated at 37° C for 24–48 h.

The cultures were purified on nutrient agar plates, pH 9.0, at 37° C. They were then classified as *Bacillus* sp. after a Gram stain, a spore stain and a catalase test gave positive results. Colonies were then transferred to Luria agar plates (10 g tryptone, 5 g yeast extract, 10 g sodium chloride and 15 g agar per litre, adjusted to pH 9.0) and supplemented with 0.1% (w/v) lichenan from *Cetraria islandica* (Sigma). After incubation at 37° C for 24–48 h, plates were flooded with 0.1% (w/v) Congo red (Cantwell and McConnell 1983). Colonies producing a clear zone were grown in Luria broth plus 0.1% lichenan for 2–3 days at 37° C and pH 9.0. The enzyme produced in the supernatant was then evaluated. Further strain identification was done by utilizing the API 50CH and 20E strips.

Endo-(1,3-1,4)-β-glucanase determination. The determination of β-glucanase activity was carried out using lichenan as a substrate (Tezuka et al. 1989). One unit was defined as the amount of enzyme that produced 1 μmol reducing sugar calculated as glucose per minute under the conditions of assay.

Bacterial strains, growth media and vectors. The *Escherichia coli* host strain for the original cloning experiment was JM101 (Messing 1979), whereas for sequencing the *E. coli* rec$^-$ strain LK112 was used (Zabeau and Stanley 1982). The cloning vector was the positive selection vector pEcoR251 (Zabeau and Stanley 1982). Subcloning for sequencing was done using the high copy number Bluescript (SK) vector. *E. coli* LK112 and JM101 were grown in Luria broth, pH 7.2, and the donor *B. brevis* strain was grown in Luria broth, pH 9.0, and maintained on Luria agar plates, pH 9.0. All strains were grown at 37° C.

Preparation of DNA and recombinant DNA techniques. Chromosomal DNA was isolated from *B. brevis* according to the meth-

od of Lovett and Keggins (1979). Partial *Sau*3A restriction of the *B. brevis* DNA and isolation of size fractionated fragments from low-melting-point agarose gels using agarase (CalBiochem) were according to the methods of Sambrook et al. (1989). DNA fragments ranging between 1.5 kb and 4 kb were isolated. Plasmid DNA was prepared by the method of Gryczan et al. (1978) and purified by cesium chloride-ethidium bromide equilibrium centrifugation as described by Sambrook et al. (1989). A *B. brevis* genomic library was constructed by ligating the partially digested *Sau*3A chromosomal DNA to *Bgl*II-endonuclease-digested p*E-co*R251. The *E. coli* JM101 and LK112 cells were made competent and transformed according to the method of Sambrook et al. (1989). DNA fragments for subcloning were isolated from 0.8% agarose TRIS/acetate gels using the Geneclean kit (Bio 101, Calif., USA). Positive transformants were selected for their ability to form clear halos on Luria agar plates containing 0.1% (w/v) lichenan in addition to ampicillin (100 µg/ml) after flooding with Congo red.

Preparation of supernatant and cell lysate fractions. The recombinant *E. coli* strain was grown overnight in Luria broth plus 0.1% lichenan and ampicillin (100 µg/ml). The supernatant as well as the periplasmic and sonicated fractions were assayed separately. The recovery of extracellular, periplasmic and cytoplasmic enzymes was done as described by Cornelis et al. (1982).

Substrate specificity of the recombinant β-glucanase. In order to determine enzyme specificity, the activity of the sonicated fraction of the recombinant *E. coli* strain JM101 containing the cloned β-glucanase gene was assayed according to Tezuka et al. (1989) at pH 9.0 using, in addition to lichenan, other high molecular mass substrates such as carboxymethylcellulose (CMC), avicel, laminarin and xylan at a final concentration of 0.5% (w/v). For calculating specific activities, protein concentration of the sonicated fraction was measured using the biuret method (Herbert et al. 1971).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) protein assay. Proteins were analysed on 15% SDS-polyacrylamide gels (Laemmli 1970). To visualize enzyme activity, samples were pre-treated by incubating for 40 min at 60° C in sample buffer and run on SDS-PAGE activity gels containing 0.1% lichenan (Zverlov and Velikodvorskaya 1990). To remove the SDS the gels were washed in phosphate buffer, pH 6.3 (Beguin 1983). The bands of enzyme activity were detected by staining the lichenan/PAGE gel with Congo red.

Restriction mapping and nucleotide sequencing. Restriction enzymes were from Boehringer Mannheim (FRG) and used according to the manufacturers specifications. The recombinant plasmids were characterized by restriction mapping using standard procedures (Sambrook et al. 1989). The Exo-III Mung bean nuclease technique was used to create a deletion series for sequencing (Heinikoff 1987). The nucleotide sequence[1] of the β-glucanase gene was determined by the dideoxynucleotide-chain-termination method (Sanger et al. 1977) using the Sequenase II Kit (USB) according to the manufacturers specifications. The nucleotide and deduced amino acid sequences were analysed using the Genetics Computer Group (GCG) software package (version 7.0). All the current databases accompanying the GCG package were screened for related nucleotide and amino acid sequences.

Results

Isolation of B. brevis ALK 36

Approximately 100 *Bacillus* spp. were evaluated in liquid culture and of these, one strain, designated ALK 36, was found to produce an alkaline endo-(1,3-1,4)-β-glucanase. This strain was identified as belonging to the species *B. brevis*. It was found to be able to grow at temperatures ranging from 37° C to 50° C and the pH growth range was between 7.0 and 11.0 with optimum growth at pH 9.0.

Effect of pH on enzyme activity and stability

The activity of the crude enzyme extract was determined at various pH values (Fig. 1). The pH was adjusted using Sorensen phosphate buffer at neutral to acid pH values and TRIS buffer at alkaline values. Other conditions were as for the standard assay method. The crude enzyme exhibited activity over a broad pH range with optimum activity at pH 9.0. Approximately 80–100% activity occurred between pH 7.0 and pH 10.0, after which the activity declined rapidly.

Effect of temperature on activity and stability

The ALK 36 endo-(1,3-1,4)-β-glucanase exhibited 95–100% activity between 65° C and 70° C (Fig. 2). At 75° C at least 75% of maximum activity was retained. Thermal stability of the enzyme was measured at pH 7.0 by incubating the enzyme at different temperatures for the indicated time period. Samples were withdrawn and the residual activity measured and results shown in Fig. 3. At 70° C, approximately 85% of activity remained after 1 h whereas at 80° C the activity was found to drop off rapidly.

Cloning of the B. brevis Alk 36 β-glucanase-encoding gene

E. coli JM101 was transformed with the genomic library of *B. brevis*. From 1800 transformants four showed hydrolysis of lichenan by plate assays. One of the transfor-

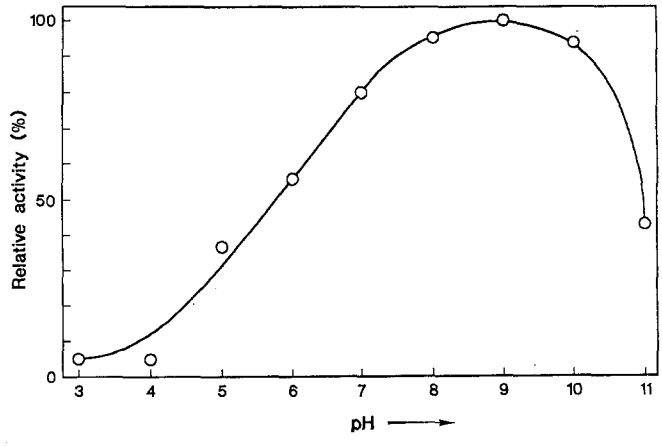

Fig. 1. Influence of pH on endo-(1,3-1,4)-β-glucanase activity. The enzyme activities at various pH values were measured by the standard assay at 40° C

[1] *Nucleotide sequence accession number.* The nucleotide sequence reported has been assigned GenBank accession number M84339

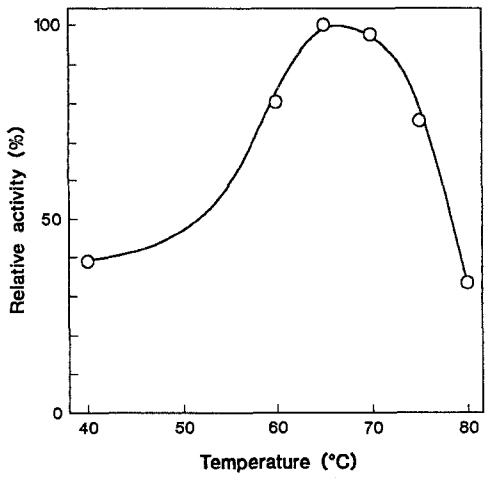

Fig. 2. Influence of temperature on endo-(1,3-1,4) β-glucanase activity. The enzyme activities at various temperatures were measured by the standard assay at pH 7.0

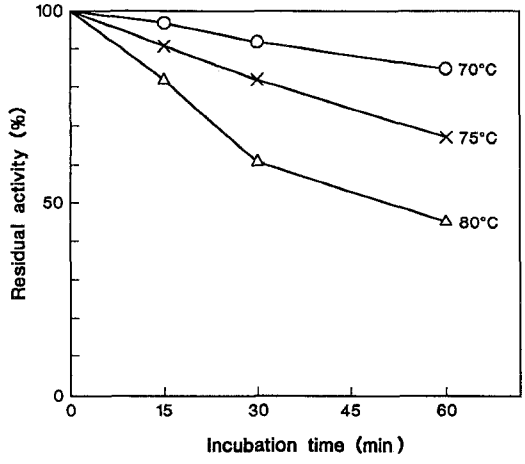

Fig. 3. Influence of temperature on the stability of endo-(1,3-1,4) β-glucanase. The enzyme was assayed at pH 7.0

mants was selected and the residing plasmid was designated pNA3. From preliminary mapping pNA3 was shown to harbour a 1.65-kb insert containing the β-glucanase-encoding (*bglBB*) gene.

Substrate specificity of the enzyme

The cloned *B. brevis* ALK 36 enzyme was characterized as an endo-(1,3-1,4)-β-glucanase or lichenase on the basis of its substrate specificity (Table 1).

Cellular localization of β-glucanase activity in E. coli JM101

In order to study the distribution of the β-glucanase synthesized in *E. coli* (pNA3) extracellular, periplasmic and cytoplasmic fractions were isolated and enzyme activity assayed (Table 2). Most of the β-glucanase enzyme activity was located in the cytoplasmic fraction and periplasmic region with very little enzyme being secreted into the extracellular medium.

Table 1. Substrate specificity of the β-glucanase enzyme produced by *Escherichia coli* JM101 (pNA3)

Substrates	U/ml	U/mg protein
Lichenan	279.90	39.78
Laminarin	0.89	0.13
Carboxymethylcellulose	0.29	0.04
Xylan (birchwood)	0.43	0.06
Avicel	0.24	0.03

Table 2. Localization of the β-glucanase enzyme produced by *E. coli* JM101 (pNA3)

Cell fraction	Distribution of total activity (%)
Extracellular	18
Periplasmic	36
Cytoplasmic	46

SDS-PAGE protein analysis

Zymograms that detected β-glucanase activity of the renatured proteins separated by SDS-PAGE gave a molecular mass of approximately 29 kDa. When the gels

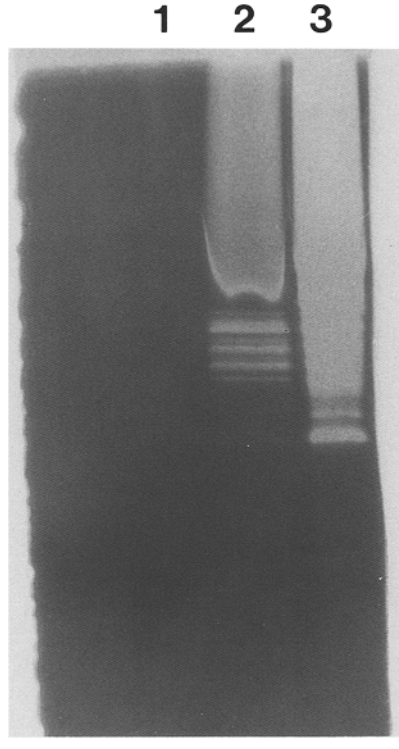

Fig. 4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis activity gel stained with Congo red: *lane 1*, sonicated extract of *Escherichia coli* JM101 (pEcoR251); *lane 2*, supernatant from *Bacillus brevis* Alk 36 grown for 24 h in Luria broth plus 0.1% lichenan; *lane 3*, sonicated extract of *E. coli* JM101 (pNA3)

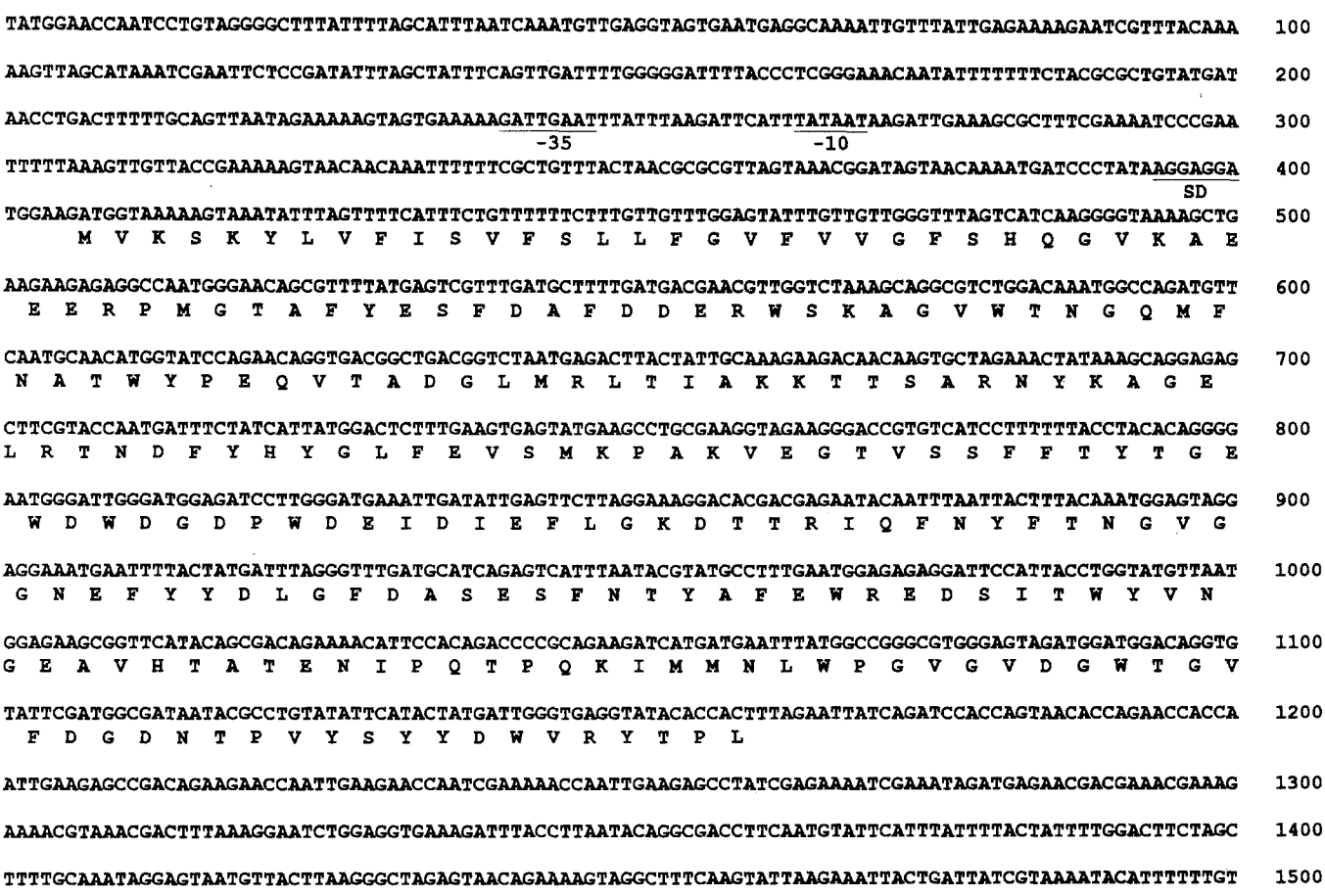

Fig. 5. Nucleotide sequence of the insert in pNA3, containing the *B. brevis bglBB* gene. The nucleotide sequence is *numbered* throughout. The deduced amino acid sequence of the β-glucanase is shown in *single-letter code* below the coding sequence. Putative promoter sequences (-35 and -10 regions) and the ribosome binding site (*SD*) are *underlined*

were stained, a zone of activity was detected running the length of the PAGE gel (Fig. 4). This indicated SDS-resistance of the enzyme, and it was found that SDS-PAGE gels could be stained directly with Congo red without the removal of SDS. Enzyme activity was assayed in the presence of different concentrations of SDS (ranging from 0.1% to 10%). It was found that no inhibition of enzyme activity occurred up to a concentration of 10% SDS.

Nucleotide sequence of the bglBB gene

The nucleotide sequence of the insert in plasmid pNA3 was determined (Fig. 5) and found to contain a single open reading frame (ORF) of 759 nt, which began with an ATG at position 407 and ended with a TAG at position 1165. The predicted size of the polypeptide encoded by this ORF was 252 amino acids, which has a calculated relative molecular mass (M_r) of 29000. A classical ribosome binding site was located 6–14 nt upstream of the ATG initiation codon (5′-AAGGAGGA-3′). A putative promoter sequence, (5′-GATTGAAT-N_{16}-TATAAT-3′) was located upstream from the ATG start codon from position 241 to 270 (Fig. 5). Sequence comparisons at the nucleotide and amino acid levels indicated that this ORF was a β-glucanase-encoding gene. The similarity of the deduced amino acid sequence of *B. brevis* β-glucanase to the other reported β-glucanases was determined on the basis of identical amino acid sequence comparisons (Table 3). The highest similarity was found to the polypeptide sequence from *B. macer-*

Table 3. Similarity of nucleotides in different endo-(1,3-1,4)-β-glucanase genes

	Bm	Bs	Bs2	Bl	Ba	Bp	Ct
Bb	53	51	50	51	49	54	46
Bm	—	69	69	68	70	88	49
Bs	—	—	99	87	91	72	48
Bs2	—	—	—	86	90	72	47
Bl	—	—	—	—	84	73	54
Ba					—	71	46
Bp							46

Sequence data of the proteins were taken from Tezuka et al. (1989) for *Bacillus subtilis* (Bs) and Murphy et al. (1984) for a second *B. subtilis* strain (Bs2), Lloberas et al. (1991) for *B. licheniformis* (Bl), Hofemeister et al. (1986) for *B. amyloliquefaciens* (Ba), Borris et al. (1990) for *B. macerans* (Bm), Gosalbes et al. (1991) for *B. polymyxa* (Bp), Zverlov et al. (1990) for *Clostridium thermocellum* (Ct) and *B. brevis* (Bb) (this paper)

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                                                                    60
Bl    MSYRVKRMLMLLVTGLFLSLSTFAASASAQT--------GGSFYEPF-NNYNTGLWQKADG
Bs    MPY-LKRVLLLLVTGLFMSLFAVTSTASAQT--------GGSFFDPF-NGYNSGFWQKADG
Ba    M----KRVLLILVTGLFMSLCGITSSVSAQT--------GGSFFEPF-NSYNSGLWQKADG
Bs2   MPY-LKRVLLLLVTGLFMSLFAVTATASAQT--------GGSFFDPF-NGYNSGFWQKADG
Bm    M--KKKSC-FTLVTTFAFSLI---FSVSALA--------GSVFWEPL-SYFNRSTWEKADG
Bp    M--MKKKSWFTLMITGVISLF---FSVSAFA--------GNVFWEPL-SYFNSSTWQKADG
Bb    MV-KSKYLVFISVFSLLFGVFVVGFSHQGVKAEEERPMGTAFYESF-DAFDDERWSKAGV
Ct    M---KNRVISLLMASLLLVLSVIVAPF--YKAEAATVVNTPFVAVFRSNFDSVQWKK---
      *    .      .      .                        *   .   ..   * *
                                                                   120
Bl    YSNGNMFNCTWRANNVSMTSLGEMRLSLTSP--SYNKFDCGENRSVQTYGYGLYEVNMKP
Bs    YSNGNMFNCTWRANNVSMTSLGEMRLALTSP--SYNKFDCGENRSVQTYGYGLYEVRMKP
Ba    YSNGDMFNCTWRANNVSMTSLGEMRLALTSP--SYNKFDCGENRSVQTYGYGLYEVRMKP
Bs2   YSNGNMFNCTWRANNVSMTSLGEMRLALTSP--AYNKFDCGENRSVQTYGYGLYEVRMKP
Bm    YSNGGVFNCTWRANNVNFTNDGKLKLGLTSS--AYNKFDCAEYRSTNIYGYGLYEVSMKP
Bp    YSNGQMFNCTWRANNVNFTNDGKLKMSLTSP--ANNKFDCGEYRSTNNYGYGLYEVSMKP
Bb    WTNGQMFNATWYPEQVTADGLMRLTIAKKTT--SARNYKAGELRTNDFYHYGLFEVSMKP
Ct    --RWAKFVSTVLEAFTGDISNGKMILTLDREYGGSYPYKSGEYRTKSFFGYGYYEVRMKA
            *  *              . .       .   .  .* *.   . ** .** **.
                                                                   180
Bl    AKNVGIVSSFFTYTGPTD--GTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKIVNLGFD
Bs    AKNTGIVSSFFTYTGPTD--GTPWDEIDIEFLGKDTTKVQFNYYTNGAGNHEKIVDLGFD
Ba    AKNTGIVSSFFTYTGPTE--GTPWDEIDIEFLGKDTTKVQFNYYTNGAGNHEKFADLGFD
Bs2   AKNTGIVSSFFTYTGPTD--GTPWDEIDIEFLGKDTTKVQFNYYTNGAGNHEKIVDLGFD
Bm    AKNTGIVSSFFTYTGPAH--GTQWDEIDIEFLGKDQTKVQFNYYTNGVGGHEKVISVGFD
Bp    AKNTGIVSSFFTYTGPSH--GTQWDEIDIEFLGKDTTKVQFNYYTNGVGGHEKIINLGFD
Bb    AKVEGTVSSFFTYTGEWDWDGDPWDEIDIEFLGKDTTRIQFNYFTNGVGGNEFYYDLGFD
Ct    AKNVGIVSSFFTYTGPS--DNNPWDEIDIEFLGKDTTKVQFNWYKNGVGGNEYLHNLGFD
      **   * *********        ************ *..*** . ** * .*    .***
                                                                   240
Bl    AANSYHTYAFDWQPNSIKWYVDGQLKHTATTQIPQTPGKIMMNLWNGAGVDEWLGSYNGV
Bs    AANAYHTYAFDWQPNSIKWYVDGQLKHTATNQIPTTPGKIMMNLWNGTGVDEWLGSYNGV
Ba    AANAYHTYAFDWQPNSIKWYVDGQLKHTATTQIPAAPGKIMMNLWNGTGVDDWLGSYNGV
Bs2   AANAYHTYAFDWQPNSIKWYVDGQLKHTATNQIPTTLGKIMMNLWNGTGVDEWLGSYNGV
Bm    ASKGFHTYAFDWQPGYIKWYVDGVLKHTATANIPSTPGKIMMNLWNGTGVDDWLGSYNGA
Bp    ASTSFHTYAFDWQPGYIKWYVDGVLKHTATTNIPSTPGKIMMNLWNGTGVDSWLGSYNGA
Bb    ASESFNTYAFEWREDSITWYVNGEAVHTATENIPQTPQKIMMNLWPGVGVDGWTGVFDGD
Ct    ASQDFHTYGFEWRPDYIDFYVDGKKVYRGTRNIPVTPGKIMMNLWPGIGVDEWLGRYDG-
      *.  ..**.*.*.   *  **.*     .* .** .  ******* * *** * * ..*
                  256
Bl    T-PLSRSLHWVRYTKR
Bs    N-PLYAHYDWVRYTKK
Ba    N-PIYAHYDWMRYRKK
Bs2   N-PLYAHYDWVRYTKK
Bm    N-PLYAEYDWVKYTSN
Bp    N-PLYAEYDWVKYTSN
Bb    NTPVYSYYDWVRYTPL
Ct    RTPLQAEYGICKILS-
        *.       .
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Fig. 6. Alignment of the amino acid sequence of the *B. brevis* (Bb) beta-glucanase with enzymes from different *Bacillus* strains. Identical and conserved amino acids among the eight proteins are marked with *asterisks* and *dots,* respectively

ans (53%) and to that of *B. polymyxa* (54%), but all sequences showed significant sequence similarities. It is interesting to note that these two sequences from *B. macerans* and *B. polymyxa* are very closely related (88% indentity), whereas the mesophilic β-glucanases form a separate cluster based on homology as previously reported (Hofemeister et al. 1986). The *B. brevis* β-glucanase gene and the *Clostridium thermocellum* laminarinase show a greater divergence in sequence similarity. Alignment of the predicted polypeptides from the different *Bacillus* β-glucanases and the *C. thermocellum* laminarinase (Fig. 6) revealed considerable homology in a number of highly conserved blocks, particularly in the central and the C-terminal parts of the proteins. The N-terminal region has the most pronounced variation between all the different sequences.

Discussion

The endo-(1,3-1,4)-β-glucanase isolated from *B. brevis* Alk 36 has some unique properties. It has a higher temperature optimum then previously reported for *Bacillus* lichenases, with the exception of *B. macerans*. The temperature profiles of the *B. macerans* and *B. brevis* lichenases are similar, but the *B. brevis* lichenase exhibits a greater residual activity at a higher temperature than that of the *B. macerans* enzyme, viz 75% activity after 1 h at 75° C for *B. brevis* lichenase as opposed to approximately 10% activity after 1 h at 70° C for *B. macerans* enzyme.

The pH optimum of the enzyme is unusual as the pH optima for bacterial lichenases tend to fall between pH 6.0 and pH 8.0, whereas that of the *B. brevis* lichenase exhibits activities of greater then 80% from pH 7.0 to pH 10.0. This pH profile is, however, similar to that of the β-1,3-glucanase isolated from an alkalophilic *Bacil-*

lus sp. by Nogi and Horikoshi (1990) and to the β-1,3-glucanase or laminarinase isolated from *C. thermocellum* by Zverlov and Velikodvorskaya (1990). These alkalophilic laminarinase enzymes both have similar thermostability profiles to that of the *B. brevis* lichenase. The distinction between β-1,3-glucanase and (1,3-1,4)-β-glucanases can be rather tenuous as it is based on substrate specificity for laminarin as opposed to lichenan, respectively. Since the substrate specificity of the *B. brevis* enzyme was found to be 300-fold more for lichenan than laminarin, it was classified as a (1,3-1,4)-β-glucanase. The laminarinase from *C. thermocellum* was, however, classified as such despite a 100-fold higher activity on lichenan (Zverlov and Velikodvorskaya 1990).

Another novel characteristic of the *B. brevis* (1,3-1,4)-β-glucanase enzyme is its resistance to SDS; this is the first report of an SDS-resistant β-glucanase. A molecular mass of approximately 29 kDa was derived by SDS-PAGE. This agrees with the molecular mass calculated from the sequence of 29050 Da and is similar to the previously published endo-(1,3-1,4)-β-glucanases, which are estimated to be between 25 and 30 kDa. An exception is the endo-(1,3-1,4)-β-glucanase cloned from *B. circulans,* which was reported to have a molecular mass of 40.5 kDa (Bueno et al. 1990). There is, however, some discrepancy as to whether this enzyme is a true lichenase due to the absence in the sequence of the conserved active centre found in all bacterial lichenases. In addition, its broad enyzme substrate specificity suggests that it might no be a true lichenase (Gosalbes et al. 1991).

The N-terminal portion of the β-glucanase polypeptide deduced from the nucleotide sequence contains a typical signal sequence. A positively charged N-terminus, with lysine residues at position 3 and 5, is followed by a highly hydrophobic region from residues 7 to 24. There is a valine at position 29 followed by an alanine at 31 which conforms with the "-3, -1 rule" for cleavage sites proposed by von Heijne (1988). This evidence points to a possible signal sequence of approximately 31 amino acids that may be cleaved from the mature protein. However, the *B. brevis* β-glucanase protein from cell extracts of *E. coli* or supernatants of *B. brevis* had an M_r of 29 kDa, which agrees with the size calculated from the nucleotide sequence. Localization experiments indicated that the protein was found mostly in the cytoplasmic and periplasmic fractions and to a lesser extent in the extracellular medium. It has been suggested that the production of β-glucanase alters the permeability of the outer membrane of *E. coli* and so allows leakage of the periplasmic space proteins (Cantwell et al. 1988).

Comparison of the amino acid sequences of the different β-glucanases showed that the genes are strongly conserved and that regions of identical amino acids are found throughout the sequence. The most extensive of these is the block (amino acids 143 to 161) proposed by Borris et al. (1990) to contain the active site of β-glucanases. This was found to be conserved in all the β-glucanase enzymes. The N-terminal region is least conserved between the all different sequences, in agreement with Hofemeister et al. (1986) who showed a great variation in the signal sequences of the highly homologous *B. subtilis* and *B. amyloliquefaciens* β-glucanase genes. Comparisons of the sequences of different β-glucanase proteins exhibiting novel characteristics should facilitate the identification of the roles played by particular amino acid residues and, ultimately, the modification of these proteins to suit specific industrial processes.

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References

Béguin P (1983) Detection of cellulase activity in polyacrylamide gels using Congo Red-stained agar replicas. Anal Biochem 131:333–336

Borriss R, Buettner K, Maentsaelae P (1990) Structure of the beta-1,3-1,4 glucanase gene of *Bacillus macerans*: homologies to other beta-glucanases. Mol Gen Genet 222:278–283

Bueno A, Vazquez de Aldana CR, Correa J, Villa TG, Rey F del (1990) Synthesis and secretion of a *Bacillus circulans* WL-12 1,3-1,4-β-glucanase in *Escherichia coli*. J Bacteriol 172:2160–2167

Cantwell BA, McConnell DJ (1983) Molecular cloning and expression of *Bacillus subtilis* β-glucanase gene in *Escherichia coli*. Gene 23:211–219

Cantwell BA, Sharp PM, Gormley E, McConnell DJ (1988) Molecular cloning of *Bacillus* β-glucanases. In: Aubert J-P, Béguin P, Millet J (eds) Biochemistry and genetics of cellulose degradation. Academic Press, London, pp 181–201

Cornelis P, Digneffe G, Willemot K (1982) Cloning and expression of a *Bacillus coagulans* gene in *Escherichia coli*. Mol Gen Genet 186:507–511

Gosalbes MJ, Pérez-González JA, González R, Navarro A (1991) Two β-glycanase genes are clustered in *Bacillus polymyxa*: molecular cloning, expression, and sequence analysis of genes encoding a xylanase and a endo-β-(1,3)-(1,4)-glucanase. J Bacteriol 173:7705–7710

Gryczan TJ, Contente S, Dubnau D (1978) Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J Bacteriol 134:318–329

Heijne G von (1988) Transcending the impenetrable: how proteins come to terms with membranes. Biochim Biophys Acta 947:307–333

Heinikoff S (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28:351–359

Herbert D, Phipps PJ, Strange RE (1971) Chemical analysis of microbial cells. Methods Microbiol 5B:209–344

Hofemeister J, Kurtz A, Borriss R, Knowles J (1986) The β-glucanase gene from *Bacillus amyloliquefaciens* shows extensive homology with that of *Bacillus subtilis*. Gene 49:177–187

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lloberas J, Perez-Pons JA, Querol E (1991) Molecular cloning, expression and nucleotide sequence of the endo-β-1,3-1,4-D-glucanase gene from *Bacillus licheniformis*. Eur J Biochem 197:337–343

Lovett PS, Keggins KM (1979) *B. subtilis* as a host for molecular cloning. Methods Enzymol 68:342–357

Messing J (1979) A multipurpose cloning system based on single stranded DNA bacteriophage M13. Recomb DNA Tech Bull 2:43

Murphy N, McConnell DJ, Cantwell BA (1984) The DNA sequence of the gene and genetic control sites for the excreted *B. subtilis* enzyme β-glucanase. Nucleic Acids Res 12:5355–5367

Nogi Y, Horikoshi K (1990) A thermostable alkaline β-1,3-glucanase produced by alkalophilic *Bacillus* sp. AG-430. Appl Microbiol Biotechnol 32:704–707

Norris JR, Berkeley RC, Logan NA, O'Donnell AG (1981) The genera *Bacillus* and *Sporolactobacillus*. In: Starr MP, Stolp H, Trüper HG, Barlows A, Schlegel HG (eds) The Prokaryotes, vol II: Springer, Berlin Heidelberg New York, pp 1724–1725

Sambrook J, Fritsch EF, Maniatis DF (1989) Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N. Y.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Nat Acad Sci USA 74:5463–5467

Takeuchi K, Nishino T, Oders M, Shiiuogaki H (1989) Alkaline proteases and microorganisms producing same. US patent no. 4797362

Tezuka H, Yuuki T, Yabuuchi (1989) Construction of a β-glucanase hyperproducing *Bacillus subtilis* using the cloned β-glucanase gene and a multi-copy plasmid. Agric Biol Chem 53:2335–2339

Zabeau M, Stanley KK (1982) Enhanced expression of cro-β-galactosidase fusion proteins studied under the control of the P_R promoter of bacteriophage λ. EMBO J 1:1217–1224

Zverlov VV, Velikodvorskaya GA (1990) Cloning the *Clostridum thermocellum* thermostable laminarinase gene in *Escherichia coli*, the properties of the enzyme thus produced. Biotechnol Lett 12:811–816