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PROPERTIES OF A THERMOACTIVE ß-1,3-1,4-GLUCANASE (LICHENASE) FROM *CLOSTRIDIUM THERMOCELLUM* EXPRESSED IN *ESCHERICHIA COLI*

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Summary: A *Clostridium thermocellum* gene (*licB*) encoding a thermoactive 1,3-1,4-ß-glucanase (lichenase) with a molecular weight of about 35 000 was localized on a 1.5-kb DNA fragment by cloning and expression in *E. coli*. The enzyme acts on ß-glucans with alternating ß-1,3- and ß-1,4-linkages such as barley ß-glucan and lichenan, but not on ß-glucans containing only 1,3- or 1,4-glucosidic bonds. It is active over a broad pH range (pH 5-12) and has a temperature optimum around 80°C. The *C. thermocellum* lichenase is unusually resistant against inactivation by heat, ethanol or ionic detergents. These properties make the enzyme highly suitable for industrial application in the mashing process of beer brewing.

Mixed-linkage ß-glucans containing alternating ß-1,3- and ß-1,4-linkages constitute a major component of the cell walls of the endosperm of cereal grains (barley ß-glucan) and of Icelandic moss (lichenan). Endo-ß-glucanases capable of degrading these ß-glucans can be grouped into 1,3-ß-glucanases (laminarinases, EC 3.2.1.39), 1,4-ß-glucanases (cellulases, EC 3.2.1.4), and 1,3-1,4-ß-glucanases (lichenases, EC 3.2.1.73). Lichenases specifically hydrolyze mixed-linkage glucans, but do not attack ß-1,4-linkages in carboxymethylcellulose or ß-1,3-linkages in laminarin.

Barley ß-glucan can cause problems in the brewing process such as reduced rates of wort separation and beer filtration. It can also lead to haze and gel formation in stored beer. These problems can be alleviated by supplementing malt enzymes

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with microbial ß-glucanases during mashing and fermentation (1). For this purpose enzymes are required which are active and stable under mashing conditions (pH 5.2-5.5, 50-70°C) (2).

Commercial enzyme preparations suitable for use in industrial fermentations have been produced mainly from bacilli (3,4). Genes encoding 1,3-1,4-ß-glucanases have been cloned from *Bacillus subtilis* (5), *B. amyloliquefaciens* (6), and *B. macerans* (7). Gene cloning revealed that such enzymes are also produced by the cellulolytic bacteria *Fibrobacter succinogenes* (8) and *Clostridium thermocellum* (9). In this communication we describe the isolation of the *C. thermocellum* lichenase gene *licB* and biotechnologically relevant properties of the encoded enzyme.

Materials and Methods

Bacterial strains and phages. *C. thermocellum* DSM 1237 (ATCC 27405) was obtained from the German Collection of Microorganisms. *E. coli* DH1 (*rec*A1, *end*A1, *hsd*R17) was obtained from the *E. coli* Genetic Stock Center, and grown in LB medium (10). The recombinant phage lambda LIC42 has been described previously (9).

Recombinant DNA techniques and cloning procedure. Preparation of plasmid DNA, endonuclease digestion, ligation, and transformation were performed by standard procedures. Restriction enzymes and T4 DNA ligase were used according to the specifications of the supplier (Boehringer, Mannheim). Transformed cells were plated on LB agar containing 0.2% (w/v) lichenan and ß-glucanase producing clones were detected by staining with Congo red.

Preparation of cell extracts. Cell-free extracts were prepared by freeze-thaw lysis of lysozyme-treated cells (10). Debris were removed by centrifugation at 45 000 *g* for 20 min. The supernatant was heated for 10 min by shaking in a 60°C water bath. The heat-precipitated proteins were removed by centrifugation for 15 min at 16 000 *g*. Protein concentrations of extracts were determined by the method of Sedmark and Grossberg (11).

Enzyme assay. ß-Glucanase activity was assayed by incubation for 30 min at 80°C in a 0.25% (w/v) solution of barley ß-glucan in 0.01 M Mes, pH 6.5. Reducing sugars released from the substrate were determined with the 3,5-dinitrosalicylic acid reagent (12). One enzyme unit corresponds to the release of 1 µmole of glucose equivalent per min.

Electrophoresis and zymogram technique. Polyacrylamide gel electrophoresis was performed in 10% polyacrylamide gels containing 0.1% (w/v) barley ß-glucan in the presence of 0.1% SDS. ß-Glucanase activity was detected by in situ staining with Congo red as described previously (13).

Materials. Barley ß-glucan was purchased from Biocon (Cork, Ireland). Lichenan, laminarin, Avicel, and carboxymethylcellulose (CMC) were from Sigma. Curdlan was obtained from Serva (Heidelberg). Phosphoric acid swollen Avicel was prepared as described by Wood (14).

Results and Discussion

The recombinant lambda phage LIC42 previously isolated from a genomic library of *C. thermocellum* DNA (9) was found to encode both a ß-glucosidase and a ß-glucanase, respectively. These genes are located on two *Pst*I fragments of roughly equal size (4.0 kb), which could be separated by subcloning in pBR322. A restriction map of the *Pst*I fragment carrying the *licB* gene is shown in Fig. 1. The approximate position of this gene was narrowed down by deletion analysis to the 1.5 kb DNA fragment between the unique *Sma*I site and the rightmost *Hin*dIII site. Expression studies using the 2.1 kb *Hin*dIII fragment indicate that the gene is transcribed in *E. coli* from its own promoter and that the direction of transcription is from left to right on the physical map presented in Fig. 1. Analysis of *E. coli* extracts by SDS-polyacrylamide gel electrophoresis and activity staining revealed a major ß-glucanase band of about 35 kDa and a minor band of 28 kDa, which presumably results from proteolytic processing of the primary gene product (data not shown).

The recombinant *C. thermocellum* enzyme was characterized as a 1,3-1,4-ß-glucanase (lichenase) on the basis of its substrate specificity. As shown in Table 1, the enzyme was highly active towards barley ß-glucan and lichenan but lacked activity towards laminarin, curdlan, and cellulosic substrates. No activity was detectable on xylan, amylose, pullulan, or p-nitrophenyl-ß-D-cellobioside. The specific activity of the crude enzyme preparation towards mixed-linkage glucans was about twice as high as that reported for purified *B. subtilis* lichenase (4). Analysis of the degradation products of barley ß-glucan by thin-layer chromatography suggested an endo-mode of action yielding a mixture of tri- and tetrasaccharides (data not shown).

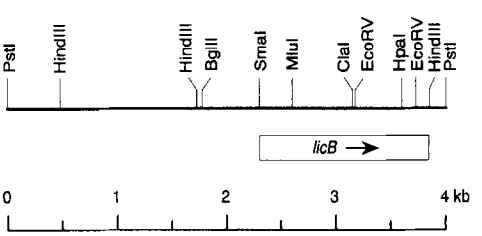


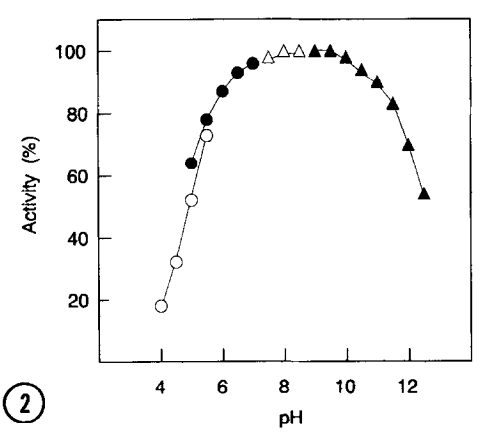
Fig. 1. Physical map of cloned *C. thermocellum* DNA expressing ß-1,3-1,4-glucanase activity. The open box indicates the approximate position of the *licB* gene. The arrow denotes the direction of transcription.

Table 1. Substrate specificity of ß-1,3-1,4-glucanase

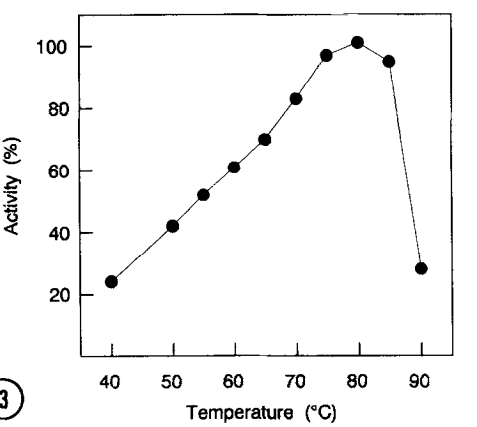
Substrate	Linkage	Specific activity (U/mg)
Barley ß-glucan	ß-1,3; ß-1,4	214
Lichenan	ß-1,3; ß-1,4	202
Laminarin	ß-1,3; ß-1,6	0
Curdlan	ß-1,3	0
Carboxymethylcellulose	ß-1,4	0
Avicel, acid swollen	ß-1,4	0

The pH profile shown in Fig. 2 indicates that the lichenase is active over an exceptionally broad pH range. The crude enzyme has optimal activity at pH 8-9 with half maximal values at pH 5.0 and 12.5. It is also remarkably thermoactive with optimal activity around 80°C (Fig. 3). The temperature optimum was not affected by the pH of the incubation mixture and nearly identical temperature profiles were observed at pH 5.0 and 9.5.

The enzyme exhibited a pronounced thermostability retaining 65% of its activity during a 24-h incubation at 75°C in the absence of substrate. The inactivation kinetics at 70 and 80°C (Fig. 4) indicate a loss of about 20% of the total activity during the first hour of incubation followed by a much slower decline in activity upon further incubation. These biphasic



(2)



(3)

Fig. 2. pH profile of ß-1,3-1,4-glucanase activity. Incubations were carried out for 30 min at 80°C in reaction mixtures containing 0.1 M acetate (o), 0.01 M Mes (●), 0.01 M Mops (Δ), or 0.01 M Chaps (▲) at the indicated pH.

Fig. 3. Temperature profile of ß-1,3-1,4-glucanase activity. Incubations were carried out at the indicated temperatures for 30 min in 0.01 M Mes, pH 6.5.

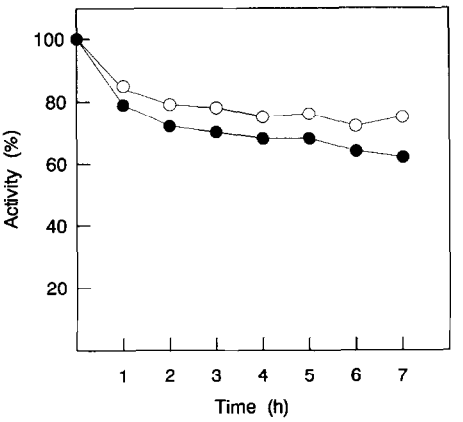


Fig. 4. Thermal stability of ß-1,3-1,4-glucanase in the absence of substrate. Extracts were incubated at 70°C (o) or 80°C (●) at pH 6.5 and assayed for residual activity.

kinetics point to a heterogeneity in the crude enzyme preparation and is consistent with the presence of two enzyme species detected by activity staining. Conceivably, proteolytic conversion of the 35-kDa enzyme to the 28-kDa form could affect the thermostability of the enzyme.

The data presented in Table 2 demonstrate that the activity of and/or stability of the lichenase is not dependent on the presence of divalent cations or reducing agents. The enzyme was not inhibited by ethanol concentrations up to 10% (w/v). Furthermore, the recombinant lichenase was exceptionally stable against denaturation by ionic detergents retaining 72% of its activity in the presence of 2.8% (w/v) SDS.

Table 2. Effect of chemicals on ß-1,3-1,4-glucanase activity

Compound added	Concentration (mM)	Activity (%)
None		100
$CaCl_2$	10	67
$MgCl_2$	10	94
$HgCl_2$	10	99
EDTA	10	82
Dithiothreitol	1	79
2-Mercaptoethanol	10	72
Ethanol	2000	110
SDS	10	92
SDS	100	72

In conclusion, the lichenase encoded by the *licB* gene of *C. thermocellum* clearly differs with respect to substrate specificity from the previously characterized *C. thermocellum cel* gene products (15,16) and from the recently identified laminarinases of the same organism (17,18). Its high activity at the pH and temperature range of the mashing process as well as its exceptional stability against denaturing conditions make it particularly suitable for reducing the viscosity of brewing mashes.

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