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CHARACTERIZATION AND PURIFICATION OF THERMOSTABLE β-GLUCOSIDASE FROM CLOSTRIDIUM THERMOCELLUM

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SUMMARY

A β-glucosidase was isolated from Clostridium thermocellum ; the enzyme was localized in the periplasmic space.

It was purified in a five-step procedure including ion-exchange chromatography on DEAE-Cellulose, chromatography on HA-Ultrogel and DEAE-Sephadex, gel filtration on AcA 34 Ultrogel and isoelectric focusing.

The final preparation was purified 944-fold with a recovery of about 5% of the initial enzyme activity.

Polyacrylamide disc electrophoresis of the purified enzyme gave a single band at pH 8.3. The enzyme is active towards cellobiose and p-nitrophenyl-β-D-glucoside(PNPG) and developed maximum activities at pH 6.0 and 65°C. A molecular weight of 50,000 daltons was estimated by gel filtration and the enzyme was isoelectric at pH 4.68.

INTRODUCTION

Studies on microbial degradation of cellulosic material have mainly been confined to mesophilic fungi and bacteria.

A thermophilic, anaerobic, cellulolytic bacteria, Clostridium thermocellum, produces large amounts of cellulase, comparable to that described for fungal cellulase (1,2). β-1,4 endo and exoglucanases were detected in the supernatant of the culture of this strain and their concerted action on cellulose produced cellodextrins and cellobiose.

The pathways by which cellodextrins and cellobiose are further metabolized are not yet known. The demonstration of the presence of cellodextrin and cellobiose phosphorylases in C. thermocellum (3,4) suggest that these products, after transport into the cells, could be phosphorolyzed with the formation of glucose 1-phosphate. However, most of the cellulolytic micro-

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organisms possess β-glucosidase (EC 3.2.1.21) (5-8) hydrolysing cellodextrins and cellobiose with formation of glucose. It seemed of interest to look for such an enzyme in C. thermocellum.

The purpose of the present work has been to isolate and purify the β-glucosidase from culture of C. thermocellum.

MATERIALS AND METHODS

Chemicals : Glucose oxidase, peroxidase, desoxyribonuclease, para nitrophenyl-β-D-glucopyranoside (PNPG), glucose-1-phosphate were obtained from Sigma; cellobiose and cystein from Fluka, 2-2'-Azino-di-3-(äthyl-benzthiazolin-sulfonat (6)) (ABTS) from Boehringer; AcA 34 Ultrogel and Ampholine pH 4-6 were purchased from LKB; DEAE Sephadex A 50 was from Pharmacia and Hydroxylapatite-Ultrogel (HA-Ultrogel) from Pharmindustrie; DEAE Cellulose (DE-52) was obtained from Wathman and xylose from Merck.

Culture and preparation of crude extracts :

Clostridium thermocellum NCIB 10 682 was grown anaerobically at 60°C on CM3 medium as described by Weimer and Zeikus (1) with cellobiose at $2gl^{-1}$. The cells were harvested during stationary phase by centrifugation and disrupted by sonication (Raytheon) in 4 volumes 50 mM potassium phosphate buffer (pH 7.0).

Enzyme assays :

β-glucosidase activity was estimated by measuring the release of p-nitrophenol from the substrate p-nitrophenyl-β-D-glucopyranoside (PNPG). A digest containing an appropriate amount of enzyme, 4 mM PNPG and 50 mM citrate-phosphate buffer (pH 6.0) was incubated at 60°C. Samples (1 ml) were removed during the first 30 min and were added to 2 ml of 1 M Na_2CO_3. The optical density of the liberated p-nitrophenol was measured at 400 nm.

One unit of β-glucosidase activity was defined as the amount of enzyme liberating one μmole of p-nitrophenol per min under the assay conditions.

β-glucosidase activity was also determined with cellobiose as a substrate. A reaction mixture containing an appropriate amount of enzyme, 40 mM cellobiose, 100 mM citrate-phosphate buffer (pH 6.0) was incubated at 60°C. Samples were removed during the first 30 min and were heat inactivated. The glucose content was determined using glucose oxidase, peroxidase and ABTS according to Werner et al (9).

One unit was defined as the amount of enzyme producing one μmole of glucose per min under the assay conditions.

Cellobiose phosphorylase was determined by measuring the release of Pi, upon incubation with glucose 1-phosphate and xylose as described by Alexander (4).

Column fractions were assayed for β-glucosidase activity in a 1.0 ml digest containing 4 mM PNPG, 50 mM citrate phosphate buffer (pH 6.0) and 5 μl of fractions at 60°C for 15 min.

Analytical methods :

Proteins were determined by the method of Lowry et al (10) or by the absorbance at 280 nm.

Analytical disc gel electrophoresis was carried out at pH 8.3 by the method of Davis (11) on 7.5% acrylamide gels with bromophenol blue as a tracking dye. A current of 3 mA/gel was applied , protein was stained with Coomassie blue.

To locate the β-glucosidase activity, the gel was incubated in 4 mM PNPG, 50 mM citrate-phosphate (pH 6.0) 15 min at 60°C; the position of the β-glucosidase showed as a yellow band.

Procedure for osmotic shock of cells :

C. thermocellum was grown at 60°C on a cellobiose medium and harvested in exponential phase. They were washed twice with 10 mM potassium phosphate buffer (pH 7.0), containing 30 mM NaCl.

Part of the sedimented cells were suspended in a measured volume of 50 mM potassium phosphate buffer (pH 7.0) and treated by ultrasonication. The rest of the cells were suspended in 10 mM potassium phosphate buffer (pH 7.0) containing 20% sucrose and 0.5 M EDTA. After 10 min, the suspension was centrifuged at 10,000 x g for 15 min at 4°C. The supernatant fluid was decanted and the pellet was rapidly dispersed in a volume of cold water (containing 1 mM $MgCl_2$) equal to that of the original suspension. The suspension was gently stirred in an ice bath for 10 min and next centrifuged at 10,000 g for 10 min at 4°C. The supernatant was termed "shock fluid". The pellet was suspended in 8 volumes of the original wet weight of bacteria and sonicated.

Phosphoglucose isomerase was assayed as described by Patni and Alexander (12).

Isoelectric Focusing :

A column of 110 ml capacity (LKB) was used for electrofocusing and the separations were essentially performed at 10°C as described by Vesterberg and Svensson (13). The density gradient was formed with sucrose. The concentration of the carrier ampholytes, Ampholine pH 4-6 (LKB) was 1% (w/v) and the anode was placed at the bottom of the column.

The experiments were performed with a final voltage of approximately 1000 v giving a maximum load of about 3 w. After focusing for 20 h, fractions of 2.1 ml were removed from the bottom of the column. The pH values of the fractions were immediately measured at 10°C.

RESULTS

Evidence for the periplasmic location of β-glucosidase.

When suspensions of intact cells of C. thermocellum were used as a source of β-glucosidase and compared with equivalents amounts of cell extracts, 80 to 100% of this activity was measurable with intact cells (Table I). This result was a first indication that the β-glucosidase seems localized outside the cytoplasmic membrane. In order to demonstrate the localization of this enzyme, a cell suspension was subjected to osmotic shock as described by Nossal and Heppel (14). Table I shows that 68% of this enzyme was released into the shock fluid. Only 6.5% of the phophoglucose isomerase,

TABLE I - CELLULAR LOCALIZATION OF β-GLUCOSIDASE IN CLOSTRIDIUM THERMOCELLUM

Fractions	Protein		β-glucosidase		Phosphoglucose isomerase	
	mg	%	total units	%	total units	%
Extracellular medium	-	-	44	7.2	-	-
intact cells	-	-	553	91	-	-
sonicated cells	156	100	608	100	105	100
shock fluid	130	88.3	415	68.2	6.8	6.5
lysate of shocked cells	15.4	6.8	144	23.7	79	75.2
phosphate sucrose EDTA	~0		48.6	8.0	3	2.8

TABLE II - PURIFICATION OF β-GLUCOSIDASE FROM CLOSTRIDIUM THERMOCELLUM

Purification steps	Total protein mg	Total activity U	Specific activity	Purification factor fold	Yield %
Crude extract	5 809	685	0.12	1.0	100
DEAE-Cellulose	168	302	1.80	15	44
HA-Ultrogel	53	283	5.33	44.4	41
DEAE-Sephadex	27.4	212	7.74	64.5	31
Ultrogel AcA 34	3	117.4	39	325	17
Isoelectric focusing	0.27	30.6	113.3	944	5

which may be considered as an intracytoplasmic enzyme, was found in the shock fluid. These results provide evidence for the presence of β-glucosidase in the periplasm of the cells.

Purification of β-glucosidase

The procedure developed for the isolation of this enzyme is shown in Table II. The washed cells were disrupted by sonication ; desoxyribonuclease at a final concentration of 10 µg/ml and 10 mM $MgCl_2$ were added to the suspension which was incubated for 2 h at + 4°C. The inactive precipitate obtained by centrifugation at 27,000 x g for 20 min was discarded. The extract was

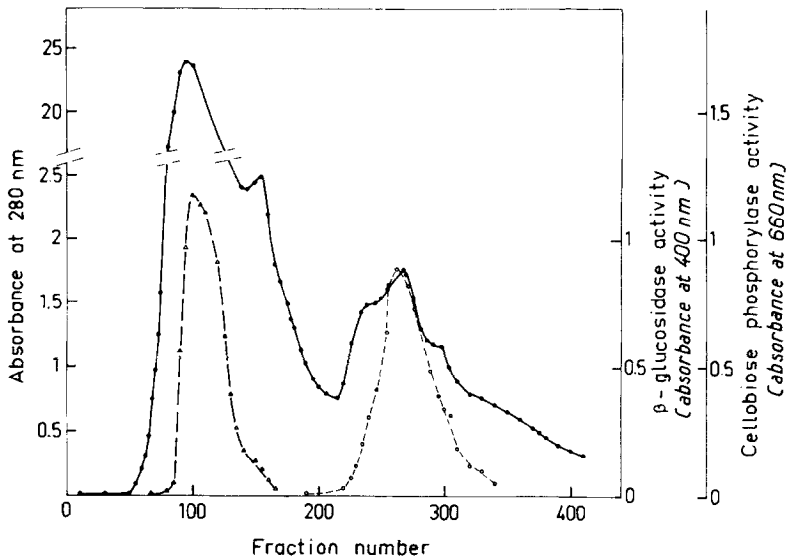

Figure 1 - Ion-exchange DEAE-cellulose DE 52 chromatography of crude extract. Column dimension : 2.6 x 63 cm, elution with 50 mM potassium phosphate buffer (pH 7.5), 3.3 ml fractions were collected. 280 nm absorbance (●—●); cellobiose phosphorylase activity (▲—▲); β-glucosidase activity (o—o).

dialyzed against 50 mM potassium phosphate (pH 7.5) and poured on to a column of DEAE Cellulose DE 52 which was previously equilibrated against the same buffer. Elution with this buffer separates the cellobiose phosphorylase emerging first from the column and the β-glucosidase which is retarded (Fig. 1). The active fractions were pooled and concentrated using an Amicon Ultrafiltration cell fitted with a PM10 membrane.

The solution dialyzed against 5 mM potassium phosphate buffer (pH 7.0) was applied to an HA-Ultrogel column equilibrated with the same buffer. The column was eluted with a linear potassium phosphate gradient from 5 mM to 200 mM. The β-glucosidase emerged from the column at 54 mM phosphate. The active fractions were pooled, concentrated, dialyzed and applied to a DEAE Sephadex A-50 column equilibrated with 50 mM potassium phosphate buffer (pH 7.0), the elution was realized with a linear gradient phosphate (50 mM-500 mM). After this step, the specific activity increased 1.5 fold.

Further purification of the β-glucosidase was achieved by gel filtration on a AcA 34 Ultrogel column equilibrated with 50 mM potassium phosphate

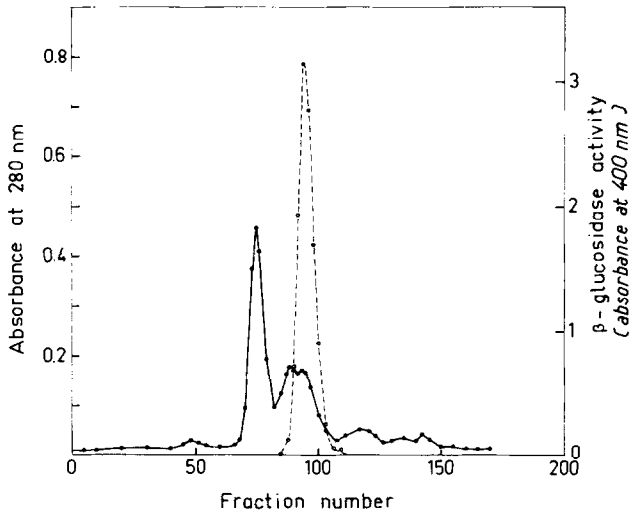

Figure 2 - Gel filtration pattern of β-glucosidase from DEAE Sephadex A 50 on an AcA 34 Ultrogel. The column (2.6 x 70 cm) was eluted with 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl; 3.3 ml fractions were collected. Absorbance at 280 nm (●—●); β-glucosidase activity (o—o).

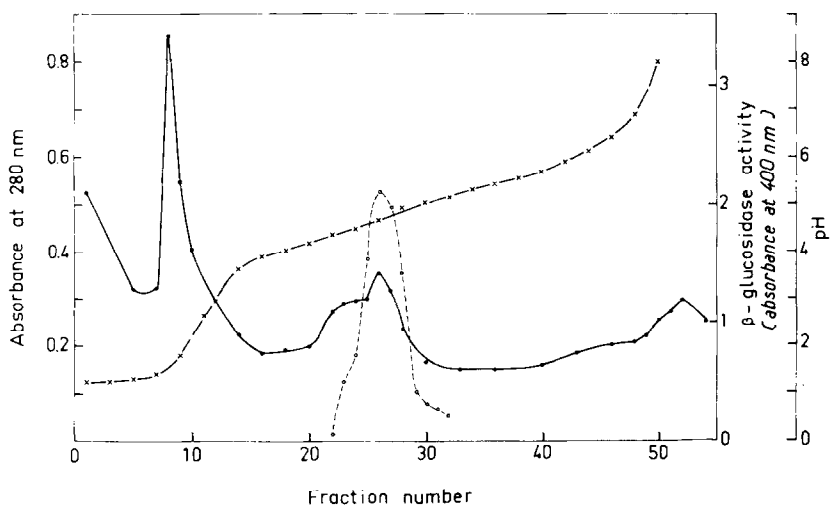

Figure 3 - Isoelectric focusing of β-glucosidase from AcA 34 Ultrogel. The concentration of carrier ampholytes (pH 4-6) was 1% (w/v). 2.1 ml fractions were collected ; fractions 25 to 29 were pooled. Details described in "Materials and Methods". Absorbance at 280 nm (●—●); β-glucosidase activity (o—o); pH of the fractions (x——x).

buffer (pH7.0) containing 0.1 M NaCl. The elution profil of the proteins is shown in Fig. 2 ; this step results in a good increase in specific activity.

The final step of purification was an isoelectric focusing in the pH range 4-6 (Fig. 3). The β-glucosidase activity was mainly focused at

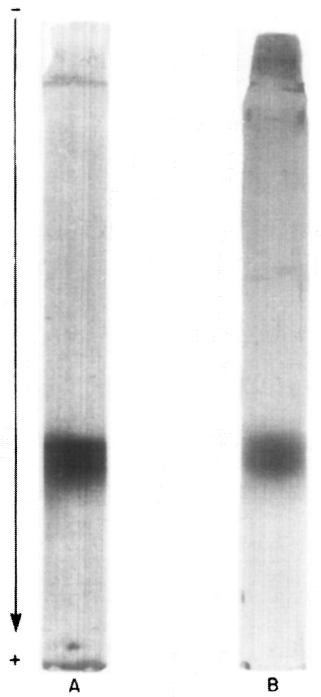

Figure 4 - Disc gel electrophoresis of β-glucosidase on 7.5% (w/v) polyacrylamide gels at pH 8.3. Samples (15 μg each) were applied. The gels were stained by Coomassie blue (A) and the enzyme activity detected as described in "Materials and Methods"(B).

pH 4.68. The carrier ampholytes and sucrose were removed from the enzyme by dialysis against 50 mM potassium phosphate buffer (pH7.0) and by molecular sieve chromatography on a column of Bio-Gel P.10.

Based on the crude starting enzyme preparation, the overall increase in specific activity of β-glucosidase was 944 fold with 5% yield of activity. The enzyme obtained from the final step of purification migrated as a single band in polyacrylamide gel electrophoresis at pH 8.3, showing β-glucosidase activity on PNPG, thus indicating a homogeneous protein (Fig. 4).

Some properties of β-glucosidase

- Enzyme assay conditions were chosen so that liberation of p-nitrophenol was linear with respect to both time and enzyme concentration.

- The effect of pH on the activity of β-glucosidase was examined at 60°C. The optimum pH was pH 6.0.

- The enzyme activity was assayed at various temperatures at pH 6.0. The optimum temperature was 65°C.

- From the elution position in a chromatographic run on AcA 34-Ultrogel, the molecular weight of the β-glucosidase was estimated to be about 50,000 daltons. The isoelectric point of the isolated β-glucosidase was determined to be at pH 4.68 from preparative electrofocusing.

- Preliminary determinations of the Michaelis constants show a Km value 2.10^{-3}M for PNPG and $8.3.10^{-2}$M for cellobiose showing that the affinity of β-glucosidase towards the natural substrat is smaller than for the synthetic substrate PNPG.

- Thermal stability of β-glucosidase : enzyme solution in 0.1 M citrate-phosphate buffer (pH 6.0) was incubated at 55°C. During the first 30 hours the β-glucosidase is quite stable and after 53 hours only 26% of its activity was lost. At 60°C, the β-glucosidase loses about 40% of its activity in 7 hours.

DISCUSSION

Although the multiplicity of β-glucosidase has been reported in some bacteria (7, 15), we found only one β-glucosidase in C.thermocellum. The greater part of this enzyme was found associated with cells. Similar observations have been reported for two other bacteria (7, 15). Thus, it appears that in bacteria, unlike fungi, the β-glucosidase is associated with cells and not released into the medium. The results of osmotic shock experiments demonstrate that the β-glucosidase of C. thermocellum is localized within the periplasmic space of the cells as reported in another bacteria (15); however the enzyme of Pseudomonas (8) was found mostly in cytoplasmic fraction. The purified enzyme from C.thermocellum is able to attack both PNPG and cellobiose, although the Km for PNPG is lower than for cellobiose. In this respect, the isolated enzyme resembles the β-glucosidase from other microorganisms

(5, 6, 17,16), but is different from the β-glucosidase of Stachybotrys atra (18) which has no activity towards cellobiose.

The molecular weight of 50,000 daltons estimated by gel filtration is in agreement with the values of 47,000 daltons (5) and 48,000 daltons (19) reported for the β-glucosidases from some fungi but it is different from the enzyme of Alcaligenes faecalis (17). β-glucosidase of C. thermocellum has been shown to be highly heat stable. In recent years, the β-glucosidase have attracted more and more interest mainly due to their potentiel importance in industrial saccharification of cellulose. The isolation of C. thermocellum β-glucosidase, considerably more thermostable than the analogous enzyme from different fungi and bacteria, could be of great interest in this industrial process.

The finding of the presence of a β-glucosidase in C. thermocellum and the capacity of this enzyme to hydrolyze cellobiose suggest that, besides the phosphorolytic cleavage of cellodextrins and cellobiose, another pathway may exist in this strain for the cellulose degradation. Cellobiose and cellodextrins, produced by cellulase from cellulose, may be hydrolyzed into glucose by the action of the β-glucosidase. Furthermore the location of this enzyme in the periplasmic space may afford that this hydrolysis takes place at the surface of the cells. The importance of this enzyme in the degradation of cellulose in C. thermocellum has to be demonstrated.

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