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PRODUCTION OF 1,3-β-GLUCANASE BY *BACILLUS* NO. 221 AN ALKALOPHILIC MICROORGANISM

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Summary

A 1,3-β-glucanase of *Bacillus* No. 221 has been extensively purified by a DEAE-cellulose column followed by a Sephadex G-75 gel filtration, and crystallized in ammonium sulfate solution. The crystalline enzyme is homogenous on the basis of polyacrylamide gel electrophoresis, sedimentation in ultracentrifuge (3.2 S), Ampholine electrofocusing (pI = 4.1) and dodecylsulfate-polyacrylamide gel electrophoresis (M_r = 36 000). The enzyme has an optimum pH for enzyme action at 8.5 which is higher than those of other 1,3-β-glucanases so far reported. The enzyme is very thermostable; about 90% of activity remains after being heated at 70°C for 10 min, and no effect of Ca^{2+} is observed. The enzyme does not hydrolyse laminaritriose, but hydrolyses laminaritetraose, and yields glucose and laminaritriose. The enzyme splits laminaran at random and yields glucose, laminaribiose, laminaritriose and higher oligosaccharides. From these results, this enzyme is a type of endo-1,3-β-glucanase.

Introduction

In the previous papers of this series [1—3], it has been reported that certain species of bacteria isolated from soil grow in alkaline media containing a high concentration of sodium carbonate. One of these bacteria, *Bacillus* No. 221, accumulates a large amount of alkaline protease having a pH optimum at 11.5—12. This strain also produces a thermostable 1,3-β-glucanase (1,3-β-D-glucan glucanohydrolase, EC 3.2.1.39) in a medium containing 1% sodium carbonate.

This paper deals with purification and some properties of 1,3-β-glucanase of this bacteria, and compares the *Bacillus* No. 221 enzyme and the *Bacillus circulans* enzyme [4].

Materials and Methods

Microorganism and medium

Bacillus No. 221 (ATCC 21522) [1] was grown aerobically for 3 days at 37°C in the following medium: pachyman, 20 g; polypeptone, 5 g; Difco yeast extract, 5 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; Na_2CO_3, 10 g; made up to 1 liter with water. Sodium carbonate was sterilized separately and added to the medium. Pachyman powder was treated with ether for 20 h in a Soxhlet apparatus.

Assay of 1,3-β-glucanase activity

The enzyme (0.02 ml) containing up to 0.02 unit in 0.01 M Tris · HCl buffer (pH 7.0) was mixed with 0.2 ml of laminaran solution (made up with 0.05 M Tris · HCl buffer, pH 8.5). After 10 min incubation at 40°C, 1 ml of dinitrosalycilic acid solution was added, and the mixture was heated in a boiling water bath for 5 min. Water (4 ml) was added to it and an absorbance at 510 nm was measured. One unit of the enzyme activity is defined as the amount of enzyme which produces one mg of reducing sugar calculated as glucose per min under the conditions described above.

Other procedures

Protein concentration was estimated by the method of Warburg and Christian [5]. Ampholine electrofocusing was done by the method of Vesterberg and Svensson [6]. Disc electrophoresis was carried out by the method of Davis [7]. Molecular weight of the enzyme was estimated by the dodecylsulfate-polyacrylamide gel electrophoresis method [8]. The hydrolysate was chromatographed on Whatman No. 1 paper with the solvent system of pyridine/ethylacetate/water (5 : 12 : 4, by vol.) at room temperature. Ammoniacal $AgNO_3$ was used as a spraying reagent. Preparation of the cell walls were reported previously [9]. Laminaran was obtained from K. and K. Laboratory, U.S.A., and Ampholine carrier ampholyte (pH 3—10) was from LKB-Producter AB, Sweden.

Results

Purification of the enzyme

Step 1. 5 l of the culture fluid were centrifuged at 6000 × *g* for 10 min. The supernatant fluid (about 1.2 units/ml) was passed through a DEAE · cellulose column (3 cm × 30 cm) equilibrated with 0.1 M phosphate buffer (pH 8.0). The column was successively washed with the same buffer containing 0.3 M NaCl. The enzyme was eluted with 0.2 M phosphate buffer (pH 5.0) containing 0.5 M NaCl.

Step 2. The enzyme was precipitated with 70% saturation of $(NH_4)_2SO_4$ and dissolved in 0.01 M Tris · HCl buffer (pH 7.0). The enzyme solution (35 ml) was passed through a Sephadex G-75 column (2.5 cm × 70 cm) equilibrated with Tris · HCl buffer (pH 7.5) containing 0.1 M NaCl.

Step 3. The enzyme was precipitated with 70% saturation of $(NH_4)_2SO_4$ and dissolved in minimum amount of water ($(NH_4)_2SO_4$ concentration was

TABLE I

Steps	Volume (ml)	Units/ml	Protein (mg/ml)	Specific activity (Units protein)	Recovery (%)
Cluture fluid	5000	1.2	1.9	0.6	100
DEAE—cellulose	450	8.7	1.2	7.1	65
Sephadex G-75	45	60.0	5.9	10.2	45
Crystals			210 mg*	11.0	39

* Expressed as total protein.

about 30% saturation). The columnar crystals were observed after a few hours. Table I summarizes the results of the purification of *Bacillus* No. 221 glucanase. Unless stated otherwise, the following experiments were carried out by using this preparation.

Physical properties of the enzyme

The purified enzyme showed only one symmetrical peak throughout duration of a run of 90 min at 201 000 × *g*. The sedimentation constant of the enzyme dissolved in 0.01 M Tris · HCl buffer (pH 8.0) containing 0.1 M NaCl (A_{280nm} approx. 5) was 3.2 S.

The enzyme migrated as a single band when subjected to disc electrophoresis by the method of Davis [7]. The molecular weight estimated by the method of dodecylsulfate-polyacrylamide gel electrophoresis was $3.6 \cdot 10^4$. The ultraviolet spectrum of the enzyme showed the typical ultraviolet absorption of protein. The ratio of absorption at 280 nm to 260 nm was about 1.9 which indicated the absence of nucleic acid.

The Ampholine electrofocusing showed that a single peak of the activity which had an isoelectric point at pH 4.1 was detected.

The results described above indicate that the enzyme preparation is homogenous.

Time course of hydrolysis

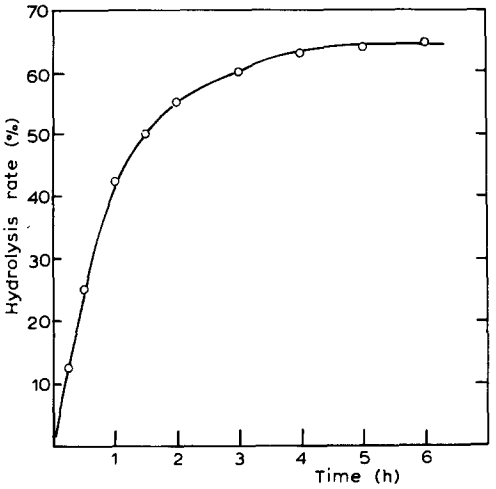
About 0.4 unit of the enzyme (0.15 ml) was mixed with 3 ml of the substrate solution (pH 8.0, 0.05 M Tris · HCl buffer). Aliquots (0.2 ml) of the reaction mixture were periodically taken, and reducing sugars were determined. As shown in Fig. 1, maximum degree of hydrolysis was about 65%. Addition of the enzyme (0.2 unit) did not cause further liberation of reducing sugars.

Effect of pH on activity

The glucanase activity of the enzyme was determined at various pH values. The pH was adjusted with McIlvaine buffer (pH 3.5—8.0); 0.05 M Tris/maleate buffer (pH 6.0—8.5); 0.05 M Tris · HCl (pH 7.0—9.0) and 0.05 M glycine/NaOH/NaCl buffer (pH 9.0—10.5). The enzyme is most active at pH 8.5, and as a reference, glucanase of *B. circulans* [4] was also tested under the same condition (Fig. 2).

Stability of the enzyme

Stability of the enzyme was investigated in buffer solutions of various pH



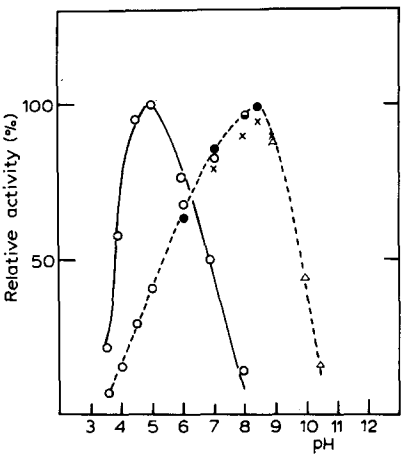


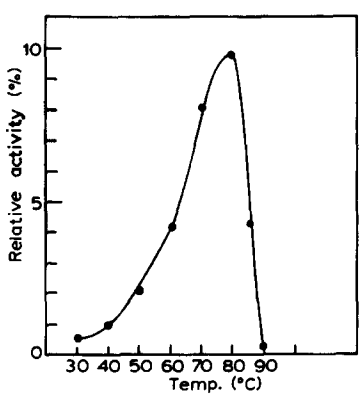
Fig. 1. Time course of hydrolysis. About 0.4 unit of the enzyme (0.15 ml) was mixed with 3 ml of the laminaran solution and incubated at 40° C.

Fig. 2. pH optimum. The pH was adjusted with the following buffer systems: ○———○, McIlvaine buffer; ●———●, Tris/maleate buffer; X———X, Tris · HCl buffer; △———△, glycine buffer. Solid line expresses pH vs activity curve of the *B. circulans* enzyme [4]; dotted line, that of the *Bacillus* No. 221 enzyme.

values. The mixtures were incubated at 75° C for 10 min and the residual activity was determined. The following buffer systems were used: 0.05 M Tris · HCl buffer (pH 7.5—9.5) and 0.05 M Tris/maleate buffer (pH 5.0—8.5). The enzyme was most stable at pH 7.0 and about 75% of the activity still remained.

Optimum temperature was determined by varying the incubation temperature. As shown in Fig. 3, maximum activity took place at 80° C.

The enzyme was dissolved in 0.05 M Tris · HCl buffer (pH 7.0) and heated at the indicated temperatures for 10 min in the presence or absence of Ca^{2+} (10 mM) or EDTA (10 mM). As shown in Fig. 4, the enzyme is quite stable up to 70° C and no effect of Ca^{2+} or EDTA was observed.



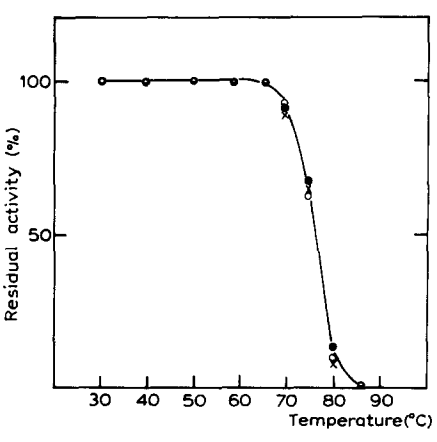


Fig. 3. Optimum temperature for enzyme action.

Fig. 4. Thermal stability. See text for the experimental details. ○———○, in the presence of Ca^{2+}; ●———●, in the absence of Ca^{2+}; X———X, in the presence of EDTA.

TABLE II

EFFECTS OF METAL IONS AND REAGENTS

Reagents added	Concentration (M)	Relative activity	
		Bacillus No. 221	*B. circulans* [4]
$HgCl_2$	$2 \cdot 10^{-3}$	0	0
Lead (II) acetate	$2 \cdot 10^{-3}$	100	100
$AgNO_3$	$2 \cdot 10^{-3}$	60	55
$MnSO_4$	$2 \cdot 10^{-3}$	120	130
$MgCl_2$	$2 \cdot 10^{-3}$	100	100
$CuSO_4$	$2 \cdot 10^{-3}$	130	95
$CoCl_2$	$2 \cdot 10^{-3}$	85	120
$FeSO_4$	$2 \cdot 10^{-3}$	100	98
$Fe_2(SO_4)_3$	$3 \cdot 10^{-3}$	115	96
$CaCl_2$	$2 \cdot 10^{-3}$	95	88
KCl	$2 \cdot 10^{-3}$	100	91
$AlCl_3$	$2 \cdot 10^{-3}$	100	85
$ZnSO_4$	$2 \cdot 10^{-3}$	0	65
$CdSO_4$	$2 \cdot 10^{-3}$	0	85
NaCl	$2 \cdot 10^{-3}$	100	100
P-Chloromercuribenzoate	$1 \cdot 10^{-4}$	100	95
EDTA	$1 \cdot 10^{-2}$	93	90

Effects of metal ions and inhibitors on enzyme action

To the comparative studies, the *Bacillus* No. 221 enzyme was incubated with metal ions in 0.05 M Tris · HCl buffer (pH 8.5) and the *B. circulans* enzyme in 0.05 M acetate buffer (pH 5.6) with metal ions. After 30 min incubation at 25° C, the residual activities were determined. The assay method of the *B. circulans* enzyme was the same as that of the *Bacillus* No. 221 enzyme except the pH of the substrate (pH 5.6, 0.05 M acetate buffer). As shown in Table II, the *B. circulans* enzyme is different from the *Bacillus* No. 221 enzyme, because the *Bacillus* No. 221 enzyme is completely inhibited by Cd^{2+} and Zn^{2+}, but the *B. circulans* enzyme is not. Neither *p*-chloromercuribenzoate nor EDTA inhibited both enzyme activities.

Hydrolysis of fungal cell walls

Fungal cell walls (5 mg) were mixed with 0.5 ml of 0.05 M Tris · HCl buffer (pH 8.5) containing 1.5 units of the enzyme and incubated at 40° C for 5 h. Hexoses liberated from the cell walls were determined by the anthrone method. The cell walls tested (*Mucor* sp., *Fusarium* sp. and *Aspergillus oryzae*) were not hydrolysed with the enzyme, although the *B. circulans* enzyme did (*Fusarium* and *A. oryzae*, 25—30%; *Mucor*, 15%).

Mode of action of the enzyme

About 1 mg of oligosaccharides (laminaribiose, laminaritriose, laminaritetraose and laminaripentaose) was dissolved in about 20 µl of the enzyme solution (0.5 unit in 10 mM Tris · HCl buffer, pH 8.5) and incubated for 2 h at 40° C. The reaction mixtures were chromatographed on Whatman No. 1 papers. Neither laminaribiose nor laminaritriose were hydrolyzed. Laminaritetraose was

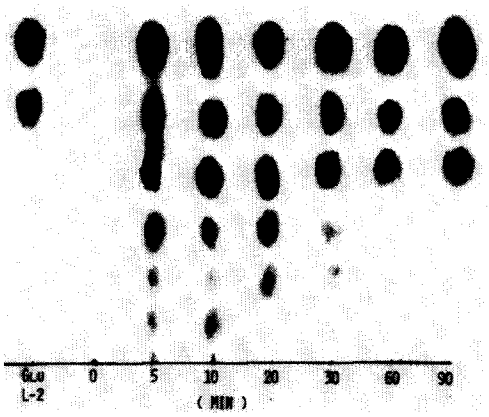


Fig. 5. Mode of action of the enzyme. About 10 mg of laminaran were dissolved in 1 ml of 10 mM Tris · HCl buffer (pH 8.5) containing 7 units of the enzyme and incubated at 40°C. The hydrolysates were chromatographed on Whatman No. 1 paper and sprayed with ammoniacal $AgNO_3$. Glu expresses glucose; L-2, laminaribiose.

hydrolyzed, and glucose and laminaritriose were detected. Laminaribiose was not, however, found in the hydrolysate. The enzyme hydrolyzed laminaripentaose and yielded glucose, laminaribiose, laminaritriose and laminaritetraose. After 20 h incubation, glucose, laminaribiose and laminaritriose were the final products. These results indicate that the enzyme does not hydrolyze laminaritriose. To confirm this result, the following crucial experiments were carried out. Laminaritriose (200 μg) was dissolved in 0.2 ml of 0.05 M Tris · HCl buffer (pH 8.5) containing 0.3 unit of the enzyme and incubated at 40°C for 3 h. Glucose liberated was determined by the glucose oxidase method [10]. As a reference, 1,3-β-glucanase of *B. circulans* was tested under the same conditions except pH (0.05 M Tris/maleate buffer, pH 5.6). The *B. circulans* enzyme hydrolyzed the substrate and yielded 42 μg of glucose but the *Bacillus* No. 221 enzyme only yielded 5—8 μg of glucose. These results sustain the results of paper chromatography of the enzyme digests described above.

About 10 mg of laminaran were dissolved in 1 ml of 10 mM Tris · HCl buffer (pH 8.5) containing 7 units of the enzyme and incubated at 40°C. Aliquots of the reaction mixture were periodically withdrawn and chromatographed on Whatman No. 1 paper. As is suggested by Fig. 5, it is a type of endo-1,3-β-glucanase.

Discussion

It is of interest that *B. circulans* 1,3-β-glucanase is stabilized by Ca^{2+}, but that the enzyme produced by alkalophilic bacteria *Bacillus* No. 221 is not. The action of the two enzymes on laminaritriose is entirely different: the *B. circulans* enzyme can hydrolyse laminaritriose and yields glucose and laminaribiose, whereas the *Bacillus* No. 221 enzyme cannot hydrolyse it. From the results, it is concluded that this enzyme is an unusual type of endo-1,3-β-glucanase which cannot hydrolyse laminaritriose. The effects of metal ions are also different: the *Bacillus* No. 221 enzyme is strongly inhibited by Cd^{2+} and Zn^{2+},

but the *B. circulans* enzyme is not inhibited. It is well-known that fungal cell walls are hydrolysed by the *B. circulans* enzyme [11]. However, the *Bacillus* No. 221 enzyme cannot hydrolyse the cell walls of *A. oryzae*. Although we do not have crucial experiments to solve this point, it might be due to the substrate specificity of the enzyme. From these results, we wish to conclude that the *Bacillus* No. 221 enzyme is different from other bacterial enzymes especially in its thermal stability and pH optimum for enzyme action.

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