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PURIFICATION AND SOME PROPERTIES OF ALKALINE PULLULANASE FROM A STRAIN OF BACILLUS NO. 202-1, AN ALKALOPHILIC MICROORGANISM*

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Summary

Pullulanase (pullulan 6-glucanohydrolase EC 3.2.1.41) was purified about 290-fold from the culture fluid of *Bacillus* No. 202-1 by DEAE-cellulose adsorption, acetone fractionation, $(NH_4)_2SO_4$ precipitation and DEAE-cellulose column chromatography followed by Sephadex G-200 molecular sieve chromatography. The enzyme gave a single band of protein by disc polyacrylamide gel electrophoresis. The molecular weight was estimated as 92 000 by sodium dodecyl sulfate gel electrophoresis. The isoelectric point was lower than pH 2.5. The optimum pH for enzyme action was about 8.5—9.0. The action of the enzyme on amylopectin and glycogen resulted in increase in the iodine coloration of 85 and 70%, respectively. The enzyme completely hydrolyzed 1,6-α-glucosidic linkages in amylopectin, glycogen and pullulan.

Introduction

Enzymes (EC 3.2.1.41, pullulan 6-glucanohydrolase) hydrolyzing 1,6-α-glucosidic linkages in glycogen and amylopectin are known to be present in yeast [1,2], higher plants [3] and some microorganisms [4—8].

We have found that a newly isolated soil bacterium (*Bacillus* sp. strain No. 202-1) produces an extracellular debranching enzyme in a high alkaline pH medium containing Na_2CO_3. Action of this enzyme on glycogen was different from pullulanase of *Aerobacter aerogenes*.

The present paper deals with a procedure for purification of this enzyme from the culture fluid and some of the physical and chemical properties of the purified enzyme are also presented.

* This paper is Part 9 of "Production of Enzymes by Alkalophilic Microorganisms". Part 8 is ref. 19.

Materials and Methods

Preparation of crude pullulanase

Bacillus No. 202-1 was grown aerobically at 37° C for 50—70 h in the medium containing soluble starch, 2%; yeast extracts (Difco), 0.5%; polypeptone, 0.5%; K_2HPO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.02%; and Na_2CO_3, 1%. Na_2CO_3 sterilized separately and added to the medium. The culture broth was centrifuged and the supernatant fluid was used as a crude pullulanase.

Assay of pullulanase activity

Pullulanase activity was determined by the method of Abdullah et al. [9] with modification. Reaction mixture (containing 0.3 ml of 1% pullulan adjusted to pH 8.0 with 0.1 M phosphate buffer and 0.05 ml of the enzyme) was incubated at 40° C for 30 min, unless stated otherwise.

The amount of reducing sugar liberated was determined and 1 unit of the enzyme is defined as the amount of the enzyme which liberates 1 μmol of reducing sugar calculated as glucose per min under above conditions.

Other procedures

Protein concentration was estimated by the method of Warburg and Christian [10]. Ampholine electrofocusing was done by the method of Vesterberg and Svensson [11]. Disc polyacrylamide gel electrophoresis was carried out by the method of Davis [12]. Molecular weight of the enzyme was estimated by sodium dodecyl sulfate -polacrylamide gel electrophoresis [13]. Total carbohydrate was determined by the anthrone-H_2SO_4 method described by Morris [14]. Reducing sugar was determined by 3,5-dinitrosalicylic acid method described by Summer and Somers [15].

Pullulan was obtained from Hayashibara Co., Japan. Potato amylopectin and oyster glycogen were from Wako Pure Chemicals Co., Japan. Crystalline pullulanase of *A. aerogenes* was from Amano Pharmaceutical Co., Japan. Crystalline β-amylase was from Schwarz Mann Research, U.S.A. Ampholine carrier ampholite (pH 2.5—4) was from L.K.B.-Producter, Sweden. Ficoll was from Pharmacia Fine Chemicals Co., Sweden. All other chemicals were of the highest quality available commercially.

Results

Purification of the enzyme. Purification was carried out at room temperature unless stated otherwise. The crude pullulanase (5000 ml) was adjusted to pH 8.0 with H_3PO_4. DEAE-cellulose powder (25 g) was suspended in the crude pullulanase solution and stirred vigorously for 30 min. The DEAE-cellulose was collected by filtration and washed with 2 l of 0.01 M phosphate buffer (pH 8.0). The enzyme adsorped on DEAE-cellulose was eluted with 900 ml of the same buffer containing 1 M NaCl. The enzyme (880 ml) was precipitated with 3 volumes of cold acetone (−20° C). The precipitate was collected by centrifugation and dissolved in 100 ml of 80% saturated $(NH_4)_2SO_4$ solution to remove contamined Na_2CO_3. The enzyme solution was dialyzed against 7 l of the same buffer containing 0.2 M NaCl for 30 h at 4° C. The insoluble material formed was removed by centrifugation.

The diffusate was passed through a column (2.3 × 45 cm) of DEAE-cellulose equilibrated with 0.01 M phosphate buffer (pH 8.0) containing 0.2 M NaCl and the column was washed with excess of the same buffer.

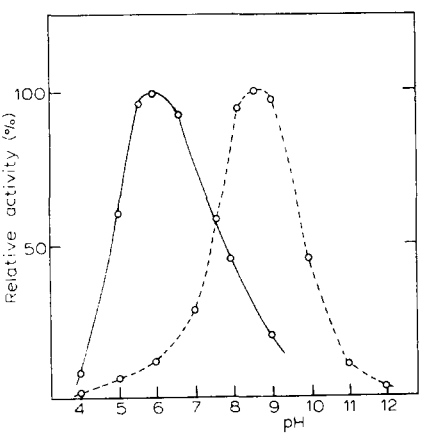
The enzyme was eluted by a linear gradient of NaCl concentration from 0.2 to 0.8 M in 750 ml of the same buffer (pH 8.0) each. Active fractions (250 ml) were combined and concentrated to 10 ml by using Ficoll. The concentrate was then subjected to gel filtration on Sephadex G-200 column (2.5 × 100 cm) equilibrated with 0.01 M phosphate buffer containing 0.1 M NaCl. Elution was performed with the same buffer. Active fractions (65 ml) were combined and concentrated. This enzyme was used for the following experiments.

Homogeneity of the enzyme. The enzyme preparation gave a single band of protein by disc polyacrylamide gel electrophoresis (pH 9.4). The molecular weight was 92 000 estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric point was lower than pH 2.5. In 0.01 M phosphate buffer (pH 8.0), the enzyme showed a typical absorption spectrum of protein. The ratio of absorption at 280 nm to 260 nm was about 1.9. The preparation was free from α-amylase and β-amylase.

Effect of pH on pullulanase activity. The pH vs activity curve of the purified enzyme is shown in Fig. 1. The optimum pH of this enzyme was from 8.5 to 9.0. As a reference, pullulanase of *A. aerogenes* was also tested under the same conditions.

Effect of temperature on pullulanase activity. The effect of temperature on pullulanase activity was measured at various temperatures. As shown in Fig. 2, the optimum temperature was 55° C at pH 8.0.

Thermal stability. Thermal stability of the enzyme was measured in 0.1 M phosphate buffer (pH 8.0) by heating at various temperatures for 15 min. As shown in Fig. 3, inactivation occurred gradually and the residual activity at 55° C was about 83%.



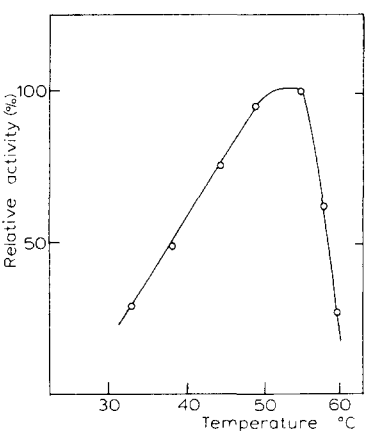


Fig. 1. Effect of pH on pullulanase activity. The pH was adjusted with the following buffer systems: acetate buffer (pH 4), phosphate buffer (pH 5—8.5), carbonate buffer (pH 9—11), borate buffer (pH 12). Solid line expresses pH vs activity curve of *A. aerogenes* enzyme and dotted line expresses *Bacillus* No. 202-1 enzyme.

Fig. 2. Effect of temperature on pullulanase activity.

pH stability. The enzyme solution at various pH values were incubated for 24 h at 4° C. After incubation, the enzyme was adjusted to pH 8.0 and residual activity was measured by the standard method. As shown in Fig. 4, the enzyme was stable at 4° C for 24 h at the range of pH 6.5—11, but inactivation occurred at below pH 6.5 and above pH 11.

Effect of various reagents on pullulanase activity. Results are expressed as inhibition percent compared with the activity in control. As shown in Table II, the enzyme was not inhibited by SH-group reagents and chelating agent such as iodoacetate, *p*-chloromercurybenzoate and EDTA. The enzyme was partially inhibited by $HgCl_2$ and $ZnSO_4$, but not sodium laurylsulfate, NaF, urea and ammonium molybdate.

Increase in iodine coloration by pullulanase of Bacillus No. 202-1 and A. aerogenes. The action of *Bacillus* No. 202-1 pullulanase on glycogen and amylopectin were compared with those of *A. aerogenes* pullulanase. The iodine coloration was measured as described by Yokobayashi et al. [7] with modification. Polysaccharide (2 mg) was first incubated at 40° C with either *Bacillus* No. 202-1 pullulanase (170 μg) at pH 8.5 or *A. aerogenes* pullulanase (450 μg) at pH 5.7 in a total volume of 1.0 ml for 5 h. The volume of 0.2-ml aliquots were mixed with 0.2 ml of 0.4% iodine/4% Kl solution. The mixture was diluted to 10 ml with water and used for determination of iodine coloration. *Bacillus* No. 202-1 and *A. aerogenes* pullulanases increased 85 and 45% in iodine coloration of potato amylopectin, respectively. *Bacillus* No. 202-1 pullulanase caused high increase (70%) in the iodine coloration, but low increase (13% at 470 nm) was observed with *A. aerogenes* pullulanase when oyster glycogen was used as substrate.

Effects of pullulanase of Bacillus No. 202-1 and A. aerogenes on amylopectin and glycogen. Polysaccharide (2 mg) was first treated with *Bacillus* No. 202-1 pullulanase (170 μg) at pH 8.5 or *A. aerogenes* pullulanase (450 μg) at pH 5.7 for 5 h at 40° C in a total volume of 1.0 ml. After heat inactivation, the reaction mixture were adjusted to pH 5.7 with 0.5 M phosphate buffer in a

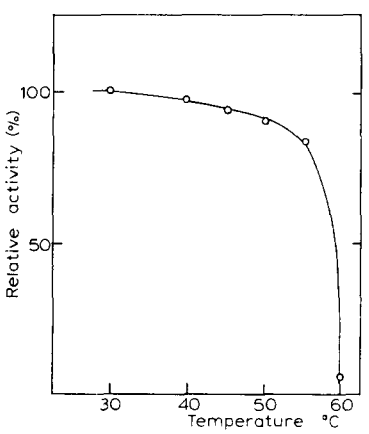


Fig. 3. Thermal stability. See the text for the experimental details.

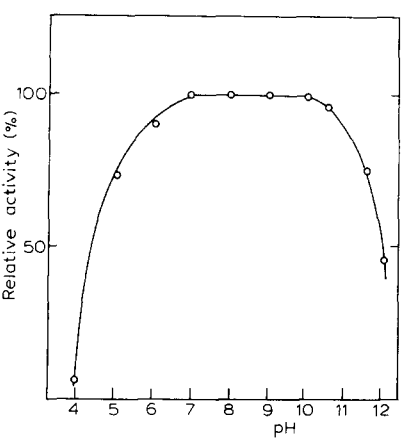


Fig. 4. pH stability. See the text for the experimental details.

TABLE I

PURIFICATION OF THE ENZYME

Steps	Volume (ml)	Protein (mg)	Activity (units)	Spec. act. (units/mg protein)	Recovery (%)
Cultured fluid	5000	69100	9450	0.14	100
DEAE-cellulose	880	5420	5720	1.05	61
Acetone (0—70%)	100	2590	4880	1.88	52
$(NH_4)_2SO_4$ (0—80%)	100	1350	3710	2.75	39
DEAE-cellulose	250	152	3070	20.2	32
Sephadex G-200	65	41	1650	40.2	17

TABLE II

EFFECTS OF VARIOUS REAGENTS ON PULLULANASE ACTIVITY

Reagents	Concentration (M)	Inhibition (%)
$HgCl_2$	10^{-3}	100
$ZnSO_4$	10^{-3}	96
$MgSO_4$	10^{-3}	44
$CuCl_2$	10^{-3}	35
EDTA	10^{-2}	0
Urea	7	0
Monoiodoacetate	10^{-2}	0
Ammonium molybdate	10^{-2}	0
p-Chloromercurybenzoate	10^{-3}	4
Sodium lauryl sulfate	0.1%	9
NaF	10^{-3}	9

total volume of 3 ml. Then the reaction mixture was incubated with 50 μl of β-amylase (500 μg) solution at 40° C for 30 h. As shown in Table III, amylopectin and glycogen were completely hydrolyzed to maltose by β-amylase and *Bacillus* No. 202-1 pullulanase. However, although pullulanase of *A. aerogenes* caused complete degradation of amylopectin on successive action with β-amylase, glycogen was partially hydrolyzed at this conditions.

TABLE III

EFFECTS OF PULLULANASE OF *BACILLUS* No. 202-1 AND *A. AEROGENES* ON AMYLOPECTIN AND GLYCOGEN

See the text for the experimental details.

Substrates	Conversion to maltose (%)		
	β-Amylase alone	Succesive action with β-amylase	
		Bacillus No. 202-1	*A. aerogenes*
Potato amylopectin	49	100	96
Glutinous rice amylopectin	52	102	103
Oyster glycogen	35	97	49

Discussion

A strain of *Bacillus* No. 202-1, newly isolated soil bacterium, produced an extracellular alkaline pullulanase in a high alkaline medium containing Na_2CO_3. This strain could not grow in neutral and acidic media. The morphological and cultural characteristics of this strain will be compared with other strains elsewhere.

The alkaline pullulanase of *Bacillus* No. 202-1 purified was homogeneous as judged by disc polyacrylamide gel electrophoresis. This enzyme had an optimum pH at 8.5—9.0. This value is higher than those of other debranching enzyme. The enzyme was not inhibited by SH-group reagents such as iodoacetate and *p*-chloromercurybenzoate, by which *Pseudomonas* isoamylase was slightly inhibited. Ammonium molybdate, which inhibites R-enzyme [16] and yeast isoamylase [2], did not inhibit *Bacillus* No. 202-1 pullulanase.

The iodine coloration of oyster glycogen was increased by *Bacillus* No. 202-1 pullulanase (70%), but low increase (13%) was observed with *A. aerogenes*.

These results suggest that *Bacillus* No. 202-1 pullulanase can hydrolyze all 1,6-α-linkages of oyster glycogen as well as of potato and glutinous rice amylopectin. These results were also sustained by β-amylase hydrolysis of glycogen after debranching with *Bacillus* No. 202-1 pullulanase as shown in Table III. The enzyme of *Bacillus* No. 202-1 is different from those of *A. aerogenes* and *Escherichia intermedia* [5] as to the action on glycogen and those of *Pseudomonas* sp. [18] and yeast [2] as to the action on pullulan. The *Streptococcus mitis* pullulanase [6] also hydrolyses both glycogen and amylopectin, but this enzyme was rapidly inactivated at temperatures higher than 40° C. The pH optimum for enzyme action was 5.4—5.8 which is lower than that of *Bacillus* No. 202-1 pullulanase.

As the result, we wish to conclude that the pullulanase of *Bacillus* No. 202-1 is different from the other pullulanase so far reported, especially in optimum pH.

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