Characterization of Endo β -1,3-Glucanase IV from *Flavobacterium dormitator* var. *glucanolyticae* FA-5

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Endo β -1,3-glucanase IV (E.C. 3.2.1.6, endo-1,3(4)- β -D-glucanase) from *Flav. dormitator* var. *glucanolyticae* FA-5 was shown to be a glycoprotein by gel filtration and sodium dodecyl sulfate gel electrophoresis. The carbohydrate moiety was composed of 17 hexose units. The enzyme had an apparent molecular weight of 3.3×10^4 , determined by gel filtration, sodium dodecyl sulfate gel electrophoresis and ultracentrifugation. The enzyme showed maximum reactivity at pH 6.0 and 6.5 for living yeast cells and laminaran, respectively. The enzyme predominantly released laminaripentaose from a variety of linear β -1,3-glucans and showed transglucanoylation activity. The amino acid composition of the enzyme and some of its physicochemical and enzymatic properties are described.

Flavobacterium dormitator var. *glucanolyticae* FA-5 has been shown to produce five endo β -1,3-glucanases (B, I, II, III and IV**).¹⁾ In order to extend the previous work on these enzymes, and to answer the question of whether one or two enzymes are responsible for the lytic activity on living yeast cells, homogeneous and pure enzymes are needed.

One of them, endo β -1,3-glucanase II, has been purified and characterized to be a "lower oligosaccharide-producing type" of endo β -1,3-glucanase (E.C. 3.2.1.6, endo-1,3(4)- β -D-glucanase), and found to be responsible for lysis of living yeast cells and hydrolysis of yeast glucan.²⁾ The other two of them (endo β -1,3-glucanases I and III) are minor and varied components. The B form of endo β -1,3-glucanase will be reported on elsewhere.

Endo β -1,3-glucanase IV has been purified and characterized in part.¹⁾ The present paper describes determination of properties of the enzyme.

MATERIALS AND METHODS

Enzyme preparation. Endo β -1,3-glucanase IV was prepared from *Flav. dormitator* var. *glucanolyticae* FA-5 as described in the previous paper.¹⁾ The purified enzyme was shown to be homogeneous by disc gel electrophoresis. Homogeneity of the enzyme was also demonstrated by ultracentrifugation. The enzyme sedimented as a single symmetrical peak during the sedimentation velocity run. The specific activity of the purified enzyme was 0.58.

Substrates. Laminaran, yeast glucan and pachyman were obtained and prepared as described in the previous paper.²⁾ All other substrates were either prepared by us or taken from the collection of carbohydrate substances available in this laboratory.

Enzyme assay. Endo β -1,3-glucanase IV was assayed using insoluble yeast glucan as substrate. The incubation mixtures contained the following components in a total volume of 2.0 ml: 50 mM potassium phosphate buffer, pH 6.5, 5 mg of yeast glucan and an appropriate amount of the enzyme. After incubation at 40°C for 10 min with vigorous shaking, the reaction was terminated by the addition of 2.0 ml of 1 M trichloroacetic acid. After centrifugation at $1,000 \times g$ for 30 min, total sugar content of the supernatant fluid was determined by the method of Dubois *et al.*³⁾ One unit of the enzyme was defined as that amount catalyzing the liberation of $1 \mu\text{mol}$ of total sugar as glucose per min under the conditions described.

Analytical disc gel electrophoresis. Polyacrylamide gel

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** They are denoted as B (breakthrough), I, II, III and IV; assignment based on SP-Sephadex C-50 (0.01 M potassium phosphate buffer, pH 5.0) absorbability.

electrophoreses were performed essentially as described by Ornstein,⁴⁾ Davis⁵⁾ and Fairbanks *et al.*⁶⁾ Polyacrylamide gel isoelectric focusing was carried out as described by Wrigley⁷⁾ with LKB pH 3~10 Carrier Ampholytes.

Amino acid analysis. For amino acid analysis, the enzyme was hydrolyzed in 6N HCl for periods of 20, 40, and 60 hr at 110°C under conditions which rigidly excluded oxygen. Amino acid analyses were performed with a Hitachi 034 automatic amino acid analyzer. Tryptophan was determined spectrophotometrically.⁸⁾

Titration of sulfhydryl groups with 5,5'-dithio bis(2-nitrobenzoic acid) (DTNB).⁹⁾ Total sulfhydryl groups in the enzyme protein were determined by titration with DTNB after reduction of disulfide bridges in the denatured enzyme protein. About 5mg of the enzyme was incubated at 40°C for 1 hr at pH 10.5 in 0.8ml of a mixture of 5M guanidinium hydrochloride, 50mM disodium ethylenediamine tetraacetic acid (EDTA), 50mM dithiothreitol and 0.1M potassium bicarbonate. The mixture was then passed through a Bio-Gel P-10 column (1 × 10 cm) previously equilibrated at pH 7.5 with a solution containing 5M guanidinium hydrochloride, 20mM potassium phosphate, pH 7.5, and 50mM EDTA. The resulting dithiothreitol-free protein was titrated with DTNB immediately after the gel filtration.

The titration was started by the addition of 0.02 ml of 10mM DTNB solution in 0.05M potassium phosphate buffer, pH 7.5. The reaction was followed at 412 nm with a spectrophotometer; the number of sulfhydryl groups reacted was based on a molar absorbance of 13,600 for the reduction product of DTNB.

Analytical ultracentrifugation. Sedimentation velocity and sedimentation equilibrium studies were done with a Hitachi 282 ultracentrifuge equipped with a photoelectric scanner.

Sugar analysis. For sugar analysis, a sample (0.5ml) was placed on a Bio-Gel P-2, -400 mesh, column (0.9 × 140 cm) equilibrated with H₂O. The sugar was eluted with H₂O and the concentration of sugar in the eluate was determined by continuous recording of the absorbance at 420 nm, after colorimetric reaction with orcinol-sulfuric acid¹⁰⁾ with a Hitachi 034 automatic analyzer.

RESULTS AND DISCUSSION

1. Physicochemical properties

The purified enzyme migrated as a single band in polyacrylamide disc gel electrophoresis at pH 4.0 and 9.5. The location of the protein band corresponded to the location of the carbohydrate band which was visualized

by staining a duplicate gel with Schiff base (Fig. 1-A). As determined by the phenol-sulfuric acid method, with glucose as standard, the enzyme contained 10.3% (w/w) carbohydrate, which corresponded to 17 hexose units when calculated from the molecular weight of the enzyme described below.

The ultracentrifugal Schlieren pattern for the enzyme showed a peak with a sedimentation coefficient of 4.05 $S_{20,w}$. $D_{20,w}$ was calculated to be $10.9 \times 10^{-7} \text{ cm}^2 \cdot \text{sec}^{-1}$ from $S_{20,w}$ and the molecular weight of the enzyme.

The absorption spectrum of the enzyme showed a maximum at 278 nm, and no significant absorption in the visible range was observed. A value of 14.75 for $E_{280 \text{ nm}, 1 \text{ cm}}^{1\%}$ was obtained by measuring the absorbance of the enzyme in 0.01M potassium phosphate buffer, pH 7.0.

The molecular weight of the enzyme was determined by the sedimentation equilibrium method (Fig. 1-B). A straight line was ob-

FIG. 1. (A) Polyacrylamide Gel Electrophoresis of Endo β -1,3-Glucanase IV.

Gel 1, pH 9.5 gel was stained with periodic acid-Schiff reagent, and Gel 2, pH 9.5 gel was stained with Coomassie brilliant blue.

(B) Sedimentation Equilibrium of Endo β -1,3-Glucanase IV.

The protein concentration was 0.3mg per ml of 0.01M potassium phosphate buffer, pH 6.0, containing 0.1M KCl. The log concentration versus r^2 data was calculated directly from ultraviolet light scans of the sample. The rotor was maintained at 15,000 rpm at 22°C for 40 hr.

tained on plotting log concentration against the square of the distance from the center of rotation, further confirming the homogeneity of the enzyme. From the slope of this straight line a molecular weight of 32,700 was calculated using a partial specific volume of $0.726 \text{ ml} \cdot \text{g}^{-1}$ obtained from the amino acid composition.

By gel filtration on Bio-Gel P-100 and sodium dodecyl sulfate disc gel electrophoresis, the molecular weight and Stokes radius of the enzyme were determined to be 33,000 and $27 \text{ \AA}$, respectively.

The complete amino acid composition of the enzyme is presented in Table I. The minimum molecular weight calculated on the basis of the amino acid content was approximately 31,900, which agreed closely with those determined by gel filtration, sedimen-

tation equilibrium and sodium dodecyl sulfate disc gel electrophoresis. A partial specific volume of $0.726 \text{ ml} \cdot \text{g}^{-1}$ was calculated from the amino acid composition.

Analytical isoelectric focusing of the enzyme in a gradient pH 3 to 10 Carrier Ampholytes showed that the isoelectric point of the enzyme, pI , was 6.5.

Native enzyme did not react with DTNB. However, 1.82 sulfhydryl groups were found in the denatured and reduced enzyme protein on DTNB titration, in good agreement with the value obtained by amino acid analysis within experimental error. The two titratable sulfhydryl groups of the denatured and reduced enzyme protein exist as an intramolecular disulfide bridge in the native enzyme protein, and are exposed by dithiothreitol and denaturing reagents such as guanidinium

TABLE I. AMINO ACID COMPOSITION OF THE ENZYME

Amino acid	Hydrolysis				Residues/ 32,000 <i>g</i> protein	Integral number/ 32,000 <i>g</i> protein
	20 hr	40 hr	60 hr	Average		
	$\mu\text{mol}/275 \mu\text{g}$					
Asp	0.290	0.270	0.279	0.280	33.2	33
Thr	0.146	0.139	0.127	0.154*	20.6	21
Ser	0.167	0.149	0.132	0.185*	21.8	22
Glu	0.180	0.185	0.181	0.182	21.6	22
Pro	0.156	0.159	0.161	0.159	18.8	19
Gly	0.298	0.286	0.295	0.293	34.7	35
Ala	0.265	0.257	0.267	0.263	31.1	31
Cys	—	—	—	—	(1.9)**	2
Val	0.145	0.156	0.150	0.150	17.7	18
Met	—	—	—	—	(2.0)**	2
Ile	0.069	0.067	0.073	0.070	8.3	8
Leu	0.128	0.133	0.132	0.131	15.5	16
Tyr	0.077	0.079	0.076	0.078	9.2	9
Phe	0.058	0.056	0.061	0.058	6.9	7
Lys	0.063	0.060	0.064	0.064	7.6	8
His	0.052	0.054	0.049	0.053	6.3	6
Arg	0.102	0.100	0.096	0.099	11.8	12
Trp	—	—	—	—	(4.8)***	5
Hexose						17
Partial specific volume, 0.726 ml · g ⁻¹ Molecular weight, 31,912						

* Extrapolated to 0 time hydrolysis.

** Determined as cysteic acid and methionine sulfoxide, respectively.

*** Determined spectrophotometrically.

TABLE II. SUBSTRATE SPECIFICITY

Substrates (2.5 mg per ml in 0.05 M sodium succinate buffer, pH 5.8) were incubated with the enzyme (16.5 μ g of protein) for 60 min at 40°C, after which samples were taken for measurement of increases in sugar reducing equivalents.

Substrate	Predominant bond	μ mol/ml
Pachyman	β -1,3-Glucosidic	1.37
Yeast glucan	β -1,3-; β -1,6-Glucosidic	0.47
Laminaran	β -1,3-; β -1,6-Glucosidic	0.16
Curdlan, paramylon	β -1,3-Glucosidic	+
Lichenan	β -1,3-; β -1,4-Glucosidic	0.08
Crown gall	β -1,2-Glucosidic	0.00
Cellulose	β -1,4-Glucosidic	0.00
Luteose, pustulan	α -1,6-Glucosidic	0.00
Pullulan	α -1,4-; α -1,6-Glucosidic	0.00
Amylose, amylopectin	α -1,4-; α -1,6-Glucosidic	0.00
Dextran	α -1,6-Glucosidic	0.00
Yeast mannan	α -1,2-; α -1,3-; α -1,6-Mannosidic	0.00
Xylan	β -1,4-Xylosidic	0.00
Gentiobiose, trehalose, maltose lactose, cellobiose, sucrose, raffinose		0.00

hydrochloride. Independent evidence for the occurrence of a disulfide bridge in the enzyme protein was obtained by comparing the amounts of carboxymethyl cysteine released by acid hydrolysis of preparations which had been carboxymethylated in 5 M guanidinium hydrochloride with and without dithiothreitol. The number of carboxymethyl cysteine residues increased from zero residues in the unreduced enzyme protein to 1.8~2.1 residues in the reduced enzyme protein, in good agreement with the value obtained by DTNB titration.

2. Enzymatic properties

Substrate specificity. The enzyme was found to be specific for β -1,3-glucans, such as pachyman (28.2 nKat), yeast glucan (9.7 nKat), laminaran (3.3 nKat), curdlan and paramylon (Table II). Figure 2 shows representative chromatograms obtained for the hydrolysates of β -1,3-glucans, after enzymatical exhaustive hydrolysis of them. The main product was laminaripentaose which was identified by methylation, gel filtration, paper chromatographic and enzymatic analyses.

Lichenan was hydrolyzed by the enzyme, but the reaction product could not be detected on a paper chromatogram even after exhaus-

FIG. 2. The Elution Pattern of Total Sugar from a Bio-Gel P-2 Column (-400 mesh, 1×150 cm) after Digestion of β -Glucans with Endo β -1,3-Glucanase IV.

The conditions for the enzyme reaction were described in Table II, except for the reaction time. After 10 hr incubation, the reaction was stopped by heating in a boiling water bath for 2 min. Total sugar released from insoluble substrate was determined by the orcinol reaction with a Hitachi 034 autoanalyzer.

tive hydrolysis of lichenan.

Optimal-pH and -temperature and stability. Figure 3-A shows that the optimum pH with log phase cells of *Saccharomyces cerevisiae*

FIG. 3. Effect of pH and Temperature on Endo β -1,3-Glucanase IV.

(A) Activity-pH curve: Buffers were as indicated in the figure. For the lytic activity assay, protoplast forming buffer was adjusted with KOH or HCl to the prescribed pH.

(B) Activity-temperature curve: The activity was measured under the standard assay conditions at various temperatures.

(C) Stability-pH curve: The enzyme, in the various buffers described in (A), was heated at 55°C for 10 min and then the remaining activity was measured under the standard assay conditions.

(D) Stability-temperature curve: The enzyme in 10 mM potassium phosphate buffer, pH 7.0, was incubated for 10 min at the prescribed temperature and then the remaining activity was measured under the standard assay conditions.

—●—, β -1,3-glucanase activity; —○—, lytic activity.

LK_2G_{12} as substrate (lytic activity) was 6.0. With laminaran as substrate (β -1,3-glucanase activity), the optimum pH shifted to 6.5. This discrepancy may result from the difference in structure between the two substrates. Similar results were obtained for the optimum temperature for both activities (40°C for lytic activity and 55°C for β -1,3-glucanase activity) (Fig. 3-B). The optimum temperature for lytic activity was not reflected in the temperature effect on the enzyme. Since the cytoplasm of *Sacch. cerevisiae* LK_2G_{12} was denatured and insolubilized when the cells were treated for 10 min at a temperature higher than 45°C, the apparent enzyme activity was decreased to a low level even after the cell walls were com-

FIG. 4. Double Reciprocal Plot of the Activity of Endo β -1,3-Glucanase IV as a Function of Yeast Glucan Concentration.

TABLE III. EFFECT OF VARIOUS METALS ON ACTIVITY OF THE ENZYME

Endo β -1,3-glucanase IV, 263 μ g, was incubated at 5°C for 3 hr with 1 mM metal salts before introduction of the substrate. Metal salts, enzyme and substrate were dissolved in 0.01 M sodium acetate buffer, pH 6.0.

Metal	Relative activity	
	β -1,3-Glucanase activity	Lytic activity
None	(100)	(100)
Zn	79	89
Cu	93	69
Ca	72	92
Co	102	100
Ni	95	100
Mn	84	100
Mg	80	115
Al	77	95
Ag	28	46
Hg	4	39

pletely removed by the enzyme action.

The effect of pH on the stability of the enzyme was studied by incubating the enzyme for 10 min at 55°C in buffers ranging from pH 4 to 10. In the range of pH 6 to 9 in potassium phosphate or Tris·HCl buffer, lytic and β -1,3-glucanase activities were not affected, whereas at higher pH values (9~10) in sodium carbonate buffer, both activities were completely lost (Fig. 3-C). Figure 3-D shows that the enzyme retained 80% of its original activity even after treatment at 50°C for 10 min.

Kinetics. Using yeast glucan as substrate at concentrations varying from 0.1 to 10 mg per

FIG. 5. Inactivation of Endo β -1,3-Glucanase IV with Hg or *p*-Chloromercuribenzoate, and Effect of β -Mercaptoethanol on Hg Inhibition.

Endo β -1,3-glucanase IV (26.3 μ g) was incubated at 5°C for 1 hr in the presence of the reagent indicated in 1.0 ml of 0.01 M potassium phosphate buffer, pH 7.0. After the incubation, the remaining activity was measured under the standard assay conditions with 1.3 μ g of the enzyme.

—●—, Hg; —○—, Hg + 0.0125 M β -mercaptoethanol; —△—, Hg + 0.1% laminaran; —×—, *p*-chloromercuribenzoate.

ml of 0.01 M potassium phosphate buffer, pH 6.5, a Lineweaver-Burk plot was obtained. At low concentrations of substrate, the reaction rate followed hyperbolic Michaelis-Menten kinetics, but deviated at higher concentrations of substrate (Fig. 4). The systematic deviation from Michaelis-Menten kinetics may be rationalized if the enzyme has the activity of transglucosidation and/or transglucanosylation. The transglucanosylation activity will be reported in the following paper.

Inhibitors. The enzyme was insensitive to

metal ions, such as Zn, Cu, Ca, Co, Ni, Mn, Mg and Al at concentrations of 1 mM. Hg and Ag caused 95% and 80% inhibition, respectively, at the same concentration (Table III). Figure 5 shows that Hg did not inhibit the enzyme in the presence of β -mercaptoethanol. However, the activity of the enzyme inactivated with Hg could not be restored by the addition of β -mercaptoethanol. Laminaran could not protect the enzyme from the Hg inhibition. The enzyme was not inhibited by the mercurous inhibitor, *p*-chloromercuribenzoate. From these results, it seems that the inhibition by mercuric ions is due to the formation of an inactive dimer enzyme connected to mercuric ions and that no sulfhydryl residue is involved in the catalytic process.

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