Purification and Some Properties of β -1, 3-Glucanase of Suspension-cultured Tobacco Cells

Kunio KATŌ, Ayako YAMADA and Masao NOGUCHI

Central Research Institute, Japan Monopoly Corporation, Umeaoka, Midori-ku, Yokohama, Japan

Received September 8, 1972

A laminaran-hydrolyzing enzyme was purified from the homogenate of suspension-cultured tobacco cells by the treatment with ion-exchangers and gel filtration. The purified enzyme was homogenous in disc-electrophoresis and was a basic protein. The optimal pH of the enzyme was 5.0. The enzyme was stable at temperature below 40°C. The inhibitory effect of Hg^{2+} , Cu^{2+} and Ag^+ was observed. Investigation of the hydrolysis product revealed that the enzyme attacked laminaran endo-wise to form laminari-tetraose, -triose, -biose and glucose.

Glucanases have been reported in a variety of bacteria, fungi, animals and higher plants. Recently, the interests have been focused on glucanases. One of the major reasons for the increased interests in these enzymes has been their usefulness as tools for studying the structure of oligo- and polysaccharides. Further interests in these enzymes stem from possibilities that they may be involved in growth of plant cells. Masuda^{1,2)} has presented the evidence that cell-wall degrading enzymes, especially β -1,3-glucanase, may play an important role in auxin induced growth. β -1,3-Glucanase has been reported to be widespread in higher plants.³⁾ There are three reports concerning the enzyme activity in suspension-cultured plant cells.^{4~6)}

The present report deals with purification and some properties of the β -1,3-glucanase from suspension-cultured tobacco cells.

MATERIALS AND METHODS

1. *Cultivation of tobacco cells.* Suspension-cultured tobacco cells, *Nicotiana tabacum* var. BY-2, were used, which had been transferred at least 100 times over 3 years. The culture medium of pipettable cell suspension consisted of Murashige and Skoog's inorganic salts⁷⁾ and contained 0.2 mg of 2,4-D/liter, 0.4 mg of thiamine-HCl/liter and 30 g of sucrose/liter. The general techniques and growth measurements were described in a previous paper.⁸⁾

For the experiments, cells were grown in a 3 liters Erlenmeyer flasks, containing 100 ml of cell suspensions

and 1000 ml of the medium. The vessels were incubated in the dark at 28°C, on a rotary shaker, operating continuously with a 4.5 cm displacement at 90 rpm. After a week, the culture was filtered and the cells were collected on a nylon cloth and washed with water.

2. *Preparation of crude enzyme solution.* The washed cells were suspended in 0.1 M phosphate buffer, pH 7.0, containing 0.01 mg of cysteine/ml, using 1 ml of buffer per gram of fresh weight of cells, and homogenized for 10 min in a Waring blender. The homogenate was centrifuged ($20000 \times g$, for 30 min) to remove solid materials. The enzyme in the supernatant was precipitated by adding solid ammonium sulfate to make 80% saturation with respect to ammonium sulfate. The precipitate was collected by centrifugation and dissolved in about 5 volumes of water. The resulting solution was dialyzed against water and against the changing solution of 0.01 M acetate buffer, pH 5.0. After the precipitate was removed by centrifugation, the supernatant was used as a crude enzyme solution.

3. *Purification of β -1,3-glucanase.* The purification of crude glucanase was performed by a combination of column chromatographies on DEAE- and CM-Sephadex (Pharmacia), and gel filtration on Sephadex G-75 (Pharmacia). The flow diagram of an outline of the purification of β -1,3-glucanase is shown in Fig. 1. These operations were carried out at 5°C and the enzyme in crude or purified state was stable for more than 1 month when it was stored at 5°C.

4. *Substrates used.* Laminaran and xylan were purchased from Tokyo Kasei Co., Ltd. and were washed several times with 80% ethanol until no sugars were detected in washings. One mg of purified laminaran gave 1.02 mg of glucose by acid hydrolysis.

Arabino-galactan was prepared from coffee beans by the method of Hashimoto *et al.*⁹⁾ Acid hydrolysis

Tobacco cultured cells
 ↓ homogenized using Waring blender
 Extract
 ↓ saturated with ammonium sulfate to 80%
 Precipitate
 ↓ dissolved in water
 ↓ dialyzed against tap water and 0.01 M acetate buffer (pH 5.0)
 Supernatant (Crude enzyme preparation)
 ↓ chromatographed on DEAE-Sephadex A-50 (2.5 × 45 cm)
 ↓ equilibrated with 0.01 M acetate buffer (pH 5.0),
 ↓ eluted with the same buffer
 Break-through solution
 ↓ chromatographed on CM-Sephadex C-50 (2.5 × 45 cm)
 ↓ equilibrated with 0.01 M acetate buffer (pH 5.0)
 Adsorbed fraction
 ↓ eluted linearly gradiently with 500 ml of 0.01 M acetate buffer and 500 ml of 2.0 M sodium chloride,
 ↓ and additional 500 ml of 2.0 M sodium chloride
 Active fraction
 ↓ chromatographed on Sephadex G-75 (2.5 × 95 cm)
 ↓ equilibrated with 0.01 M acetate buffer (pH 5.0)
 Purified glucanase

FIG. 1. Outline of the Separation and Purification of β -1,3-Glucanase.

of arabino-galactan gave only two spots of arabinose and galactose on paper chromatogram. Cell wall fraction were prepared from suspension-cultured tobacco cells according to the method of Nevins *et al.*¹⁰⁾ Yeast-glucan was prepared from fresh baker's yeast according to the method of Bell and Northcote.¹¹⁾

5. *Determination of enzyme activity.* The reaction mixture contained 0.5 ml of 1% laminaran solution, 0.5 ml of 0.1 M acetate buffer, pH 5.0, and 1 ml of enzyme solution. After the mixture was kept at 40°C for 10 min, the reducing sugar produced was determined by the method of Somogyi-Nelson.¹²⁾ One unit of enzyme activity was defined as the amount of enzyme that produced reducing sugars equivalent to 1 mg of glucose per 10 min under above conditions.

6. *Determination of protein.* The amount of protein was determined according to the method of Lowry *et al.*,¹³⁾ using Bovine serum albumin (Tokyo Kasei Co. Ltd.) as a reference. The relative amount of protein was tentatively expressed with the absorbance at 280 m μ per cm of enzyme solution.

7. *Paper chromatography.* An aliquot of the reaction mixture was passed through a Dowex 50W-X4 (H⁺ type) column. The eluate was concentrated in a vacuum evaporator to a syrup (1 ml) at below 45°C. Paper chromatography was carried out by the descending method using Whatman filter paper No. 1 with a solvent system, ethyl acetate: pyridine: water (8: 2: 1).

The sugars on paper chromatograms were visualized by spraying aniline hydrogen phthalate reagent.

8. *Disc-electrophoresis.* Disc-electrophoresis of the purified glucanase was carried out on pH 8.3^{14,15)} and 4.3¹⁶⁾ gels, respectively, under following conditions: current, 4 mA/column; time, 90 min; temperature, 5°C; staining, Amido black.

RESULTS

1. Degradation of cell walls

The progressive curve of hydrolysis of isolated cell wall by crude enzyme is shown in Fig. 2. As shown in Fig. 2, isolated cell walls from tobacco cultured cells were partially hydrolyzed by crude enzyme of cells.

FIG. 2. Partial Degradation of Isolated Cell Walls by Crude Enzyme Solution.

The reactions were run by adding 10 ml of enzyme solution to a test tube containing 200 mg of isolated tobacco cell walls in 0.1 M acetate buffer, pH 5.0. The reaction flasks were placed on a reciprocal shaker at 40°C for the desired length of time. The reactions were stopped by boiling. The precipitate was removed by filtration and washed with water. The combined filtrate was made up to 50 ml with water, and the amounts of reducing sugars in 2 ml of the solution were measured. Control; the reaction mixture was boiled before the reaction was run in the same manner as above.

●—●, cell wall; ○—○, control.

2. Degradation of soluble polysaccharides

The crude enzyme preparation showed

arabino-galactan, xylan and laminaran hydrolyzing activities. The results are shown in Table I. As shown in Table I, the activity of β -1,3-glucanase was more than 150 times higher than xylan or arabino-galactan hydrolyzing activity. Carboxymethyl cellulose has not been hydrolyzed by crude enzyme preparation from cells, although the culture broth showed cellulase activity.

TABLE I. DEGRADATION OF SOLUBLE POLYSACCHARIDES BY CRUDE ENZYME PREPARATION

Substrates	Activity ^{a)}	
	Cells $\mu\text{g/g}$ (f.w.)	Culture broth $\mu\text{g/ml}$
Laminaran	4855.5	207.1
Arabino-galactan	9.9	13.1
Xylan	25.2	33.3
CMC	— ^{b)}	10.8

^{a)} To 0.5 ml of 0.5% substrate solution and 0.5 ml of 0.1 M acetate buffer (pH 5.0), was added 1 ml of crude enzyme solution, and after 60 min incubation at 40°C, the reducing sugars produced was determined as glucose and expressed as $\mu\text{g/g}$ of fresh cells and $\mu\text{g/ml}$ of broth, respectively.

^{b)} not detected.

3. Purification of β -1,3-glucanase

3.1. Chromatography on DEAE- and CM-Sephadex. After a column of DEAE-Sephadex A-50 (2.5 $\times$ 45 cm) was equilibrated with 0.01 M acetate buffer (pH 5.0), the crude enzyme solution was passed through the column. No glucanase was adsorbed on the resin. The break-through solution from the column was concentrated to 100 ml with polyethylene glycol (# 6.000). The concentrated enzyme solution was passed through a column of CM-Sephadex C-50 (2.5 $\times$ 45 cm) which had been equilibrated with 0.01 M acetate buffer (pH 5.0). The enzyme was adsorbed on the resin and after washing the column with 0.01 M acetate buffer, the enzyme was desorbed with a linear gradient starting with 500 ml of the same buffer in a mixing flask and 500 ml of the same buffer containing 2.0 M sodium chloride, and the additional 500 ml of the latter solution. As shown in Fig. 3, the enzyme was eluted from the

resin with 2.0 M sodium chloride solution. Active fractions as glucanase were concentrated with polyethylene glycol and taken into next step.

3.2. Gel filtration on Sephadex G-75. The enzyme purified by column chromatography on CM-Sephadex, was subjected to gel filtration

FIG. 3. Chromatography of Glucanase of Tobacco Cells on a CM-Sephadex C-50 Column.

Column, 2.5 $\times$ 45 cm. Flow rate, 10 ml/hr. $\circ$ --- $\circ$, Protein; $\bullet$ — $\bullet$, Enzyme activity. Active fractions, Nos of 67~97 were collected.

FIG. 4. Gel Filtration of Glucanase of Tobacco Cells on a Sephadex G-75 Column.

Column, 2.5 $\times$ 95 cm. Flow rate, 20 ml/hr. $\circ$ --- $\circ$, Protein; $\bullet$ — $\bullet$, Enzyme activity. Active fractions, Nos of 24~30 were collected.

TABLE II. SUMMARY OF THE YIELDS OF ACTIVITY AND PROTEIN AT EACH STEP OF PURIFICATION

Tobacco cells (922 g)	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)
Ext. from tobacco cells	1540	4900	3200	1.53	100
Salting out	150	3670	2000	1.84	72.8
Dialyzed sol.	160	1970	370	5.32	40.2
DEAE-Sephadex A-50	235	1520	230	6.61	31.0
CM-Sephadex C-50	20	1220	34	35.88	24.9
Sephadex G-75	20	1010	15	67.33	20.7

on a column of Sephadex G-75 which had been equilibrated with 0.01 M acetate buffer (pH 5.0). Figure 4 shows the results. Active fractions as glucanase were concentrated to 20 ml with polyethylene glycol. The results of overall purification procedures are summarized in Table II. By these purification procedures, the specific activity of the enzyme was increased 45 fold compared with that of extract of cultured cells. The yields of purified enzyme were 15 mg from 922 g of fresh cells (0.5 mg of protein or 33.7 units/g dry weight of the cells). The recovery of activity was 20.7%. No action of the purified glucanase upon substrates such as arabino-galactan, hemicellulose, cellulose and cell walls of tobacco cells was recognized.

4. Properties of the purified enzyme

4.1. *Homogeneity of purified enzyme.* As shown in Fig. 5, disc-electrophoretic pattern

FIG. 5. Homogeneity of Purified Glucanase by Disc-electrophoresis.

pH 4.3 gel was used.

of the glucanase on pH 4.3 gel showed a single band, which was not seen on pH 8.3 gel. From the results, it is considered that the purified glucanase is a highly basic protein. The purified enzyme contained 6.7% of sugars as

glucose.

4.2. *pH optimum for enzyme action.* The enzyme activity was investigated at various pH of 3.0 to 8.0 with MacIlvaine buffer. As shown in Fig. 6, the optimal pH was found to be 5.0.

FIG. 6. Effect of pH on the Activity of β -1,3-Glucanase.

Laminaran, 0.5 ml of 1% solution, 0.5 ml of MacIlvaine buffer, and 1 ml of purified enzyme solution were mixed and incubated at 40°C, for 10 min.

4.3. *Thermal stability.* Thermal stability of the purified enzyme in 0.1 M acetate buffer (pH 5.0) was examined. One ml of the enzyme solution was mixed with 0.5 ml of buffer solution, and the mixture was kept at each temperature for 20 min. After heat treatment, the enzyme solution was rapidly cooled in ice-cold water. The mixture of this heat

treated solution and 0.5 ml of 1% laminaran solution was incubated at 40°C for 10 min. As shown in Fig. 7, the enzyme is stable at temperatures below 40°C.

4.4. Initial reaction of the glucanase. The progressive curve of hydrolysis of laminaran by the glucanase is shown in Fig. 8. The hydrolysis of laminaran was related linearly

FIG. 7. Thermal Stability of β -1,3-Glucanase.

Heated for 20 min at the temperature indicated, in 0.1 M acetate buffer, pH 5.0.

FIG. 8. Time Course of Hydrolysis of Laminaran by β -1,3-Glucanase.

Laminaran, 0.5 ml of 1% solution, 0.5 ml of 0.1 M acetate buffer (pH 5.0) and 1 ml of enzyme solution (3.75 μ g protein, 0.26 units) were mixed and incubated at 40°C, for desired length of time.

to reaction time for first 10 min, and 5% of substrate was hydrolyzed.

The variation of enzyme activity with various concentrations of the enzyme was also examined at pH 5.0. The glucanase activity was linear function of enzyme concentration up to 3.8 μ g of protein in the reaction mixture.

4.5. Effect of substrate concentration. Double reciprocal plots of initial velocity of glucanase for laminaran concentration is shown in Fig. 9, which indicates that the reciprocal of initial velocity is quite proportional to that of the concentration of laminaran. From the graph, the K_m value for laminaran was calculated to be 0.22%.

FIG. 9. Double Reciprocal Plots of Initial Velocity of β -1,3-Glucanase against Laminaran Concentration.

5. Products of enzyme action on laminaran

The reaction mixture composed of 5 ml of 1% laminaran solution, 5 ml of 0.1 M acetate buffer (pH 5.0), and 1 ml of glucanase (5.5 units) was incubated at 40°C. The paper chromatograms of the reaction products from laminaran are shown in Fig. 10, which revealed the formation of glucose and oligosaccharides such as laminari-biose, -triose, and -tetraose. Polymerization degree of the oligomers was determined by the graphical method reported by French.¹⁷⁾ While at earlier stage of hydrolysis, the reaction products gave no glucose spot on paper chromatograms, glucose was detected after three hours incubation, and increased with prolonged incubation. Yeast-glucan was also hydrolyzed by the purified glucanase to give glucose and laminari-

FIG. 10. Time Course of Hydrolysis of Laminaran as Shown by Paper Chromatography.

biose after 18 hr incubation, but did not give laminari-triose and -tetraose.*

6. Effect of metal ion on enzyme activity

Laminaran, 0.5 ml of 1% solution, 0.5 ml of 0.1 M acetate buffer, pH 5.0, 0.5 ml of salt solution (final concentration 1×10^{-3} M) and 0.5 ml of enzyme solution were mixed and incubated at 40°C for 10 min. As shown in Table III, enzyme activity was inhibited by Hg^{2+} , Cu^{2+} and Ag^+ . The enzyme activity was not affected by the presence of metal chelator (EDTA).

DISCUSSION

There are some reports describing that plant cell walls have an ability to undergo autolysis *in vitro*,¹⁸⁻²⁰⁾ and that cultured-cell walls are degraded by wall-associated enzymes.⁶⁾ The crude enzyme solution from tobacco cells

* It was shown by further studies with enzymic action that laminaran was hydrolyzed to glucose and laminari-biose by a large amount of the purified glucanase after 18 hr incubation. Laminari-tetraose was hydrolyzed to laminari-triose, biose and glucose, and laminari-triose to laminari-biose and glucose. No laminari-biose was hydrolyzed to glucose by this glucanase. These results will be reported later.

TABLE III. EFFECT OF METAL ION ON ENZYMIC ACTIVITY

Salt (1×10^{-3} M)	Relative activity (%)
None	100
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	102.7
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	105.3
$\text{Fe}_2(\text{SO}_4)_3 \cdot 9\text{H}_2\text{O}$	78.7
AgNO_3	0.2
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	23.4
$\text{Ca}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$	115.2
$\text{Hg}(\text{CH}_3\text{COO})_2$	0
EDTA (5×10^{-3} M)	103.9

also degrades isolated cell walls partially, and shows several polysaccharide degrading activities. Among these a very active glucanase is found. β -1,3-Glucanase of endo type has been found by Reese *et al.*⁵⁾ in callus tissues of bean, lettuce, carrot and pepper.

As shown in Fig. 10, laminaran was hydrolyzed by the purified glucanase to give laminari-tetraose, -triose, -biose and glucose. From the above results it is suggested that this glucanase is also an endo-wise enzyme, but further studies is required to elucidate the mode of action of this enzyme.

As shown in Fig. 2, and Table I, crude enzyme hydrolyzed isolated cell walls from cultured cells and showed arabino-galactan and xylan hydrolyzing activities. But no such action of the purified glucanase upon above substrates was observed. Although Masuda^{1,2)} has presented that β -1,3-glucanase may play an important role in auxin induced growth, the physiological role of this glucanase on growth of cultured cells is not known.

REFERENCES

- 1) Y. Masuda and S. Wada, *Bot. Mag. Tokyo*, **80**, 100 (1967).
- 2) S. Wada, E. Tanimoto and Y. Masuda, *Plant & Cell Physiol.*, **9**, 369 (1968).
- 3) A. E. Clarke and B. A. Stone, *Phytochemistry*, **1**, 175 (1962).
- 4) O. L. Gamborg and D. E. Eveleigh, *Can. J. Biochem.*, **46**, 417 (1968).
- 5) M. M. Frederick, W. Parrish and E. T. Reese, *Phytochemistry*, **6**, 1097 (1967).
- 6) K. Keegstra and P. Albersheim, *Plant Physiol.*,

- 45, 675 (1970).
- 7) T. Murashige and F. Skoog, *Physiol. Plant.*, **15**, 473 (1962).
- 8) T. Matsumoto, K. Okunishi, K. Nishida, E. Tamaki and M. Noguchi, *Agr. Biol. Chem.*, **35**, 543 (1971).
- 9) Y. Hashimoto, Y. Tsujisaka and J. Fukumoto, *Nippon Nogeikagaku Kaishi*, **43**, 831 (1969).
- 10) D. J. Nevins, P. D. English and P. Albersheim, *Plant Physiol.*, **42**, 900 (1967).
- 11) D. J. Bell and D. H. Northcote, *J. Chem. Soc.*, **1950**, 1944.
- 12) M. Somogyi, *J. Biol. Chem.*, **160**, 69 (1945); **195**, 19 (1952).
- 13) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 14) L. Ornstein, *Ann. New York Acad. Sci.*, **121**, Art. 2, 321 (1964).
- 15) B. J. Davis, *ibid.*, 404 (1964).
- 16) K. A. Reisfeld, U. J. Lewis and D. E. Williams, *Nature*, **195**, 281 (1962).
- 17) D. French and G. M. Wild, *J. Am. Chem. Soc.*, **75**, 2612 (1953).
- 18) M. Katz and L. Ordin, *Biochim. Biophys. Acta*, **141**, 126 (1967).
- 19) A. Kivilaan and S. Lee, *Plant Physiol.*, **41**, lxxix (1966).
- 20) S. Lee, A. Kivilaan and R. S. Bandurski, *ibid.*, **42**, 968 (1967).
-