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FOUR GLYCOSIDASES SECRETED BY *COLLECTOTRICHUM LINDEMUTHIANUM**

K. KEEGSTRA,† P. D. ENGLISH and P. ALBERSHEIM

Department of Chemistry, University of Colorado, Boulder, CO 80302, U.S.A.

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Abstract—The fungal plant pathogen, *Collectotrichum lindemuthianum*, was grown in culture with either galactose, arabinose or pectin as the carbon source resulting in the selective secretion of α-galactosidase, α-arabinofuranosidase and exopolygalacturonase, respectively. Each enzyme has been purified by ion exchange and gel permeation chromatography. In addition, a β-glucosidase was purified from the culture filtrate or arabinose grown fungus. The purified α-galactosidase and α-arabinosidase preparations were found to be essentially free of other carbohydrases while the β-glucosidase and exopolygalacturonase preparations contain contaminating activities.

INTRODUCTION

ONE OF the major problems in studying the structure of plant cell walls has been the lack of purified cell wall degrading enzymes for use in fractionating the wall material.[1] Plant pathogens are an excellent source of such degradative enzymes, since the plant cell wall is the natural substrate for the degradative enzymes secreted by these pathogens.

Previous work from this laboratory has demonstrated that the bean pathogen, *Collectotrichum lindemuthianum*, can be induced to secrete large amounts of cell wall degrading enzymes into the culture medium.[2,3] Thus we began a study of the polysaccharide degrading enzymes from this fungus. The purification and properties of an endopolygalacturonase from *C. lindemuthianum* has been reported previously.[4] In this report we describe the production and partial purification of several glycosidases secreted by this fungus.

RESULTS

Purification of α-Galactosidase

The crude culture filtrate from galactose grown fungus was dialyzed against 10 mM potassium phosphate, pH 7·0. The dialyzed solution was passed through a DEAE-cellulose column equilibrated in the phosphate buffer. The column was washed with this buffer and then subjected to a linear 0–0·3 M sodium chloride gradient in the buffer. The enzyme activity eluted at the end of the gradient. The pooled fractions were desalted and concentrated by pressure dialysis.

The enzyme solution was then placed on an Agarose-0·5 m column (2·0 × 110 cm, V_0 = 105 ml). The column was eluted with 0·1 M potasium phosphate, pH 7·0. The enzyme eluted at the void volume of the column. These steps yielded a highly purified protein which chromatographed as a single species on Agarose-15 m as shown in Fig. 1. A summary of the purification is presented in Table 1.

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† National Institutes of Health Predoctoral Fellow.

[1] D. T. A. LAMPORT, *Ann. Rev. of Plant Physiol.* **21**, 235 (1970).
[2] P. D. ENGLISH and P. ALBERSHEIM, *Plant Physiol.* **44**, 217 (1969).
[3] P. D. ENGLISH, J. B. JURALE and P. ALBERSHEIM, *Plant Physiol.* **47**, 1 (1971).
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The α-galactosidase is very stable, losing only about 10% of its activity after 30 min at 50°. It has been stored for 18 months at 2° or −20° with no messurable loss of activity. The α-galactosidase has a very large MW, as shown by its elution position on Agarose-15 m. The α-galactosidase may be a glycoprotein since the purified preparation contains 250 μg of glucose equivalents of carbohydrate (determined by the anthrone test[5]) per mg of protein. The enzyme is active over a broad pH range with an optimum near pH 5 as described earlier.[3] The K_m of the enzyme for *p*-nitrophenyl-α-D-galactoside is $5{\cdot}4 \times 10^{-4}$. The α-galactosidase enzyme degrades raffinose, but its affinity for raffinose is approximately 100 × less than its affinity for *p*-nitrophenyl-α-D-galactoside.

TABLE 1. SUMMARY OF THE PURIFICATION OF α-D-GALACTOSIDASE

Sample	Total activity (units)	Specific activity (units/mg)	Relative purification	% yield
Crude	32 600	25·0	1	100
Dialyzed	27 000	148	59	83
DEAE-cellulose	11 750	1820	73	36
Agarose-0·5 m	11 100	7670	310	34
Agarose-15 m	10 700	7700	310	33

Purification of α-Arabinose and β-Glucosidase

The culture filtrate from arabinose grown fungus was chromatographed on DEAE-cellulose equilibrated in 0·1 M potassium phosphate, pH 7·0. A linear gradient from 0 to 0·2 M sodium chloride in this buffer was used. The elution pattern of several enzymes is shown in Fig. 2. The α-arabinofuranosidase containing fractions, 27–40, were combined and the resulting solution was concentrated and desalted by pressure dialysis.

TABLE 2. SUMMARY OF THE PURIFICATION OF α-ARABINOFURANOSIDASE

Sample	Total activity (units)	Specific activity (units/mg)	Relative purification	% yield
Crude	3800	2·0	1	100
DEAE-cellulose	2500	21·6	10·8	65
Agarose-0·5 m	1300	73	36·5	35
Agarose-0·5 m	760	110	55	20

The concentrated solution was placed on an Agarose-0·5 m column (2·0 × 110 cm, V_0 = 105 ml) and the column eluted with 0·1 M potassium phosphate, pH 7·0. The α-arabinofuranosidase activity eluted just after the column void volume. The fractions with highest specific activity were pooled and concentrated by pressure dialysis. This concentrated solution was rechromatographed on Agarose-0·5 m as shown in Fig. 3. The fractions of highest specific activity were pooled and saved. A summary of the purification

[5] Z. DISCHE, in *Methods in Carbohydrate Chemistry* (edited by R. L. WHISTLER and M. L. WOLFROM), Vol. I p. 479, Academic Press, New York (1962).

of α-arabinofuranosidase is presented in Table 2. The α-arabinofuranosidase, like the α-galactosidase, is a very stable enzyme and can be stored at 2° or −20° for long periods with little loss of activity. The purified α-arabinosidase is able to degrade a commercial araban polymer.

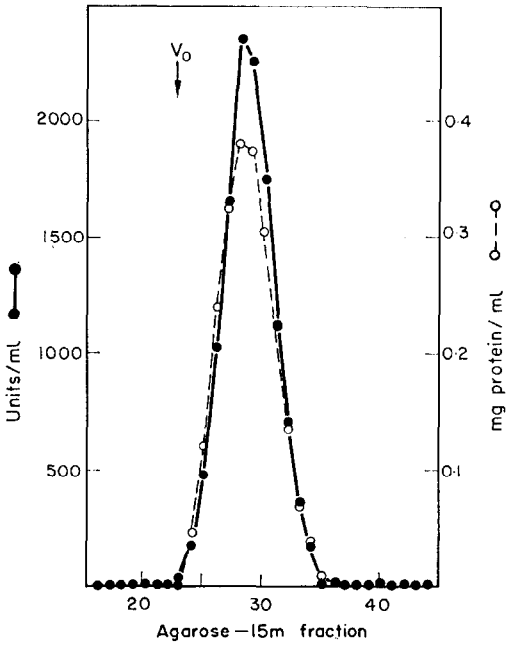

FIG. 1

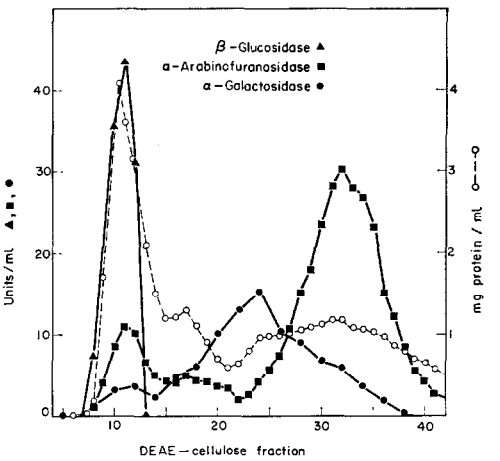

FIG. 2

FIG. 1. CHROMATOGRAPHY OF α-GALACTOSIDASE ON AGAROSE-15m.

The α-galactosidase in 2 ml of 0·1 M potassium phosphate buffer, pH 7·0, was placed on an Agarose-15 m column (1·6 × 85 cm calc. V_0 = 65 ml) and eluted with the same buffer. Three ml fractions were collected. The fractions were assayed for α-galactosidase and protein as described in Experimental.

FIG. 2. CHROMATOGRAPHY OF ARABINOSE GROWN CULTURE FILTRATE ON DEAE-CELLULOSE.

The culture filtrate (12 l.) was adjusted to pH 7·0 and passed through a column of DEAE-cellulose (3·0 × 22 cm) which had been equilibrated with 0·1 M potassium phosphate, pH 7·0. The column was eluted first with 200 ml of 0·1 M potassium phosphate buffer, pH 7·0, and then with a 0–0·2 M linear NaCl gradient (fraction 1–26). The gradient was followed by elution with 0·2 M sodium chloride in buffer. Ten ml fractions were collected. The column was assayed for β-glucosidase, α-galactosidase, α-arabinosidase, β-galactosidase, β-xylosidase and protein as described in Experimental.

Fractions 8–12, containing the β-glucosidase (Fig. 2), were combined, concentrated, and desalted by pressure dialysis. The β-glucosidase containing solution was placed on an Agarose-0·5 m column (2·0 × 110 cm, V_0 = 105 ml). The enzyme activity eluted just past the void volume in a manner very similar to the α-arabinosidase (Fig. 3). The enzyme solution was concentrated by pressure dialysis and then placed on an Agarose-1·5 m column (1·6 × 76 cm, V_0 = 58 ml). In this case, the enzyme eluted at 1·6 V_0 as shown in Fig. 4. A summary of the purification of the β-glucosidase is presented in Table 3. The purified β-glucosidase contains no cellulase activity when measured on carboxymethyl-cellulose.

TABLE 3. SUMMARY OF THE PURIFICATION OF β-GLUCOSIDASE

Sample	Total activity (units)	Specific activity (units/mg)	Relative purification	% yield
Crude	3200	0·41	1	100
DEAE-cellulose	1780	11·0	27	55
Agarose-0·5 m	1230	25·2	62	39
Agarose-1·5 m	800	36	88	25

Purification of Exopolygalacturonase

The culture filtrate of the pectin grown fungus (β-strain) was dialyzed against 50 mM sodium acetate, pH 5·2. The dialyzed solution was then passed through a column of DEAE-cellulose (Whatman D.E.-52) that had been equilibrated with the 50 mM sodium acetate, pH 5·2. The enzyme activity was not absorbed onto the column. The effluent from the DEAE-

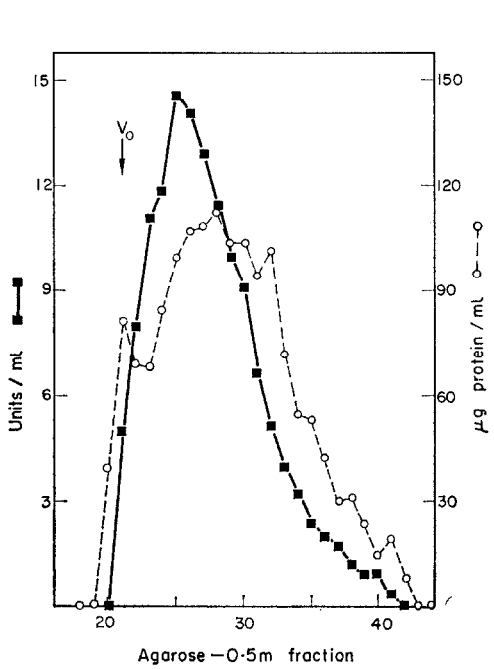

FIG. 3

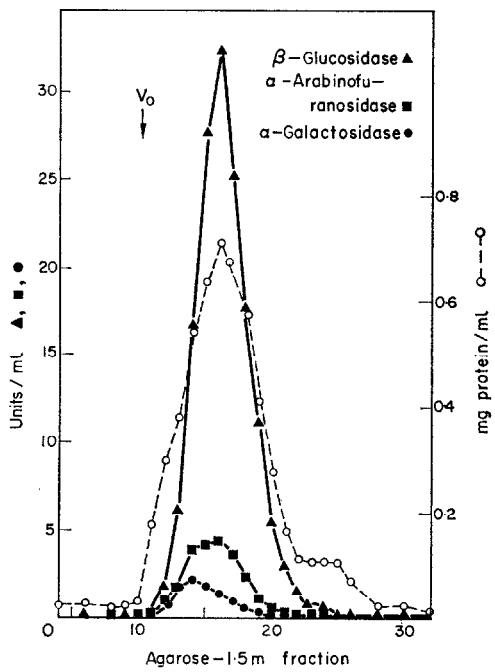

FIG. 4

FIG. 3. CHROMATOGRAPHY OF α-ARABINOFURANOSIDASE ON AGAROSE-0·5 m.

The α-arabinofuranosidase in 5 ml of 0·1 M potassium phosphate, pH 7·0, was placed on an Agarose 0·5 m column (2·0 × 110 cm, V_0 = 105 ml) and eluted with buffer. Five ml fractions were collected. The fractions were assayed for α-arabinofuranosidase and protein as described in Experimental.

FIG. 4. CHROMATOGRAPHY OF β-GLUCOSIDASE ON AGAROSE-1·5 m.

The β-glucosidase in 3 ml of 0·1 M potassium phosphate, pH 7·0, was placed on an Agarose-1·5 m column (1·6 × 76 cm, V_0 = 58 ml) and eluted with this buffer. Fractions of 5·7 ml were collected. The fractions were assayed for β-glucosidase, α-galactosidase, α-arabinosidase and protein as described in Experimental.

cellulose column was then passed through a column of Bio Rex 70 which had been equilibrated in the same buffer. Again the activity was not absorbed onto the column. This effluent was concentrated by pressure dialysis. The concentrated enzyme was placed on a Bio-Gel P-100 column (1·3 × 110 cm, V_0 = 55 ml) and eluted with 50 mM sodium acetate, pH 5·2. The enzyme activity eluted at the void volume of the column. The activity was again

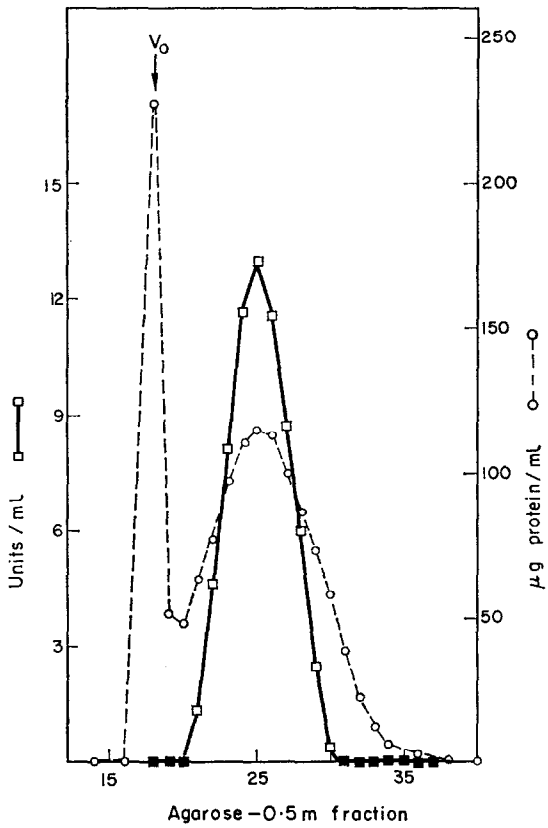

FIG. 5. CHROMATOGRAPHY OF THE EXOPOLYGALACTURONASE ON AGAROSE-0·5 m.
The exopolygalacturonase in 1·7 ml of 50 mM sodium acetate, pH 5·2, was placed on an Agarose-0·5 m column (1·4 × 98 cm, calc. V_0 = 57 ml) and eluted with the same buffer. Fractions of 3·2 ml were collected and assayed for polygalacturonase and protein as described in Experimental.

concentrated by pressure dialysis and placed on an Agarose-0·5 m column (1·4 × 98 cm, V_0 = 57 ml). The column was eluted with 50 mM sodium acetate, pH 5·2. The enzyme eluted at 1·8 V_0 as shown in Fig. 5. Fractions 24–26 were combined and used as the exopolygalacturonase. A summary of the purification of the exopolygalacturonase is shown in Table 4. This enzyme produces little reduction in the viscosity of polygalacturonic acid solutions while hydrolyzing 10% of the galacturonic acid glycosidic linkages. Thus, the enzyme is assumed to attack polygalacturonic acid in an exo manner. This was confirmed by paper chromatography of the products where, even very early in the reaction, monogalacturonic acid is found as the only product.

TABLE 4. SUMMARY OF THE PURIFICATION OF EXOPOLYGALACTURONASE

Sample	Total activity (units)	Specific activity (units/mg)	Relative purification	% yield
Crude-dialyzed	—	—	—	—
DEAE-cellulose	975	41·5	1·0	100
BioRex-70	975	48·5	1·1	100
P-100	430	64·1	1·5	44
Agarose-0·5 m	198	183	4·4	20

Measure of Contaminating Glycosidase Activities

Each of the purified enzyme preparations was tested for a variety of glycosidase and hydrolase activities which might interfere with its usefulness in controlled degradation of plant cell wall material. The results of this experiment are summarized in Table 5. It is important to note that these comparisons of the amounts of the various activities are for these model substrates only and may not be proportional to the actual amounts of the various enzymes present.

TABLE 5. THE ACTIVITIES OF OTHER ENZYMES IN THE PURIFIED GLYCOSIDASE PREPARATIONS

Substrate	Enzyme preparation used: α-Galacto-sidase	α-Arabino-furanosidase	β-Gluco-sidase	Exopoly-galacturonase
p-Nitrophenyl-α-D-galactoside	1663	0·33	1·2	0
p-Nitrophenyl-α-L-arabinofuranoside	0	12·2	1·0	0
p-Nitrophenyl-β-D-glucoside	0	0	18·1	0
p-Nitrophenyl-β-D-xyloside	1·3	0	1·5	0
p-Nitrophenyl-β-D-galactoside	1·3	0	1·6	0·8
Araban	0	15·0	29·0	30·0
CM cellulose	0	0	0	0
Polygalacturonic acid	0	0	7·0	20·6

Each glycosidase preparation was tested for its ability to degrade the listed substrates. The reactions were carried out as described in Experimental. The values given are expressed as units of activity per ml of enzyme solution.

DISCUSSION

Glycosidases have been isolated and purified from a wide variety of sources.[6–17] We have searched for a source of enzymes capable of degrading isolated plant cell walls. The enzyme

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mixture secreted by *C. lindemuthianum* when this fungus is grown in culture with cell walls as the carbon source is capable of extensive degradation of isolated plant cell wall material.[2]

The ability to induce selectively a desired enzyme is advantageous during subsequent purification attempts. *C. lindemuthianum* when grown in culture permits such selective induction. For example, when galactose is used as the carbon source for growth of *C. lindemuthianum*, large quantities of α-galactosidase are secreted while essentially no α-arabinofuranosidase, polygalacturonase or cellulase is detectable in the medium. A similar situation exists for the other enzymes, although their induction is not as specific as that observed with galactose as the carbon source.

In addition to the enzymes reported here and previously,[4] it is possible to induce this fungus to secrete two xylanases by growth of the fungus on xylan, a β-xylosidase by growth on xylose, an arabanase by growth on araban, and two cellulases by growth on cellulose (unpublished results of this laboratory).

The α-galactosidase described in this paper appears to be nearly homogeneous and contains no contaminating enzyme activities which might interfere with its use on cell wall polysaccharides. The α-arabinofuranosidase is not homogeneous, but it is highly purified and, like the α-galactosidase, does not contain any other enzymes which might interfere with its use on cell wall polysaccharides. The exopolygalacturonase still contains arabanase activity which will limit the exopolygalacturonase's usefulness on any pectic polysaccharides which contain arabinose residues. The β-glucosidase preparation contains several contaminating enzyme activities despite the appreciable purification obtained. These enzymes elute as a single peak during gradient elution of the DEAE cellulose column as shown in Fig. 2, and these enzymes are still present after fractionation on two gel filtration columns. It may be that these activities are contained in a single particle which prevents their separation. This also would prevent their being used to degrade cell wall polysaccharides in a controlled manner.

None of the purified enzyme preparations reported here are capable of degrading isolated plant cell walls. The *C. lindemuthianum* endopolygalacturonase reported previously[4] is capable of removing pectic materials from cell walls isolated from bean hypocotyls or cultured sycamore cells. A cellulase purified from this fungus is able to release carbohydrate material from isolated walls pretreated with the endopolygalacturonase, but is unable to attack the walls that have not been pretreated (unpublished results of this laboratory). The glycosidases reported here will be useful in studying the structure of the fragments released by these endo enzymes.

EXPERIMENTAL

All materials for column chromatography were purchased from Bio-Rad Laboratories with the exception of the Whatman DEAE-cellulose which was purchased from Sigma. The araban and all of the *p*-nitrophenyl glycosides, except for the *p*-nitrophenyl-α-L-arabinofuranoside, were purchased from Koch-Light. The *p*-nitrophenyl-α-L-arabinofuranoside was synthesized by Dr. J. B. Jurale of this laboratory using the method of Fielding and Hough.[18] Citrus pectin was a gift of Sunkist Growers Inc. *C. lindemuthianum* was maintained as described previously.[2] The α-strain of the fungus was used for the production of all of the enzymes except the exopolygalacturonase where the β-strain was used. For production of the enzymes, the fungus was grown in a 2800-ml Fernbach flask fitted with a three-way stopcock as described.[2] The fungus was grown in basal salts medium[2] with the carbon source varying according to the enzyme desired. For production of α-galactosidase, the carbon source was 20% galactose, while for α-arabinosidase production 2% arabinose was used. The β-glucosidase was isolated from the arabinose medium, but can be specifically induced by growth on glucose medium. For production of the exopolygalacturonase, the β-strain of the fungus was grown with 1% citrus pectin as the carbon source.

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Samples of the culture medium were withdrawn as described[2] and assayed for the desired enzyme activity. When the activity in the medium had reached a maximum (usually 2–3 weeks), the culture was harvested. The mycelia were removed by filtering through a coarse sintered glass funnel or through GF/C glass filter paper.

The activities of the glycosidases were determined by measuring the hydrolysis of the respective *p*-nitrophenyl glycosides as described[3] except that all reactions were run at 30°. One unit of activity is defined as that amount of enzyme which releases 1 μmol of *p*-nitrophenol in a 1 hr-reaction.

Polygalacturonic acid for the assay of the polygalacturonase was prepared by the base-catalyzed de-esterification of a 0·1% solution of citrus pectin at pH 12 and 0° for 6 hr followed by reduction with sodium borohydride (10 mg/l. at pH 12 and 0° for 1 hr). To assay for the polygalacturonase, 1·0 ml of a 0·1% solution of this polygalacturonic acid in 50 mM sodium acetate, pH 5·2, was reacted with the enzyme at 30°. The increase in reducing groups was assayed by the Nelson–Somogyi method.[19] The degradation of other polysaccharides was assayed in a similar manner. One unit of activity is defined as the amount of enzyme which releases 1 μmol of reducing sugar in a 1 hr reaction. Protein was measured by the method of Lowry *et al.*[20] All purifications were carried out at 4°.

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Key Word Index—Collectotrichum lindemuthianum; fungi plant pathogen; α-galactosidase; α-arabinofuranosidase; exopolygalacturonase; β-glucosidase.