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Production and Properties of Galactosidases from *Corticium rolfsii*

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When *Corticum rolfsii* was grown in a medium containing bran extract under aerobic conditions, it secreted α-D-galactosidase and β-D-galactosidase into the culture fluid. Pectin also stimulated the production of these enzymes, whereas galactose, glucose, and sucrose stimulated their production to a lesser degree. *C. rolfsii* produced greater amounts of both enzymes than *Aspergillus niger*. Both galactosidases in the culture medium hydrolyzed α- and β-*p*-nitrophenyl-D-galactosides as well as lactose, stachyose, melibiose, and raffinose. Both exhibited optimal activity at *p*H 2 to 4 and were quite stable under acidic conditions. α-Galactosidase was separated from β-galactosidase by column chromatography.

Corticium rolfsii is a plant-pathogenic, oxalate-forming basidiomycete. Culture fluid from this fungus has been demonstrated to contain an endo-polygalacturonase (5) and an α-L-arabinofuranosidase (6). These enzymes are characterized by their acid stability and by their low *p*H optima. The present paper describes the elaboration of α-D-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) and β-D-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) by *C. rolfsii*. The ability of this pathogen to secrete these enzymes under a variety of culture conditions is demonstrated, and some of the properties of these enzymes are described.

MATERIALS AND METHODS

Organism. Strain K2 of *C. rolfsii* was originally isolated in the laboratory of Plant Pathology, Kyoto University, and was kindly supplied by N. Naito of Kagawa University. The strain of *Aspergillus niger* used was ATCC 10577.

Cultivation. The fungus was cultured in a medium containing 20 g of the sugar under investigation or 12.5 g of pectin or bran extract, 10 g of peptone, 0.5 g of NH_4NO_3, 0.5 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.3 ml of 2% $FeCl_3$ in 1 liter of water. When bran extract was used, it was prepared by boiling 50 g for 1 hr. The solid matter was removed from the suspension; the supernatant fluid was adjusted to a total volume of 1 liter and used in place of the water in the fungal medium. The fungal medium was, in all cases, adjusted to *p*H 5.4.

The fungus was routinely grown in 50 ml of medium in a 500-ml shaken flask at 28 C on a reciprocal shaker. As described in a previous paper (6), the culture fluid was withdrawn daily, the mycelium was removed by filtration, and the clear solution was dialyzed against 0.01 M citrate-0.02 M Na_2HPO_4 buffer (*p*H 4.0). The dialyzed solution was used as the enzyme source.

Greater amounts of the enzymes were obtained by growing the fungus in the bran extract medium in a 200-liter aerated fermentation tank (1 liter of air per liter of medium per 1 min) at 28 C for 122 hr. The culture fluid (170 liters) was obtained by removing the mycelium by filtration and subsequent centrifugation. Ammonium sulfate was added to the culture fluid to 90% saturation. The resulting precipitate was collected and dissolved in 5.1 liters of water. The enzyme solution was dialyzed against water and then against 0.01 M citrate-0.02 M Na_2HPO_4 buffer, *p*H 4.0. The dialyzed solution was used to study the purification and properties of the α- and β-galactosidases.

Enzyme assay. A standard reaction mixture contained 0.8 ml of 1 mM *p*-nitrophenyl-α- or -β-D-galactopyranoside, 0.8 ml of buffer, and 0.8 ml of enzyme solution and was incubated at 30 C for 1 hr. The reaction was terminated by the addition of 2.4 ml of 0.1 M Na_2CO_3. The amount of *p*-nitrophenol released was determined by measuring the absorbance at 400 nm in a Hitachi spectrophotometer (model 139). One unit of α- or β-D-galactosidase was defined as the amount of enzyme which released 1 nmole of *p*-nitrophenol in 1 min under the above conditions. The α- and β-*p*-nitrophenyl galactosides were purchased from Pierce Chemical Co., Rockford, Ill., and Sigma Chemical Co., St. Louis, Mo., respectively. When di- and trisaccharides were used as substrates, the reducing sugars produced were determined by the Somogyi-Nelson method (10, 12). Lactose was purchased from De-Meijerij-Meghel Co., The Netherlands, stachyose and melibiose were purchased from Sigma Chemical Co., and raffinose was purchased from E. Merck AG, Darmstadt, Germany. Pectin was assayed by the carbazole method (8, 13). When

reaction mixtures were analyzed for sugars, the macromolecules were first removed by precipitation with ethyl alcohol (80% final concentration). The resulting precipitate was removed by centrifugation, the supernatant fluid was passed through a small column (1.8 by 10 cm) of mixed Amberlite CG-120 (H^+ form) and IR-45 (OH^- form), ratio 1:2, and the effluent was then evaporated to dryness under reduced pressure. The residue was dissolved in 1 ml of deionized water and applied to a Toyo no. 50 A filter paper. The chromatograms were developed by descending chromatography in ethyl acetate-pyridine-water (12:5:4). The location of reducing sugar on the chromatograms was detected as reported by Trevelyan et al. (15) and modified by Smith (11).

Stability of enzymes. The stability of the galactosidases at various *p*H values was determined by adjusting 5 ml of enzyme solution containing 20 mg of protein to the appropriate *p*H value (*p*H 1.0 to 8.5) with 0.1 N HCl or 0.1 N NaOH. The enzyme solution was then mixed with 2.0 ml of buffer (0.2 N KCl-HCl buffer or 0.1 N sodium citrate-HCl buffer for *p*H 1.0 to 2.0; 0.1 M citrate-0.2 M Na_2HPO_4 buffer for *p*H 2.0 to 8.5). Toluene (0.2 ml) was added to each solution, and the solutions were incubated at 2 C for 72 hr. After incubation, each mixture was adjusted to *p*H 2.0, and deionized water was added to each to bring the volume to 20 ml. Relative activity of each solution was determined by using 0.05 mg of protein for α-galactosidase assays and 0.2 mg of protein for β-galactosidase assays.

Separation of α-galactosidase. Partial purification of both enzymes was achieved by gel filtration on Sephadex G-100 and column chromatography on QAE-Sephadex A-50. The crude enzyme solution was further dialyzed against 0.01 M citrate-0.02 M Na_2HPO_4 buffer (*p*H 4.0) for 24 hr. The dialyzed solution (400 ml) containing 3.36 g of protein was poured over a column of Sephadex G-100 (4.4 by 70 cm) which had been equilibrated with 0.01 M citrate-0.02 M Na_2HPO_4 buffer, *p*H 4.0. The column was irrigated by the same buffer, and the eluate was collected in 20 ml-fractions. Active fractions were combined and dialyzed against 0.01 M sodium phosphate buffer (*p*H 7.5) for 24 hr; the dialysate was charged on a column of QAE-Sephadex A-50 and chromatographed as described in Fig. 4. Sephadex G-100 and QAE-Sephadex were purchased from Pharmacia Co., Uppsala, Sweden.

Protein in the enzyme solution was calculated from the absorbance at 280 nm by using crystalline α-L-arabinofuranosidase from *A. niger* as standard.

RESULTS

Properties of α- and β-galactosidases produced by C. rolfsii. Figure 1 shows that the amounts of α- and β-*p*-nitrophenyl galactoside, hydrolyzed under standard conditions, were directly proportional to the amount of protein in the culture fluid. One milligram of protein in the crude culture fluid liberated 185 nmoles of galactose from *p*-nitrophenyl-α-galactoside and 33 nmoles of galactose from *p*-nitrophenyl-β-galactoside per 1 min.

The *p*H optima of the α- and β-galactosidases in the culture fluid of *C. rolfsii* were determined

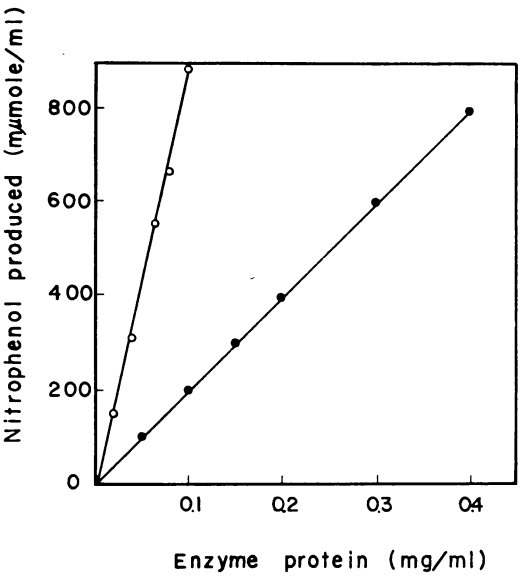

FIG. 1. *Effect of enzyme concentration on activity of α- (○) and β-galactosidases (●). Sodium citrate (0.1 M)-HCl buffer (pH 2.0) was used. The reactions were incubated at 30 C for 1 hr.*

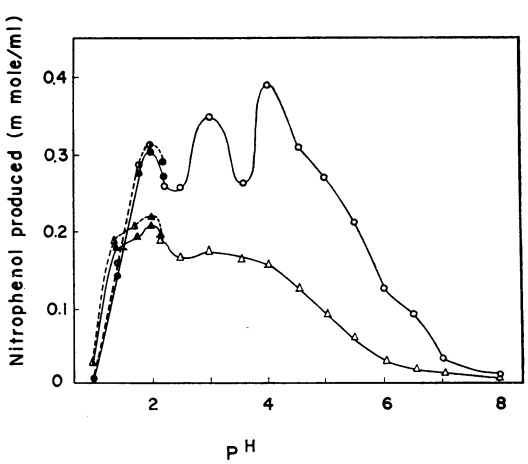

FIG. 2. *Effect of pH on the activity of α- and β-galactosidases. We used 0.2 N KCl-HCl buffer (◑, ▲) and 0.1 M sodium citrate-HCl buffer (●, ▲) for pH 1.0 to 2.2, and 0.1 M citrate-0.2 M Na_2HPO_4 buffer (○, △) for pH 2.2 to 8.0; concentration of enzyme protein was adjusted to 0.05 mg per ml of reaction mixture for α-galactosidase assay and 0.2 mg for β-galactosidase assay. Symbols: ◑, ●, ○, α-galactosidase activity; ▲, ▲, △, β-galactosidase activity.*

(Fig. 2.). Since the relative enzyme activity was determined over the pH range of 1 to 8, several buffers were employed as detailed in the legend of Fig. 2. The *p*H optima of the galactosidases appeared to be approximately *p*H 2 to 4, and three independent peaks were detected in case of α-galactosidase.

The stability of both galactosidases at various *p*H values indicates that 84% of the original α-galactosidase and 94% of the original β-galactosidase remain after the treatment at *p*H 2.0 and that both enzymes are stable at *p*H values 2 to 7.

The rate of *p*-nitrophenyl galactoside hydrolysis was linear with time during 40 min of incubation. The initial rate of *p*-nitrophenyl-α-galactoside hydrolysis, 183 nmoles per min per mg of protein, may be compared to that of *p*-nitrophenyl-β-galactoside, 33 nmoles per mg of protein per min.

The α- and β-galactosidases of *C. rolfsii* hydrolyzed β-lactose, stachyose, melibiose, and raffinose with a relative efficiency of 2.8:4.1:5.3:11.0 (Fig. 3). Paper chromatographic data show that galactose was liberated from these oligosaccharides. Galactose was also released from the *p*-nitrophenyl galactosides.

The ability of *C. rolfsii* to secret α- and β-galactosidases is dependent upon the carbon source on which it grows. The data of Table 1 compare the relative effectiveness of L-arabinose, D-xylose, D-glucose, D-galactose, sucrose, lactose, sodium galacturonate, pectin, and bran extract in inducing the secretion of α- and β-galactosidases. The *p*H of the culture fluid, the amount of reducing sugar present in the culture fluid, and the activities of α- and β-galactosidases were assayed for each culture at 24-hr intervals. The greatest growth was obtained when the fungus was grown on bran extract; an intermediate amount of growth was obtained with galactose, glucose, sucrose, lactose, and pectin (Table 1).

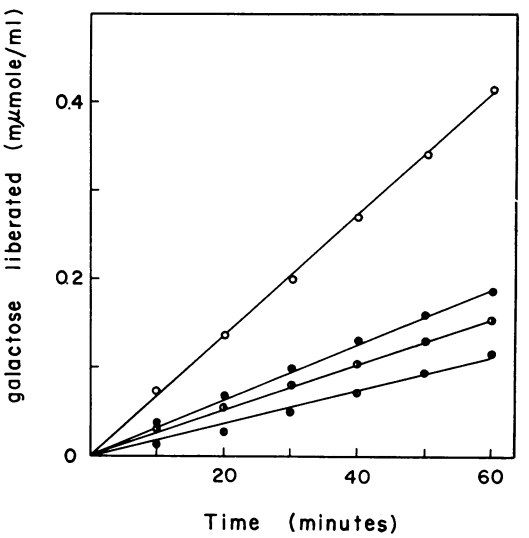

FIG. 3. *Hydrolysis of lactose, melibiose, stachyose, and raffinose. A 1-ml amount of 10 mM sugar solution, 3 ml of 0.1 M sodium citrate-HCl buffer (pH 2.0), and 1 ml of enzyme solution containing 0.3 mg of protein were mixed and incubated at 30 C. Liberated galactose was determined by the Somogyi-Nelson method. Symbols:* ○, *raffinose;* ●, *melibiose;* ◑, *stachyose;* ◐, *lactose.*

TABLE 1. *Influence of carbon source on the production of α- and β-galactosidases by C. rolfsii*

Carbon source (2%)	Percentage of carbon source consumed	Relative growth	Amt (milliunit) of galactosidases per ml of culture fluid		Ratio of α-galactosidase to β-galactosidase
			α-Galactosidase	β-Galactosidase	
Arabinose	40	+++[a]	340 (120)[b]	102 (144)	2.8
Xylose	33	+++	400 (160)	54 (144)	7.4
Glucose	74	++++	359 (168)	38 (168)	9.4
Galactose	71	++++	713 (144)	90 (144)	7.9
Sodium galacturonate	68	+	39 (144)	39 (144)	1.0
Sucrose	87	++++	632 (168)	41 (168)	4.0
Lactose	72	++++	275 (168)	0	
Pectin	96	++++	1,060 (144)	440 (144)	2.4
Bran extract[c]	68	+++++	2,310 (168)	587 (144)	3.9

[a] At the end of 192 hr of incubation, the mycelia were collected, washed with hot water, dried, and weighed. Symbols: +++++, abundant mycelial growth (6 mg/ml, dry weight); +, very small amount of dry mycelium (0.2 mg/ml); +++, 0.6 mg/ml; ++++, 0.7 to 1.4 mg/ml.

[b] The numbers in parentheses represent the time of incubation (hr) necessary to obtain maximal production of enzymes.

[c] Water-extractable material from 5% bran suspension.

Only a small amount of mycelium was obtained when the fungus was grown on arabinose, xylose, or sodium galacturonate. The acidity of the culture fluid dropped from *p*H 5.4 to 2.0 to 2.2 because of the production of oxalic acid. When the fungus was grown in a medium containing bran extract, the *p*H dropped from 5.4 to 2.3 after 72 hr of incubation but increased gradually to 4.4 by 192 hr of incubation. When bran extract was the only carbon source, the greatest production of α- and β-galactosidases resulted; pectin was also an effective substrate with regard to its ability to stimulate the production of these enzymes. Galactose, glucose, and sucrose stimulated the production of intermediate levels of these enzymes, and lactose stimulated the production of α-galactosidase but not β-glactosidase.

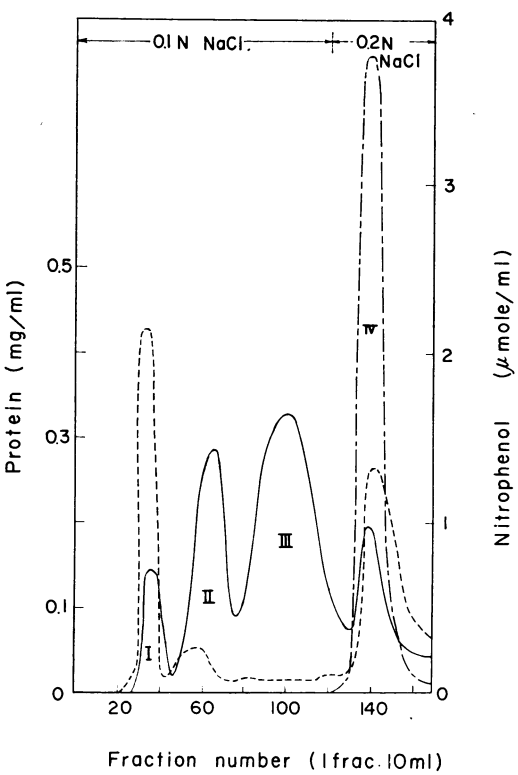

FIG. 4. *Chromatography on QAE-Sephadex A-50. QAE-Sephadex A-50 was equilibrated with 0.01 M sodium phosphate buffer (pH 7.5) and packed in a column (2.8 by 40 cm). The enzyme solution, 150 ml containing 2,190 units of α-galactosidase and 405 units of β-galactosidase, was poured onto the column. The enzymes were eluted by a stepwise addition of 0.1 to 0.2 N NaCl with a constant addition of 0.01 M sodium phosphate buffer, pH 7.0. Symbols: solid line, activity of α-galactosidase; line of alternating long and short dashes, β-galactosidase; dashed line, protein.*

C. rolfsii produced, under these conditions, 27.5 times as much α-galactosidase and 3.7 times as much β-galactosidase than *A. niger* ATCC 10577.

In the first gel filtration on Sephadex G-100, the activity of α-galactosidase was detected in fractions 10 to 45 and the activity of β-galactosidase was detected in fractions 10 to 25. Fractions 13 to 35 were collected, and ammonium sulfate was added to the solution up to 0.8 saturation. The resulting precipitate was subjected to the second gel filtration on Sephadex G-100, and both enzymes were recovered. The active fractions were collected and layered on a column of QAE-Sephadex A-50. α-Galactosidase was eluted into three peaks (I, II, III) and was clearly separated from β-galactosidase (Fig. 4). β-Galactosidase was detected in peak IV, but small amounts of α-galactosidase were also recovered in the same fractions. The optimal *p*H range of α-galactosidase in peaks I, II, and III appeared to be from *p*H 2.0 to 4.5, but the *p*H curves were not parallel with one another.

DISCUSSION

C. rolfsii produces α- and β-galactosidases which are stable and optimally active under acidic conditions. The characteristics of these enzymes indicate that they are acid glycosidases (6). Other organisms have been shown to produce α-galactosidase with optima at *p*H 2.8 to 5.7 (17) and *p*H 4.8 (16) for bottom yeast, *p*H 3.5 to 3.6 for *A. oryzae* (17), *p*H 5.2 for *Streptomyces olivaceus* (14), and *p*H 4.0 for *A. niger* (2). β-Galactosidases produced by a variety of organisms have been reported to be active at the following *p*H values: 7.2 to 7.3 for *Escherichia coli* (4, 7) 7.0 for *Paracolobactrum aerogenoides* (1), 7.0 for *Streptococcus lactis* (3), and 3.8 for *A. niger* (2). The α- and β-galactosidases from *C. rolfsii* are most active at *p*H 2 to 4 and also at *p*H 1.5 to 2.0. In the *p*H curve of α-galactosidase, three independent peaks were found to appear at *p*H 2.0, 3.0, and 4.0. α-L-Arabinofuranosidase of *C. rolfsii* is most active at *p*H 2.5 and fairly active at *p*H 1.0 (6), and endopolygalacturonase from the same organism is most active at *p*H 2.5 (5). These optima are much lower when compared with general microbial glycosidases and glycanases.

The enzymes of *C. rolfsii* are able to hydrolyze several naturally occurring di- and trisaccharides as well as α- and β-*p*-nitrophenyl galactosides.

The data in Table 1 demonstrate that maximal production of the enzymes with the various carbon sources used is achieved at 120 to 168 hr of incubation. More importantly, the ratio of the maximal amounts of α-galactosidase and β-galac-

tosidase varies according to the carbon source used for growth. This is demonstrated by the fact that, when pectin was the only carbon source, 2.4 times more α-galactosidase was produced than β-galactosidase, whereas glucose resulted in the production of 9.4 times more α-galactosidase than β-galactosidase. When lactose was used as the carbon source the production of β-galactosidase was not stimulated, although it is well known that *E. coli* produces β-galactosidase exclusively in the presence of lactose (9). It is interesting that *A. niger* produces almost twice as much β-galactosidase as α-galactosidase under conditions in which *C. rolfsii* produces 3.9 times as much α-galactosidase as β-galactosidase.

As shown in Fig. 4, α-galactosidase of *C. rolfsii* was completely separated from β-galactosidase.

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