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α-L-Arabinofuranosidase from *Rhodotorula flava*

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An α-L-arabinofuranosidase (EC 3.2.1.55) from the culture fluid of *Rhodotorula flava* IFO 0407 grown on beet arabinan as a carbon source has been highly purified. The purified enzyme has a pH optimum of 2.0. The enzyme is unusually acid stable, retaining 82% of its activity after being maintained for 24 h at pH 1.5 and at 30°C. The apparent K_m and V_{max} values of the enzyme for phenyl α-L-arabinofuranoside were determined to be 9.1 mM and 72.5 μmol per min per mg of protein, respectively.

Ehrlich and Schubert (3) reported in 1928 that an enzyme in Takadiastase liberates arabinose from beet arabinan. In 1964, Kaji and Tagawa (9) reported that *Aspergillus niger* produces an arabinanase in its culture fluid. This enzyme was purified to homogeneity (12), crystallized (10), and demonstrated to be an α-L-arabinofuranosidase (EC 3.2.1.55) which attacks terminal α-L-arabinofuranosides (11). More recently, Kusakabe et al. (15) reported that a yeast, *Rhodotorula flava,* produces an arabinanase in the culture fluid. The present paper describes the purification and some properties of the arabinanase produced by *R. flava.*

MATERIALS AND METHODS

Organism and cultivation. *R. flava* has also been called *Cryptococcus flava, Torula flava,* and *Chromotorula flava.* The strain, IFO 0407, used here was obtained from the Institute for Fermentation of Osaka, Japan. The organism was cultured in a medium containing 20 g of beet arabinan, 6 g of peptone, 5 g of yeast extract, 3 g of NH_4NO_3, 1 g of KH_2PO_4 and 0.5 g of $MgSO_4 \cdot 7H_2O$ in 1 liter of deionized water. The organism was grown routinely in 50 ml of medium in a 500-ml flask and was incubated at 28°C on a reciprocal shaker (120 strokes per min) for 4 days. The supernatant fluid, obtained from the culture fluid by centrifugation, was dialyzed against 0.01 M citrate-sodium phosphate, pH 4.0. The dialyzed solution was used for an estimation of the amount of enzyme present in the culture fluid.

Assay methods. A reaction mixture containing 0.5 ml of 1.0% purified beet arabinan, 0.4 ml of 0.1 M citrate-phosphate buffer, pH 3.0, and 0.1 ml of enzyme solution was incubated routinely at 50°C for 30 min. The reaction was stopped by the addition to the reaction mixture of 1 ml of 0.1 M Na_2CO_3. The reducing sugar released by the action of the enzyme was determined as L-arabinose equivalents by the Nelson-Somogyi methods (16, 17). One unit of the enzyme is the amount of enzyme which liberates 1 μmol of L-arabinose equivalents from beet arabinan per min under the above conditions. The enzymatic activity on phenyl α-L-arabinofuranoside and on *p*-nitrophenyl α- and β-D-galactopyranosides was assayed under the same conditions as with the beet arabinan. The amount of *p*-nitrophenol released by the enzyme was determined spectrophotometrically by determining the increase in absorbancy at 400 nm.

The amount of protein in the enzyme solution was estimated from the absorbancy at 280 nm. The crystalline (10) α-L-arabinofuranosidase, which had been prepared in our laboratory from the culture fluid of *A. niger,* was used as a standard in the absorbancy assay. Sugar content in the culture fluid was determined as L-arabinose by the Nelson-Somogyi method after acid hydrolysis of polysaccharide in the culture fluid. The degree of cell growth was measured by the increase in absorbancy at 510 nm of 10-fold-diluted culture fluid. A 10-fold dilution of the sterile culture medium was used as a control.

Purification of enzyme. The centrifuged culture fluid was made 90% saturated with respect to ammonium sulfate. After the solution was allowed to stand overnight at 2°C, the resulting precipitate was collected by centrifugation and dissolved in 300 ml of deionized water. This solution was dialyzed for 24 h against 20 liters of deionized water and then for 24 h against 20 liters of 0.01 M citrate-phosphate buffer, pH 4.0. This solution was then chromatographed, first on SP-Sephadex C-50 and then on diethylaminoethyl (DEAE)-cellulose in 0.01 M citrate-phosphate, pH 4.0 and 8.0, respectively. In each case, the enzyme was eluted from the columns in a linear 0 to 0.5 M NaCl gradient in the same buffer. Then the enzyme solution from the DEAE-cellulose column was chromatographed on DEAE-Sephadex A-50 and on Sephadex G-100. The last two procedures were done twice. Before the gel filtration, the enzyme solution was concentrated in a collodion bag. The details of the second chromatography on DEAE-Sephadex A-50 and the second gel filtration on Sephadex G-100 are described in the legends of Fig. 2 and 3, respectively. The above purification procedures were performed at 2°C.

Substrates. Crude beet arabinan was extracted from beet pulp by the method of Hirst and Jones (5) and was purified as described previously (18). Arabinoxylan (18), 1,5-arabinan (18), and bran extract (14) were also prepared as described previously. Pectin was purchased from Sunkist Growers, Inc. Bran extract contains various sugar units such as arabinose, xylose,

galactose, and glucose. Pectin contains minor components of arabinose, galactose, and rhamnose as sugar unit. Phenyl α-L-arabinofuranoside was prepared by the method of Börgeson et al. (1). p-Nitrophenyl α- and β-D-galactopyranosides were purchased from Sigma Chemical Co.

Gel electrophoresis. Polyacrylamide-gel electrophoresis was carried out in tris(hydroxymethyl)-aminomethane-glycine buffer (pH 8.3) by the method of Davis (2) with a current of 3 mA for 2 h. Protein was stained with 0.25% Coomassie Brilliant Blue R. Gels were destained by diffusion for 48 h in acetic acid-methanol-water (75:50:875).

RESULTS

Production of arabinanase. The effect of carbon source on the production of arabinanase is shown in Table 1. The enzyme was produced specifically when polysaccharides containing arabinose such as beet arabinan, arabinoxylan, pectin, and bran extract were used as the sole carbon source. No enzyme activity was detected when L-arabinose and other mono- and oligosaccharides were used as the carbon source. Figure 1 shows enzyme activity in the culture fluid as a function of culture age, culture density, and the amount of sugar consumed when the organism was cultured in the medium containing beet arabinan as the carbon source. The maximum amount of enzyme production, cell growth, and sugar consumption was obtained after 4 days of growth.

Purification of enzyme. The crude enzyme solution was prepared from the culture fluid by precipitation with ammonium sulfate. When the crude enzyme acted on beet arabinan, only L-arabinose was detected by paper chromatography throughout various times of incubation. The results of chromatographing the enzyme on the second DEAE-Sephadex A-50 column and on the second Sephadex G-100 column are shown in Fig. 2 and 3, respectively. The results of the overall purification procedures are summarized

TABLE 1. *Effect of carbon sources on the production of arabinosidase by R. flava IFO 0407*

Carbon source	Growth (4 days)[a]	Arabinosidase[b]
Glycerol	0.06	0
L-Arabinose	0.36	0
D-Xylose	0.32	0
D-Glucose	0.36	0
Maltose	0.38	0
Soluble starch	0.16	0.1
Arabinan (beet)	0.12	5.1
Arabinoxylan	0.08	1.4
Pectin (citrus)	0.08	0.4
Bran extract	0.02	0.2

[a] Optical density at 510 mm.
[b] Units per milliliter of culture fluid.

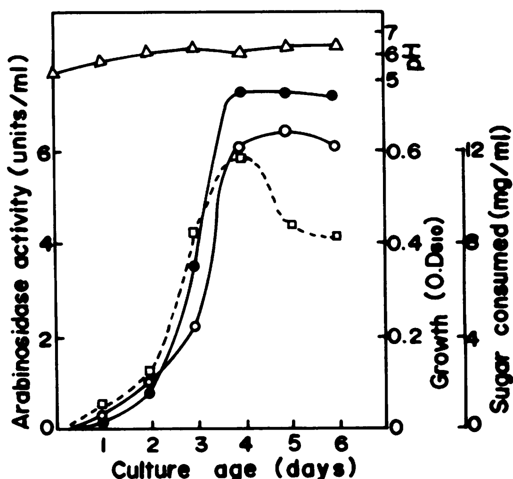

FIG. 1. *Time course of enzyme production.* (●) *Arabinosidase activity;* (○) *sugar consumed;* (□) *growth;* (△) *pH of culture medium.*

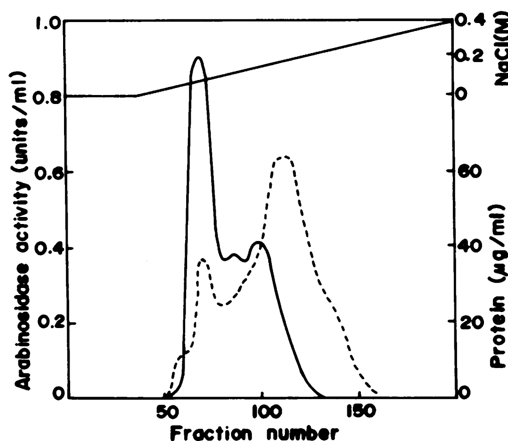

FIG. 2. *Chromatography on DEAE-Sephadex A-50 (II). A DEAE-Sephadex A-50 column (2.8 by 8 cm) was equilibrated with 0.01 M citrate-sodium phosphate, pH 7.0. The enzyme solution, 94 ml, containing 180 units or 12.9 mg of protein, was placed on the column. The enzyme was eluted with a linear gradient between 0 and 0.4 M NaCl in the citrate-phosphate buffer. Fractions (5 ml) were collected. Fractions 58 to 80 were combined.* (——) *Enzyme activity;* (----) *protein.*

in Table 2. The enzyme was purified 125-fold in specific activity from the crude extract. The purity of the enzyme was demonstrated by disc electrophoresis as shown in Fig. 4.

Effect of pH on activity and stability of enzyme. The effect of pH on the activity of the purified enzyme is shown in Fig. 5. The pH optimum of the enzyme activity appears to be approximately 2.0. The enzyme was still active

at pH 1.1. The enzyme was quite stable for 72 h between pH 2.0 and 5.0 when maintained at 2°C. Stability of the enzyme at low pH values and at 30°C was also examined by use of 0.1 M HCl-sodium citrate buffer (Fig. 6). The enzyme lost 77% of the initial activity after 24 h at pH 1.1, but at pH 1.5 and 2.0 the loss of enzymatic activity was only 18% and 5%, respectively.

Inhibition studies. After the enzyme was preincubated in 0.1 M acetate buffer (pH 4.0) containing salts or inhibitors for 20 min, the substrate was added to the enzyme solution and the enzymatic activity was determined. The blank tests were run with boiled enzyme in each case of salts or inhibitors. Ca^{2+}, Co^{2+}, Mg^{2+}, Fe^{2+}, CN^-, ethylenediaminetetraacetate and 2-mercaptoethanol did not exhibit inhibition when tested at a final concentration of 1 mM. As shown in Table 3, the arabinosidase was partially inhibited by Ag^+, Sn^{2+}, Cu^{2+}, and Hg^{2+}.

Action of the purified enzyme on various substrates. In these experiments, the ability of the arabinosidase to degrade various substrates was determined by incubation at pH 2.0 and at 30°C for 10 min. As shown in Table 4, the purified enzyme was found to be active on phenyl α-L-arabinofuranoside, beet arabinan (α-1,5-, 1,3-arabinan), 1,5-arabinan, and arabinoxylan (arabinofuranosyl 1 → 3 xyloglycan), but inactive on the *p*-nitrophenyl α- and β-D-galactopyranosides.

The Michaelis constant (K_m) and maximum velocity (V_{max}) of the purified enzyme for phenyl α-L-arabinofuranoside were calculated to be 9.1 mM and 72.5 μmol per min per mg of protein, respectively, from Lineweaver-Burk plots at pH 2.0 and 30°C.

Reaction products from beet arabinan hydrolyzed by the purified enzyme were analyzed by

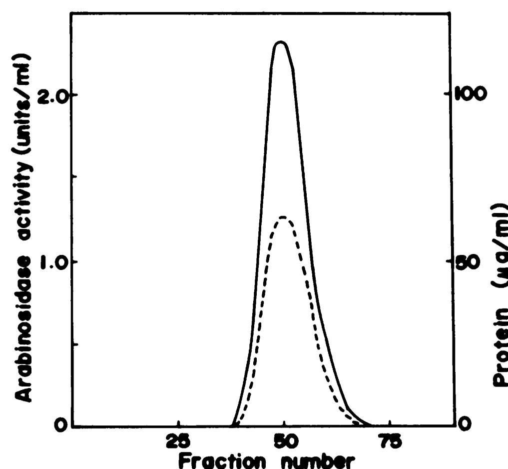

FIG. 3. *Gel filtration on Sephadex G-100 (II). A Sephadex G-100 column (1.6 by 85 cm) was equilibrated with 0.01 M citrate-sodium phosphate buffer, pH 4.0. The enzyme solution from the DEAE-Sephadex column, 2.5 ml, containing 54 units or 2.0 mg of protein, was placed on the column. The enzyme was eluted with the same buffer. Fractions (1.8 ml) were collected, and fractions 40 to 65 were combined.* (——) *Enzyme activity;* (----) *protein.*

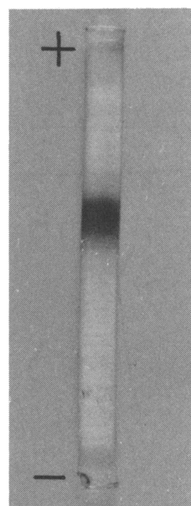

FIG. 4. *Disc gel electrophoresis of the purified enzyme. The purified enzyme solution containing 50 μg of protein was layered on a column of 7% polyacrylamide gel.*

TABLE 2. *Purification of arabinosidase*

Purification step	Vol (ml)	Total protein (mg)	Total activity (units)	Yield (%)	Sp act (units/mg)	Size of column (cm)
Ammonium sulfate	450	13,800	3,580	100	0.26	
SP-Sephadex C-50	800	4,590	1,610	45	0.35	5.0 × 30
DEAE-cellulose	860	589	1,500	41	2.54	2.8 × 40
DEAE-Sephadex A-50 (I)	238	146	500	14	3.42	2.8 × 20
Sephadex G-100 (I)	94	12.9	180	5.0	14.0	1.6 × 100
DEAE-Sephadex A-50 (II)	110	2.0	54	1.5	27.0	2.8 × 18
Sephadex G-100 (II)	45	1.6	52	1.5	32.5	1.6 × 85

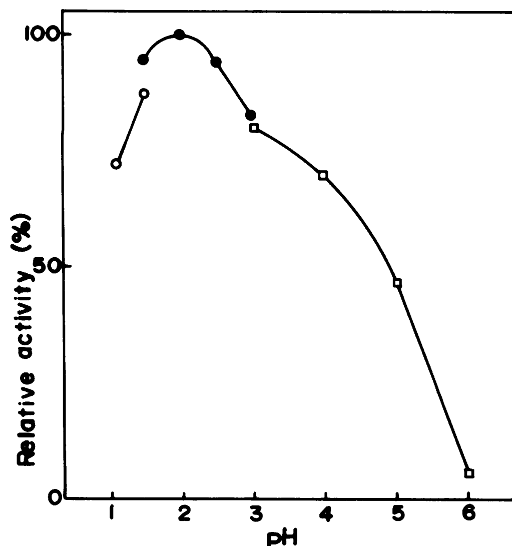

FIG. 5. *Effect of pH on the activity of the enzyme. A reaction mixture consisting of 0.5 ml of 1% beet arabinan, 0.4 ml of 0.1 M buffer, and 0.1 ml of enzyme solution was incubated at 50°C for 30 min.* (○) *KCl-HCl buffer;* (●) *HCl-sodium citrate buffer;* (□) *citrate-sodium phosphate buffer.*

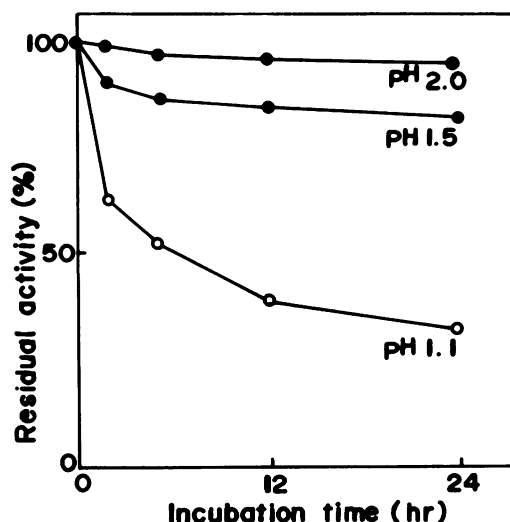

FIG. 6. *Effect of pH on stability of enzyme. The enzyme solution, 0.2 ml containing 5 μg of protein, was mixed with 0.8 ml of 0.1 M buffer, and each mixture was allowed to stand at 30°C. Residual activity was determined and represented as percentage of untreated enzyme solution.* (●) *HCl-sodium citrate buffer;* (○) *KCl-HCl buffer.*

the paper chromatographic method described previously (8). After 5 min of incubation, only arabinose was detected. The amount of arabinose released increased as the reaction proceeded. No other sugars or oligosaccharides were detected during a 24-h incubation.

TABLE 3. *Effects of salts or inhibitor on enzymatic action*

Salt or inhibitor	Concn in reaction mixture (mM)	Relative activity (%)
None	1.0	100
$AgNO_3$	1.0	36
$HgCl_2$	1.0	77
$SnCl_2$	1.0	54
$CuSO_4$	1.0	59
$MnSO_4$	1.0	85
p-CMB[a]	0.01	91

[a] Sodium *p*-chloromercuribenzoate.

TABLE 4. *Action of the purified enzyme on various kinds of substrates*

Substrate	Concn[a]	Reaction rate[b]
Phenyl α-L-arabinofuranoside	20 mM	49.8
Beet arabinan	0.5%	10.4
1,5-Arabinan	0.15%	3.6
Arabinoxylan	0.5%	4.9
p-Nitrophenyl α-D-galactopyranoside	0.5 mM	0
p-Nitrophenyl β-D-galactopyranoside	0.5 mM	0

[a] Concentration of substrate in reaction mixture.
[b] Micromoles per minute per milligram of enzyme protein.

DISCUSSION

By screening many microorganisms for their ability to hydrolyze arabinan, *A. niger* (9), *Corticium rolfsii* (14), and the other following fungi have been reported to produce arabinanase: *Botrytis cinerea, Sclerotinia libertiana, Gloeosporium kaki, Coniothyrium diplodiella* (13), *B. alli, Fusarium oxysporum* f. *lupini, S. sclerotiorum*, etc. (4). Bacteria can also degrade arabinan. *Clostridium felsineum* var. *sikokianum* produces an arabinanase which randomly attacks arabinan (6). Kaji and Saheki (8) demonstrated that enzyme purified to homogeneity from the culture fluid of *Bacillus subtilis*, is an endo-arabinanase (EC 3.2.1.~) with a mode of action quite different from that of the α-L-arabinofuranosidase reported here. Kusakabe et al. (15), in 1975, were the first to demonstrate the ability of a yeast, *R. flava*, to produce an arabinanase.

The present results show that *R. flava* IFO 0407 secretes an α-L-arabinosidase when polysaccharides containing arabinose, such as beet arabinan, are used as the sole carbon source, but not when L-arabinose is the carbon used (Table

1). In contrast, other fungi produce arabinanase when L-arabinose is used as the sole carbon source (13, 14).

The α-L-arabinosidase of *R. flava* has now been highly purified (Fig. 4). The purified enzyme has a very low pH optimum (Fig. 5) and also has acid stability (Fig. 6). The purified arabinosidase is, in fact, a typical acid glycosidase.

Among substrates tested, phenyl α-L-arabinofuranoside was the most active tested with the purified enzyme. In addition, the enzymatic product was paper chromatographically demonstrated to be only arabinose when the purified enzyme acted on beet arabinan. The purified enzyme was also able to attack the 1,3-L-arabinofuranosyl linkage in arabinoxylan and the 1,5-linkage in 1,5-arabinan. From these results, it is concluded that this enzyme is an exo-α-L-arabinofuranosidase. We consider that the main component of arabinan-degrading enzymes secreted by *R. flava* is the α-L-arabinofuranosidase because the sole reaction product from beet arabinan hydrolyzed by the crude enzyme is also arabinose.

We previously found that *C. rolfsii* also produces an acid α-L-arabinofuranosidase (14) as well as some other acid glycosidases (7). It is particularly interesting to learn that *C. rolfsii* is not the only organism capable of producing acid arabinosidase.

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