An α -L-Arabinofuranosidase from *Streptomyces purpurascens* IFO 3389

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By screening 46 strains of Actinomycetes for their ability to hydrolyze arabinan, 16 strains were found to have α -L-arabinofuranosidase activity, and *Streptomyces purpurascens* IFO 3389 was selected as the most promising of the sixteen. An α -L-arabinofuranosidase [EC 3.2.1.55] has been highly purified from the culture fluid of this organism grown on beet arabinan as the carbon source. The molecular weight of the native enzyme was determined to be 495,000 by gel filtration and that of the subunit to be 62,000 by SDS polyacrylamide gel electrophoresis. The *pI* value was 3.9. The purified enzyme was active on *p*-nitrophenyl α -L-arabinofuranoside and arabino-oligomers, and inactive on arabinan, arabinoxylan and arabinogalactan. The optimum pH was 6.5. The enzyme was inhibited by Hg^{2+} , Ag^+ and L-arabino- γ -lactone. The values of *K_m* and *V_{max}* for *p*-nitrophenyl α -L-arabinofuranoside were determined to be 8.2×10^{-5} M and 89.3 μmol per min per mg of protein, respectively.

Since the report by Kaji *et al.*¹⁾ in 1963 on the production of arabinosidase by *Aspergillus niger*, purification procedures for this enzyme have been steadily developed in our laboratory. It was purified to homogeneity²⁾ and demonstrated to be α -L-arabinofuranosidase.³⁾ The enzyme was crystallized and its properties were studied in detail.⁴⁾ Many fungi other than *A. niger* have been reported to produce a similar arabinosidase, but only one species of yeast, *Rhodotorula flava*,⁵⁾ was found to be a good source of this enzyme. *Bacillus subtilis*⁶⁾ elaborates α -L-arabinofuranosidase and endo-arabinase [EC 3.2.1.99] in the same culture fluid, and the two arabinosidases from *Clostridium felsineum*⁷⁾ may be exo- and endo-type arabinosidases. The present paper describes the results of screening of Actinomycetes, the production and purification of α -L-arabinofuranosidase from *Streptomyces purpurascens*, and its properties, particularly that this arabinosidase is exclusively active on a low-molecular weight substrate.

MATERIALS AND METHODS

Organisms and cultivation. A total of 46 strains of Actinomycetes were screened for the ability to hydrolyze arabinan. The strains used here were stock cultures obtained from the Institute for Fermentation of Osaka, the Laboratory of Fermentation Physiology, Kyoto University, and the Institute of Kitazato. The organisms were cultured in a medium containing 10 g of beet arabinan, 5 g of peptone, 1 g of yeast extract and 0.05 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in one liter of 0.05 M potassium phosphate buffer, pH 7.0. The organisms were grown in 5 ml of medium in a test tube and incubated at 28°C on a reciprocal shaker for 3 days. In the case of cultivation in flasks, the organisms were grown in 50 ml of medium in a 500-ml flask or 750 ml of medium in a 3-liter flask for 6 days. Of 6 species showing remarkable ability, *S. purpurascens* IFO 3389 was selected for the present investigation.

Assay methods. α -L-Arabinofuranosidase activity was assayed using beet arabinan as a substrate. The reaction mixture containing 0.5 ml of 0.5% purified beet arabinan, 0.4 ml of 0.1 M citrate-0.2 M sodium phosphate buffer, pH 6.5, and 0.1 ml of enzyme solution was incubated routinely at 30°C for 60 min. The reaction was stopped by the addition of 1 ml of 0.1 M Na_2CO_3 . The reducing sugar released by the action of the enzyme was determined by the Nelson-Somogyi method^{8,9)} using L-arabinose as a standard. When *p*-nitrophenyl α -L-arabinofuranoside was used as substrate, a reaction mixture containing 0.5 ml of 1 mM substrate, 0.4 ml of 0.1 M citrate-0.2 M sodium phosphate buffer, pH 6.5, and 0.1 ml of enzyme solution was

incubated at 30°C for 10 min. The reaction was stopped and the amount of *p*-nitrophenyl released was determined spectrophotometrically at 400 nm. The enzymatic activity on *p*-nitrophenyl α - and β -D-galactopyranosides was assayed under the same conditions. One unit of enzyme activity was defined as the amount of enzyme which liberates 1 μ mol of L-arabinose or *p*-nitrophenol per min under the above conditions.

The amount of protein in the enzyme solution was estimated from the absorbance at 280 nm. The crystalline α -L-arabinofuranosidase, which had been prepared in our laboratory from the culture fluid of *A. niger*, was used as a standard in the protein assay. The degree of cell growth was measured by weighing the cells after washing and drying them. Identification of the reaction products was carried out by paper chromatography. Ethanol was added to the reaction mixture and the precipitate was removed by centrifugation. The supernatant fluid was evaporated and the residue dissolved in deionized water. Then the dissolved solution was spotted on a paper and developed descendingly with a solvent system of *n*-butanol-pyridine-water=6:4:3. The sugars were visualized with alkaline AgNO_3 or aniline hydrogen phthalate reagent.

Purification of enzyme. The centrifuged culture filtrate, 45 liters, was made to 50% saturation with respect to ammonium sulfate and stood overnight. The resulting precipitate was removed by centrifugation at $9,600 \times g$. The supernatant was made to 70% saturation, and the resulting precipitate was collected by centrifugation and dissolved in deionized water, and then dialyzed for 24 hr against 0.01 M citrate-0.02 M sodium phosphate buffer, pH 7.5. The dialyzed solution was applied to a column of DEAE-cellulose previously equilibrated with 0.01 M citrate-0.02 M sodium phosphate buffer, pH 7.5. The enzymes were eluted stepwisely with 0.1, 0.2 and 0.5 M NaCl in the same buffer. Fractions rich in the arabinosidase which was active on *p*-nitrophenyl α -L-arabinofuranoside were collected and dialyzed successively against water and 0.01 M citrate-0.02 M sodium phosphate buffer for 24 hr each. The second step was QAE-Sephadex A-50 column chromatography which was performed under the same conditions as mentioned above except that a linear gradient of 0.2 to 0.6 M NaCl was used. The active fractions were collected and dialyzed against 0.01 M citrate-0.02 M sodium phosphate buffer, pH 7.0. The solution was concentrated with an Amicon ultrafiltration cell (DIAFLOW membrane PM-30) and poured onto a column of Sepharose 6B in 0.01 M citrate-0.02 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The same buffer solution was applied to the column for gel filtration. This procedure was repeated twice and fractions rich in arabinosidase were collected and dialyzed against deionized water and 0.001 M sodium phosphate buffer, pH 7.0, for 24 hr each. The dialyzed solution was applied to a column of hydroxylapatite previously equilibrated with the same buffer. The enzyme was eluted with a linear gradient of

0.001 to 0.2 M the same buffer. The active fractions were collected and dialyzed against 0.01 M citrate-0.02 M sodium phosphate buffer, pH 7.0. The dialyzed solution was concentrated with a collodion bag. The last step of purification was Sepharose 6B gel filtration as described above. All the experiments were performed in a cold room at 2°C.

Substrates. Crude beet arabinan was extracted from beet pulp by the method of Hirst and Jones¹⁰) modified as follows. One hundred grams of beet pulp was washed and swollen overnight in deionized water. The samples were heated in 2% Ca(OH)_2 at 100°C for 24 hr. After removal of the solid material by filtration and centrifugation at $9,600 \times g$ for 10 min, the supernatant was concentrated to 250 ml below 50°C with a rotary evaporator and adjusted to pH 3 with acetic acid. After standing for 24 hr at room temperature, the precipitate was removed by centrifugation under the same conditions. By adding 4 volumes of ethanol to the supernatant solution, arabinan was precipitated for 24 hr and collected by centrifugation. The precipitate was dissolved in 100 ml of deionized water and the insoluble matter was removed by centrifugation. Activated charcoal powder was added to the supernatant, followed by standing overnight at 60°C. The charcoal was removed and the supernatant lyophilized. Crude beet arabinan thus obtained was purified as described previously.¹¹) Arabinoxylan, 1,5-arabinan and wheat bran extract were also prepared as described previously.¹¹) Arabino-biose and triose (α -1,5-linkage) were prepared by Mr. Yoshihara from 1,5-arabinan with endo-arabinase as reported previously.⁶) Phenyl β -D-arabinoside and *p*-nitrophenyl glycosides were purchased from Koch-Light Laboratories, Ltd.

Electrophoresis. Polyacrylamide gel electrophoresis (7.5%) was performed according to the method of Davis¹²) with pH 8.3 buffer system. SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Weber and Osborn¹³) with a polyacrylamide (12.5%) slab gel containing 0.1% sodium dodecyl sulfate. Protein was stained with 0.1% Coomassie Brilliant Blue R. Gels were destained in 7% acetic acid. Isoelectric focusing electrophoresis was carried out with carrier ampholine (pH 4~6) by the method of Vesterberg and Svensson¹⁴) with the small scale column designed by Doi and Ohtsuru.¹⁵)

RESULTS

1. Screening

By screening 46 strains of Actinomycetes for their ability to hydrolyze arabinan, 16 strains were found with enzymatic activity. Six strains with significant activity are listed in Table I. *S. purpurascens* IFO 3389 was selected as a suit-

TABLE I. ARABINOSIDASE ACTIVITY OF ACTINOMYCETES

Organism	Arabinosidase ^a activity (m units/ mg of cells)	Reaction ^a products
<i>Streptomyces aureofaciens</i> KA 204	5.0	Arabinose Galactose Unidentified sugar
<i>S. flaveolus</i> KA 1143	5.5	Arabinose Unidentified sugar
<i>S. lavendulae</i> KA 140	5.3	Arabinose Galactose Unidentified sugar
<i>S. massaporeus</i> IFO 3841	5.2	Arabinose
<i>S. olivochromogenes</i> IFO 3178	4.7	Arabinose
<i>S. purpurascens</i> IFO 3389	6.3	Arabinose

^a Beet arabinan was used as substrate in the enzyme assay.

able organism for studying arabinosidase, because its activity was high, the enzymatic product was arabinose only, and growth was good, in particular it elaborated a new type of arabinosidase.

2. Production of arabinosidase

After judging the effects of the nitrogen source, phosphate concentration and the initial pH of the medium on the production of arabinosidase, a medium containing 10.0 g of carbon source, 5 g of peptone, 0.05 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.0 g of yeast extract in one liter of deionized water was chosen as the basal medium. The effect of the carbon source on the production of arabinosidase was investigated. The organism was grown in 50 ml of the medium in a 500-ml flask at 28°C for 5 days. The amount of arabinosidase was largely dependent on the carbon source used by the organism for growth. As to the relative production of the enzyme on eight different carbon sources, the greatest amount of enzyme

was found in the culture fluid when beet arabinan was used, and the activity was estimated to be 10.5 m units per mg of dry cells. Arabinose was also a fairly good carbon source and arabinosidase activity was 5.9 m units per mg of dry cells. Pectin, soy bean powder, wheat bran extract, gum arabic and galactose were poor carbon sources. Soluble starch was not effective for the production of arabinosidase activity.

3. Purification of enzyme

The crude enzyme solution, 1.4 liters, was poured onto a column of DEAE-cellulose. The results of the chromatography are shown in Fig. 1. Arabinosidases were eluted as peaks I, II and III. The ratio of the enzymatic activity toward *p*-nitrophenyl α -L-arabinofuranoside to that on arabinan in peak III is larger than those of peaks I and II. The arabinosidases in the latter two peaks were the same type as the reported α -L-arabinofuranosidase from various organisms, that is to say, these arabinosidases were active not only on *p*-nitrophenyl α -L-arabinofuranoside but also on arabinan. Thus, the enzyme in peak III was further purified by column chromatography on QAE-Sephadex A-50. The arabinosidase was eluted with 0.2 to 0.6 M NaCl in 0.01 M citrate-0.02 M sodium phosphate buffer, pH 7.5. It was highly active on *p*-nitrophenyl α -L-arabinofuranoside, and slightly active on arabinan. After the active fractions were combined, dialyzed and concentrated, the enzyme was further purified by gel filtration on Sepharose 6B. The enzyme solution was poured onto a column of hydroxylapatite, and the activity was, subsequently, recovered in two peaks (Fig. 2). The enzyme in the minor peak was different from the enzyme in major one in *pI*.

The enzyme solution in the main peak was dialyzed and the dialyzed solution was concentrated. The solution ran through a column of Sepharose 6B and the activity curve was parallel with the curve of protein in a single peak. The results of the overall purification procedures are summarized in Table II. The enzyme was purified 120-fold. A single band in

FIG. 1. Column Chromatography on DEAE-Cellulose.

A DEAE-cellulose column was equilibrated with 0.01 M citrate-0.02 M sodium phosphate buffer, pH 7.5. The enzyme solution, 1,400 ml, containing 4,470 units or 6,540 mg of protein, was placed on the column. The enzyme was eluted by stepwise addition of 0.1, 0.2 and 0.5 M NaCl in the same buffer. Fractions of 20 ml were collected. Fractions 311 to 324 were combined. ○—○, arabinosidase activity on *p*-nitrophenyl α -L-arabinofuranoside; ●—●, arabinofuranosidase activity on beet arabinan; -----, protein; —, NaCl.

FIG. 2. Column Chromatography on Hydroxylapatite.

A hydroxylapatite column was equilibrated with 0.001 M sodium phosphate buffer, pH 7.0. The enzyme solution, 5.8 ml, containing 418 units or 14.5 mg of protein, was placed on the column. The enzyme was eluted with a linear gradient of 0.001 to 0.2 M the same buffer. Fractions of 2 ml were collected. Fractions 49 to 54 were combined.

○—○, arabinosidase activity on *p*-nitrophenyl α -L-arabinofuranoside; -----, protein; —, concentration of buffer.

TABLE II. PURIFICATION OF α -L-ARABINOFURANOSIDASE

Step	Volume (ml)	Total protein (mg)	Total activity ^a (units)	Yield (%)	Specific activity (units/mg)	Column size (cm)
(NH ₄) ₂ SO ₄	1,400	6,540	4,470	100	0.68	
DEAE-cellulose	265	2,200	3,930	88	1.78	5.0 × 50
QAE-Sephadex A-50	130	430	2,600	58.2	6.0	4.6 × 40
Sephacrose 6B (I)	15.2	49	770	17.2	15.7	1.6 × 100
Sephacrose 6B (II)	5.8	14.5	418	9.4	28.8	1.6 × 100
Hydroxylapatite	11.8	2.75	180	4.0	65.5	2.0 × 12
Sephacrose 6B (III)	5.9	0.89	74	1.65	82.9	1.6 × 100

^a *p*-Nitrophenyl α -L-arabinofuranoside was used as substrate in the enzyme assay.

Fig. 3 demonstrates the homogeneity of the purified α -L-arabinofuranosidase.

4. Properties of the purified enzyme

(1) *Molecular weight.* The molecular weight of α -L-arabinofuranosidase was determined to be 495,000 by gel filtration on Sepharose 6B. The molecular weight of the subunit was calculated to be 62,000 by SDS polyacrylamide gel electrophoresis for which the standard proteins were thyroglobulin, ferritin, catalase, phosphorylase, albumin and lactate dehydrogenase (Fig. 4). The native enzyme may consist

of 8 subunits.

(2) *Isoelectric point.* The *pI* value was determined to be 3.9 by isoelectric focusing electrophoresis.

(3) *Effect of pH on the activity of the purified enzyme.* The effect of pH on the activity of the purified enzyme is shown in Fig. 5. The optimum pH for the enzymatic activity is 6.5.

(4) *Substrate specificity of the purified enzyme.* As shown in Table III, the enzyme was remarkably active on *p*-nitrophenyl α -L-arabinofuranoside. After the purified enzyme had acted on arabinobiose and triose, re-

FIG. 3. Polyacrylamide Gel Electrophoresis of the Purified Enzyme.

The purified enzyme solution containing 1.6 μg of protein was layered on a 7.5% polyacrylamide slab gel.

FIG. 4. Molecular Weight Estimation by SDS-Polyacrylamide Gel Electrophoresis.

Standard proteins: (A) phosphorylase (mol. wt. 94,000); (B) albumin (67,000); (C) catalase (60,000); (D) lactate dehydrogenase (36,000).

FIG. 5. Effect of pH on Activity of the Purified Enzyme.

A reaction mixture consisting of 0.5 ml of 1 mM *p*-nitrophenyl α -L-arabinofuranoside, 0.4 ml of 0.1 M buffer and 0.1 ml of enzyme solution was incubated at 30°C for 10 min. $\circ$, citrate-sodium phosphate buffer; $\bullet$, NH_4OH - NH_4Cl buffer; $\triangle$, Na_2CO_3 - NaHCO_3 buffer.

TABLE III. SUBSTRATE SPECIFICITY OF THE PURIFIED α -L-ARABINOFURANOSIDASE

Substrate	Concn. ^a	Reaction time	Reaction rate ^b
<i>p</i> -Nitrophenyl α -L-arabinofuranoside	0.5 mM	10 min	71.5
<i>p</i> -Nitrophenyl β -D-galactopyranoside	0.5 mM	1 hr	0
Beet arabinan	0.15%	6 hr	0
Arabinoxylan	0.15%	6 hr	0
Arabinogalactan	0.15%	6 hr	0
Gum arabic	0.15%	6 hr	0

^a Concentration of substrate in the reaction mixture.

^b Micromoles per minute per milligram of enzyme protein.

arabino-oligomers. The enzyme was inactive on arabinan, arabinoxylan, arabinogalactan, *p*-nitrophenyl β -D-galactopyranoside, phenyl β -L-arabinopyranoside and the following *p*-nitrophenyl glycosides; α -D-galactopyranoside, α - and β -D-glucopyranosides, α - and β -D-xylopyranosides, α -L-fucopyranoside, α -D-mannopyranoside and *N*-acetyl β -D-glucosaminide.

(5) *Inhibitory studies.* After the enzyme was preincubated in 0.1 M citrate-0.2 M sodium phosphate buffer (pH 6.5) containing the salts and inhibitors for 10 min, the substrate was added to the enzyme solution and the enzymatic activity was determined. Blank tests were run with boiled enzyme. The purified enzyme was completely inhibited by Hg^{2+} and Ag^+ at 1 mM. It was also potently inhibited by L-arabonic- γ -lactone (10 mM).

(6) *The values of K_m and $V_{\max}$.* The Michaelis constant (K_m) and maximum velocity ($V_{\max}$) of the purified enzyme for *p*-nitrophenyl α -L-arabinofuranoside were calculated to be 8.2×10^{-5} M and 89.3 μmol per mg of protein, respectively, from Lineweaver-Burk plots at pH 6.5 and 30°C.

DISCUSSION

spectively, at 30°C for 5 hr, the reaction product was found to be only L-arabinose by paper chromatography. Thus, the arabinosidase was confirmed to be active not only on synthetic arabinofuranoside but also on

The present paper is the first to describe a wide screening of Actinomycetes for their ability to produce arabinosidase. About one third of the type cultures tested had arabinosidase activity. As reported elsewhere,¹⁶ we isolated

150 strains of wild-type Actinomycetes from soil in Kagawa Prefecture on a medium containing beet arabinan as the sole carbon source. From them about 50 strains were selected as having arabinosidase activity. Most of these organisms seemed to belong to *Streptomyces*. It was, therefore, shown that Actinomycetes with arabinosidase activity occur frequently, especially of the genus *Streptomyces*.

Monomeric as well as polymeric arabinose was a good carbon source for the production of arabinosidase by *S. purpurascens* IFO 3389, a typical enzyme producer. Similar results were observed for the production of the same enzyme by fungi such as *A. niger* and *Botrytis cinerea*,¹⁷⁾ while for production by *R. flava*⁵⁾ arabinose was not a good carbon source. Therefore, *S. purpurascens* appears to be similar to fungi rather than to yeast in this regard.

α -L-Arabinofuranosidases produced by various fungi, i.e. *A. niger*,¹⁾ *C. rolfsii*,¹⁸⁾ *B. cinerea*,¹⁷⁾ *Sclerotinia libertiana*,¹⁷⁾ *Botrytis fabae*,¹⁹⁾ *Sclerotinia fructigena*,²⁰⁾ *Myrothecium verrucaria*,²¹⁾ *R. flava*,⁵⁾ or bacteria, i.e. *B. subtilis*,^{22,23)} *Streptomyces massapoensis*, are active on high-molecular weight substrates such as beet arabinan, arabinoxylan and arabinogalactan, as well as on low-molecular weight synthetic substrates, hydrolyzing 1,3- and 1,5-arabinosyl linkages.

The authors have found, while screening a great number of Actinomycetes, that one component of arabinosidases of *S. purpurascens* IFO 3389, unlike the arabinosidase mentioned above, is inactive on polymers on incubation for 6 hr at 30°C, though active on *p*-nitrophenyl α -L-arabinofuranoside and arabino-oligomers. On the basis of this specificity, it was concluded that the highly purified arabinosidase of *S. purpurascens* is a new type of α -L-arabinofuranosidase [EC 3.2.1.55] in regard to its substrate specificity. As the organism produced a great deal of this arabinosidase in the culture fluid, it is a good source of the enzyme.

In the present studies, an arabinosidase pro-

duced by *S. purpurascens* was found to be exclusively active on a low-molecular weight substrate, but it may be, conversely, that an arabinosidase exclusively active on a high-molecular weight substrate, such as beet arabinan, can be produced by *Bacillus subtilis* and *Erwinia carotovora*, according to recent work in our laboratory (unpublished).

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