Tech. Bull. Fac. Agr. Kagawa Univ. Vol. 34, No. 1, 79～85, 1982

α-L-ARABINOFURANOSIDASE FROM *STREPTOMYCES MASSASPOREUS* IFO 3841

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Streptomyces massasporeus IFO 3841 からの α-L-アラビノフラノシダーゼ

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An α-L-arabinofuranosidase (EC 3.2.1.55) from the culture fluid of *Streptomyces massasporeus* grown on beet arabinan as a carbon source has been highly purified. The purified enzyme has a pH optimum of 5.0, and was demonstrated to be highly active on *p*-nitrophenyl α-L-arabinofuranoside. The enzyme was potently inhibited by Hg^{2+} or L-arabonic γ-lactone. The apparent Km values of the enzyme for *p*-nitrophenyl-α-L-arabinofuranoside and arabinan were determined to be 1.67 mM and 25 g per liter, and Vma values were calculated to be 8.33 and 4.88 μ moles per min per mg of protein, respectively. The molecular weight of the enzyme was estimated to be 54,000.

Streptomyces massasporeus はビートアラビナンを炭素源とする培養により，α-L-アラビノフラノシダーゼ (EC 3.2.1.55) を生産し，その酵素はカラムクロマトグラフィーにより高度に精製された．精製酵素は分子量 54,000で，pH 5.0に至適 pH を持ち，Hg^{2+} および L-アラビノ-γ-ラクトンにより活性が阻害された．*p*-ニトロフェニル α-L-アラビノフラノシドに高い活性を示し，ビートアラビナンにも作用した．この両基質に対する Km 値はそれぞれ 1.67mM および 25g/*l* であり，Vmax は8.33および 4.88μmol/min/mg であった．

Introduction

Since an arabinosidase from *Aspergillus niger* was purified and demonstrated to be an α-L-arabinofuranosidase (EC 3.2.1.55) by Kaji *et al.*,[1-3] some fungi such as *Corticium rolfsii*[4] and *Rhodotorula flava*[5] were reported to produce the same type of arabinosidase. *Bacillus subtilis*[6] elaborates α-L-arabinofuranosidase and endo-arabinanase in the same culture fluid, and *Clostridium felsineum*[7] might produce exo- and endo-type of arabinosidases. Many known and unknown strains of Actinomycetes were found to produce arabinosidases,[8,9] and an arabinosidase from *Streptomyces purpurascens*[9] were purified and demonstrated to be a new type of α-L-arabinofuranosidase. The present paper describes the production, purification and properties of α-L-arabinofuranosidase from *Streptomyces massasporeus*.

Materials and methods

Organism and cultivation. *Streptomyces massasporeus* IFO 3841 obtained from the Institute for Fermentation of Osaka was used in this study. The culture medium containing 10 g of beet arabinan, 5 g of peptone, 1 g of yeast extract and 0.05 g $MgSO_4 \cdot 7H_2O$ in 1 liter of 0.05 M potassium phosphate buffer, pH

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7.0, was used for the enzyme production. The organism was grown in 50 m*l* of the medium in a 500-m*l* flask or 750 m*l* of the medium in a 3-liter flask for 5 days. In large scale cultivation, the organism was grown in 5 liter of the medium in a 10-liter jar fermenter for 8 days.

Assay methods. α-L-Arabinofuranosidase activity was mainly assayed by using beet arabinan as the substrate. A reaction mixture containing 0.5 m*l* of 0.5% purified beet arabinan, 0.4 m*l* of 0.1 M citrate-0.2 M sodium phosphate buffer, pH 5.0, and 0.1 m*l* of enzyme solution was incubated routinely at 30℃ for 60 min. The reaction was stopped by the addition of 1 m*l* of 0.1 M Na_2CO_3 to the reaction mixture. The reducing sugar released by the action of the enzyme was determined by the Nelson-Somogyi method[10,11] using L-arabinose as a standard. When *p*-nitrophenyl α-L-arabinofuranoside was used as the substrate, a reaction mixture containing 0.5 m*l* of 1 mM substrate, 0.3 m*l* of 0.1 M citrate-0.2 M sodium phosphate buffer, pH 5.0, and 0.2 ml of enzyme solution was incubated at 30℃ for 30 min. The reaction was stopped and the amount of *p*-nitrophenol 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 the enzyme activity was determined as the amount of enzyme which liberated 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 absorbancy at 280 nm. The crystalline α-L-arabinofuranosidase,[12] which had been prepared in our laboratory from the culture fluid of *A. niger*, was used as a standard in the absorbancy 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 as described in the previous paper.[8]

Purification of enzyme. The centrifuged culture filtrate, 2 liter, was made 90% saturated with respect to ammonium sulfate and stood overnight. The resulting precipitate was collected by centrifugation and dissolved in deionized water. This solution was dialyzed against deionized water and then 0.01 M potassium phosphate buffer, pH 7.0. The dialyzed solution, 170 m*l*, was poured onto a column of Sephadex G-50 in 0.04 M potassium phosphate buffer, pH 7.0, and the same buffer was applied to the column for the gel filtration. The active fractions were collected and concentrated with an Amicon ultrafiltration cell (DIAFLOW membrane UM-10). The enzyme solution was dialyzed against 0.04 M phosphate buffer, pH 7.0, and chromatographed on DEAE-Sephadex A-50 and then on hydroxylapatite. Then the enzyme solution from the latter column was concentrated by adsorption and elution on a small column of DEAE-Sephadex A-50. The concentrated solution was dialyzed against the phosphate buffer, pH 7.0, and the second gel filtration was performed as described above.

Substrates. Crude beet arabinan was extracted from beet pulp by the modified method of Hirst and Jones[13] as described follows. Beet pulp washed with water was heated in 2% Ca $(OH)_2$ at 120℃ for 45 min. After removal of the solid material with filtration and centrifugation, the supernatant was concentrated with a rotary evapolator and abjusted to pH 3 with acetic acid. By adding ethanol to the solution, arabinan was precipitated, collected by centrifugation and decolorized by activated charcoal powder. Crude beet arabinan thus obtained was purified as described previously.[14] Arabinoxylan, 1, 5-arabinan and bran extract were also prepared as described previously.[14] Phenyl β-D-arabinoside and *p*-nitrophenyl glycosides were purchased from Sigma Chemical Co. L-Arabonic acid γ-lactone was purchased from Koch-Light Laboratories Ltd.

Results

Production of arabinosidase. After judging the effect of nitrogen sources, phosphate concentration and initial pH of the medium on the production of arabinosidase, a medium containing 10.0 g of carbon source, 5 g of peptone and 0.5 g of $MgSO_4 \cdot 7H_2O$ in 1 liter of 0.01 M potassium phosphate buffer, pH 7.0, was chosen as a basal medium. The effect of carbon source on the production of arabinosidase is shown in

Table 1. Effect of carbon source on the production of arabinosidase by *Streptomyces massasporeus*

Carbon source	Growth (mg/ml)	Arabinosidase (m units/ml)
Glycerol	3.1	0
L-Arabinose	4.6	17.3
D-Galactose	4.0	7.0
D-Glucose	4.2	0
Beet arabinan	4.2	22.7
Soluble starch	3.1	0.7
Bran extract	2.5	1.1
Pectin	3.7	3.9
Soy been powder	—	3.3

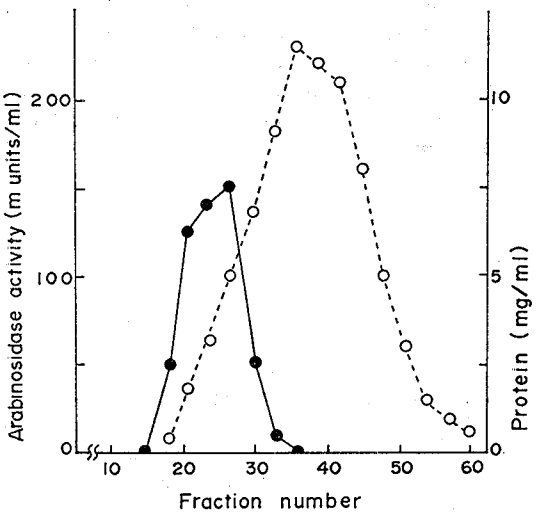

Fig. 1. Gel filtration on Sephadex G-50.
A Sephadex G-50 column (3.0 by 80 cm) was equilibrated with 0.04 M potassium phosphate buffer, pH 7.0. The enzyme solution, 170 ml, containing 16.1 units or 1.48 g of protein, was placed on the column. The same buffer was applied to the column. Fractions (15 ml) were collected. Fractions 18 to 30 were combined.
(●——●) Enzyme activity; (○·····○) protein.

Table 1. The enzyme was produced remarkably when beet arabinan was used as the sole carbon source, and arabinose was also fairly good carbon source.

Purification of enzyme. The result of the gel filtration on Sephadex G-50 column is shown in Fig. 1. The result of the chromatography on DEAE-Sephadex A-50 is shown in Fig. 2. Arabinosidases were eluted in peaks I and II, and their enzymatic properties were similar each other. The enzyme in peak I, which was higher than in peak II in specific activity, was further purified by column chromatography on hydroxylapatite. Its result is shown in Fig. 3. The result of the second gel filtration is shown in Fig. 4. The active curve of the enzyme is appeared parallel with the curve of protein in a single peak. The results of the overall purification procedures are summarised in Table 2. The enzyme was purified 71-fold in specific activity from the crude extract.

Molecular weight. The molecular weight of the purified enzyme was determined by the gel filtration chromatography. Chromatography on Sephadex G-100 (2.6 by 82 cm) in 0.02 M potassium phosphate

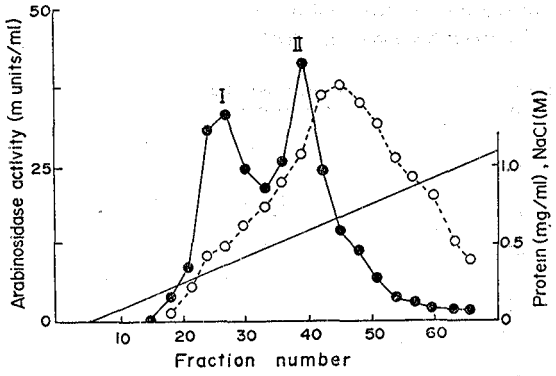

Fig. 2. Chromatography on DEAE-Sephadex A-50.
A DEAE-Sephadex A-50 column (2.6 by 30cm) was equilibrated with 0.04 M potassium phosphate buffer, pH 7.0. The enzyme solution, 42 ml, containing 15.3 units or 559 mg of protein, was placed on the column. The enzyme was eluted with a linear gradient between 0 and 1.0 M NaCl in the same buffer. Fractions (10ml) were collected. Fractions 22 to 31 were combined. (●——●) Enzyme activity; (○ ····○) protein; (——) NaCl.

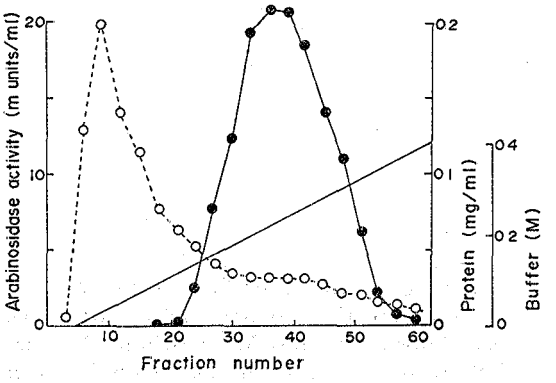

Fig. 3. Chromatography on hydroxylapatite.
A hydroxylapatite column (2.0 by 20 cm) was equilibrated with 0.005 M potassium phosphate buffer, pH 6.5. The enzyme solution, 100 ml, containing 2.36 units or 39.6 mg of protein, was placed on the column. The enzyme was eluted with a linear gradient between 0.005 and 0.4 M phosphate concentration. Fractions (10ml) were collected. Fractions 31 to 47 were combined. (●——●) Enzyme activity; (○ ····○) protein; (——) buffer.

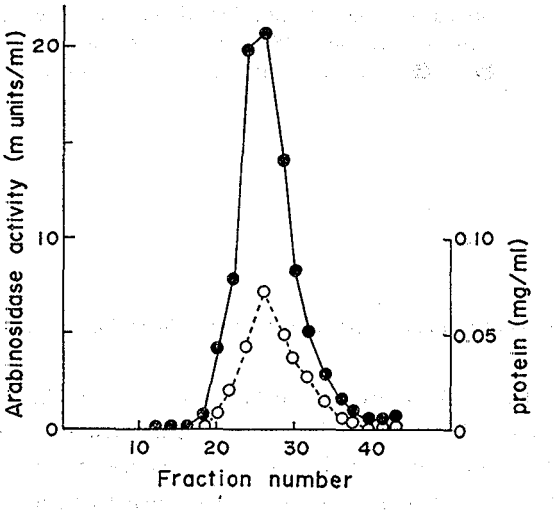

Fig. 4. Gel filtration on Sephadex G-100.
A Sephadex G-100 column (2.6 by 80 cm) was equilibrated with 0.04 M potassium phosphate buffer, pH 7.0. The enzyme solution, 20 ml, containing 1.19 units or 2.09 mg of protein, was placed on the column. The same buffer was applied to the column. Fractions (8 ml) were collected. Fractions 23 to 28 were combined. (●——●) Enzyme activity: (○ ····○) protein.

buffer containing 0.1 M NaCl, pH 7.0, indicated a molecular weight of 54,000.

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 optimum pH of the enzyme activity appears to be 5.0. The enzyme was active over the wide range of pH 3.0 to 8.0. The enzyme was quite stable for 20 h between pH 6.0 and 8.0 when maintained at 30℃, but lost 29% of the initial activity at pH 4.0 and 9.0.

Table 2. Purification of arabinosidase

Step	Total protein (mg)	Total activity (units)	Yield (%)	Specific activity (units/mg)
Ammonium sulfate	1,480	16.1	100	0.011
Sephadex G-50	569	15.6	96.7	0.027
DEAE-Sephadex A-50	39.6	2.36	14.6	0.060
Hydroxylapatite	3.31	1.77	11.0	0.54
DEAE-Sephadex A-50 (concentration)	2.09	1.19	7.4	0.57
Sephadex G-100	0.95	0.74	4.6	0.78

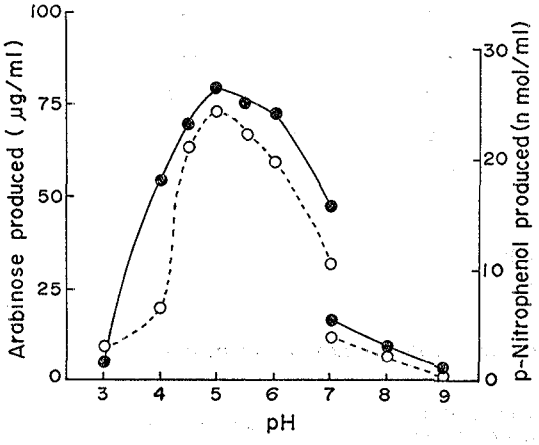

Fig. 5. Effect of pH on the activity of the enzyme.
Assays were carried out as described under Materials and Methods. Between pH 3.0 and 7.0, 0.1 M citrate-0.2 M sodium phosphate buffer was used, between pH 7.0 and 9.0, 0.1 M tris-HCl buffer. Beet arabinan (○·····○) and *p*-nitrophenyl α-L-arabinofuranoside (●——●) were used as the substrate.

Inhibition study. After the enzyme was preincubated in 0.1 M citrate-0.2 M sodium phosphate buffer (pH 5.0) 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 the boiled enzyme. As shown in Table 3, the arabinosidase was potently inhibited by Hg^{2+} (1 mM) and L-arabonic γ-lactone (10 mM), and partially inhibited by Mn^{2+}, Cu^{2+} and Pb^{2+} (1 mM).

Action of the purified enzyme on various substrates. As shown in Table 4, the purified enzyme was found to be active on *p*-nitrophenyl α-L-arabinofuranoside, beet arabinan (α-1, 5-and 1, 3-arabinan), 1, 5-arabinan, arabinogalactan (arabinofuranosyl 1→3 galactoglycan), and arabinoxylan (arabinofuranosyl 1→3 xyloglycan) but inactive on gum arabic and *p*-nitrophenyl α-and β-D-galactopyranosides.

The Michaelis constants (Km) were calculated to be 25 g per liter for arabinan, 1.67 mM for *p*-nitrophenyl α-L-arabinofuranoside, and the maximum velocity (V_{max}) was 4.88 μ mol or 8.33 μ mol per min per mg of protein, respectively, from Lineweaver-Burk plots at pH 5.0 and 30°C.

Reaction products from beet arabinan hydrolyzed by the purified enzyme were analyzed by the paper chromatographic method. After 5 min of incubation, only arabinose was detected and the amount of arabinose released increased as the reaction proceeded. No other sugars or oligosaccharides were detected during a 24-h incubation.

Table 3. Effect of salts and aldonic acid on enzymatic activity

Salt or aldonic acid	Concentration in reaction mixture (mM)	Relative activity (%)
None	0	100
$MgSO_4$	1.0	88
$MnSO_4$	1.0	84
$CuSO_4$	1.0	81
$ZnSO_4$	1.0	88
$Pb(CH_3COO)_2$	1.0	50
$Hg(CH_3COO)_2$	1.0	41
$FeSO_4$	1.0	78
p-CMB[1]	0.1	100
	1.0	100
EDTA[2]	1.0	100
L-Arabonic acid *r*-lactone	1.0	68
	10	23

1) Sodium *p*-chloromercuribenzoate.
2) Ethylenediaminetetraacetate, sodium salt.

Table 4. Action of the purified enzyme on various kinds of substrates

Substrate	Concentration in reaction mixture	Reaction rate[1]
Arabinan	0.5%	833
1,5-Arabinan	0.5%	450
Arabinogalactan	0.5%	408
Arabinoxylan	0.5%	192
Gum arabic	0.5%	0
p-Nitrophenyl α-L-arabinofuranoside	0.5mM	1,810
Phenyl β-D-arabinopyranoside	0.5mM	0
p-Nitrophenyl α-D-galactopyranoside	0.5mM	0
p-Nitrophenyl β-D-galactopyranoside	0.5mM	0

1) Nanomoles per minute per milligram of enzyme protein.

Discussion

Monomeric as well as polymeric arabinose was a good carbon source for the production of arabinosidase by *S. massasporeus* IFO 3841. The similar results were observed for the production of the same type of enzyme by *S. purpurascens*[9] and fungi such as *A. niger* and *Botrytis cinerea*,[15] while for the production by

R. flava[5] arabinose was not a good carbon source. Therefore, *S. massasporeus* as well as *S. purpurascens* appears similar to fungi rather than to yeast in this regard.

An arabinosidase produced by *S. massasporeus* was highly purified and shown maximum activity at pH 5.0. The enzyme was relatively stable between pH 4.0 and 9.0. The enzyme was clearly inhibited by L-arabonic γ-lactone as well as Hg^{2+}. This inhibition by L-arabonic γ-lactone suggests that the purified arabinosidase hydrolyzes specifically α-L-arabinofuranosidic linkages. Other divalent cations also inhibited partially the enzymatic action. These results in the inhibition studies are similar to those observed with the arabinosidase of *R. flava*[5].

The apparent Michaelis constant (Km) and maximum velocity (V_{max}) of the enzyme for *p*-nitrophenyl α-L-arabinofuranoside were 1.67 mM and 8.33 μmol per min per mg of protein, respectively. These values were lower than those of arabinosidases of *A. niger*[3], *C. rolfsii*[4] and *R. flava*[5]. The value of Km for beet arabinan was estimated to be 25 g per liter and this value was larger than those of the enzymes from the three organisms. Although the purified enzyme of *S. massasporeus* was active on *p*-nitrophenyl α-L-arabinofuranoside as well as polysaccharides containing arabinose (Table 4), the relative small value of Km for the synthetic substrate and the large value for beet arabinan suggest that the best substrate for this enzyme is the low-molecular weight arabinoside.

The molecular weight of the enzyme was estimated to be 54,000, which was similar to that (53,000) of the arabinosidase of *A. niger*[12]. In addition, the enzymatic product was paper chromatographically demonstrated to be only arabinose when the purified enzyme acted on beet arabinan. From these results it is concluded that the enzyme from *S. massasporeus* is an α-L-arabinofuranosidase (EC 3.2.1.55).

This work had been performed from 1978 to 1980 in laboratory of microbial biochemistry, Kagawa University.

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(Received May 31, 1982)