Title	Bacillus sp. No. 430 のα-L-アラビノフラノシダーゼの精製と性質(農芸化学科)
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Purification and Properties of α-L-Arabinofuranosidase II from *Bacillus* sp. No. 430†+

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Summary

This study was carried out for an effective utilization of agricultural wastes by microbial enzymes. In order to liberate arabinose from arabinose-containing polysaccharides such as arabinan, arabinoxylan, and arabinogalactan, α-L-arabinofuranosidase was investigated. An α-L-arabinofuranosidase II was purified from the culture fluid of *Bacillus* sp. No. 430, which was isolated from the soil of sugar cane fields. The process of purification was as follows ; salting out by ammonium sulfate, first DEAE-cellulose, second DEAE-cellulose, QAE-Sephadex A-50, DEAE-Toyopearl 650 S, and Sephadex G-75 column chromatography. The enzyme was highly purified by the method of disc gel electrophoresis. The purified enzyme had the maximum reactivity at pH 7.0 and 40°C. The enzyme was partially inhibited by Cu^{2+}, Cd^{2+}, α,α′-dipyridyl, iodoacetic acid, and *p*-chloromercuribenzoic acid. The highly purified enzyme was confirmed to liberate L-arabinose from beet arabinan, arabinogalactan, arabinoxylan, and *p*-nitrophenyl α-L-arabinofuranoside. The reaction product was paper chromatographically demonstrated to be L-arabinose only. The purified enzyme was inactive for *p*-nitrophenyl α-, β-D-galactopyranosides and *p*-nitrophenyl β-D-xylopyranoside. And thus, it was characterized as α-L-arabinofuranosidase which was exo-arabinanase. High purity of xylan from sugar cane bagasse or galactans from coffee bean and soybean was obtained with the enzyme.

Introduction

Ehrlich and Schubert[2] reported in 1928 that an enzyme in Takadiastase liberated arabinose from beet arabinan. In 1964, Kaji and Tagawa[4] reported that *Aspergillus niger* produced arabinanase in its culture fluid. The enzyme was purified to homogeneity, crystallized, and demonstrated to be an α-L-arabinofuranosidase which attacked terminal α-L-arabinofuranoside[5,6,8]. There are reports

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on bacterial arabinanase from *Clostridium felsineum*[3] and *Bacillus subtilis*[7,16].

The authors have isolated and screened useful microorganisms for establishing methods of effective utilization of agricultural wastes by microbial enzymes. In the previously paper[20,21], the bacterium (*Bacillus* sp. No. 430) which produced arabinan-degrading-enzyme, was isolated from soil of sugar cane fields, and two types of the enzyme, which were different in ionic properties, were separated by DEAE-cellulose column chromatography. And one of them (α-L-arabinofuranosidase I) was purified and characterized.

In the present paper, it is described that an α-L-arabinofuranosidase II from *Bacillus* sp. No. 430 has been isolated in a highly purified form, and some of its properties have been compared to those of the α-L-arabinofuranosidase I from this strain.

Materials and Methods

Organism and cultivation

The strain, *Bacillus* sp. No. 430 used here was isolated from the soil of sugar cane field in Okinawa prefecture as previously reported[20]. The organism was cultured in a medium, containing 2.5g of ammonium sulfate, 0.1g of yeast extract, 0.5g of K_2HPO_4, and 0.1g of $MgSO_4 \cdot 7H_2O$, in 1L of beet pulp extract. After the organism was grown in 5ml of the medium in a test tube and incubated at 30°C for 24 hr, it was transfered into the medium (50 ml) in a 500-ml flask and incubated at 30°C for 24 hr. The supernatant fluid, obtained from the culture fluid by centrifugation, was dialyzed against 10mM potassium phosphate buffer (pH7.0). 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% purified beet arabinan, 0.25 ml of McIlvaine buffer (pH6.5), and 0.25 ml of enzyme solution was incubated routinely at 40°C for 60 min. The reaction was terminated by the addition to the reaction mixture of 1 ml of 0.1M sodium hydroxide. The reducing sugar released by the action of the enzyme was determined as L-arabinose equivalents by the Nelson Somogyi[11,13]. 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. Specific activity was expressed as units per mg of protein.

The amount of protein in the enzyme solution was determined by the method of Lowry *et al.*[19] with serum albumin as a standard or estimated from the absorbancy at 280 nm.

Disc gel electrophoresis of the purified enzyme was performed by the procedure of Davis[1].

Paperchromatography of the sugars

Paperchromatography of the enzymatic digest of beet arabinan or arabinoxylan of sugar cane was carried out by the following method. The paper was developed two times by ascending methods, using the solvent system of n—BuOH : EtOH : H_2O (9 : 1 : 10, v/v/v). The spots were detected by aniline hydrogen phthalate[12] or alkaline silver nitrate.

Substrates

Crude arabinan[2,4] from beet pulp, crude arabinogalactans[10,21] from soybean or coffee bean, and crude arabinoxylan[17] from sugar cane bagasse were prepared and purified by the method of Tagawa and Kaji[14,21]. *p*-Nitrophenyl α-L-arabinofuranoside, *p*-nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl β-D-galactopyranoside, and *p*-nitrophenyl β-D-xylypyranoside were purched from

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Results and Discussion

Purification of the enzyme

Bacillus sp. No. 430 isolated from soil was grown in a medium containing beet pulp extract solution. The culture fluid was used as crude enzyme for enzyme solution. All subsequent operations were carried out at 4°C.

Step 1. Fractionation of ammonium sulfate

Ammonium sulfate was added to the culture filtrate to 90% saturation. After the mixture had stood for 60 min, the resulting precipitate was collected by centrifugation and was dissolved in 10 mM potassium phosphate buffer(pH 7.0). The solution was dialyzed for 15 hr against two changes of 100 volumes of the same buffer. The insoluble materials formed during the dialysis were removed by centrifugation.

Step 2. First DEAE-cellulose column chromatography

The enzyme solution was placed on a DEAE-cellulose column (ϕ 3.0 X 21.2cm) equilibrated with the dialysis buffer. Elution was carried out with the buffer solution stated above (arabinanase I). After the column was washed thoroughly with the same buffer and then with the buffer containing 0.15 M NaCl, the enzyme was eluted with the buffer supplemented with 0.50 M NaCl (arabinanase II). And then, arabinanase II was separated from I in this step. The active fractions were combined, and the enzyme solution was dialyzed for 15 hr against two changes of 100 volumes of 10 mM potassium phosphate buffer (pH 7.0).

Step 3. Second DEAE-cellulose column chromatography

The enzyme solution was placed on a DEAE-cellulose (ϕ2.0 X 10 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with the same buffer containing with 0.15 M sodium chloride, the enzyme was eluted with a linear gradient between 0.15 and 0.6 M NaCl in the same buffer. The active fractions were combined, and the enzyme solution was dialyzed against 100 volumes of the same buffer.

Step 4. QAE-Sephadex A-50 column chromatography

The enzyme solution was subjected to QAE-Sephadex A-50 column (ϕ 1.8 X 7.5 cm) in the same manner as mentioned above. The active fractions were collected, and the enzyme solution was dialyzed against 100 volumes of the same buffer.

Step 5. DEAE-Toyopearl 650 S column chromatography

The enzyme solution was chromatographed on a DEAE-Toyopearl 650 S column (ϕ1.0 X 7.5 cm) equilibrated with the dialysis buffer. After applying the enzyme and washing the column with the same buffer, the enzyme was eluted with a linear gradient between 0 and 0.5 M NaCl in the same buffer. The active fractions were concentrated by ultrafiltration.

Step 6. Sephadex G-75 column chromatography

The enzyme was applied to a Sephadex G-75 column (ϕ 1.7 X 103 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) and eluted with the same buffer. The active fractions were concentrated by ultrafiltration. Approximately 2-fold purification was achieved with an over-all yield about 2%. A protocol of the purification is presented in Table 1.

Table 1. Purification of arabinanase II of *Bacillus* sp. No. 430

Purification steps	Total protein (mg)	Total units	Specific activity (unit/mg)	Yield (%)
Crude enzyme	973	216	0.22	100
Ammonium sulfate	960	182	0.19	84
First DEAE-cellulose				
P-I	243	115	0.47	53
P-II*	377	30	0.08	14
Second DEAE-cellulose	120	20	0.17	9.3
QAE-Sephadex A-50	40.0	14	0.35	6.5
DEAE-Toyopearl 650 S	20.2	8.5	0.42	3.9
Sephadex G-75	10.1	4.4	0.44	2.0

*P—II was used for next steps.

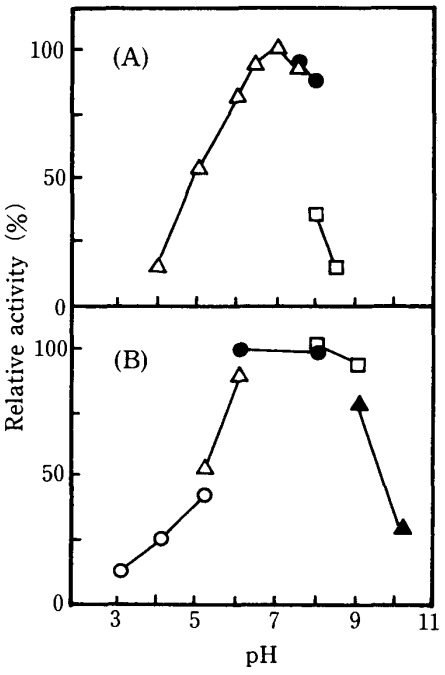

Fig. 1

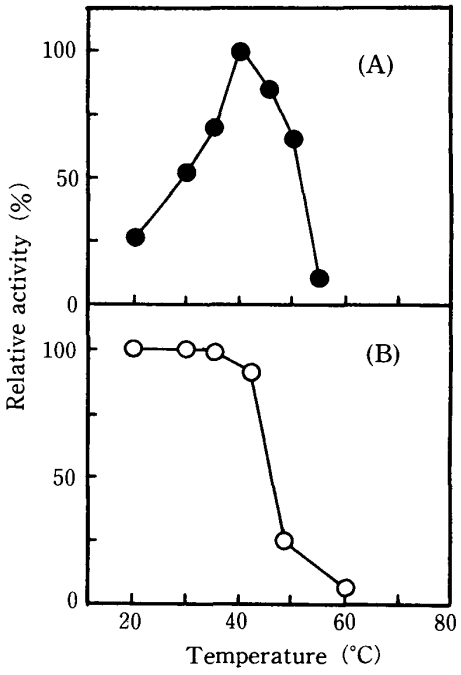

Fig. 2

Fig. 1. Effect of pH on the activity of arabinanase II (A) and the enzyme stability (B)

The enzyme activity was assayed at various pHs (A). The enzyme was incubated at pHs and 40°C for 5 min, and the activity was then assayed at pH 7.0 (B). Buffers : -O- ; Sodium phosphate—HCl buffer, -△-; McIlvaine buffer, -●- ; Potassium phosphate buffer, -□- ; Tris—HCl buffer, -▲- ; Na_2CO_3—$NaHCO_3$ buffer.

Fig. 2. Effect of temperature on activity of arabinanase II (A) and the enzyme stability (B).

The enzyme activity was assayed at various temperature (A). The enzyme was incubated at various temperatures and at pH 7.0 for 5 min, and the activity was then assayed. (B).

The purified enzyme was shown to be nearly homogeneous by criteria of disc gel electrophoresis.

Properties of the enzyme

Effect of pH on activity and stability

The effect of pH on the activity of the purifed arabinanase II from *Bacillus* sp. No. 430 was shown in Fig. 1. The optimum pH of the enzyme activity appears to be 7.0. The enzyme was active over the wide range of pH 4.0 to 9.0. As described previously, optimum pH of arabinanase I by this strain was 6.5[21]. It was reported that the optimum pH arabinanase from *Bacillus subtilis* for activity was 6.5[16]. and then, it was found that the optimum pH of arabinanase I was similar to that of *Bacillus subtilis*[16,21] but that of arabinanase II was different slightly from them. It is known that the pH optima of the enzymes originated in plant-phathogenic fungi and yeast[15] are from 2 to 3. The enzyme studied in this work was quite stable for 5 min treatment in a range of pH 6.0 to 8.0 when it was maintained at 40°C, but lost 13% and 31% of the initial activity at pH 4.0 and 9.0, respectively. The result obtained was similar to that of arabinanase I[21].

Effect of temperature on activity and stability

The effect of temperature on the activity and stability of the enzyme was shown in Fig. 2. The optimum temperature of the enzyme for activity was 40°C. This value was similar to that of arabinanase I[21]. The enzyme was stable for 5 min at a range of 0 to 40°C when it was maintained at pH 6.0, but lost 25% of the initial activity at 50°C.

Inhibition study

After the enzyme was preincubated for 10 min in McIlvaine buffer (pH 7.0), containing salts or

Table 2. Effect of various compounds on enzyme activity

Compounds (1mM)	Relative activity (%)
None	100
$CaCl_2 \cdot 2H_2O$	98
$MgCl_2 \cdot 6H_2O$	100
$ZnCl_2$	102
$CuCl_2$	76
$CoSO_4 \cdot 7H_2O$	93
$NiCl_2$	96
$SnCl_2 \cdot 2H_2O$	93
$SrCl_2 \cdot 6H_2O$	93
$CdCl_2 \cdot 2 \cdot 1/2\ H_2O$	85
$HgCl_2$	89
Ethylenediaminetetraacetic acid	88
o—Phenanthroline	88
α, α'—Dipyridyl	76
Iodoacetic acid	84
p—Chloromercuribenzoic acid*	84
N—Ethyl maleinimide	100
Sodium diethyldithiocarbamate	92

**p*—Chloromercuribenzoic acid (0.01 mM)

inhibitors, the substrate was added to the enzyme solution and enzymatic activity was determined. The blank test as run with the boiled enzyme. As shown in Table 2, the arabinanase was partially inhibited by Cu^{2+}, Cd^{2+}, α, α'-dipyridyl, iodoacetic acid, and *p*-chloromercuribenzoic acid. On the other hand, the enzyme activity was not affected by Ca^{2+}, Mg^{2+}, Zn^{2+}, *N*-ethylmaleinimide and sodium diethyldithiocarbamate.

Action of the purified enzyme on various substrates

As shown in Table 3 and 4, the purified enzyme was active on *p*-nitrophenyl α-L-arabinofuranoside, arabinogalactants from coffee bean and soybean, arabinan from beet, and arabinoxylan from sugar cane bagasse, but inactive on gum arabic, xylan from wood, *p*-nitrophenyl α-and β-D-galactopyranosides, and *p*-nitrophenyl β-D-xylopyranoside.

Table 3. Substrate specificity of arabinanase II (1)

The enzyme activity was assayed as follows : The reaction mixture containing 0.25 ml of lmM substrate, 0.15 ml of McIlvaine buffer, pH 7.0, and 0.1 ml of enzyme solution was incubated at 37°C for 20 min. The reaction was terminated by the addition to the reaction mixture of 1 ml of 0.1 M Na_2CO_3 solution and the amount of *p*-nitrophenol released was determined spectrophotometrically at 400 nm.

Substrates	Relative activity (%)
p-Nitrophenyl α-L-arabinofuranoside	100
p-Nitrophenyl α-D-galactopyranoside	0
p-Nitrophenyl β-D-galactopyranoside	0
p-Nitrophenyl β-D-xylopyranoside	0

Table 4. Substrate specificity of arabinanase II (2)

The enzyme activity was determined as described in the text.

Substrates	Relative activity (%)
Arabinan (sugar—beet)	100
Arabinogalactan (Coffee bean)	150
Arabinogalactan (Soybean)	45
Arabinoxylan (Sugar—cane bagasse)	20
Xylan	0
Gum arabic	0

Limit of hydrolysis of arabinan

To arabinan solution, purified arabinanase II was added and hydrolysis curve and limit of hydrolysis were examined (Fig. 3) . The hydrolysis degree of arabinan was rapidly increased with increasing reaction time up to 12 hr, but it increased gradually after incubation for 12 hr. The limit of hydrolysis of arabinan by this enzyme was 68%. It was found that the limit of hydrolysis of arabinan by arabinanase II was higher than that of arabinanase I (50%) [21].

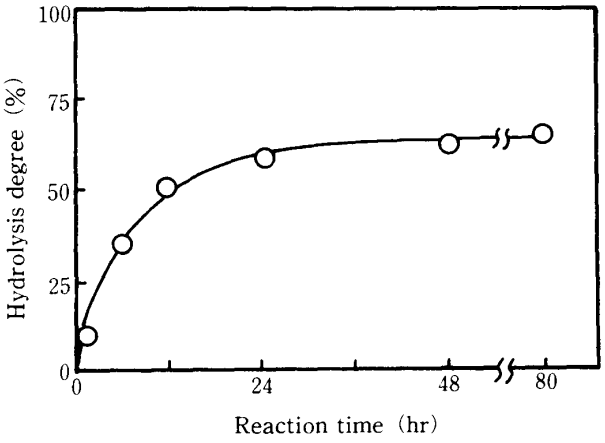

Fig. 3

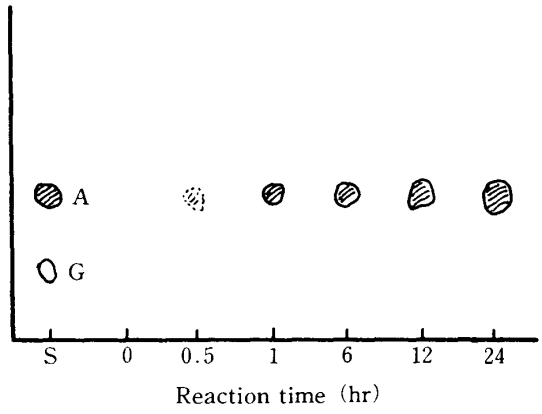

Fig.4

Fig. 3. Hydrolysis of beet arabinan by arabinanase II of *Bacillus* sp. No. 430.

The reaction mixture, containing 10 ml of 1% arabinan solution, 0.5 ml of 0.1M potassium phosphate buffer and 6 ml of enzyme solution was incubated at 40°C. An aliquot of the reaction mixture was withdrawn at intervals as indicated in figure, and the reducing sugar liberated was measured by the method of Somogyi-Nelson[11,13].

Fig. 4. Paper chromatogram of the products from beet arabian by arabinanase II of *Bacillus* sp. No. 430.

The experimental details were same as described in the legend of Fig. 3. At indicated time, an aliquot of the mixture was withdrawn and spotted on Toyo filter paper No. 50. The paper was developed two times by the ascending method using the solvent system of n-BuOH : EtOH : H_2O (9 : 1 : 10, v/v/v). The spots were detected by aniline hydrogen phthalate[12]. S represents the standard sugar solutions. A and G indicate L-arabinose and D-galactose, respectively.

Reaction products

Reaction products from beet arabinan hydrolyzed by the purified enzyme were analyzed by the paper chromatographic method. As shown in Fig. 4, after 30 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 24-hr incubation. Kaji[3,7,8] reported that the enzyme hydrolyzing arabinan to arabinose would involve, at least, two different enzymes ; one hydrolyzed arabinan exowisely to release L-arabinose only (exo-enzyme) , and the other attacked a rondom position of the arabinan molecule to give L-arabinose and its oligosaccharides as the hydrolytic products (endo-enzyme). Although there were two types of arabinanases such as exo-arabinanase and endo-arabinanase in *Clostridium felsineum*[3] or *Bacillus subtilis* [7,16], arabinanase I and II produced by this strain (*Bacillus* sp. No. 430) were characterized as α-L-arabinofuranosidase which

was exo-arabinanase[21].

Application of the enzyme

In order to liberate arabinose from arabinose containing polysaccharides such as arabinoxylan and arabinogalactan, the enzyme was investigated, The reaction products from hemicellulose of sugar cane bagasse (Fig. 5) or arabinogalactans from coffee bean and soybean, hydrolyzed by this enzyme, were also analyzed as the same manner described above. Only arabinose was detected after incubation, but any other oligosaccharides or monosaccharides such as xylose, galactose, and glucose were not detected in the reaction medium of all reaction times from 30 min to 24 hr. And thus, high purity of xylan or galactans were obtained with the enzyme. Development of the application methods which use characteristics of the enzyme is expected. However, for practical uses the enzyme is needed to be more stable under pH, temperature, and other conditions.

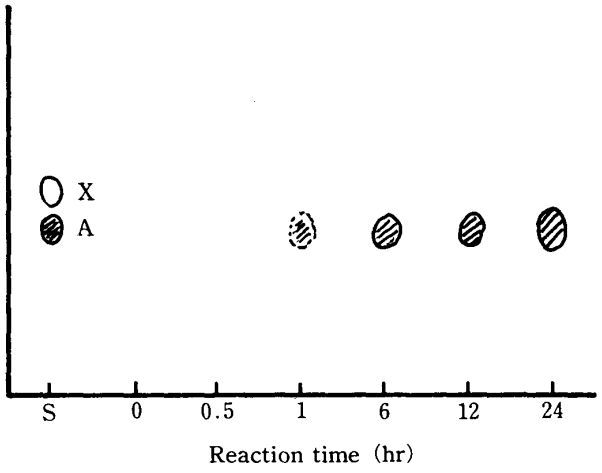

Fig. 5. Paper chromatogram of the products from hemicellulose (arabinoxylan) of sugar cane bagasse by arabinanase II of *Bacillus* sp. No. 430.

The experimental details were same except for hemicellulose as described in the legend of Fig. 4. The paper chromatography was carried out as described in the previous paper[17]. S represents the standard sugar solution. A and X indicate L-arabinose and D-xylose, respectively.

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Bacillus sp. No. 430のα-L-アラビノフラノシダーゼの精製と性質

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要　約

Bacillus sp. No. 430 の生産するアラビナン分解酵素の精製を行い，その酵素化学的性質を検討した。酵素の精製は，供試菌株の培養沪液から硫酸アンモニウム分画，DEAE—セルロース，2回めのDEAE—セルロース，QAE－セファデックスA－50，DEAE－トーヨーパール650S及びセファデックスG—75などのカラムクロマトグラフィーを組み合わせて行なった。精製酵素はディスク電気泳動的に高度に精製されていた。精製酵素の反応至適pHは7.0，反応至適温度は40°Cであった。本酵素反応はCu^{2+}，Cd^{2+}，α，α′—ジピリジル，沃度酢酸，パラクロロマーキュリーベンゾエートにより部分的に阻害を受けた。精製酵素はビートアラビナン，アラビノガラクタンやパラーニトロフェニルアラビノフラノシドに作用した。反応生成物はL－アラビノースのみが検出された。本酵素はα-L-アラビノフラノシダーゼとして特徴づけられた。本酵素を用いることにより，さとうきびバガスから高純度のキシランが得られた。

※琉球大学農学部農芸化学科