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ENDO-ARABINANASE FROM *BACILLUS SUBTILIS* F-11

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Summary

An arabinanase was purified from the culture fluid of *Bacillus subtilis* F-11. The process was as follows: salting out by $(NH_4)_2SO_4$, repeated chromatography on hydroxy apatite and gel filtration on Sepharose-6B. The purified enzyme was demonstrated to be homogeneous by disc electrophoresis. The enzyme was found to be active on arabinan and 1,5-arabinan, but inactive on phenyl α-L-arabinofuranoside, *p*-nitrophenyl β-D-galactopyranoside, arabinoxylan, gum arabic. The enzyme released arabinose, arabinobiose, arabinotriose and higher oligosaccharides during the course of hydrolysis of 1,5-arabinan. The end products were found to be arabinose and arabinobiose after 144 h of hydrolysis.

Introduction

In previous work [1,2], α-L-arabinofuranosidase (EC 3.2.1.55) was highly purified from the culture fluid of *Aspergillus niger* and obtained in crystalline form. The enzyme was found to be active exclusively on arabinofuranoside and inactive towards arabinopyranoside.

The present paper describes the method for purification of a bacterial arabinanase and some properties of the enzyme, emphasizing substrate specificity in relation to α-L-arabinofuranosidase.

Materials and Methods

The bacteria, *Bacillus subtilis* F-11, isolated from soil in our laboratory was cultured in a medium composed of 10.0 g of peptone, 0.5 g of K_2HPO_4, 0.5 g of KCl and 0.5 g of $MgSO_4 \cdot 7H_2O$ in 1 l of beet pulp extract. Beet pulp extract was prepared as described in the previous paper [2]. The initial pH of the medium was adjusted to 7.0. The cultivation was carried out at 28°C for 88 h in a 10-l jar fermenter.

$(NH_4)_2SO_4$ was added to the culture filtrate to 0.9 saturation. After the mixture had stood overnight, the resulting precipitate was collected by centrifugation and dissolved in deionized water. The solution was dialyzed, first against deionized water and then against 0.01 M sodium phosphate buffer, pH 6.8 for 48 h each. The first column chromatography was carried out as described under Fig. 1. The partially purified enzyme was concentrated with collodion bag, and dialyzed against 0.01 M sodium phosphate buffer (pH 6.8). The dialyzed solution was poured onto a column of Sepharose 6B in 0.01 M sodium phosphate buffer (pH 6.8). The procedure of gel filtration is described in Fig. 2. All the experiments were performed in a cold room at 2° C. Beet arabinan was used as substrate for the enzyme assay during the over-all purification procedures.

Beet arabinan was extracted from beet pulp by the method of Hirst and Jones [3] and purified by DEAE-cellulose column chromatography and Sephadex G-100 gel-filtration as described in the previous paper [4]. Arabinoxylan and 1,5-arabinan were also prepared as described in the same report [4]. Arabinogalactan was purchased from K and K Laboratories Inc. and purified three times by ethanol precipitation. Gum arabic was purchased from Suzu Pharmaceutical Company. Phenyl α-L-arabinofuranoside was prepared by the method of Börjeson et al. [5]. *p*-Nitrophenyl α- and β-D-galactopyranoside was purchased from Sigma Chemicals Company.

A reaction mixture, containing 3.0 ml of 0.3% 1,5-arabinan or 1.0% beet arabinan, 1.0 ml of enzyme solution, 0.5 ml of 0.2 M acetic acid/sodium acetate buffer (pH 6.0) and 2 drops of toluene, was incubated at 30° C for 15 min (1,5-arabinan) or 30 min (beet arabinan). The reaction was stopped by the addition of 0.2 M NaOH to an equal volume of reaction mixture. The reducing sugar released by the action of the enzyme was determined as arabinose by the Nelson-Somogyi method [6,11]. One unit of the enzyme is that amount of enzyme which liberates 1 μmol of aldehyde from beet arabinan per min under the above conditions. The actions on the synthetic glycosides were tested as described in the previous report [2].

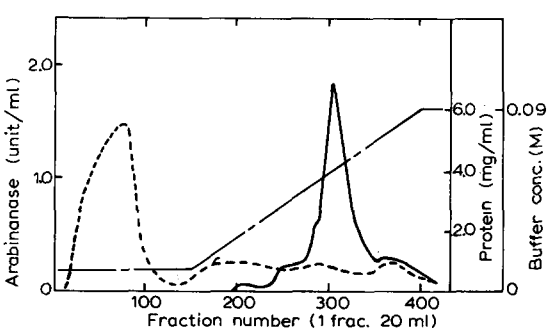


Fig. 1. Chromatography on hydroxyapatite (I). Hydroxyapatite was equilibrated with 0.01 M sodium phosphate buffer (pH 6.8) and packed in a column, 5.0 × 23 cm. The enzyme solution, 1000 ml containing 22.05 g protein or 2918 units of arabinanase, was poured on to the column which was then washed with the same buffer. The enzyme was eluted with a linear gradient of 0.01—0.09 M same buffer (pH 6.8). Fractions 281—330 were combined. ———, Enzyme activity; - - - - - -, protein.

Results

Purification of enzyme

The crude enzyme solution, 1000 ml containing 22.05 g protein or 2918 units of arabinanase, after salting out and dialyzation, was poured onto a column of hydroxyapatite. The result of the chromatography is shown in Fig. 1. Further purification was performed as described above. The last gel filtration is shown in Fig. 2. The results of the overall purification procedures are summarized in Table I and the enzyme was purified 375-fold from the crude enzyme solution.

The single band in Fig. 3 demonstrates the homogeneity of the purified enzyme.

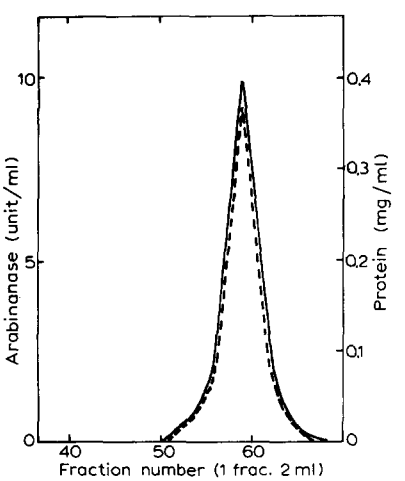
When 1,5-arabinan was used as substrate, the amount of reducing sugar produced is a linear function of enzyme concentration up to 2.0 g protein per ml of reaction medium under the conditions mentioned above.

Effect of pH on activity and stability of enzyme

The effect of pH on activity of purified enzyme is shown in Fig. 4. The maximum activity appears at pH 6.0. The purified enzyme was relatively stable at pH range of 7.0—10.0 and most stable at pH 9.0.

Action of the purified enzyme on various substrates

As shown in Table II, the purified enzyme was found to be active on beet



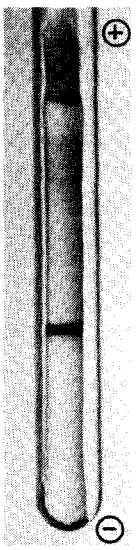


Fig. 2. Gel filtration on Sepharose-6B (III). Sepharose-6B was equilibrated with 0.06 M sodium phosphate buffer (pH 6.8) and packed in a column, 1.5 × 83 cm. The enzyme solution, 0.5 ml containing 11.3 mg protein or 540.7 units of arabinanase, was applied to the column which was then washed with the same buffer. Fractions 53—65 were combined. ———, Enzyme activity; - - - - - -, protein.

Fig. 3. Disc electrophoresis of the purified enzyme. The purified enzyme solution containing 60 μg of protein was layered on a column of 7% polyacrylamide gel and allowed to migrate in glycine-acetate buffer (pH 4.0) for 2 h [7]. Protein was stained with Amido black 10B.

TABLE I

PURIFICATION OF ARABINANASE

Step	Vol. (ml)	Protein (mg)	Activity (units)	Yield (%)	Spec. act. (unit/mg)	Size of column (cm)
$(NH_4)_2SO_4$	1000	22 050	2918	100	0.13	
Hydroxyapatite (I)	1000	832	1280	43.9	1.54	5.0 X 23.0
Hydroxyapatite (II)	1170	228.7	1122	38.5	4.91	2.0 X 14.5
Sepharose-6B (I)	60	34.9	845	29.0	24.2	1.5 X 83.0
Hydroxyapatite (III)	267	27.8	684	23.4	24.6	1.4 X 5.2
Sepharose-6B (II)	28	12.5	584	20.0	46.7	1.5 X 83.0
Hydroxyapatite (IV)	125	11.3	541	18.5	47.9	5.0 X 23.0
Sepharose-6B (III)	33	3.2	156	5.3	48.8	1.5 X 83.0

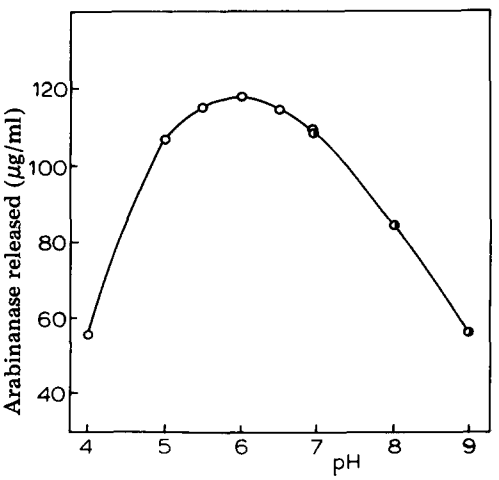


Fig. 4. Effect of pH on activity of the purified enzyme. A reaction mixture containing 3.0 ml of 0.3% 1,5-arabinan, 0.5 ml of buffer and 1.0 ml of enzyme (6.75 μg of protein) was incubated at 30° C for 15 min. 0.2 M acetate buffer was used for pH 4.0 to 7.0, 0.2 M Na_2CO_3 /$NaHCO_3$ buffer for pH 7.0 to 9.0.

TABLE II

ACTION OF THE PURIFIED ENZYME ON VARIOUS KINDS OF GLYCOSIDES

Substrate	Concentration	Sugar produced (μg/ml of reaction mixture)	
		30 min	120 h
1,5-Arabinan	0.3%	184	466
Beet arabinan	0.3%	38	60
Beet arabinan	1.0%	110	217
Arabinoxylan	1.0%	0	0
Arabinogalactan	1.0%	0	0
Gum arabic	1.0%	0	0
Phenyl α-L-arabino furanoside	25 mM	0	0
p-Nitrophenyl α-D-galactopyranoside	1 mM	0	0
p-Nitrophenyl β-D-galactopyranoside	1 mM	0	0

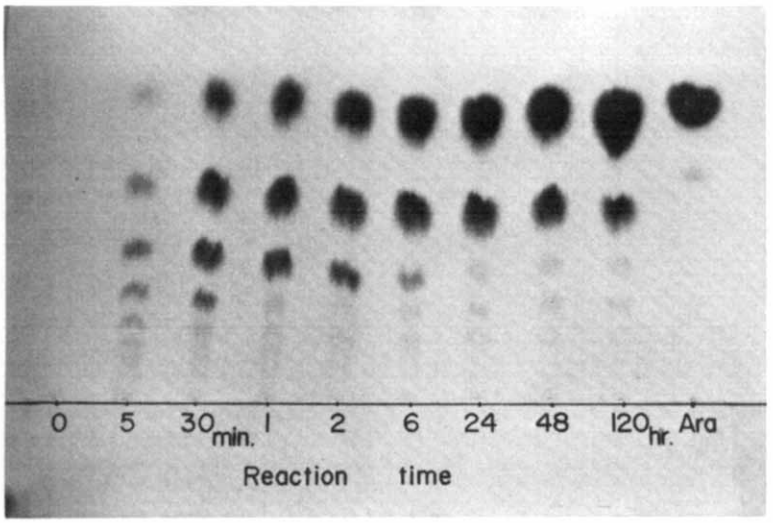


Fig. 5. Paperchromatogram of sugars produced with increasing time of hydrolysis. A reaction mixture containing 400 ml of 0.3% 1,5-arabinan solution, 133.4 ml of enzyme solution (400.2 μg of protein), 66.6 ml of 0.2 M acetate buffer (pH 6.0) and 1.0 ml of toluene was incubated at 30°C. Reaction mixture, 50 ml, was withdrawn, ethanol added and precipitate was removed. The supernatant solution was passed through a column of Dowex-50W-X4 (H^+ form) and concentrated. The chromatograms developed thrice with butanol/acetic acid/water (4 : 1 : 5). The sugars were visualized with alkaline $AgNO_3$ or aniline hydrogen phthalate reagent.

arabinan and 1,5-arabinan and inactive on arabinoxylan, arabinogalactan, gum arabic, phenyl α-L-arabinofuranoside. This result is very different from the substrate specificity previously found, i.e. that α-L-arabinofuranosidase hydrolyzes all these substrates [1,2]. It was also confirmed that the enzyme had no activity on *p*-nitrophenyl β-D-galactopyranoside.

Samples from reaction mixture containing 1,5-arabinan as substrate were withdrawn for the determination of intermediate and end products by paper chromatography after 5 min to 120 h of incubation. The products shown in Fig. 5, revealed on paper chromatograms, were as follows: after 5 min, 5 kinds of oligoarabinoses and small amounts of arabinose; after 30 min to 6 h, 4 to 2 kinds of oligoarabinoses and arabinose; after 24 to 120 h, arabinobiose and arabinose.

After 1 h of incubation, arabinose and 4 kinds of oligomers were separated by paper chromatography and each oligoarabinose was confirmed to produce only arabinose after acid hydrolysis. Degree of polymerization was also estimated on the basis of reducing values before and after hydrolysis of oligoarabinoses with α-L-arabinofuranosidase which had been prepared in our laboratory [8]. The 4 oligomers were confirmed to be arabinobiose, arabinotriose, arabinotetraose and arabinopentaose, respectively.

As regards time course of enzymatic degradation, the amount of reducing sugars is a linear function of reaction time up to 20 min and the reaction velocity was determined to 5.1 μg (as arabinose) per μg of enzyme protein per min when 1,5-arabinan was used as substrate. The rate of reaction rapidly dropped 25 min of incubation later. After prolonged incubation for 120 h, the

percentage of degradation increased gradually up to 23.3% for 1,5-arabinan and 3.3% for beet-arabinan.

Evidently the best substrate for the purified enzyme was 1,5-arabinan and the end products were arabinose and arabinobiose.

Inhibitory studies

The procedure was analogous to the previous experiment [1]. The mercuric ion was potent inhibitor.

Discussion

Ehrlich and Schubert [9] reported, in 1928, that an arabinanase liberates arabinose from beet arabinan. An arabinanase of *A. niger* was purified in homogeneous protein [10] and obtained in crystalline form [2]. This enzyme was found to be α-L-arabinofuranosidase on the basis of its properties [1,2]. The same enzyme was also obtained from the culture fluid of *Corticium rolfsii* in a highly purified state [8]. As reported in these papers from our laboratory, arabinose is rapidly liberated from phenyl α-L-arbinofuranoside and the side chains of beet arabinan, arabinoxylan, arabinogalactan by enzymatic action.

According to the present result, highly purified arabinanase of *B. subtilis* F-11 hydrolyzes 1,5-arabinan and beet arabinan within 23.3% and 3.3% extent, respectively, but none of the enzymatic activity was found on phenyl α-L-arabinofuranoside, arabinoxylan and arabinogalactan. Evidently the purified enzyme is different from α-L-arabinofuranosidase in substrate specificity. That is to say, the arabinanase is unable to hydrolyze the synthetic arabinofuranoside and terminal non reducing α-L-arabinofuranosyl residues in the side chains of polysaccharides.

Whereas hydrolysis of 1,5-arabinan yielded arabinose and arabinobiose to hexaose after incubation for 5 min to 6 h, arabinotriose and higher oligomers disappeared after prolonged incubation. The end products were found to be arabinose and arabinobiose. When beet arabinan was used as substrate, the rate of hydrolysis was very low as compared with 1,5-arabinan. It seems that side chains in beet arabinan inhibit the action of purified arabinanase on 1,5-arabinofuranosyl linkages in main chain. Thus the true substrate was found to be 1,5-arabinan.

On the basis of these data, it is concluded that the highly purified enzyme hydrolyzes 1,5-arabinan in endo-wise form. It might be called endo-1,5-α-arabinanase.

Acknowledgment

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