Arthrobacter globiformis S64-1によるβ-D-フラクトフラノシダーゼの生産

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Production of β-D-fructofuranosidase by *Arthrobacter globiformis* S64-1

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A strain of bacterium isolated from soil sampled in Mie Prefecture, was identified as *Arthrobacter globiformis* S64-1. This microorganism produced inulinase with high activity. As the enzyme hydroyzed inulin with an exo-type reaction, the enzyme was designated as β-D-fructofuranosidase. The enzyme showed maximal activity at 40°C and pH6.5.
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Introduction

Inulin is a linear β-2, 1 linked fructose polymer terminated by sucrose residue. Inulin is almost insoluble in cold water but it is soluble in hot water. In the study of inulinase, mainly enzymes from molds and yeasts were reported. Nakamura and Nakatsu[1] studied inulinase from *Penicillium* sp. which hydrolyzed inulin to fructose with an exo-type reaction. Nakamura et al.[2-3] reported on inulinase from *Aspergillus niger* which hydrolyzed inulin with an endo-type reaction. Negoro[4] described inulinase produced by *Kluyveromyces flagilis* which decomposed inulin with an exo-type reaction.

Recently we found a strain of bacterium which had been isolated from soil sampled in Mie Prefecture. This microorganism, strain S64-1, produced inulinase in culture broth. Through taxonomical studies the microorganism was identified as *Arthrobacter globiformis* S64-1. This paper summarizes our studies on the taxonomical identification of the microorganism and some characteristics of the crude enzyme.

Materials and Methods

1. Cultivation of the microorganism

For the production of the enzyme, basal medium composed of 0.4% $Na_2HPO_4 \cdot 12H_2O$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% NH_4NO_3, 0.003% $CaCl_2 \cdot 2H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.05% yeast extract (Difco) and 0.3% inulin, pH 7.0 was used. One loop of the microorganism was inoculated to 500 ml shaking flask containing 100 ml of the medium and cultured for 24 hours at 30°C with reciprocate shaking. The cells were removed by centrifugation (9,000 g, 15 min) and dialyzed against 10 mM phosphate buffer pH 7.0. The dialyzate was used as a crude enzyme solution.

For the preparation of DNA, the basal medium containing 1% glycine was used and cultured for 48 hours at 30°C.

2. Identification of the microorganism

1) Determination of DNA GC%

The chromosomal DNA of the microorganism was extracted by the method of Marmur[5]. Hyperchromity of the chromosomal DNA was monitored by a Beckman DU-8 spectrophotometer. Chromosomal DNA extracted from *Escherichia coli* K-12 was used as the standard of T_m. DNA GC content of the strain S64-1 was calculated using the formula of Marmur and Doty[6].

2) Gram staining

The microorganism was cultured on basal medium agar containing 0.5% inulin for 1 day at

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30°C. Gram staining of the cells were performed by the modified method of Hucker, and observed by a microscope.

3) Catalase test

The cells were touched by 3% H_2O_2 solution on a glass slide, and generation of O_2 was observed.

4) Oxidase test

The solution of N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride (1%) was prepared. A small part of a filter paper was dipped into this solution. The microoganism was rubbed into the filter paper with a loop made of platinum. The color of the filter paper was observed.

5) Heat resistance

The cells were suspended in 10% sterilized skim milk. This suspension was incubated at 63°C for 30min. After incubation, one loop of the suspension was streaked on basal medium agar and incubated at 30°C. And the survival of the microorganism was tested.

6) Hydrolysis of starch

One loop of the microorganism was streaked on nutrient agar plate containing 0.2% soluble starch, and cultured at 30°C for 2 days. About 10 ml of Lugor solution was added and the color of the plate was observed.

7) Liquification of gelatin

The microorganism was inoculated to 10% gelatin containing nutrient broth, and cultured for 5 days at 20°C. Then the liquification of the medium was tested.

8) Reduction of nitrate

The microorganism was cultured for 2 days at 30°C with succinate-nitrate medium composed of 1% succinic acid, 0.1% K_2HPO_4, 0.1% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.02% KCl and 0.001% $FeSO_4 \cdot 7H_2O$, pH7.2. A few drops of 0.8% sulfanilic acid in 5N CH_3COOH and 0.5% α-naphthylamine in 5N CH_3COOH were added. And the color of the medium was observed.

3. Standard enzyme assy

The enzyme solution (0.1 ml) was mixed with 0.2 ml of 0.1 M phosphate buffer pH 6.5 and 0.2 ml of H_2O. Inulin solution(0.1%, 0.5 ml) was added and incubated 30 min at 40°C. Reaction was stopped by heating 5 min at 100°C. The resultant reducing sugar was determined by Somogyi-Nelson's[7-8]method. One unit of the enzyme was defined as the amount of the enzyme which cleaved 1.0μ mol of fructosidic bond per minute.

4. Paper chromatography

Ascendicng paper chromatography was carried out using Toyo filter paper No.50, with the solvent system n-butyl alcohol/pyridine/ water (3:2:2, by, vol.) at 60°C. After irrigation, the reaction product was revealed with resorcinol-HCl reagent[9].

Results and Discussion

1. Identification of the microorganism

The taxonomical characters of the isolated microorganism are summarized in Table 1. Strain S64-1 was a gram positive, non-sporulating bacterium which showed pleomorphism during the growth cycle. And from the other characters shown in Table 1, the strain was identified as *Arthrobacter* sp.. Table 2 shows the characters of *Arthrobacter* species in the 8th edition of Bergey's manual and strain S64-1. Strain S64-1 showed no groth factor requirement, and the DNA GC% was 62.7%, so the characters of this strain ware well coincident with those of *Arthrobacter globiformis*. From these data the microorganism was identified as *Arthrobacter globiformis* S64-1.

2. Some characteristics of the crude enzyme

1) Effect of pH on enzyme activity

The enzyme activity was measured in buffers of various pH from 4.5 to 8.0 at 40°C. As shown in Fig.1, the optimal pH of the enzyme for the hydrolysis of inulin was pH 6.5. At this condition the crude enzyme showed activity of 0.2 U/ml.

2) Effect of temperature on enzyme activity

The enzyme activity was assayed at various temperature from 30°C to 60°C at pH6.5. As

shown in Fig.2, the maximal activity was obtained at 40°C.

3) pH stability

The enzyme solution was incubated at various pH from 3.0 to 9.0, for 6 hours at 10°C. After incubation, the remaining activity was assayed. As shown in Fig.3, big loss of activity was observed at the lower pH region.

4) Thermal stability

Themal stability at pH 6.5 was observed. The enzyme solution was incubated at various temperatures from 30°C to 60°C for 10 min. After incubation the remaining activity was assayed at pH 6.5, 40°C. As shown in Fig.4, the enzyme activity was relatively stable below 40°C.

Table 1. Taxonomical Characters of Strain S64-1

Gram staining	positive
pleomorphism	+
spores	−
motility	−
growth in air	+
growth anaerobically	−
catalase	+
oxidase	−
heat resistance (63°C 30min)	−
DNA GC%	62.7%

Table 2. Comparison of *Arthrobacter* Species

	A. globiformis	*A. simplex*	*A. tumescens*	*A. citreus*	*A. terregens*	*A. flavescens*	*A. duodocadis*	S64-1
Growth factor requirement								
Biotin	d	−	−	+	+	+	−	−
Thiamine	−	−	+	+	+	+	+	−
Pantothenic acid	−	−	−	−	+	−	−	−
Nicotinic acid	−	−	−	+	−	−	−	−
Vitamin B_{12}	−	−	−	−	−	−	+	−
Terregens factor	−	−	−	+	+	+	−	−
Gelatin hydrolysis	+	+	+	+	−	+	+	+
Starch hydrolysis	d	−	d	−	−	+	−	−
$NO_3 \rightarrow NO_2$	d	+	d	+	+	+	+	+
DNA GC%	60-64.4	71.7	69.8	62.9				62.7

d ; some strains positive, some negative

3. Mode of action

The enzyme solution (0.5 ml) was incubated with 0.2 ml of 0.1 M phosphate buffer pH 6.5, 0.1 ml of H_2O and 0.2 ml of 1% inulin at 37°C for 0 to 6 hours. The reaction mixture (10 μl) was applied to the paper chromatography. Fructose solution (0.5%) was used as a standard sample. As shown in Fig.5, only fructose was detected as a final reaction product. From this experiment, it is clear that the inulinase of *Arthrobacter globiformis* S64-1 hydrolyzes inulin with an exo-type reaction. So this enzyme should be classified as a β-D-fructofuranosidase. In the study of β-D-fructofuranosidase, Nakamura[1] reported a enzyme from *Aspergillus niger*. The enzyme showed maximal activity at pH 5.3. Negoro[4] studied a β-D-fructofuranosidase from *Kluyveromyces fragilis*. The enzyme showed optimal activity at pH 4.5. The enzyme produced by *Arthrobacter globiformis* S64-1 showed maximal activity at pH 6.5. So this enzyme will be advantageous to the hydrolysis of inulin in the neutral pH region.

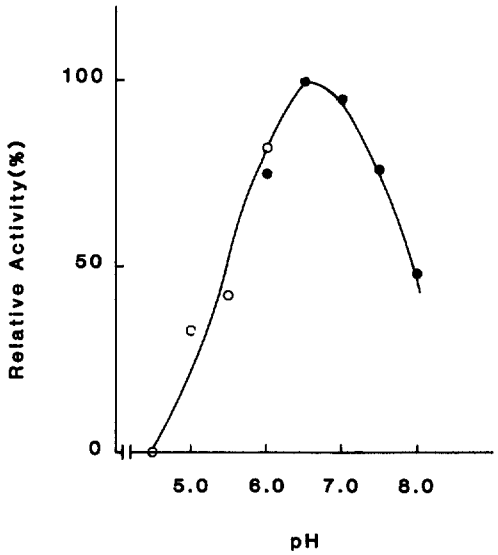

Fig. 1. Effect of pH on the Enzyme Activity.
○ ; 0.02 M citrate buffer
● ; 0.02 M phosphate buffer

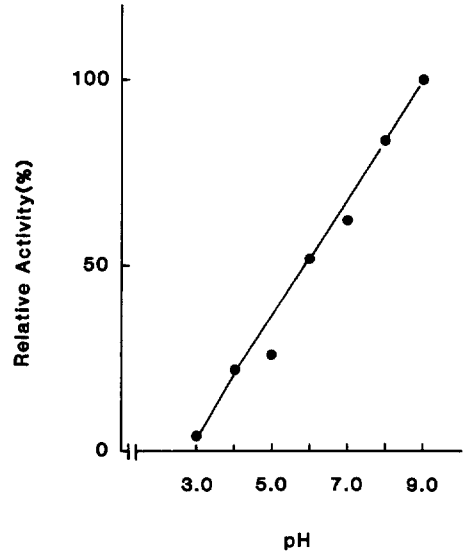

Fig. 3. pH Stability of the Enzyme.

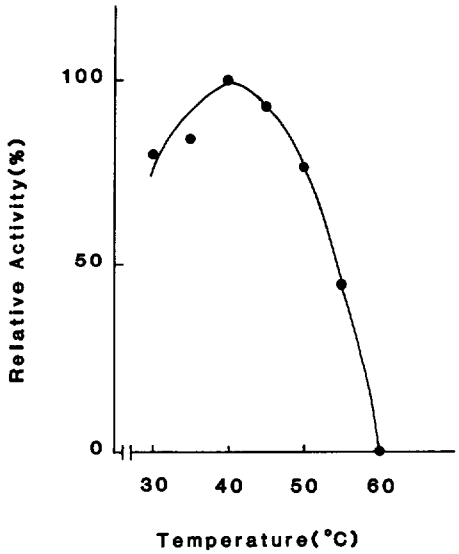

Fig. 2. Effect of Temperature on the Enzyme Activity.

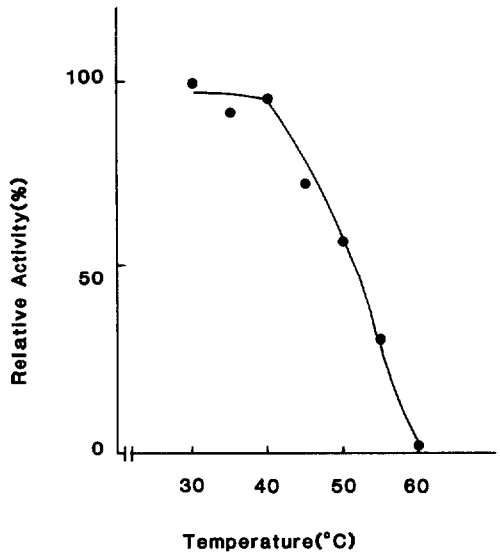

Fig. 4. Thermal Stability of the Enzyme.

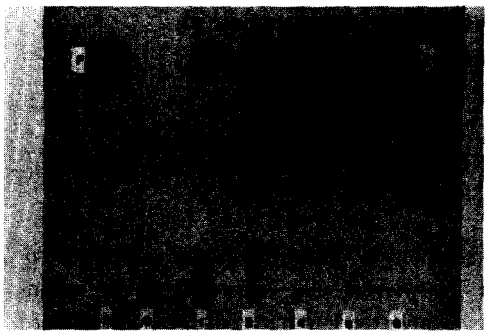

Fig. 5. Paper Chromatogram of Hydrolysis Product from Inulin by the *Arthrobacter globiformis* Inulinase.

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Arthrobacter globiformis S64-1によるβ-D-フラクトフラノシダーゼの生産

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三重県の土壌から分離された，細菌S64-1株は菌体外にイヌリン分解酵素を生産した。

1．本菌株はグラム陽性の好気性細菌でありDNA GC%,生理的性質を検討した結果*Arthrobacter*属に属することが示された。さらに，Bergey's Manual 第8版の記述にもとづいて検討したところ*Arthrobacter globiformis* S64-1と同定された。

2．本菌株の生産する菌体外イヌリナーゼの至適反応条件はpH6.5, 40°Cであった。本酵素のイヌリンに対する作用様式を検討した結果，本酵素はexo-型の反応によってフラクトースを生産する。β-D-フラクトフラノシターゼであることが明らかになった。