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INTRODUCTION

Trehalase (E.C. 3.2.1.28) is the enzyme that hydrolyzes the disaccharide trehalose into two α-D-glucose molecules. This enzyme was discovered as an extracellular enzyme from *Aspergillus niger*[1]. The substrate specificity of trehalase over trehalose was high, and it was clearly shown that there was no sugar transferase reaction. This enzyme was discovered in certain microbes, mold, yeast, insects, and so on[2]. As trehalose is a very important sugar for microorganisms, microbial trehalase has been well researched[1,3−5].

In recent years, trehalose has been produced more readily and in large quantities, and various characteristics have been clarified. However, most research on microbial trehalase has focused on cells that produce glucose as a carbon source, and there have been very few reports examining trehalose as carbon source.

The drain on the dairy farm that we have been studying provides a unique environment in which cow excrement and milk are sometimes mixed, and we thought that microorganisms using trehalose might exist there. In our previous paper, we described the isolation of *Moraxella osloensis* No. 55, which produced intracellular trehalase from a dairy farm drain[6]. In this paper the purification and the properties of trehalase from these bacterial cells, which were grown in a medium containing trehalose, are described.

MATERIALS AND METHODS

Microorganism. Moraxella osloensis No. 55 isolated from a dairy farm drain[6] was used for the enzyme purification. The bacteria were maintained on slant cultures containing standard agar medium.

Chemicals. DEAE-Sepharose CL-6B and Sephadex G-100 were purchased from Amersham Biosciences, Uppsala, Sweden and Toyopearl HW-65F from Tosoh Co., Ltd., Tokyo, Japan. Other chemicals used were of technical grade.

Cultivation. The organism was cultured in the original complex medium containing 1.0% trehalose (Hayashibara, Okayama, Japan) at 35 ℃ for 24 hours as described previously[6]. The cells (44 g wet) obtained from 8,400 ml of culture were rinsed with 10mM sodium phosphate buffer (pH 7.0) and stored at −40 ℃ until preparation of the enzyme.

Protein Measurement. The protein was measured spectrophotometrically by its absorbance at 280 nm.

Enzyme assay. To assay trehalase activity, the reaction mixture containing 0.5 ml of 0.5% trehalose, 0.4 ml of 0.1 M sodium phosphate buffer (pH 7.0), and 0.1 ml of an appropriate concentration of enzyme solution was incubated at 32 ℃ for 10 min. The measurement of glucose was performed using the glucose-oxidase-peroxidase method (Glucose-ARII kit; Wako Pure Chemical Industries Ltd., Osaka, Japan). One unit of trehalase activity was defined as the amount of enzyme that catalyzed the hydrolysis of one μmol of trehalose per min.

Purification of trehalase. All procedures were performed at 0 to 5 ℃.

Step 1. Extraction. The cells were suspended in 10mM sodium phosphate buffer (pH 7.0). One hundred ml of suspension in an ice bath was

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disrupted by ultrasonication with a Branson model 450 sonifier for 20 min at 15% of output. The supernatant obtained by centrifugation (27,700 *g*, 15 min) was used as the crude extract.

Step 2. Ammonium sulfate fractionation. The crude extract was dialyzed overnight against 40% $(NH_4)_2SO_4$ saturation in a 0.1 M sodium phosphate buffer (pH 7.0). After centrifugation (27,700 *g*, 30 min), the supernatant was dialyzed overnight against 60% $(NH_4)_2SO_4$ saturation in the same buffer. After centrifugation (27,700 *g*, 30 min), the precipitate was suspended in 10mM sodium phosphate buffer (pH 7.0), and again dialyzed overnight against same buffer.

Step 3. Chromatography on a DEAE-Sepharose CL-6B column. The active fraction was put on a DEAE-Sepharose CL-6B column (ϕ2.0×32.5 cm) equilibrated with 10mM sodium phosphate buffer (pH 7.0). After the non-adsorbed protein was eluted from the column, the enzyme was eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer.

Step 4. Chromatography on a Toyopearl HW-65F column. The active fractions were combined and dialyzed overnight against 0.1 M sodium phosphate buffer saturated with $(NH_4)_2SO_4$ to 30% (pH 7.0). The enzyme solution was put on a Toyopearl HW-65F column (ϕ1.5×35.5 cm) equilibrated with the same buffer. The elution was done with a linear gradient of 30 to 0% $(NH_4)_2SO_4$ in the same buffer.

Step 5. Chromatography on a Sephadex G-100 column. The active fractions were combined and dialyzed overnight against 10mM sodium phosphate buffer containing 2 mM 2-mercaptoethanol, and 0.1 M NaCl (pH 7.0). The dialyzed solution was concentrated to 2 ml in a collodion bag under vacuum, and was filtered on a Sephadex G-100 column (ϕ2×65 cm) equilibrated with the same buffer. The active fractions were combined as the partially purified enzyme preparation.

Disc electrophoresis. Disc electrophoresis of the enzyme preparation was performed by the method of Davis at pH 9.5[7]. The protein bands were stained with Commassie Brilliant Blue.

HPLC analysis. The products of the enzyme reaction were detected by a HPLC-AS6 Carbohydrate column (Carbo Pac PA-1, ϕ4×250 mm) with a Dionex Bio LC series apparatus using pulsed amperometric detection (PAD) (DX-300 SYSTEM, DIONEX, USA)[8]. The gradient elution was established by mixing eluent A (150 mM NaOH) with eluent B (500 mM sodium acetate in 150 mM NaOH) in various ratios using a flow rate through the column of 1.0 ml per min. The concentration of sodium acetate was as follows: 25 mM (0-1 min), 25-50 mM (1-2 min), 50-200 mM (2-20 min), 500 mM (20-22 min), and 25 mM (22-30 min). The applied PAD potentials for EI (300 ms), E2 (120 ms), and E3 (300 ms) were, 0.04, 0.06, and −0.80 V, respectively, and the output range was 1 mC.

RESULTS

Purification of trehalase.

Trehalase was partially purified from the extract of *Moraxella osloensis* as described in Material and Methods. Homogeneity of the enzyme obtained by the subsequent Sephadex G-100 column chromatography (Fig. 1) was examined by disc electrophoresis at pH 9.5, but

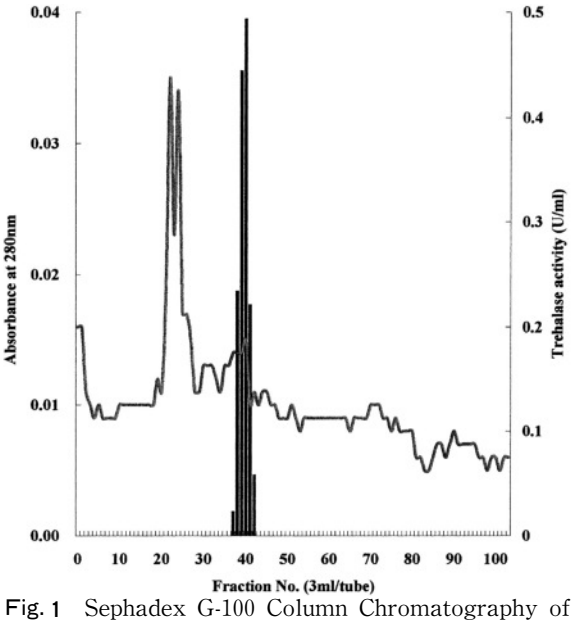

Fig. 1 Sephadex G-100 Column Chromatography of Trehalase from *Moraxella osloensis* No. 55

The enzyme (3 ml) was applied to a Sephadex G-100 column (ϕ2.0×67 cm) equilibrated with 10 mM sodium phosphate buffer containing 2 mM 2-mercaptoethanol and 0.1 NaCl (pH 7.0), and eluted with the same buffer at a flow rate of 3.0 ml / h. ■, trehalase activity; —, absorbance at 280 nm.

two or more bands were detected. The specific activity of this partially purified enzyme was 22 units per mg of protein, which was 786-fold higher than that of the crude extract, with 4.8% recovery. The purification procedures are summarized in Table 1.

Effects of pH.

The effects of pH on enzyme activity are shown in Fig. 2. The enzyme was most active at a pH of 7.1. At 4 ℃, the enzyme was stable at a pH range of 6.0 to 8.5 for 22 h (Data not shown).

Substrate specificity.

The enzyme hydrolyzed trehalose specifically, and did not hydrolyze cellobiose, maltose, melibiose, lactose, sucrose, raffinose, or soluble starch (Table 2). The products of the enzymatic hydrolysis of trehalose were tested by HPLC analysis. Only glucose was detected on the anion-exchange chromatogram (Fig. 3).

Effects of various metal ions.

Table 3 shows the effects of various metal ions on the trehalase activity of the enzyme. Activity was significantly inhibited by Ag^{2+} and Zn^{2+}, and

Table 1 Summary of Purification Procedure of Trehalase from *Moraxella osloensis* No. 55

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units / mg)	Purification (-fold)	Recovery (%)
Cell free extract	3,280	92	0.028	1	100
Ammonium sulfate (40-60%)	630	74	0.12	4.3	80
DEAE-Sepharose CL-6B	17.5	23	1.3	46	25
Toyopearl HW-65F	1.5	8.1	5.4	193	8.8
Sephadex G-100	0.2	4.4	22	786	4.8

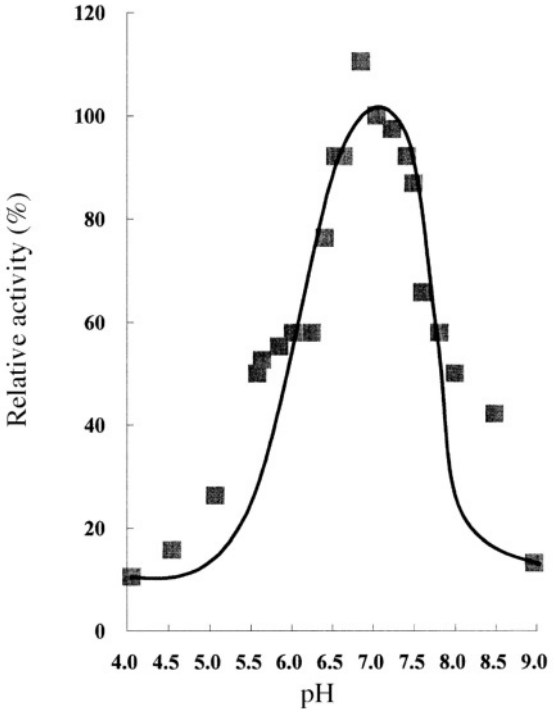

Fig. 2 Effects of pH on the Activity of Trehalase from *Moraxella osloensis* No. 55

The reaction mixture containing 0.5 ml of trehalose , 0.4 ml Britton-Robinson buffer (0.04 M H_3PO_4, 0.04 M CH_3COOH, 0.04 M H_3BO_4-0.2 M NaOH) and 0.1 ml enzyme solution was incubated at 32 ℃ for 10 min.

Table 2 Substrate Specificity of Partially Purified Enzyme

Substrate (10mM)	Relative activity (%)
Trehalose	100
Cellobiose	0.1
Maltose	0.1
Melibiose	0.2
Lactose	nd
Sucrose	nd
Raffinose	nd
Soluble starch*	nd

nd, not detected; *, 0.25%

Table 3 Effects of Various Metal Ions on Partially Purified Enzyme

Reagent (1mM)	Relative activity (%)
None	100
$MgCl_2$	93
$CaCl_2$	93
$MnCl_2$	80
$CoCl_2$	74
$NiCl_2$	70
$BaCl_2$	62
$CuSO_4$	50
$CuCl_2$	40
$ZnSO_4$	32
$AgNO_3$	0

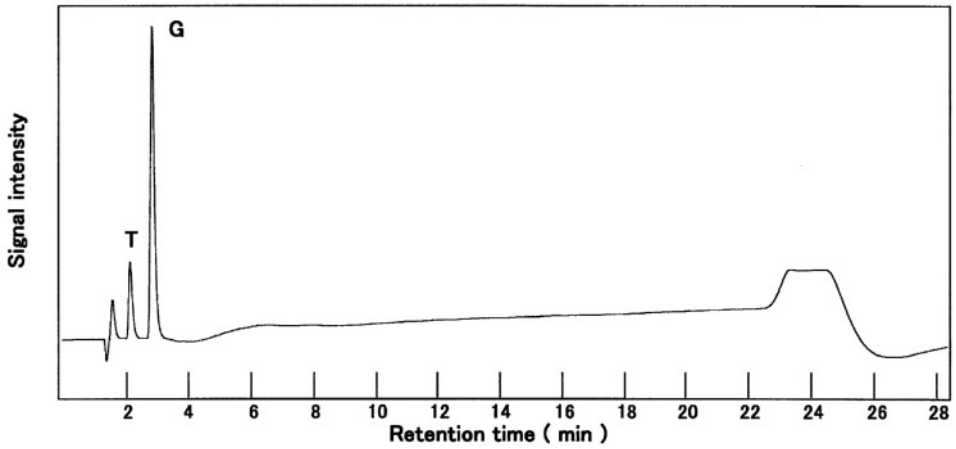

Fig. 3 Anion-exchange Chromatogram of the Products from Trehalose Hydrolyzed by the Enzyme of *Moraxella osloensis* No. 55

The reaction mixture containing 0.4 ml enzyme, 1.6 ml of 0.1 M sodium phosphate buffer (pH 7.0) and 2.0 ml of 0.5% trehalose was incubated at 32 ℃. At the 60 min, 0.5 ml of the reaction mixture was pipetted out and boiled at 100 ℃ for 3 min. HPLC analysis was described in the text. G, glucose; T, trehalose.

slightly inhibited by Cu^{2+}, Ba^{2+}, Ni^{2+} and Co^{2+}.

DISCUSSION

After gel filtration operation, the amount of protein had decreased significantly. It was thought that further purification operation was impossible, so the active protein fraction after Sephadex G-100 column chromatography was used as the final enzyme sample. When the purity of the obtained fraction was examined by native electrophoresis, a few bands were confirmed. The total activity, total protein, and specific activity of the partial purified enzyme obtained by the Sephadex G-100 column chromatography were 4.4 units, 0.2 mg of protein, and 22 units per mg of protein, respectively. The character of the partial purified enzyme was examined.

Among the purification steps, the recovery of trehalase activity was the lowest for DEAE-Sepharose column chromatography. However, through this procedure, the specific activity value rose from 0.12 U/mg to 1.3 U/mg. Thus an ion-exchange carrier with a higher recovery rate should be selected in the future.

Eight kinds of saccharide including trehalose were used, and the substrate specificity of the enzyme produced by *Moraxella osloemsis* No. 55 was examined. This bacterial trehalase hydrolyzed trehalose specifically, and did not act on any of the other seven saccharides. Moreover, when this enzyme hydrolyzed the trehalose, HPLC analysis showed that the sole product was glucose. These characteristics were matched those of the trehalase that had been reported up to now [1,3–5,9].

The enzyme was most active at a pH of 7.1. This value is similar to the value (6.8-7.0) of yeast's neutral trehalase[2]. Due to the culture method used to grown the bacteria in this experiment, no acid trehalase activity was found. In the current experiment we were able to clarify the culture conditions of the bacterium, such as nutrients, aerobic conditions, and temperature, and the enzymes were produced in large quantities. However, further work on the effective purification of the enzyme is needed in the future.

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ABSTRACT

Trehalase from *Moraxella osloensis* was partially purified about 786-fold with a recovery rate of 4.8% from the crude cell-free extract by ammonium sulfate fractionation, DEAE-Sepharose CL-6B column chromatography, Toyopearl HW-65F column chromatography, and Sephadex G-100 column chromatography. The enzyme was most active at a pH of 7.1, and was stable from pH 6.0 to 8.5 for 22 h at 4 ℃. The enzyme hydrolyzed trehalose and produced only glucose. The enzyme was specifically active against on trehalose and not against the other disaccharises tested.

要　　約

Moraxella osloensis のトレハラーゼを，細胞抽出液から，硫酸アンモニウム分画法，DEAE-Sepharose CL-6B，Toyopearl HW-65F，Sephadex G-100 の各カラムクロマトグラフィーによって，4.8%の回収率で，786 倍に部分精製した。本酵素は pH 7.1 で最も良く作用し，4 ℃，22 時間処理で，pH 6.0 から 8.5 の間で安定であった。本酵素はトレハロースを加水分解してグルコースのみを生産した。本酵素はトレハロースに特異的に作用し，他の二糖類には作用しなかった。