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Enzymatic synthesis of a novel trehalose derivative, 3,3′-diketotrehalose, and its potential application as the trehalase enzyme inhibitor

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Abstract We reported the preparation of a novel trehalose derivative based on enzymatic oxidation of trehalose by water-soluble glucose-3-dehydrogenase (G3DH) from marine bacterium *Halomonas* sp. α-15 cells. The product of G3DH enzymatic conversion was 3,3′-diketotrehalose (3,3′dkT), a novel trehalose derivative of which both third hydroxy groups of glucopyranosides were oxidized. 3,3′dkT was revealed to show an inhibitory effect toward pig-kidney and *Bombyx mori* trehalases. The IC_{50} values of 3,3′dkT were 0.8 and 2.5 mM and K_i values were 0.2 and 0.6 mM for pig-kidney and for *B. mori* trehalases, respectively. In addition, 3,3′dkT did not show any inhibitory effect on both maltase and mannosidase activities. Therefore, 3,3′dkT was a specific inhibitor of trehalases.

Key words: Glucose-3-dehydrogenase; Trehalose; Trehalase; Inhibitor; Insecticide

1. Introduction

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a disaccharide in which two glucose molecules are linked by an α-1–1-bond. Trehalose is commonly found in fungi, algae and in several invertebrate phyla but not in flowering plants and vertebrates [1]. Trehalose is present in all insects, and in many insects it is present in high concentrations and constitutes the major hemolymph (blood) sugar [2].

Trehalase (EC 3.2.1.28) specifically hydrolyzes trehalose into two glucose molecules. Trehalase is widely spread among animals, plants and microorganisms, and its important role becomes particularly apparent in biological regulation [3]. In insects, the use of sugar as an energy source is controlled by trehalase. Trehalase is the rate-limiting enzyme in the process of glycogen synthesis from hemolymph trehalose in developing ovaries of the silkworm. Hence this enzyme may be regarded as a putative new target for insect control. Therefore, the specific inhibitors for trehalases may find application in the regulation of the metabolism of this energy reserve. Several trehalase inhibitors have been reported such as validamycins [4–7], validoxylamines [7–9] deoxynojirimycin [10,11], trehalostatin [12,13], trehazolin [14,15] and suidatrestin [16]. These compounds are basically produced by actinomycetes as antibiotics, or their derivatives. Only a few instances of research have been reported utilizing trehalose as the substrate for the preparation of trehalase inhibitors based on the chemical or enzymatic method. Recent development of the enzymatic method for trehalose production [17,18], reduced the price of trehalose and enabled the application of trehalose to various kinds of applied researches, such as for the additives for enzyme lyophilization.

We have recently reported the enzymatic conversion of saccharide based on glucose-3-dehydrogenase (G3DH) isolated from a marine bacterium *Halomonas* sp. α-15 [19]. Utilizing 1,5-anhydro-D-glucitol (1,5AG), a diabetes clinical marker, as the substrate, 3-keto anhydroglucitol was prepared, which is useful as the precursor for the synthesis of 1,5AG analogues. Considering that this G3DH shows wide substrate specificity toward various saccharides, the utilization of trehalose as the substrate for the enzymatic conversion system based on G3DH will result in a novel trehalose derivative with biological activities.

In this paper, we describe the preparation of a novel trehalose derivative based on enzymatic oxidation of trehalose by G3DH, and its biological activities, particularly as a trehalase inhibitor.

2. Materials and methods

2.1. Chemicals

Potassium ferricyanide, 5-methylphenazinium methosulfate (phenazine methosulfate; PMS), 2,6-dichloro-*N*-(4-hydroxyphenyl)-*p*-benzoquinoneimine (DCIP) were purchased from Kanto Chem., Tokyo, Japan. Trehalose was kindly supplied from Hayashibara Institute Corp. (Okayama, Japan). Maltase (baker's yeast) and mannosidase (jack bean, *Canavalia ensiformis*) were purchased from Wako Pure Chemicals (Osaka, Japan). The analogue substrate for enzyme analyses, *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-α-D-mannoside were purchased from Sigma (St. Louis, MO, USA). Trehalase (pig-kidney) was purchased from Sigma. All other chemicals used in this study were reagent grade.

2.2. Enzyme preparation

G3DH was prepared from *Halomonas* sp. α-15 cells, according to our previous reports [20]. G3DH activity was determined using PMS-DCIP-mediated color development enzyme assay as described previously [20].

Trehalase from silk worm larvae, *Bombyx mori*, was prepared according to the literature [21] as a crude enzyme sample.

2.3. Enzymatic conversion of trehalose

20 mM of trehalose was added in a reaction vessel containing 15 ml 100 mM potassium phosphate buffer pH 7.5 with 5.0 U G3DH and 100 mM potassium ferricyanide as an electron mediator, and kept at 25 °C under dark conditions during the reaction. After 10 h of incubation the reaction mixture was lyophilized. The product was dissolved by adding 150 ml of methanol, and simultaneously, protein

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and potassium ferricyanide were precipitated. Methanol was then evaporated and the sample was re-dissolved in distilled water, and analyzed for trehalose and its enzyme product by HPLC (Tosoh, Tokyo, Japan) using a sugar analysis column (SUGAR SP081 8 mm ID×300 mm, Shodex, Tokyo, Japan) equipped with a refractive index (RI) detector. Potassium ferricyanide concentration in the sample was determined by forming Prussian blue color by adding ferric sulfate-Dupanol reagent, as was described elsewhere, and by measuring absorbance at 660 nm.

The product of trehalose enzyme conversion using G3DH was purified with silica gel chromatography using acetonitrile as the solvent. The purification steps were evaluated by checking eluent by thin layer chromatography (silica gel $60F_{254}$ plate (Merck) with acetonitrile:H_2O (7:3)). 10 mg of purified product was dissolved in D_2O. ^{13}C-NMR spectra were recorded with a JEOL ALPHA 500 spectrometer (500 MHz) using TMS as the internal probe.

Mass spectroscopy analysis of the product was carried out using Mariner Electrospray – TOFMAS spectrometer (Applied Biosystems, Foster City, CA, USA). The samples were dissolved at about 5 mM in 5 mM ammonium acetate:acetonitrile (1:1). The sample solution was introduced via an injection syringe at a flow rate of 5 μl min^{-1}.

2.4. Enzyme analyses

Trehalase activity was measured based on the enzymatic measurement of the amount of glucose as the hydrolytic product of the trehalase enzyme reaction. The optimized procedure for the kinetic studies with the trehalase of pig-kidney and of *B. mori* was as follows: a total volume of 100 μl containing 10 μl of trehalase (0.03 U) and 90 μl of trehalose solution was incubated at 37°C for 10 min. The reaction was terminated by heating at 95°C for 5 min followed by cooling on ice for 10 min. A volume of 10 μl of the supernatant was added to 190 μl of the following reaction reagent for glucose measurement. The measurement of glucose was carried out using PQQ glucose dehydrogenase (PQQGDH) according to the previous report [22]. The reaction reagent of glucose measurement contains 0.6 mM PMS, 0.06 mM DCIP 0.014 U of PQQGDH, 1 mM $CaCl_2$ and 1 μM PQQ in 10 mM MOPS–NaOH (pH 7.0). The reaction was carried out for 30 min at 25°C. The decrease in absorbance of DCIP at 600 nm was compared with the standard calibration curve for glucose, which was prepared with same reaction reagent using samples of known glucose concentration. One unit of trehalase activity was defined as the quantity of enzyme producing 2 μmol glucose per min.

For the inhibitor studies, 3,3′-diketotrehalose (3,3′dKT) was dissolved in water. 40 μl of the inhibitor solution was preincubated with the 10 μl of trehalase solution (0.03 U) for 5 min, followed by addition of 50 μl of the substrate solution. Kinetic plots of the inhibition were obtained by measuring trehalase activity at various concentrations of trehalose and 3,3′dKT. The amount of liberated glucose was enzymatically determined as above.

Maltase and mannosidase activities were measured using *p*-nitrophenyl-α-D-glucopyranoside or *p*-nitrophenyl-α-D-mannopyranoside as a substrate, respectively, and monitored at 400 nm due to the liberated amount of *p*-nitrophenol.

3. Results

3.1. Characterization of trehalose derivative formed by G3DH catalyzing conversion

From 0.103 g of trehalose, 0.088 g of derivative was obtained. The derivatives gave a Rf value of 0.57 and differed from those of trehalose (Rf = 0.45). Table 1 shows the assigned chemical shifts of trehalose and its derivative, recovered and purified from the reaction vessel. The product showed six peaks as trehalose, but it lacks the doublet peak at 72 ppm corresponding to the third hydroxy group of trehalose, and shows an alternative singlet peak derived from carbonyl group at 206 ppm. The TOFMAS analyses of the derivative resulted $m/z = 356.1$, which indicated that molecular weight of the derivative is 360.1, 4 smaller than trehalose. These observations confirmed that the product of G3DH conversion of trehalose was 3,3′dKT, as shown in Fig. 1. The molar purification yield of 3,3′dKT was 86.4%.

Table 1
^{13}C-NMR spectral data of trehalose and trehalose derivative in D_2O

	α,α-Trehalose (ppm)	Trehalose derivative (ppm)
C-1	93.16	96.56
C-2	70.95	73.61
C-3	72.07	206.56
C-4	69.61	72.50
C-5	72.44	75.28
C-6	60.46	60.46

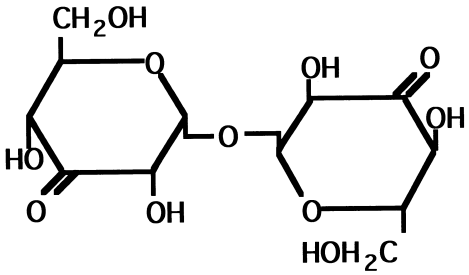

Fig. 1. Structure of 3,3′dkT.

3.2. The biological activity of 3,3′dKT

The trehalose derivative, 3,3′dkT was tested as the substrate for pig-kidney trehalase. The result was shown in Fig. 2. When 50 mM of trehalose was utilized as a substrate, the accumulated amount of glucose was observed, which was the hydrolytic product of the trehalase. However when the same experiment was carried out using 50 mM of 3,3′dkT as a substrate, no glucose was detected, even after 33 h of incubation. Therefore, 3,3′dkT was not a substrate of trehalase.

We then have investigated the inhibitory effect of 3,3′dkT on trehalases (Fig. 3a,b). With the increase of the 3,3′dkT

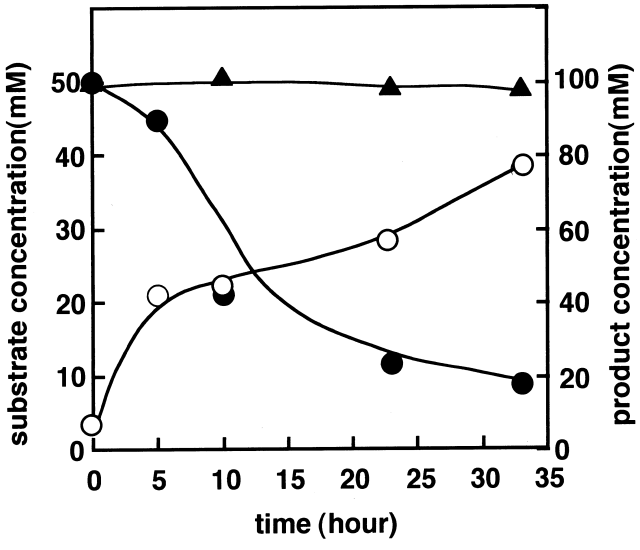

Fig. 2. Time course of trehalose and 3,3dkT hydrolysis by pig-kidney trehalase. Hydrolysis of trehalose and 3,3′dkT by pig-kidney trehalase. 3,3′dkT (▲), trehalose (●) and its hydrolytic product (○), glucose, were measured by HPLC method as described in Section 2. No product was observed from the experiment using 3,3′dkT as a substrate.

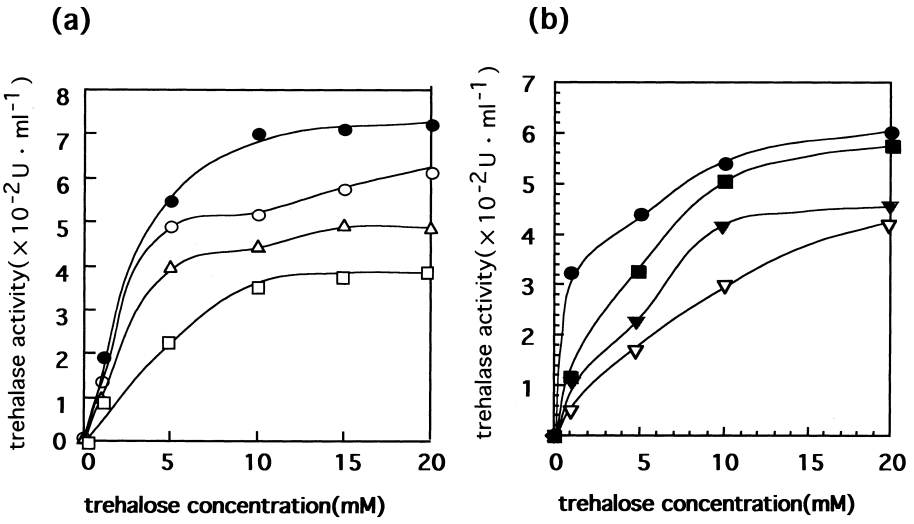

Fig. 3. Kinetic plots of the inhibition of trehalases from (a) pig-kidney and (b) *B. mori* by 3,3′dkT. 3,3′dkT concentration (a) 0 mM (●), 0.01 mM (○), 0.1 mM (△), 1.0 mM (□), (b) 0 mM (●), 0.5 mM (■), 2.0 mM (▼), 5.0 mM (▽).

concentration, both pig-kidney trehalase and *B. mori* trehalase activity decreased. IC_{50} values obtained from the dose experiments were 0.8 and 2.5 mM for pig-kidney and for *B. mori* trehalases, respectively. Fig. 4a,b shows the Dixon plots ([S] vs. $1/v$) of 3,3′dkT toward trehalases. Since the each line intersects to the left of the $1/v$ axis, the inhibition by 3,3′dkT was categorized as competitive. The K_i values obtained from Dixon plots were 0.2 and 0.6 mM for pig-kidney and for *Bombyx* trehalases, respectively (Table 2). In addition, 3,3′dkT did not show any inhibitory effects on both maltase and mannosidase catalyzing hydrolysis. Therefore, 3,3′dkT is a specific inhibitor of trehalases.

4. Discussion

In this paper, we described the preparation of a novel trehalose derivative, 3,3′dkT by G3DH catalyzing enzyme conversion, and its potential characteristics as a trehalase inhibitor.

Several naturally occurring 1–1 linked amino-disaccharide antibiotics have been reported. They included 3,3′-diamino-3,3′-dideoxy-α, α-trehalose, 3-amino-3-deoxy-α-D-mannopyranosyl 3-amino-3-deoxy-α-D-mannopyranoside [23], and 3,3′-neotrehalosadiamine [24]. These compounds were produced as the metabolites of actinomycetes. However, no one has ever reported about the structure nor existence of 3,3′dkT. It may be possible that 3,3′dkT is a key intermediate during the biosynthesis of the above-mentioned amino sugars from trehalose. Previously, enzymatic conversion of trehalose by G3DH prepared from a bacterium, *Flavobacterium saccharophilum*, was reported [25]. The product of this reaction was not 3,3′dkT but was 3-ketotrehalose, which was formed by the oxidation of one of the third hydroxy groups of α-D-glucopyranoside in trehalose. This would be due to the difference in the substrate specificity between *F. saccharophilum* G3DH and *Halomonas* sp. α-15 G3DH. At the moment, the utilization of *Halomonas* sp. α-15 G3DH is the only method for the preparation of 3,3′dkT.

Since 3,3′dkT inhibits trehalase activity but not closely related glycolytic reactions, such as maltase and mannosidase,

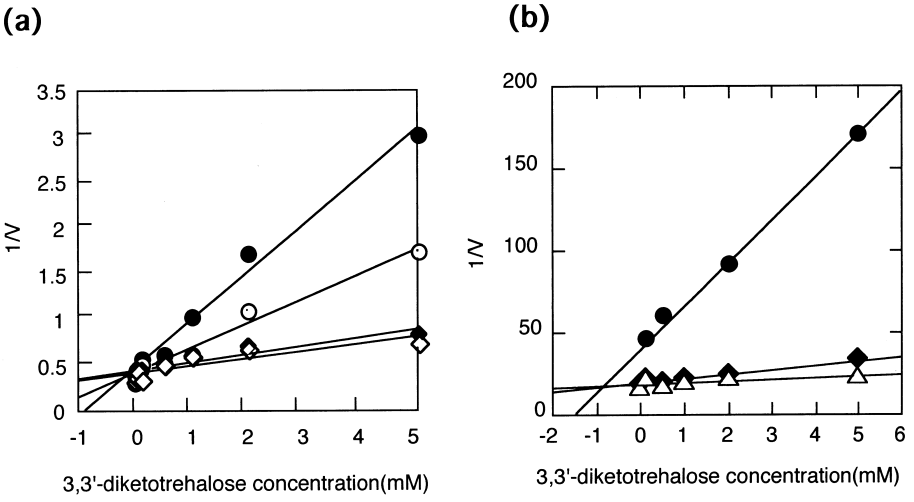

Fig. 4. Dixon plots of trehalases from (a) pig-kidney and (b) *B. mori* by 3,3′dkT. Trehalose concentration (a) 5 mM (●), 10 mM (○), 15 mM (◆), 20 mM (◇) (b) 1 mM (●), 10 mM (◆), 20 mM (△).

Table 2
K_i and IC_{50} values of 3,3′dKT of trehalase from pig-kidney and *B. mori*

Trehalase	K_i (mM)	IC_{50} (mM)
pig-kidney	0.2	0.8
B. mori	0.6	2.5

this trehalose derivative can be categorized as a specific inhibitor of trehalases. The trehalase enzyme inhibitors are expected to be utilized as the insecticides. In general, the trehalase inhibitors affect the oocyte development and oogenesis in female insect, consequently decreases the hatching efficiency. The inhibitory potential of 3,3′dkT was lower than the reported trehalase inhibitors. The K_i value of 3,3dkT is 0.2–0.6 mM, which is more than 10^3–10^5-fold higher than well known inhibitors, such as validoxylamines (4.3×10^{-10} for insects) and suidatrestin (2.0×10^{-8} for insects). These inhibitors are also competitive inhibitors. Trehalosamine [25] is a trehalose derivative and is also a competitive inhibitor with $K_i = 1.6\times10^{-2}$ for insects. Among various trehalose derivatives, 3,3′dkT shows the highest inhibition constant. The trehalase inhibitors showing significant inhibition toward trehalase are not the trehalose derivatives, but the analogues substrates. The synthesis of these inhibitors can be achieved only by the fermentation of actinomycetes producing these compounds as the antibiotics. However, the G3DH enzyme conversion system for 3,3′dkT production is a one-step route with high yield. Several insects, such as cockroaches, favor trehalose, and structurally similar 3,3′dkT will be utilized by mixing it with feed trehalose as for the oral administration, which will compensate for the low K_i value. Since 3,3′dkT is a water soluble sugar, the hydrophilic property of this compounds may not be advantageous in applying field distribution as the insecticides to be penetrated. The enhancement in the hydrophobic property of 3,3′dkT will be essential for the further application in the insecticides. It is also remarkable that the price of trehalose is lower than 300 yen kg^{-1} ($3 kg^{-1}), which is advantageous for a large scale production system, and also for large amount dosages, considering its application for insecticides. Various amino derivatives of trehalose have been reported and their pharmacological application is expected [26,27]. Although they are natural occurring compounds, some chemical synthetic methods have been reported [28], utilizing commercially available trehalose as a starting compound, but not via 3,3′dkT. Since 3,3′dkT will be readily converted to the amino derivatives by reductive amination, 3,3′dkT will be also valuable as a starting compound for the further development and preparation of several amino sugars.

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