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Enzymatic synthesis of a galactose-containing trehalose analogue disaccharide by *Pyrococcus horikoshii* trehalose-synthesizing glycosyltransferase: Inhibitory effects on several disaccharidase activities

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Abstract

Pyrococcus horikoshii trehalose-synthesizing glycosyltransferase employed a galactose as an acceptor in the glucosyl transfer reaction with an NDP-Glc donor. The reaction produced a non-reducing transfer product in a yield of more than 30% based on the molar concentration of donor used. The transfer product was purified by paper chromatography and preparative HPLC, and its glycosidic structure was confirmed by ^{13}C nuclear magnetic resonance to be α-D-glucopyranosyl α-D-galactopyranoside. Interestingly, this trehalose analogue disaccharide inhibited the action of several disaccharidases, including a trehalase. The analogue competitively inhibited porcine kidney and rat intestinal trehalases with K_i values of 0.68 and 3.7 mM, respectively. It also competitively inhibited other intestinal disaccharidases such as sucrase, maltase, and isomaltase with respective K_i values of approximately 0.66, 3.0, and 2.1 mM. Accordingly, this trehalose analogue would be a potentially indigestible disaccharide, effectively inhibiting intestinal brush border disaccharidases.

Keywords: *Pyrococcus horikoshii* trehalose-synthesizing glycosyltransferase (PhTG); Galactose acceptor; Trehalose analogue; Competitive inhibition; Disaccharidase

1. Introduction

Trehalose is a non-reducing disaccharide in which two glucose molecules are bonded in an α,α-(1 ↔ 1)-glucosidic linkage [1]. This naturally occurring sugar is widely distributed in various organisms such as bacteria, yeast, fungi, insects, invertebrates, and lower and higher plants. It may serve as an energy source or protect proteins and cellular membranes from a variety of environmental stress conditions, including desiccation, dehydration, heat, and freezing [2]. Trehalose is not very hygroscopic, stable to wide ranges of pH and heat, and a low or anti-cariogenic compound [3,4]. Generally, ingested trehalose is hydrolyzed to glucose by intestinal trehalase (EC 3.2.1.28) and absorbed in the small intestine.

Interestingly, there are several reports on synthesis of structural analogues of trehalose. Non-symmetrical trehalose derivatives including α-D-glucopyranosyl α-D-galactopyranoside have been chemically synthesized in organic solvent [5,6]. In addition, disaccharide analogues of trehalose containing xylosyl, fucosyl, or galactosyl moiety have been enzymatically synthesized by using a trehalose phosphorylase (EC 2.4.1.64) [7,8]. Trehalose phosphorylase is one of trehalose biosynthetic enzymes; it can hydrolyze trehalose in the presence of inorganic phosphate to yield G-1-P and a glucose, and reversibly synthesize trehalose from both products in vitro [9]. Properties of the trehalose analogues have not been well known.

Recently, a novel trehalose synthetic pathway was discovered involving unique trehalose-synthesizing glycosyltransferases from *Thermococcus litoralis* [10] and *Pyrococcus horikoshii* [11]. The enzymes were able to synthesize trehalose from NDP-Glc as a glucosyl donor and glucose as an acceptor.

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Moreover, we have just found that the trehalose-synthesizing glycosyltransferase from *P. horikoshii* has novel substrate specificity for galactose as an acceptor molecule in the disaccharide synthesis reaction. In this paper, therefore, we describe the enzymatic synthesis of the galactose-containing trehalose analogue disaccharide using *P. horikoshii* trehalose-synthesizing glycosyltransferase (PhTG). We also characterize the transfer product in terms of inhibition properties against disaccharide-hydrolyzing activities of several enzymes such as trehalase, α-glucosidase, sucrase, maltase, and isomaltase.

2. Experimental

2.1. Enzymes and chemicals

P. horikoshii trehalose-synthesizing glycosyltransferase (PhTG) was prepared and purified as previously reported [11]. Nucleoside diphosphate glucose (UDP-Glc, ADP-Glc, and GDP-Glc), *p*-nitrophenyl α-D-glucopyranoside (*p*NPαG), *p*-nitrophenyl β-D-galactopyranoside (*p*NPβGal), isomaltose, baker's yeast α-glucosidase, porcine kidney trehalase, and an acetone powder of rat intestinal enzymes (trehalase, sucrase, maltase, and isomaltase) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of reagent grade.

2.2. Transglycosylation reaction

The PhTG activity was spectrophotometrically assayed at 505 nm by a modified glucose oxidase/peroxidase method [12] under standard conditions at 25 °C in 50 mM sodium phosphate buffer (pH 7.0) containing 40 mM trehalose as a substrate. A transglycosylation reaction by PhTG was performed with NDP-Glc (UDP-, ADP-, or GDP-Glc) as the glucosyl donor and galactose as the acceptor. The purified enzyme (500 U) was added to a 100 ml reaction mixture containing 17.7 mM NDP-Glc and 55.6 mM galactose in 50 mM sodium acetate buffer (pH 6.0). The enzyme reaction was carried out at 37 °C for 24–36 h, and the reaction was terminated by boiling for 5 min. The reaction mixture was centrifuged at 12,000 × *g* for 10 min, and filtered using 0.45 μm membrane filter for further experiments.

2.3. Analysis of reaction product

The reaction products were analyzed by TLC on Whatman K5F silica gel plates (Whatman, Kent, UK). The plate was developed with isopropyl alcohol–ethyl acetate–water (3:1:1 v/v/v) or *n*-butanol–pyridine–water (7:3:1 v/v/v) [11]. Carbohydrates on the TLC plate were visualized by dipping method as previously reported [13]. The reaction products were quantitatively analyzed by HPAEC, using a Dionex CarboPac PA100 column (0.4 cm × 25 cm, Dionex Co., Sunnyvale, CA, USA) with a linear gradient (0–15%, v/v) of 600 mM sodium acetate and 150 mM sodium hydroxide at a flow rate of 1.0 ml/min [14].

2.4. Purification of reaction product

The transfer product was isolated by preparative paper chromatography on a Whatman 3 mm paper (23 cm × 55 cm) with a descending technique. The paper was irrigated with isopropyl alcohol–ethyl acetate–water (3:1:1 v/v/v) for 24 h. After air-drying, spots on the paper were located using an $AgNO_3$ reagent to verify separation of the reaction products. The paper was sectioned, and the product was eluted with deionized water. The eluted transfer product was further purified by Prep-HPLC (LC-918, JAI Co. Ltd., Tokyo, Japan) with a polymeric gel filtration column (W-251, 2 cm × 50 cm) [15]. ^{13}C nuclear magnetic resonance (NMR) spectra of the transfer product and standard carbohydrates were recorded with a JEOL JNM LA-400 MHz NMR spectrometer (Tokyo, Japan). The samples were dissolved in DMSO-d_6 at 24 °C using tetramethylsilane (TMS) as the chemical shift reference.

2.5. Assays of several disaccharidases

The activities of various disaccharide-hydrolyzing enzymes were assayed in the absence and presence of the transfer product. α-Glucosidase activity was spectrophotometrically determined at 420 nm using *p*NPαG (0.3–1.0 mM) in 50 mM sodium acetate buffer (pH 5.0) at 25 °C [16]. β-Galactosidase activity was also spectrophotometrically determined using *p*NPβGal (0.3–1.0 mM) in 50 mM sodium phosphate buffer (pH 7.0) at 25 °C [17]. Porcine kidney trehalase and rat intestinal enzyme activity assays were performed in 50 mM sodium phosphate buffer (pH 7.0) at 25 °C using the modified glucose oxidase/peroxidase method [12]. The porcine kidney and rat intestinal trehalase assays were performed with a final trehalose concentration of 0.5–6 and 10–160 mM, respectively. The sucrase, maltase, and isomaltase activities were assayed using sucrose at 2.9–15 mM, maltose at 0.3–8 mM, and isomaltose at 1.2–8.0 mM, respectively. The kinetic parameters (k_{cat} and K_m) for all the reactions above were calculated using software developed by Duggleby [18].

2.6. Kinetics of enzyme inhibition

The disaccharide-hydrolyzing enzyme reactions employed were performed according to the foregoing reaction conditions with various concentrations (0.1–5.8 mM) of the transfer product as an inhibitor. The type of inhibition, inhibition constant (K_i), and other kinetic parameters were determined using Dixon plots and a replot of the slope versus the reciprocal of the substrate concentrations [19].

3. Results and discussion

3.1. Analysis of the transglycosylation reaction by PhTG

We observed that the enzyme was able to employ a galactose as an acceptor in the transglycosylation reactions with UDP-, ADP-, and GDP-Glc donors, yielding a transfer product. As shown in Fig. 1, each glucosyl-transfer product was newly

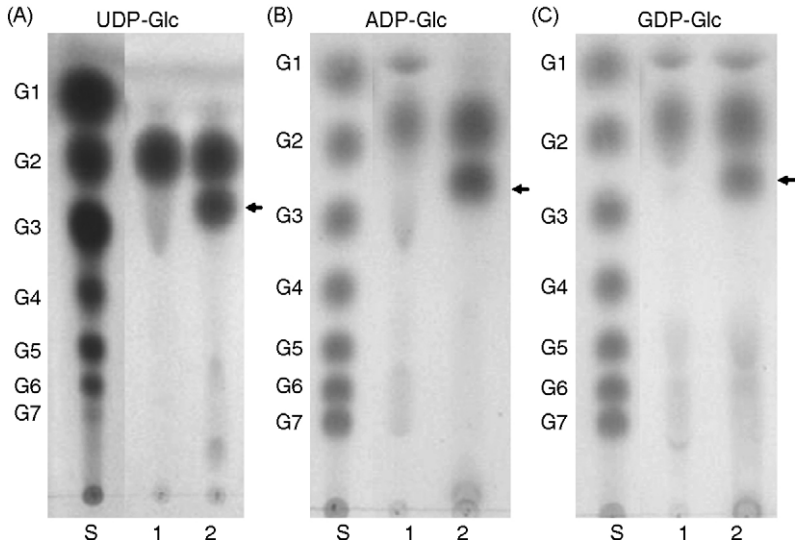

Fig. 1. TLC analysis of the transglycosylation reaction by PhTG with UDP-Glc (A), ADP-Glc (B), or GDP-Glc (C) donor and a galactose acceptor. Lane S: maltodextrin standards; lane 1: UDP-Glc (A), ADP-Glc (B), GDP-Glc (C) and galactose; lane 2: reaction mixture of PhTG with each donor and galactose.

formed and detected by TLC analysis. The transfer product for each transglycosylation, which was expected to be a galactose-containing disaccharide, had equivalent position on the TLC (arrows in Fig. 1, lane 2) and HPAEC chromatograms (data not shown). The molecular masses of the transfer products for all three transglycosylations were determined by MALDI-TOF mass analysis. Each transfer product was calculated to be an identical 342 Da from the peak at m/z 365.4 ($[M + Na]^+$), which matches the expected molecular mass of the sodium adduct of hexose disaccharide. Consequently, the enzyme was thought to have a broad donor specificity for the synthesis of the same disaccharide product containing a glucose and a galactose, just as shown for the trehalose synthesis [11].

The enzyme specificities for the glucose and the galactose acceptors were kinetically compared in the transglycosylation reaction using a UDP-Glc donor (Table 1). The K_m value (4.6 mM) for glucose was approximately 2.8 times lower than that for galactose. Glucose was also approximately 22 times more effective than galactose in terms of reaction specificity, V_{max}/K_m. This result implies that the enzyme basically prefers glucose as the acceptor in the transglycosylation. The enzyme can, however, use galactose as an acceptor even in a much lower affinity.

3.2. Purification and structural identification of the transfer product

The transfer product was repeatedly purified by paper chromatography and Prep-HPLC as described. Approximately 0.19 g of transfer product was obtained after purification from a transglycosylation reaction (36 h) with 1 g UDP-Glc and 1 g galactose. As a result, the production yields were evaluated to be approximately 31 and 10% based on the molar concentrations of UDP-Glc and galactose, respectively. The purified transfer product was confirmed to be non-reducing by using a copper-bicinchoninate method [13]. ^{13}C NMR analysis was performed to identify the glycosidic structure of the purified transfer product (data not shown). Additional carbon signals were observed in the ^{13}C NMR spectrum of the transfer product as compared to that of α-galactose. Those signals undoubtedly resulted from the transfer of an α-glucosyl moiety from UDP-Glc to α-galactose. In addition, there was relatively large change in the α-galactose C-1 chemical shift from 93.11 to 93.91 ppm. A chemical shift from 92.9 to 94.1 ppm in the transfer product was also observed in the C-1 of α-glucose transferred, which is almost the same as the α-glucose C-1 chemical shift in trehalose. These results confirmed that C-1 of transferred glucosyl moiety was specifically attached to C-1 of α-galactose in the transglycosylation, forming a trehalose analogue disaccharide through an α,α-(1 ↔ 1)-glycosidic linkage, α-D-glucopyranosyl α-D-galactopyranoside.

Table 1
Kinetic parameters for the acceptor specificity of PhTG in the transglycosylation using UDP-Glc donor

Acceptor	K_m (mM)	V_{max} (mM min^{-1})	V_{max}/K_m (min^{-1})
Glucose	4.6 ± 1.4	206 ± 19	44.78
Galactose	12.7 ± 3.7	26.1 ± 2.5	2.06

3.3. Inhibition of several disaccharidases

Trehalose is generally hydrolyzed by an intestinal trehalase to yield two glucose molecules, which are absorbed into the small intestine [3]. On the contrary, the trehalose analogue in this study was neither hydrolyzed by porcine kidney and rat intestine trehalases, nor by α-glucosidase and β-galactosidase. This is in good agreement with the previous report [8]. In addition, the analogue was not hydrolyzed by rat intestinal enzymes including maltase, sucrase, and isomaltase. Because the trehalose analogue was unhydrolyzed, it was considered that the analogue could be effective at inhibiting trehalase or other disaccharide-hydrolyzing enzyme activities.

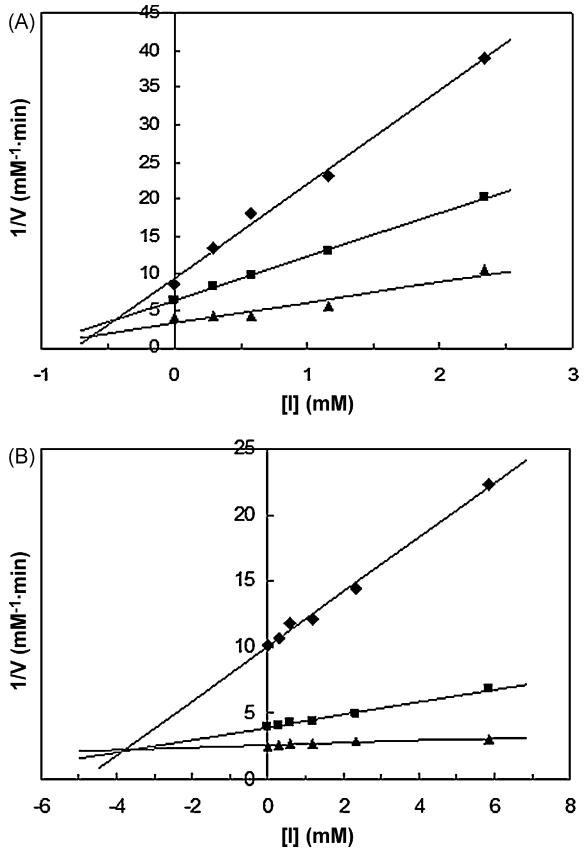

Fig. 2. Dixon plots of the activities of porcine kidney (A) and rat intestine (B) trehalases in the presence of the trehalose analogue inhibitor.

The Dixon plots and replots of the slope indicated inhibition type for the activities of the enzymes tested (Figs. 2 and 3). The inhibition constant and kinetic parameters for the substrates were also determined (Table 2). The trehalose analogue showed competitive inhibition for porcine kidney trehalase with a K_i value of 0.68 mM (Fig. 2A), and for rat intestinal trehalase with a K_i value of 3.72 mM (Fig. 2B). This indicates that the analogue resists the hydrolysis by trehalases, but favorably binds the enzyme active site, competing with the trehalose substrate. In fact, trehalase inhibition is an interesting target for development of novel insecticides, because trehalose is a vital source of energy for insect flight [20]. Sucrose, phloridzin, and tris(hydroxymethyl)aminomethane (TRIS) are known competitive trehalase inhibitors with K_i values in the 1.1–3.7 mM range [21]. Moreover, several potent inhibitors have been reported including suidatrestin, validoxylamine A, and trehazolin with micromolar to nanomolar K_i range [20,22]. Reportedly, trehalase accounts for 0.1 and 0.3% of the total brush-border membrane protein in rabbit kidney and intestine, respectively [21]. It could be inferred that the effective trehalase inhibition might delay trehalose breakdown and absorption in the small intestine.

Unexpectedly, the trehalose analogue in the present study also competitively inhibited baker's yeast α-glucosidase with a K_i value of 0.29 mM and other rat intestinal disaccharidases (sucrase, maltase, and isomaltase) with K_i values of 0.66, 3.04, and 2.14 mM, respectively (Fig. 3A–D). The inhibition constants for the analogue were approximately 2.6–11.5 times lower than the K_m value of the corresponding substrate for each enzyme. It was considered that the trehalose analogue could compete effectively against sucrose with an α-(1 → 2)-glycosidic bond, maltose with an α-(1 → 4)-glucosidic bond, and isomaltose with an α-(1 → 6)-glucosidic bond, respectively in the substrate binding sites. The analogue was only inactive against β-galactosidase. Sucrose is widely used in many foods as a sweetener and its daily intake is still large. Starch, the major dietary carbohydrate, is degraded into maltose in the small intestine. Thus, disaccharidases such as sucrase and α-glucosidase (maltase) in the brush-border membranes of the small intestine are recognized to be important targets for inhibition. This inhibition may limit the intestinal digestion of dietary carbohydrates, thus controlling diabetes and obesity [23–26]. Reportedly, L-arabinose was recommended as a good inhibitor as it inhibited only intestinal sucrase activity with a K_i value of 2 mM in vitro [24]. This monosaccharide showed no inhibition against intestinal maltase, isomaltase, trehalase, or lactase activity. Compared to L-arabinose, the trehalose analogue was three times more potent in the inhibition against intestinal sucrase, concomitantly inhibiting the activities of other intestinal disaccharidases including maltase in vitro. Recently, valienamine (an amino sugar analogue of D-glucose) and 3,4,5-trihydroxybenzoic acid (gallic acid) were also suggested to be potent inhibitors of brush-border sucrase, which were competitive inhibitors with respective K_i values of approximately 0.77 and 1 mM, similar to the trehalose analogue in this study [25,26].

Accordingly, the results showed that the trehalose analogue was very favorable for inhibiting the activities of intestinal disaccharidases such as trehalase and sucrase. Furthermore, it significantly inhibited the activities of other disaccharidases used. Thus, we suggest that the galactose-containing trehalose analogue would have a potential as indigestible disaccharide, effectively inhibiting the intestinal brush-border disaccharidases and potentially interfering with intestinal digestive functions.

Table 2
Kinetic parameters for the activities of porcine kidney trehalase, baker's yeast α-glucosidase, and rat intestinal disaccharidases such as trehalase, sucrase, maltase, and isomaltase in the presence of the trehalose analogue

Enzyme	Inhibition type	K_i (mM)	K_m (mM)	V_{max} (mM min^{-1})	V_{max}/K_m (min^{-1})
Porcine kidney trehalase	Competitive	0.68 ± 0.16	2.19 ± 1.42	0.82 ± 0.40	0.37
Baker's yeast α-glucosidase	Competitive	0.29 ± 0.14	0.75 ± 0.26	1.96 ± 0.35 × 10^{-3}	0.0026
Rat intestine trehalase	Competitive	3.72 ± 0.19	126.4 ± 11.2	0.47 ± 0.02	0.0037
Rat intestine sucrase	Competitive	0.66 ± 0.06	7.6 ± 1.2	0.09 ± 0.01	0.012
Rat intestine maltase	Competitive	3.04 ± 0.48	2.4 ± 1.6	0.19 ± 0.09	0.079
Rat intestine isomaltase	Competitive	2.14 ± 0.07	7.5 ± 0.8	7.5 ± 0.8	0.039

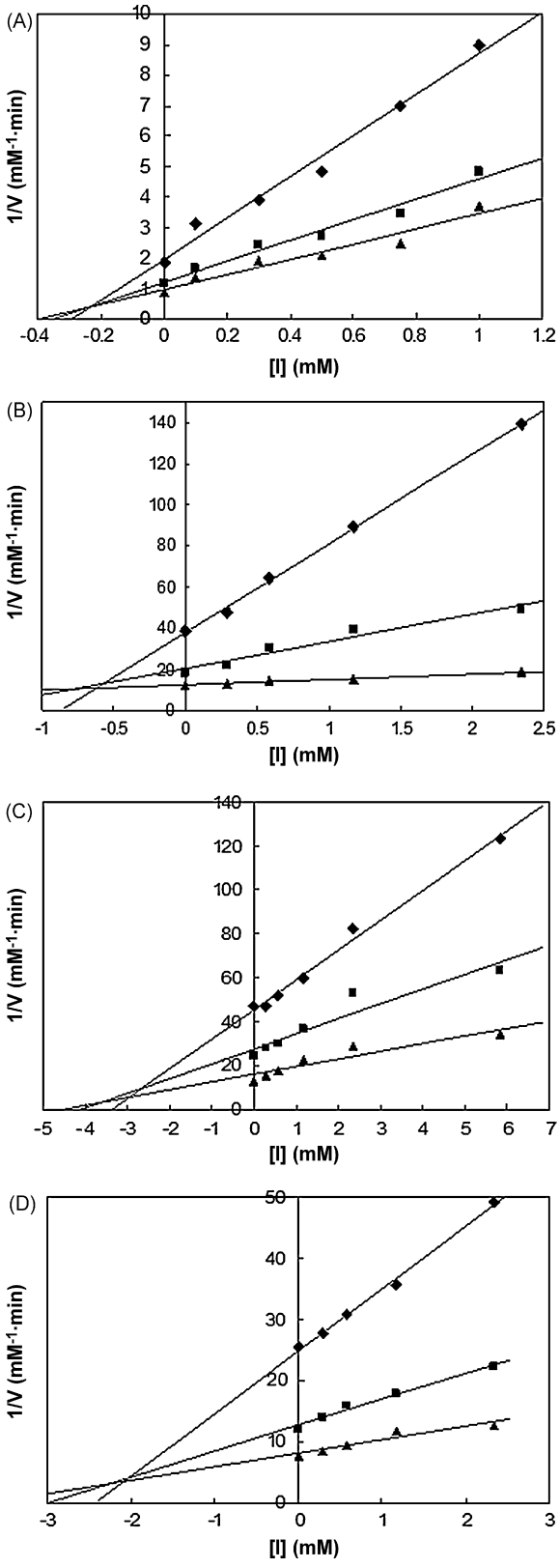

Fig. 3. Dixon plots of the activities of baker's yeast α-glucosidase (A), rat intestine sucrase (B), rat intestine maltase (C), and rat intestine isomaltase (D) in the presence of the trehalose analogue inhibitor.

Further study is needed in vivo. The unique properties of the trehalose analogue in enzyme inhibition seem to be attributed to its concurrent characteristics of structural resemblance and indigestibility.

4. Conclusions

In conclusion, a galactose was employed as an acceptor in the glucosyl transfer reaction by PhTG with an NDP-Glc donor, yielding a transfer product. The product was identified to be α-D-glucopyranosyl α-D-galactopyranoside, trehalose analogue disaccharide. The analogue showed the inhibitory effects on the activities of several disaccharidases. The analogue showed competitive inhibition against kidney and intestinal trehalases and other intestinal disaccharidases including sucrase, with low millimolar range of K_i values. Thus, the trehalase analogue might be effective at inhibiting intestinal brush-border disaccharidases.

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