Rapid Paper

Reaction Mechanism of a New Glycosyltrehalose-producing Enzyme Isolated from the Hyperthermophilic Archaeaum, *Sulfolobus solfataricus* KM1†

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An amylolytic activity, which converts soluble starch to α,α-trehalose (trehalose), was found in the cell homogenate of the hyperthermophilic, acidophilic archeaum *Sulfolobus solfataricus* KM1. Two enzymes, a glycosyltransferase and an amylase, which are essential for this activity, were purified to homogeneity. A glycosyltransferase catalyzed the conversion of maltoligosaccharides to glycosyltrehaloses. Based on a detailed analysis of the reaction products, kinetic parameters, and an experiment using *H*-labeled substrates, it was verified that glycosyltransferase transferred an oligomer segment of maltoligosaccharide to the C1–OH position of glucose, located at the reducing end of the maltoligosaccharide, to produce a glycosyltrehalose having an α-1,1 linkage. The reaction appears to be intramolecular. Nine strains of the *Sulfolobaceae* family were found to have glycosyltransferases.

Key words: trehalose; *Sulfolobus solfataricus*; glycosyltrehalose; glycosyltransferase; *Acidimius brierleyi*

Many investigations have been done on enzymes that can catalyze the transglycosylation of starch and starch hydrolysates, *i.e.*, α-glucosidase, 1–3) cyclomaltoolactrontransferase, 4) branching enzyme, and neopullulanase. 5) The transglycosylation activities of these enzymes consisted of four types: activity to form an α-1,2 linkage, α-1,3 linkage, α-1,4 linkage, or α-1,6 linkage.

The direct production of an oligosaccharide having an α-1,1 linkage from substrates such as starch or maltoligosaccharides using enzymatic transglycosylation may be practical, but it has never been reported for any enzyme that can catalyze transglycosylation to form an α-1,1 linked oligosaccharide including trehalose.

In our previous study on trehalose-producing bacteria, the cell homogenate of the hyperthermophilic, acidophilic archeaum *Sulfolobus solfataricus* KM1, was found to have amylolytic activity for converting soluble starch to trehalose. 8) Two enzymes, a new glycosyltransferase and an amylase, were purified.

In this paper, the characteristics of the glycosyltransferase were investigated based on the course of the reaction products and kinetic parameters against substrates of various chain lengths. We also searched for the mechanism of transglycosylation using a nonreducing-end *H*-labeled substrate. Furthermore, we compared characteristics of the enzyme with other purified glycosyltransferases that were isolated from various strains of the *Sulfolobaceae* family.

Materials and Methods

**Bacterial strains.** *S. solfataricus* KM1 (KM1) was isolated from an acid hot spring in Gunma Prefecture, Japan in 1993. 7) *S. solfataricus* ATCC 35091 (DSM 1616), and *S. acidocaldarius* ATCC 33909 (DSM 639) were obtained from the American Type Culture Collection, Maryland, U.S.A. *S. solfataricus* DSM 5833, and *Acidimius brierleyi* DSM 1651 were obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Federal Republic of Germany.

**Culture conditions.** Cultures of KM1, ATCC 35091, 35092, 49426, DSM 5354, 5389, and 5833 were grown at 75°C, aerobically, using Brock's medium. 9) *S. acidocaldarius* ATCC 33909 was grown using the same medium at 70°C and pH 3.0. *Acidimius brierleyi* DSM 1651 was grown, using Zillig's medium. 10)

**Enzyme assay of glycosyltransferase activity.** Glycosyltransferase activity was assayed by the method described in our previous paper. 7) Glycosyltransferase was incubated in 10% maltopentaose in 50 mM sodium acetate buffer, pH 5.5, at 60°C. The reaction was stopped by heating at 100°C for 5 min and the maltotriosyltrehalose produced was hydrolyzed with 5 U/ml glucoamylase at pH 4.5, 40°C overnight. The trehalose thus obtained was quantitatively analyzed by HPLC. One unit (U) of glycosyltransferase was defined as the amount of enzyme that would produce 1 μmol of trehalose per min at 60°C.

**Enzyme assay for glucosyltrehalose-producing activity.** The enzymatic reaction was done by the enzyme assay of the glycosyltransferase activity described above except for using maltotriose as the substrate. The glucosyltrehalose thus obtained was measured by HPLC. One unit (U) was defined as the amount of enzyme that would produce 1 μmol of trehalose per min at 60°C.

**Purification of glycosyltransferase.** The glycosyltransferase from KM1 was purified by the method described in our previous paper. 7) Based on the purification method of glycosyltransferase from KM1, glycosyltransferases from other strains were also purified.

**Substrate specificity.** The analysis was done using purified glycosyltransferase at the substrate concentration of 100 mM as an enzyme assay method for glycosyltransferase as described above with maltoligosaccharides of various chain lengths. The trehalose was measured by HPLC and estimated as that corresponding to glycosyltrehalose.

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Abbreviations: HPLC, high pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DP, degree of polymerization.
High-pressure liquid chromatography. The composition of the saccharide produced was analyzed by HPLC using a TSK-gel Amide-80 column (4.6 by 250 mm, Tosoh) at room temperature. The elution of saccharides was done with a 72.5% acetonitrile-deionized water solution and detected with a differential refractometer (RID-6A, Shimadzu, Kyoto, Japan).

Preparation of the labeled substrate. Two mm of [glucose-6-14C]UDP-Glc (4 Ci mmol) and 4 mm of maltotetraose were incubated with 1.1 U/ml of glycogen synthase in the presence of 0.5 mm of glucose-6-phosphate in 10 mm of Tris HCl (pH 8.0) at 30 C for 30 h. Radioactive maltotetraose labeled at the nonreducing end with [6-14C]glucose was purified by silica gel Kieselgel 60 thin-layer chromatography plate using a developing solvent of butanol ethanol water (5:5:3). 10

Analysis of the transglycosylation mechanisms using a 1-H-labeled substrate. The labeled maltopentaose (10 mm) was incubated with 3 U/ml of purified glycosyltransferase for 3 h at pH 5.5 and 60 C. The reaction product was treated with 5 U/ml of glucosamylase at 40 C overnight. The hydrolysate was put on a silica gel Kieselgel 60 thin-layer chromatography plate and developed by a solvent of butanol ethanol water (5:5:3). The monosaccharide and disaccharide fractions were detected using the anisaldehyde sulfate method and each spot was scraped and the radioactivity was measured with a liquid scintillation spectrometer (Wallaac 1410 liquid scintillation counter, Pharmacia, Tokyo, Japan).

Electrophoresis, isoelectric focusing. Polyacrylamide gel electrophoresis was done on a 10% polyacrylamide slab gel in the presence of SDS by the method of King and Laemmli. 1 2 The isoelectric point was identified by isoelectric focusing (ACI Japan system), using the manufacturer’s optimizing method.

Measurement of enzyme concentration. The enzyme concentration was estimated for the dry weight of purified enzyme. The molar absorbitivity was calculated by the Lambert Beer law. Molecular activity, k_m, was calculated by using the value of molar absorbitivity.

Results
Glucosyltrehalose-producing activity of strains in the Sulfolobaceae family
The activities of the cell homogenate of strains that belong to the Sulfolobaceae family were assayed. As shown in Table I, all strains tested had the glucosyltrehalose-producing activity. These strains possessed the same glucosyltransferase activity as that of KM1 when maltotriose was used as the substrate.

Purification of glucosyltransferases
These purified enzymes gave a single band on SDS-PAGE. As shown in Table II, KM1 glycosyltransferase had its maximum activity at pH 5.0–6.0 and 70–80 C. As much as 91% of the activity remained even after an incubation period of 6 h at 85 C. The physicochemical properties of enzymes isolated from other strains were almost the same as those of KM1 glycosyltransferase.

Substrate specificity
As previously described, 11 the main products from maltotriose, maltotetraose, and maltopentaose were glucosyltrehalose (α-D-maltosyl α-D-glucopyranoside), maltosyltrehalose (α-D-maltotriosyl α-D-glucopyranoside), and maltotriosyltrehalose (α-D-maltotetraosyl α-D-glucopyranoside), respectively. The DP of the product by the enzyme reaction was responsible for each maltooligosaccharide of DP3 or higher. Glucose, maltose, isomaltoolose, isomaltotriose, isomaltotetraose, isomaltopentaose, and panose were not used as substrates. A comparison of the relative reactivity among maltooligosaccharides and yields of glucosyltrehaloses can be seen in Table III. Maltopentaose was the most suitable substrate, with 20 times greater reactivity than maltotriose. The yields of maltotriosyltrehalose, maltotriosyltrehalose, maltotetraosyltrehalose, and maltotetaosyltrehalose, which have a degree of polymerization of 4 or higher, were higher than 78%, while the yield of glucosyltrehalose of DP3 had a low value of 45%.

Course of enzymatic reactions
The production of glucosyltrehalose was accompanied by side products such as glucose and maltooligosaccharide shortened by one glucose residue due to a side hydrolysis reaction as shown in Fig. 1. When maltotetraose was used as the substrate, the secondary by-products glucosyltrehalose and maltose were produced by the transglycosylation and hydrolysis of maltotriose which was in turn produced by the above-mentioned side hydrolysis reaction (Fig. 1b). When maltopentaose was used as the substrate, the secondary by-products maltotriosyltrehalose and malt-

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**Table I. Glucosyltrehalose-producing Activity of Various Strains in the Sulfolobaceae Family**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Activity (U/gram cell)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfolobus solfataricus</td>
<td>ATCC 35091 0.11</td>
</tr>
<tr>
<td></td>
<td>ATCC 35092 0.10</td>
</tr>
<tr>
<td></td>
<td>DSM 5354 0.22</td>
</tr>
<tr>
<td></td>
<td>DSM 5833 0.09</td>
</tr>
<tr>
<td></td>
<td>KM1 0.23</td>
</tr>
<tr>
<td>Sulfolobus acidocaldarius</td>
<td>ATCC 35090 0.22</td>
</tr>
<tr>
<td></td>
<td>ATCC 49426 0.04</td>
</tr>
<tr>
<td>Sulfolobus shibatai</td>
<td>DSM 5389 0.20</td>
</tr>
<tr>
<td>Acidilamna briarei</td>
<td>DSM 651 0.11</td>
</tr>
</tbody>
</table>

* Wet cell weight.

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**Table II. Physicochemical Properties of Glycosyltransferases of the Sulfolobaceae Family**

<table>
<thead>
<tr>
<th></th>
<th>KM1</th>
<th>ATCC 5833</th>
<th>ATCC 35090</th>
<th>DSM 1651</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opt. pH</td>
<td>5.0 ± 6.0</td>
<td>4.5 ± 5.5</td>
<td>4.5 ± 5.5</td>
<td>4.5 ± 5.5</td>
</tr>
<tr>
<td>pH stability</td>
<td>4.0 ± 10.0</td>
<td>4.5 ± 12.0</td>
<td>4.0 ± 10.0</td>
<td>4.0 ± 12.0</td>
</tr>
<tr>
<td>Opt. temp. (C)</td>
<td>70 ± 80</td>
<td>70 ± 80</td>
<td>70 ± 80</td>
<td>70 ± 80</td>
</tr>
<tr>
<td>Mol. wt. (SDS PAGE)</td>
<td>75,000</td>
<td>75,000</td>
<td>74,000</td>
<td>74,000</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>CuSO4 6.1</td>
<td>CuSO4 5.3</td>
<td>CuSO4 5.6</td>
<td>CuSO4 6.3</td>
</tr>
</tbody>
</table>

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**Table III. Substrate Specificity of Glycosyltransferases from KM1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Main product</th>
<th>Relative reactivity (as)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Glucosyltrehalose</td>
<td>5.0 ± 5.0</td>
<td>45.0</td>
</tr>
<tr>
<td>Maltose</td>
<td>Maltosyltrehalose</td>
<td>46.3 ± 7.8</td>
<td>78.5</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>Maltotriosyltrehalose</td>
<td>100 ± 8.7</td>
<td>87.5</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>Maltotetraosyltrehalose</td>
<td>80.0 ± 8.1</td>
<td>81.7</td>
</tr>
<tr>
<td>Maltopentaose</td>
<td>Maltopentaosyltrehalose</td>
<td>88.6 ± 8.8</td>
<td>88.5</td>
</tr>
</tbody>
</table>
Glycsoyltrehalose-producing Enzymes from *S. solfatarius* 923

![Fig. 1. Course Studies on the Production of Glycosyltrehaloses from Maltotriose (a), Maltotetraose (b), and Maltpentaose (c) by KM1 Glycosyltransferase.](image)

Each of the substrate at a 10% concentration was incubated with 4.5 U/ml glycosyltransferase and analyzed by Amide-80 HPLC as described in Materials and Methods. Symbols: ▲: glucose; ▼: maltose; ○: maltotriose; ●: glycsoyltrehalose; Δ: trisaccharidemaltotriose and glucosyltrehalose; □: maltotetraose; ■: maltosyltrehalose; ▼: tetrascarihdemaltotetraose and maltosyltrehalose; ○: maltpentaose; ●: maltpentosyltrehalose.

**Table IV. Rate Parameters of Glycosyltransferase from KM1 for Maltoooligosaccharides**

<table>
<thead>
<tr>
<th>DP of substrate</th>
<th>$K_m$ (mm)</th>
<th>$k_0$ (s$^{-1}$)</th>
<th>$k_m K_m$ (mm$^{-1}$.s$^{-1}$)</th>
<th>log($k_m$/$K_m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>228</td>
<td>48.3</td>
<td>0.211</td>
<td>0.68</td>
</tr>
<tr>
<td>4</td>
<td>21.8</td>
<td>211</td>
<td>9.67</td>
<td>0.99</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>47</td>
<td>49.0</td>
<td>1.69</td>
</tr>
<tr>
<td>6</td>
<td>2.7</td>
<td>204</td>
<td>74.6</td>
<td>1.87</td>
</tr>
<tr>
<td>7</td>
<td>1.2</td>
<td>181</td>
<td>150</td>
<td>2.18</td>
</tr>
</tbody>
</table>

trotiose were produced by transglycosylation and hydrolysis of maltotetraose; glycsoyltrehalose and maltose were subsequently produced in the same way as maltotetraose (Fig. 1c). In each case, the maltoooligosaccharide substrate was cleaved to produce an equimolar amount of glucose and a maltoooligosaccharide shortened by one glucose residue. The yield of the main products, glycsoyltrehaloses, was seen to decrease with an increase of the yield of the side products. A glycsoyltrehalose of more than DP of the parental maltoooligosaccharide could not be produced.

**Measurement of the rate parameters**

Glycosyltransferase was incubated with various concentrations of the maltoooligosaccharides and the activity was measured. As shown in Table IV, the values of the Michaelis constant, $K_m$, decreased with the increase in the length of the substrates from 228 mm (maltotriose) to 1.2 mm (maltpentaose). The values of the molecular activity, $k_0$, which was calculated by using the value of molar absorptivity of this enzyme ($ε = 9.4 × 10^4$), were increased from 48 $s^{-1}$ (maltotriose) to 247 $s^{-1}$ (maltpentaose).

**Transglycosylation mechanisms using 3H-labeled substrate**

As shown in Fig. 2a, most of the radioactivity was recovered in the monosaccharide (glucose) fraction. There was very little radioactivity in the disaccharide (trehalose) fraction; it was presumably due to background activity derived from the radioactive glucose. This indicated that the 3H-labeled glucose residue located at the nonreducing end of the maltoooligosaccharide remained as the same glucose unit of the glycsoyltrehalose (Fig. 2b).

From these results, it appeared that the glucose residue of the trehalose structure in glycsoyltrehalose was not likely to be derived from the nonreducing end of the parental maltoooligosaccharide but from the reducing end.

**Discussion**

The glycosyltransferases examined in this work possess the same action patterns, because the glycosyltransferase from KM1 and other strains can be purified by the enzyme assay method of KM1 glycosyltransferase. Also, the physicochemical properties of these enzymes were almost the same as those of KM1 glycosyltransferase. This may indicate that this enzyme is widely distributed in the *Sulfolobaceae* family, i.e., genera *Sulfolobus* and *Acidimius*.

In this report, we attempted to clarify the reaction mechanism of glycosyltransferase by a detailed analysis of the reaction products. The HPLC analysis of the reaction product indicated that the enzyme never produced a glycsoyltrehalose of longer chain length than the substrate used. Glucose and maltoooligosaccharide shortened by one glucose residue were regularly produced as by-products from maltoooligosaccharide substrates of various chain lengths by the enzymatic reaction. The experimental results using labeled substrate demonstrated that the glucose residue of the trehalose moiety was not derived from the nonreducing end of the substrate (Fig. 2). Precedents from other studies of glycosyltransferases suggest that the transglycosylation would start primarily by hydrolysis of an $α$-1,4 glycosidic linkage. The glycosyl residue would bind to the enzyme and form a covalent enzyme-substrate intermediate. And the hydrolyzed glucose molecule derived from reducing-end glucose of the parental substrate would be located at active site pocket of the enzyme. Subsequent transglycosylation occurs, rather than hydrolysis of the covalent enzyme-substrate intermediate because $H_2O$ molecule would be hard to approach the glycosyl residue owing to steric hindrance or the hydrophobic environment around the active site cleft. Therefore, the hydrolytic side reaction can be explained by the competition between the $H_2O$ molecule and the reducing-end glucose molecule as an acceptor of the glycosyl residue. A decrease in the concentration of glycsoyltrehaloses was observed after the maximal production of the glycsoyltrehaloses (Fig. 1). It was presumed that the hydrolysis of the glycsoyltrehaloses by the glycosyltransferase also occurs.

As shown in Fig. 1, and Tables III and IV, the enzyme showed low hydrolysate production, low value of $K_m$...
(=high affinity), and a high yield of glycosyltrehalose with the longer chain-length substrate. From the subsite theory, 14,15 the subsite affinities except for $A_1$, $A_2$ were evaluated using observed $\log(k_c/K_m)$ values. From the value of $\log(k_c/K_m)$ among maltooligosaccharides having a degree of polymerization from 3 to 7, it was predicted that the enzyme has 6 or 7 subsites (Table IV). Therefore, the high affinity of the enzyme with longer chain-length substrates is presumed to be due to an increased binding site.

From these results, we expected that the reaction mechanism of the enzyme is the following way. The glycosyltransferase transferred a glycosyl residue produced from maltooligosaccharide by the hydrolysis reaction to the Cl–OH position of glucose, at the reducing end of maltooligosaccharide, to produce glycosyltrehalose having an $\alpha$-1,1 linkage. There is no report of an enzyme that transfers a glycosyl residue to form an $\alpha$-1,1 linkage. This would be intramolecular transglycosylation. A detailed analysis of the reaction mechanism which may include the possibility of an intermolecular transglycosylation is now underway.

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References