Subsite Structure and Catalytic Mechanism of a New Glycosyltrehalose-producing Enzyme isolated from the Hyperthermophilic Archaeum, \textit{Sulfolobus solfataricus} KM1

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A glycosyltrehalose-producing enzyme from \textit{Sulfolobus solfataricus} KM1 catalyzes a conversion of maltooligosaccharides to glycosyltrehaloses and also hydrolyzes maltooligosaccharides to liberate glucose, as a side reaction. From the sum of the conversion and hydrolysis reaction rates, the rate parameters involved in the “splitting” of the α-1,4 glucosidic linkage were calculated. From the data obtained, the subsite structure for maltooligosaccharides was identified. From the analysis of the hydrolysate of maltotriose in [\textsuperscript{18}O] labeled H\textsubscript{2}O, the hypothesis of the C1-O bond splitting and the formation of a glycosyl (maltosyl)-enzyme intermediate was strongly supported. From the analysis of the reaction product in the presence of [\textsuperscript{3}H] labeled glucose, the occurrence of intermolecular transglycosylation was confirmed. These data strongly support the suggestion that the catalytic mechanism of this enzyme is a transglycosylation.

\textbf{Key words:} trehalose; glycosyltransferase; subsite structure; catalytic mechanism; \textit{Sulfolobus solfataricus}

In our previous paper, it was shown that there is a new pathway involving two coupled enzymes, a glycosyltrehalose-producing enzyme (glycosyltransferase) and a glycosyltrehalose-hydrolyzing enzyme (α-amylase) from \textit{Sulfolobus solfataricus} KM1, which produced an 80% yield of trehalose from starch.\textsuperscript{1-3} The glycosyltrehalose-producing enzyme catalyzes a conversion of maltooligosaccharides to glycosyltrehaloses and also hydrolyzes maltooligosaccharides to liberate glucose, as a side reaction. The hydrolyzing side reaction was conspicuous with shorter substrates, such as maltotriose. In this paper, we focused on these two reactions and identified the subsite structure using rate parameters of “splitting” of the α-1,4 glucosidic linkage, which were calculated from the sum of the conversion and hydrolysis reaction rates. Previous studies on subsite structure of both endo-type enzymes (such as α-amylases) and exo-type enzymes (such as glucoamylases, α-glucosidases, and β-amylases, which act from the nonreducing end of the substrate), were analyzed and evaluated.\textsuperscript{4-6} This is the first report of subsite structure analysis of an exo-type enzyme that acts from the reducing end of the substrate.

Previous studies of transglycosylating enzymes described the catalytic mechanism of transglycosylation by double displacement and oxocarbonium ion mechanisms.\textsuperscript{7-11} To confirm the hypothesis that the reaction proceeds according to these postulated mechanisms, we examined the possibility of the splitting of the C1-O bond by using [\textsuperscript{18}O]-labeled H\textsubscript{2}O. This event would support the existence of a glycosyl-enzyme intermediate. Furthermore, we examined the possibility of intermolecular transglycosylation by using [\textsuperscript{3}H]-labeled glucose. In this paper we present evidence for this hypothesis through the description of these experiments.

\textbf{Materials and Methods}

\textit{Bacterial strains and culture conditions.} \textit{Sulfolobus solfataricus} KM1 (KM1) was isolated from an acid hot spring in Gunma prefecture, Japan in 1993. Cultures of KM1 were grown at pH 4.0, 75°C aerobically using the standard culture medium.\textsuperscript{12}

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Purification of glycosyltransferase. The glycosyltransferase from KM1 was purified by the method described in our previous paper.¹³

Definition of glycosyltransferase activity. The glycosyltransferase activity was assayed by the method described in our previous paper.¹¹ One unit (U) of glycosyltransferase was defined as the amount of enzyme that would produce 1 μmol of trehalose after glucoamylase treatment of the reaction solution which was given from 10% maltopentaose per min in 50 mm sodium acetate buffer, pH 5.5, at 60°C.¹³

Preparation of glycosyltrehaloses. Maltotriosyltrehalose, maltotetraosyltrehalose, and maltopentaosyltrehalose were prepared using the purified glycosyltransferase reaction followed by a 0.1 N NaOH treatment at 100°C for 2 hr, to decompose the remaining reducing saccharides and deionization using a mixed bed of Amberlite IR120B and IRA411S (Organico, Tokyo, Japan). Glucosyltrehalose and maltotriosyltrehalose were prepared by a partial hydrolysis of maltotriosyltrehalose by glucoamylase followed by purification using HPLC (Tosoh TSK-gel Amide-80 column, 72.5% acetonitrile).

Measurement of rate parameters. The assay method for glycosyltransferase previously described¹¹ was modified to stop the reaction by adding HCl. After neutralization with NaOH, the reaction product was hydrolyzed overnight by glucoamylase at 40°C. The sample was then deionized on Sep-Pak Plus QMA and CM (Waters, Tokyo, Japan) and the trehalose thus obtained was analyzed by HPLC as described above.

The hydrolysis reaction was the same as that described above except that there was no glucoamylase treatment and the liberated glucose was measured using the Glucose C II-Test Wako (Wako Pure Chemical, Osaka, Japan).

As described before, it was considered that the "sum" of the rate of both conversion and hydrolysis was appropriate for the calculation of accurate evaluation of subsite affinities. For the calculation of the rate parameters of the "sum" of conversion and hydrolysis, the rates of both reactions at the same substrate concentration were added and used.

From these data, Kₙ and kₙ for each substrate were evaluated from an S/S/V plot. Each value was calculated from the mean of 4 to 8 measurements.

Measurement of enzyme concentration. The enzyme concentration was estimated by using the value of molar absorption (ε = 9.4 x 10⁴).²¹

Evaluation of subsite affinities and kₙ. The subsite affinities (Aᵢ) of glycosyltransferase were evaluated based on Hiromi's subsite theory.¹²,¹³ We referred to the method for glucoamylases and α-glucosidases that act on the nonreducing end side and have only one major nonproductive binding mode (j = 2).²³ However, we modified the dealing of the major nonproductive binding mode as j = 3 (Fig. 1), because this enzyme acts from the side of the reducing end. From the precedent of some glycoenzymes, the A₂ was expected to be a negative value so as to cause a distortion to a glucose residue located at A₂. In fact, when the subsite affinities were calculated with the assumptions of negative A₁ and major nonproductive binding mode as j = 3, the rate parameters observed agreed with the parameters conversely calculated from the A₁ and kₙ. This is except for the values for maltose, otherwise, they were in complete disagreement. The details of the calculation methods are described in an appendix.

Identification of the splitting position using [¹⁸O] labeled H₂O. Ten percent of maltotriose in 50% [¹⁰O] labeled H₂O (Isotec Inc., USA), 20 mm sodium acetate (pH 5.5), and 6.6 U/ml glycosyltransferase were incubated at 70°C for 1 hr. The reaction products of glucose and maltose were separated by HPLC (TSK-gel amide-80, 75% acetonitrile as solvent). After lyophilization, each sample was analyzed by FAB negative mass spectrometry (JMS-SX102/SX102 DA 7000 Tandem Mass, Joel Datum).

Analysis of intermolecular transglycosylation using [¹³C] labeled glucose. The [¹³C] labeled glucose (5 μCi, DuPont, USA) was dissolved in 25 μl of 20 mm
sodium acetate (pH 5.5), 160 U/ml glycosyltransferase, 10% maltotriose, and glucose at the concentrations shown in Fig. 5. The reaction mixtures were incubated at 60°C and the samples were withdrawn at the times shown in Fig. 5. The reaction was stopped by adding HCl. After neutralization with NaOH, reaction products were separated by descending paper chromatography on Whatman No. 3 paper and eluted three times with propan-1-ol (75%), v/v at ambient temperature.\(^{15}\) According to the position of standard mono, di, and tri-saccharides, which were recognized by silver staining,\(^{16}\) the area of paper containing the reaction product was cut out and put into a vial with liquid scintillator, and the radioactivity was measured (Wallac 1410 liquid scintillation counter, Pharmacia, Tokyo, Japan). Zero time samples were taken as an indication of background and were measured using the same procedure.

**Results**

1. **Measurement of rate parameters and evaluation of subsite affinities**

   From the measurement of rate parameters, the enzyme showed low hydrolysate production, low \(K_m\) (high affinity), high \(k_o\), and a high yield of glycosyltrehalose with the longer chain-length substrate. The contribution of hydrolysis was conspicuous with shorter substrates such as maltotriose (Table 1). Furthermore, in this study, we found that maltose was only weakly converted to trehalose and weakly hydrolyzed. The rate parameters were measured from 0.09 M to 2.7 M because of a limitation of the solubility of the maltose. The \(K_m\) and \(k_o\) for the conversion, the hydrolysis, and the “sum” of these reactions were \((4.9 \pm 0.8) \times 10^{-3} \text{M}, 2.6 \pm 0.5 \text{sec}^{-1}, 0.5 \pm 0.05) \times 10^{3} \text{M}, 0.4 \pm 0.02 \text{sec}^{-1}, (1.7 \pm 0.2) \times 10^{3} \text{M}, 1.9 \pm 0.2 \text{sec}^{-1}\), respectively. As shown above, maltose reacted very weakly, and the values were considered to involve much error. The subsite affinities were calculated by the methods described in the appendix (Table 2 and Fig. 2). The subsites were numbered starting from the reducing end side. As shown in Fig. 3, the rate parameters conversely calculated using \(A_i\) and \(K_{int}\) values agreed with the observed values except for the values for maltose. Thus, positive affinities were observed at subsites 1, 3 – 8. Negative affinities were observed at subsites 2, 9, and 10. The catalytic site is considered to be between subsites 1 and 2.

![Fig. 2. Subsite Structure of Glycosyltransferase from S. solfatarius KM1](image)

The wedge is the catalytic site of the enzyme.

\[ \text{Fig. 2. Subsite Structure of Glycosyltransferase from } S. \text{ solfatarius KM1} \]

<p>| Table 1. Rate Parameters of Glycosyltransferase for Maltooligosaccharides |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>DP ((\text{mm}))</th>
<th>Conversion (1)</th>
<th>Hydrolysis (2)</th>
<th>Sum of (1) and (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_m) (mM)</td>
<td>(k_o) (s(^{-1}))</td>
<td>(K_m) (mM)</td>
<td>(k_o) (s(^{-1}))</td>
</tr>
<tr>
<td>3</td>
<td>161 ± 13</td>
<td>46.6 ± 3.4</td>
<td>72.6 ± 5.1</td>
</tr>
<tr>
<td>4</td>
<td>21.8 ± 1.7</td>
<td>211 ± 12</td>
<td>11.7 ± 3.1</td>
</tr>
<tr>
<td>5</td>
<td>5.04 ± 0.19</td>
<td>247 ± 14</td>
<td>9.53 ± 0.90</td>
</tr>
<tr>
<td>6</td>
<td>2.73 ± 0.27</td>
<td>204 ± 11</td>
<td>3.98 ± 0.15</td>
</tr>
<tr>
<td>7</td>
<td>0.905 ± 0.144</td>
<td>215 ± 17</td>
<td>1.77 ± 0.54</td>
</tr>
<tr>
<td>8</td>
<td>0.634 ± 0.026</td>
<td>302 ± 7</td>
<td>0.978 ± 0.271</td>
</tr>
<tr>
<td>9</td>
<td>0.687 ± 0.093</td>
<td>190 ± 10</td>
<td>0.487 ± 0.192</td>
</tr>
<tr>
<td>10</td>
<td>0.849 ± 0.021</td>
<td>133 ± 5</td>
<td>—</td>
</tr>
</tbody>
</table>

| Table 2. Subsite Structure of Glycosyltransferase of S. solfatarius KM1 |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Subsite number (i) | 10 | 9 | 8 | 7 | 6 | 5 | 4 | 3 | 2 | 1 |
| Subsite affinity \((A_i)\) (kcal/mol) | —0.38 | —0.36 | 0.46 | 0.76 | 0.28 | 1.06 | 1.69 | 1.18 | —0.53 | 2.72 |
| \(K_{int}\) (s\(^{-1}\)) | 268 |

\(^a\) The subsites are numbered counting from the subsite at which the reducing-end glucose residue of the substrate is bound in the productive mode.
Fig. 3. Dependence of the Michaelis Constant $K_m$, the Molecular Activity $k_0$, and their Ratio $k_0/K_m$ on the Degree of Polymerization of the Substrates for a Glycosyltransferase Catalyzed Reaction.
Symbols: □, pK_m; ○, log k_0; △, log ($k_0/K_m$). The solid lines are the theoretical ones conversely calculated using $A$, and $k_{int}$ values.

Table 3. Rate Parameters of Glycosyltransferase for Hydrolysis of Glycosyltrehaloses

<table>
<thead>
<tr>
<th>DP</th>
<th>$K_m$ (mm)</th>
<th>$k_0$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>86.5 ± 9.0</td>
<td>13.0 ± 1.5</td>
</tr>
<tr>
<td>4</td>
<td>41.2 ± 9.2</td>
<td>3.90 ± 0.49</td>
</tr>
<tr>
<td>5</td>
<td>8.89 ± 0.58</td>
<td>1.44 ± 0.06</td>
</tr>
<tr>
<td>6</td>
<td>2.29 ± 0.11</td>
<td>0.487 ± 0.006</td>
</tr>
<tr>
<td>7</td>
<td>1.23 ± 0.08</td>
<td>0.510 ± 0.020</td>
</tr>
</tbody>
</table>

In addition, the rate parameters for the hydrolysis of glycosyltrehaloses were measured. As shown in Table 3, the effect of hydrolysis of glycosyltrehaloses became insignificant and could be omitted under these reaction conditions.

2. Confirmation of transglycosylation using labeled compounds
Identification of the splitting position using $^{18}$O labeled H$_2$O

The FAB negative mass spectral data showed that maltose only had a molecular mass 2 units greater

Fig. 4. FAB Negative Mass Spectra of Hydrolysates of Maltotriose by Glycosyltransferase.
Arrow indicates the peak at which the molecular mass is 2 units greater than the unlabeled maltose.
than the unlabeled compound, thus confirming that the water molecule is incorporated into the maltose (Fig. 4, d). The hypothesis of the splitting of the C1-O bond was confirmed. A peak that was expected to be derived from $[^1H]$ in glucose (Fig. 4, b) was considered to be a spontaneous substitution between water and the OH residues of glucose, as it was also observed under the same conditions but without the enzyme (data not shown).

**Analysis of intermolecular transglycosylation to a $[^1H]$ labeled glucose**

From the results (Fig. 5) it can be seen that the increase in incorporation of $[^1H]$ labeled glucose into the tri-saccharide fraction depended on the glucose concentration. Maximum incorporation was observed at 70% glucose after 8 h. It was confirmed that 24% of the substrate maltotriose resulted in intermolecular transglycosylation.

**Discussion**

1. **Measurement of rate parameters and evaluation of substrate affinities**

Although the rate parameters for maltose could be measured, it reacted very weakly and the values were considered to involve much error. Therefore, these values were not used for the calculation of substrate affinities.

By the methods in the appendix, we found that the main affinities are to subsites 1, 3, 4, and 5, and that subsite 2 has a negative affinity ($-0.53$ kcal/mol, Table 2). The shorter substrates were believed to bind with difficulty because of the effect of subsite 2. The reaction was considered to be mainly intramolecular transglycosylation. It is probable that the high affinity of subsite 1 (2.72 kcal/mol) and the steric hindrance located next to the reducing end side of subsite 1, caused the retention of the glucose from the reducing end of the parental substrate, and intramolecular transglycosylation to occur. Longer chain substrates with a degree of polymerization of 4 or longer, indicated low hydrolyzing activity. These substrates may block the entry of water molecules to the catalytic center. Nevertheless, hydrolysis by transglycosylation to a water molecule was clearly observed.

2. **Confirmation of transglycosylation using labeled compounds**

Previous studies of transglycosylating enzymes describe the catalytic mechanism of transglycosylation by double displacement and oxocarbonium ion mechanisms. The postulated mechanisms are believed to proceed via a glycosyl-enzyme intermediate.

From the analysis of the reaction manner, the conversion of maltopentose to maltotrioseyltrehalose was an equilibrium reaction directed towards the formation of maltotrioseyltrehalose at an approximate 80% yield (data not shown). When glucose was added to the mixture of enzyme and maltotriose (which shows the strongest hydrolyzing reaction among maltooligosaccharides), it inhibited the decrease of trisaccharide by hydrolysis. On the other hand, maltose did not have this inhibitory effect. Moreover, by-products of condensation with longer chain length of oligosaccharides than the parental maltooligosaccharides, were not detected by the enzymatic reaction between glucose and maltooligosaccharides (degree of polymerization was 2 to 7, data not shown). These observations suggest the existence of a glycosyl-enzyme intermediate.

Furthermore, gene analysis showed that glycosyltransferase contained regions of highly conserved sequences found in the $\alpha$-amylase family, which includes transglycosylating enzymes. We therefore suspect that this enzyme has a similar catalytic center and catalytic mechanism.

If the glycosyltrehalose-producing reaction was explained as a double displacement mechanism, for the sake of convenience, it would be expected to proceed according to the reaction model described below with maltotriose as the model compound (Fig. 6). First, the C1-O bond (not the O-C4 bond) of the glucosidic linkage at the reducing end of the substrate is split. The glycosyl residue (in this case the maltosyl residue) binds to the enzyme to form a glycosyl-enzyme intermediate. The split glucose residue, which is derived from the reducing-end glucose, would receive a proton from one of the enzyme's catalytic amino acid residues located at the catalytic cleft. The glycosyl residue would then be transferred to the glucose in the catalytic cleft of the enzyme by $\alpha$-1,1 linkage. The hydrolytic side reaction occurs when the glycosyl residue is instead transferred to a H$_2$O molecule.

Even if the reaction proceeds by an oxocarbonium ion mechanism, it is possible to explain that the catalytic mechanism proceeds via step “b” in Fig. 6.

Our results strongly support the hypothesis described above. From the identification of the split-
Fig. 6. Possible Catalytic Mechanism of Glycosyltransferase from *S. solfataricus* KM1 with Maltotriose as the Model Compound.

The circles mean glucose residues. The shaded portion of circles means the direction of C1-OH positions. The opened portion of the circles means the direction of C4-OH positions. The asterisk means the position of H-labeled glucose residues. "HA" and "B" mean an acid group and a nucleophilic group of enzyme.

Process a-b-c-d; intramolecular transglycosylation. Process a-b-c'-d'; intermolecular transglycosylation. Process a-b-c'-d'; hydrolysis.

The reaction position using $[^1]H$ labeled H$_2$O, the splitting of the C1-O bond was confirmed. This suggests the formation of a glycosyl (maltosyl)-enzyme intermediate. Subsequently, the glycosyl residue is transferred to the glucose in the catalytic cleft of the enzyme to form an α,1,1 linkage. The hydrolytic side reaction occurs when the glycosyl residue is instead transferred to a H$_2$O molecule. Furthermore, from the analysis of incorporation of $[^1]H$ labeled glucose into maltotriose, we conclude that the enzyme can catalyze an intermolecular transglycosylation.

The reaction of this glycosyltrehalose-producing enzyme apparently proceeds by intramolecular transglycosylation. From this point of view, the enzyme might be an isomerase. However, from the data in this paper, we consider the catalytic mechanism of this enzyme to be essentially a transglycosylation. From the situation of the use of this enzyme, it is much more comprehensive to consider the enzyme as a kind of transferase. Therefore, we called this enzyme glycosyltransferase.

Acknowledgments

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References

Appendix

Calculation method for subsite affinities and $k_{cat}$

Step 1. Evaluation of $A_1$ to $A_4$ was made using the equation which involves only the productive terms ($R$ means gas constant, $T$ means absolute temperature):

$$\ln \left( \frac{k_0}{K_m} \right)_n - \ln \left( \frac{k_0}{K_m} \right)_{n-1} = \left[ \sum_{i=n}^{r} A_i \right] - \left[ \sum_{i=n-1}^{r} A_i \right] / RT$$

$$= A_n / RT.$$  \hspace{1cm} (1)

Step 2. This enzyme acts on the substrate at reducing end. $A_3$ should be larger than $A_2$, because of the weak reactivity of maltose. Furthermore, from the precedent of some glycoenzymes, $A_3$ was expected to be negative so as to cause a distortion to a glucose residue located at $A_2$. Hence it is reasonable for any $n$-mer substrate, that only one productive ($j=1$) and one nonproductive binding mode ($j=3$) are taken into account for a good approximation (Fig. 1, a). As seen from Fig. 1, a, the $B_{m,j}$ (the molecular binding affinity) give an equation as follows;

$$B_{m,3} - B_{m,1} = (A_{n+1} + A_{n+2}) - (A_1 + A_2).$$  \hspace{1cm} (2)

Then Eq. (2) becomes;

$$\exp \{(A_{n+1} + A_{n+2}) / RT \} = \left[ \frac{k_m}{k_0} \right] - 1$$

$$\exp \{(A_1 + A_2) / RT \}. \hspace{1cm} (3)$$

Thus the vertical and horizontal intercepts of a plot of $\exp \{(A_{n+1} + A_{n+2}) / RT \}$ vs. $(1/k_0)$ will give the values of $\exp (A_1 + A_2 / RT)$ and $1/k_{cat}$, respectively. The observed values from maltose was not used, because it reacted very weakly and they were considered to involve much error. Thus, $k_{cat}$ (268 s$^{-1}$) and $A_1 + A_2$ (2.19 kcal/mol) were evaluated.

Step 3. Evaluation of $A_2$ was made by using the values of $k_{cat}$ and $A_1 + A_2$ from step 2, and the $(1/k_m)$ of maltotetraose, according to the equation shown below (the factor 0.0179 arises from the correction for the change in free energy of mixing upon the binding of enzyme and substrate at 60°C in aqueous solution).

$$(k_0/k_m)_s = 0.0179 \exp \{(A_1 + A_2 + A_3 + A_4) / RT \}. \hspace{1cm} (4)$$

Step 4. For the evaluation of $A_1$ and $A_3$, the association constant, $K_{A_2}$ was necessary. Thus, $(1/K_m)_h$ was used for the evaluation of $K_{A_2}$ as shown below;

$$(1/K_m)_h = K_{6,1} + K_{6,2} + K_{6,3} + K_{6,4} + K_{6,5} + K_{6,6} + K_{6,7} + K_{6,8} + K_{6,9} + K_{6,10}.$$  \hspace{1cm} (5)

Deriving the equation for $K_{A_2}$, Eq. (5) becomes;

$$K_{A_2} = (1/K_m)_h - (K_{6,1} + K_{6,2} + K_{6,3} + K_{6,4} + K_{6,5} + K_{6,6} + K_{6,7} + K_{6,8} + K_{6,9} + K_{6,10}.$$  \hspace{1cm} (6)
\[ 0.0179 \exp \left\{ \frac{(A_2 + A_4 + A_6 + A_7)}{RT} \right\} \] (7)

As shown in Fig. 1, b, \( A_1 \) can be solved from the equation below. The \( K_{7,1} \) was calculated from the evaluated affinities from steps 1, 2, and 3;

\[ K_{7,2} - K_{6,2} = 0.0179 \exp \left\{ \frac{(A_4 + A_2 + A_3 + A_4 + A_6 + A_7)}{RT} \right\} \]
\[ - \exp \left\{ \frac{(A_3 + A_2 + A_4 + A_6 + A_7)}{RT} \right\} \] (8)

\[ = 0.0179 \left[ \exp \left\{ \frac{(A_2 + A_4 + A_6 + A_7)}{RT} \right\} \times \exp \left( \frac{A_1}{RT} \right) \right] - \exp (0) \] (9)

The evaluated \( K_{6,2} \) (Eq. (7)) was applied to Eq. (9) to obtain a value for \( A_1 \). \( A_2 \) was then calculated from the value of \( A_1 + A_2 \) which was obtained in step 2.