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Review

Trehalose production with a new enzymatic system from *Sulfolobus solfataricus* KM1 [1]

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Abstract

An amylolytic activity that converts soluble starch to α,α-trehalose (trehalose) was found in the cell homogenate of the hyperthermophilic, acidophilic archaeum *Sulfolobus solfataricus* KM1. Two enzymes, a glycosyltransferase and an α-amylase, which are essential for this activity, were purified to homogeneity. A glycosyltransferase catalyzed the conversion of maltooligosaccharides to glycosyltrehaloses and an α-amylase catalyzed the hydrolysis of glycosyltrehaloses to trehalose. The glycosyltransferase transferred an oligomer segment of maltooligosaccharide to the C1−OH position of glucose, located at the reducing end of the maltooligosaccharide, to produce a glycosyltrehalose having an α-1,1 linkage. The α-amylase hydrolyzed only the α-1,4 glucosidic linkage adjacent to the trehalose unit of the glycosyltrehaloses. Their activities were maximal at 70–80°C and 70–85°C, with high thermostability, respectively. The genes encoding for both enzymes were cloned and expressed in *Escherichia coli*. The regions highly conserved in α-amylase family exist in the amino acid sequences of these enzymes. A new process for trehalose production from starch was developed using the purified enzymes. The yield of trehalose from starch was 81.5% using these two enzymes. This review describes our efforts to reveal in detail the characters of these enzymes involved in practical trehalose production.

Keywords: Trehalose productio; Enzymatic system; *Sulfolobus solfataricus* KM1

1. Introduction

Trehalose (*O*-α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside) is a nonreducing disaccharide with two glucose residues bound by an α-1,1 linkage. Trehalose is widely distributed among microorganisms, plants, and insects, and has many biological functions not only for storage, but also for protection of proteins and cell membranes from heat and osmotic changes [1,2], and it is also a source of mobile energy for insects. Trehalose might have various industrial applications as a preservative for unstable foods, cosmetics, and medicines. Thus, much attention has been directed to inexpensive means for preparing trehalose. A number of methods for enzymatic production of trehalose have been studied previously; reaction of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase with UDP-Glc and Glc-6-P [3,4], condensation of glucose by trehalose [5,6], coupled reaction of maltose phosphorylase and trehalose phosphorylase with maltose as substrate [7,8],

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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and transglucosylation reaction of trehalose synthase with maltose as substrate [9,10]. However, none of these methods are satisfactory for industrial application owing to the difficulty of large scale preparation of these enzymes and separation of trehalose, as well as low production yield.

The direct production of trehalose from starch using enzymatic transglycosylation may be the most practical method, but an enzyme that creates an α-1,1 linkage between glucose residues has never been reported. Previously, Lama et al. [11,12] reported a thermostable amylolytic activity in cell homogenate of *Sulfolobus solfataricus* that produces trehalose from starch. If the enzyme that catalyzes this reaction was available for industrial production of trehalose, it would provide a useful method to produce trehalose on a commercial scale because of the following reasons; first, the enzyme, derived from hyperthermophilic archaeum, can be applied to high temperature process enhancing reaction and decreasing the risk of retrogradation of starch or contamination of reaction mixture by microorganisms; second, it will decrease the production cost because the enzyme can use an inexpensive substrate, starch.

In order to determine what type of enzyme were involved in the reaction and how they act on starch to produce trehalose, we purified the target enzymes from the strain *S. solfataricus* KM1. From the characterization of the reaction, we elucidated a new coupled pathway of two enzymes, a glycosyltransferase and an α-amylase. Here we describe the purification, characterization, and mode of action of these new trehalose producing enzymes. We also show an optimal condition for trehalose production from starch using the recombinant glycosyltransferase and α-amylase from *S. solfataricus* KM1. In addition, another research group has reported investigations of similar enzymes from *Arthrobacter* sp. [13–15] and *S. acidocardarius* [16,17]. A few comments will be made on the difference between the enzyme systems of the two groups.

2. Enzyme purification and characterization

2.1. Glycosyltransferase

S. 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 [18].

The cell homogenate of KM1 had amylolytic, trehalose-producing activity with 10% starch as substrate at 60°C. Phenyl Toyopearl column chromatography separated the amylolytic, trehalose-producing activity as a single peak when starch was used as the substrate. Further purification by DEAE Toyopearl column chromatography showed a single peak with only amylolytic activity (Fig. 1) [19].

However, trehalose-producing activity could not be detected in any of the DEAE chromatography fractions in the case of the enzyme assay using starch as the substrate. Therefore, a method for detecting trehalose-producing activity in DEAE chromatography fractions was developed in which a shorter maltooligosaccharide, maltotriose was used as the substrate instead of starch. An unknown trisaccharide in the reaction product was detected by this method.

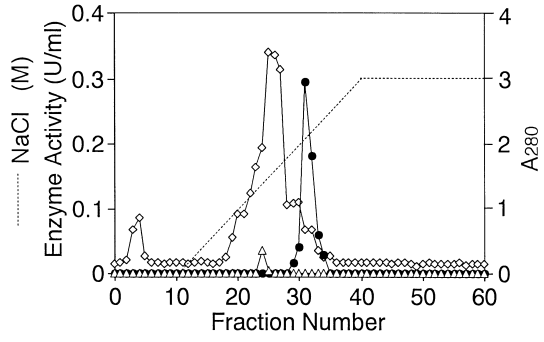

Fig. 1. DEAE Toyopearl column chromatography of trehalose-producing enzymes of *S. solfataricus* KM1. (Δ) unknown trisaccharide producing activity when used maltotriose as the substrate. One unit corresponds to the amount of enzyme produced 1 μmol of unknown trisaccharide per min; (●) amylolytic activity. One unit corresponds to the amount of enzyme reduced the value of absorbance for iodine–starch complex at 620 nm by 10% per 10 min; (◇) protein, absorbance at 280 nm.

Table 1
Physicochemical properties of glycosyltransferase and α-amylase

	Glycosyl-transferase	α-Amylase
Opt. pH	5.0–6.0	4.5–5.5
pH stability	4.0–10.0	3.5–10.0
Opt. temp. (°C)	70–80	70–85
Thermal stability (for 6 h at 85°C)	91%	100%
Mol. wt. (SDS-PAGE)	76,000	61,000

From this trisaccharide, glucose and trehalose were liberated by hydrolysis using glucoamylase. Based on the NMR spectral data [19] of this trisaccharide, it was identified as glucosyltrehalose (α-D-maltosyl, α-D-glucopyranoside), in which two glucose residues located at the middle and the reducing end of triose were bound by an α-1,1 linkage.

From these results, it was considered that the enzyme hydrolyzed the maltotriose to produce maltose and glucose, and then transferred the maltose residue to the C1–OH position of glucose residue to form glucosyltrehalose.

In a preliminary study, maltopentaose was found to be the preferred substrate for the enzyme instead of maltotriose. Therefore, purification of the enzyme was done by enzymatic assay using maltopentaose as the substrate.

On SDS-PAGE, the molecular mass of the purified enzyme was approximately 76 kDa. The isoelectric point was estimated as pH 6.1 by isoelectric focusing. Maximal activity was observed at pH 5.0–6.0 and 70–80°C. As much as 91% of the activity remained even after 6 h of incubation at 85°C (Table 1) [19].

Analysis of the reaction products using ^{1}H and ^{13}C NMR spectral data indicated that the enzyme catalyzes the conversion of maltooligosaccharides to the corresponding glycosyltrehaloses (the word 'glycosyltrehalose' is defined as an oligosaccharide of more than DP3, having a trehalose structure as an end unit).

A comparison of the relative reactivity among maltooligosaccharides and yields of glycosyltrehaloses can be seen in Table 2 [20]. Maltopentaose was the most suitable substrate, with 20 times greater reactivity than maltotriose. The yields of maltosyltrehalose, maltotriosyltrehalose, maltotetraosyltrehalose, and maltopentaosyltrehalose, which have a degree of polymerization of 4 or higher, were higher than 78%, while the yield of glucosyltrehalose of DP3 had a lower value of 45%.

2.2. α-Amylase

The mixture of the amylolytic activity from the DEAE chromatography fraction and the purified glycosyltransferase produced trehalose directly from starch (data not shown). Therefore, the amylolytic activity is expected to hydrolyze glycosyltrehaloses to trehalose. Actually, only trehalose and maltotriose were found in the hydrolysate of maltotriosyltrehalose.

Based on these results, the activity was purified by enzyme assay of amylase using maltotriosyltrehalose, not starch, as the substrate. SDS-PAGE indicated the molecular mass of the purified enzyme to be 61 kDa. The isoelectric point was pH 4.8 by isoelectric focusing. Maxi-

Table 2
Substrate specificity of glycosyltransferase from KM1

Substrate (10%)	Main product	Relative reactivity (%)	Yield (%)
Glucose	–	0	0
Maltose	–	0	0
Maltotriose	Glucosyltrehalose	5.0	45.0
Maltotetraose	Maltosyltrehalose	46.3	78.5
Maltopentaose	Maltotriosyltrehalose	100	87.3
Maltohexaose	Maltotetraosyltrehalose	80.0	81.7
Maltoheptaose	Maltopentaosyltrehalose	88.6	88.5

Table 3
Substrate specificity of α-amylase from KM1

Substrate (10 mM)	Product	Relative reaction rate (%)
Maltose	G1	0.7
Maltotriose	G1, G2	1.1
Maltotetraose	G1, G2, G3	1.1
Maltopentaose	G1, G2, G3, G4	6.6
Maltohexaose	G1, G2, G4, G5	6.4
Maltoheptaose	G1, G2, G5, G6	6.6
Trehalose	–	0
Glucosyltrehalose	G1, Trehalose	0.2
Maltosyltrehalose	G2, Trehalose	14.4
Maltotriosyltrehalose	G3, Trehalose	91.6
Maltotetraosyltrehalose	G4, Trehalose	100
Maltopentaosyltrehalose	G5, Trehalose	93.4

G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose.

mal activity was observed at pH 5.0–6.0 and 70–80°C. As much as 91% of the activity remained even after 6 h of incubation at 85°C (Table 1).

From the analysis of the reaction product, the enzyme was shown to hydrolyze glycosyltrehaloses only by cleavage of the α-1,4 bond adjacent to the α-1,1 bond and to liberate trehalose and maltooligosaccharide, the degree of polymerization of which was reduced by two glucose residues.

As shown in Table 3 [21], glycosyltrehaloses (more than DP3) and maltooligosaccharides (more than DP2) were used as substrates for the enzyme. Glycosyltrehaloses were hydrolyzed in preference to the corresponding maltooligosaccharides with approximately 6–15 times greater reactivity. Glycosyltrehaloses with more than five glucose residues would be even more suitable.

3. The reaction mechanism of trehalose-producing enzymes

3.1. Glycosyltransferase

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 glycosyltrehalose of longer chain length than the substrate used. Glucose and maltooligosaccharide shortened by one glucose residue were regularly produced as by-products from maltooligosaccharide substrates of various chain lengths by the enzymatic reaction.

The experimental results using nonreducing end [^{3}H] glucose labeled substrate demonstrated that the glucose residue of the trehalose moiety was not derived from the nonreducing end of the substrate (Fig. 2) [20]. Precedents from other studies of glycosyltransferases suggest that the transglycosylation would start primarily by hydrolysis of an α-1,4 glycosidic linkage [22]. The glycosyl residue would bind to the enzyme and form a covalent enzyme-substrate intermediate. And the hydrolyzed glucose molecule derived from the reducing-end glucose of the parental substrate would be located at the active site pocket of the enzyme. Subsequent transglycosylation occurs, rather than hydrolysis of the covalent enzyme-substrate intermediate because H_2O

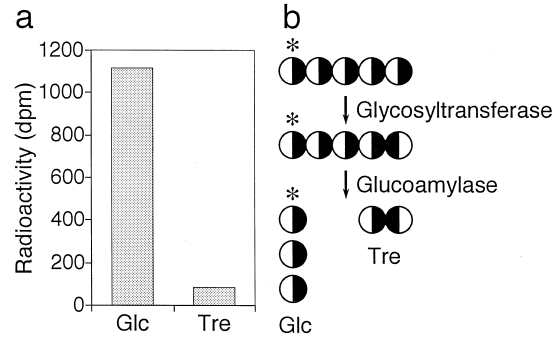

Fig. 2. An efficient model for the transglycosylation mechanism using ^{3}H-labeled maltopentaose. (a) Nonreducing-end [^{3}H]-labeled maltopentaose was incubated with glycosyltransferase from KM1 followed by glucoamylase treatment. The hydrolysate was chromatographed on a TLC plate. Radioactivity of glucose and trehalose fractions were measured [20]. (b) The circles mean glucose residues. The shaded portion of circles mean the direction of C1–OH positions. The opened portion of the circles mean the direction of C4–OH positions. The asterisks mean the position of ^{3}H-labeled glucose residues. Glc, glucose; Tre, trehalose.

molecules could not 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 glycosyltrehaloses was observed after the maximal production of the glycosyltrehaloses. It was presumed that the hydrolysis of the glycosyltrehaloses by the glycosyltransferase also occurs.

As shown in Tables 2 and 4, the enzyme showed low hydrolysate production, low value of K_m (= high affinity), and a high yield of glycosyltrehalose with longer chain-length substrates. The high affinity of the enzyme for longer chain-length substrates is presumed to be due to an extended binding site.

From these results, we expected that the reaction mechanism of the enzyme is the following: the glycosyltransferase transfers a glycosyl residue produced from maltooligosaccharide by the hydrolysis reaction to the C1–OH position of glucose, at the reducing end of maltooligosaccharide, to produce glycosyltrehalose.

To confirm the hypothesis that the enzyme is a glycosyltransferase, we examined the incorporation of [^{3}H] labeled glucose into the product. We may conclude that the enzyme catalyze a intermolecular transglycosylation (data not shown). Therefore, the enzyme is considered to form a glycosyl-enzyme intermediate during the reaction. On the other hand, Nakada et al. reported that MTSase (maltooligosyl trehalose synthase), which corresponds to glycosyltransferase, does not catalyze a intermolecular transglycosylation (unpublished data).

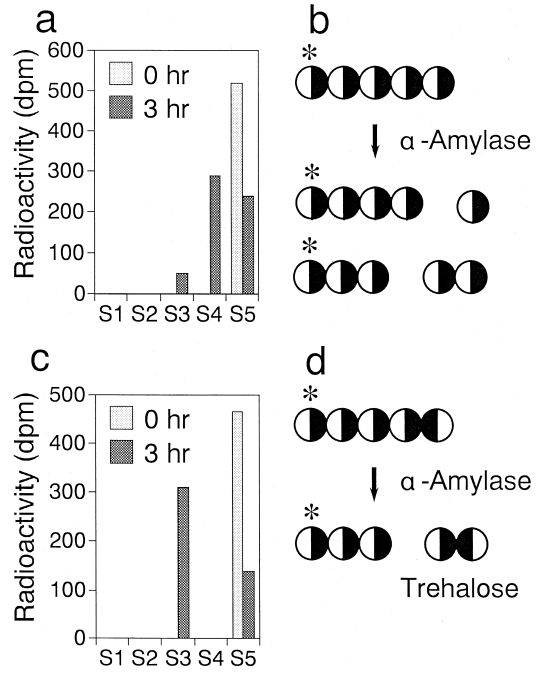

Fig. 3. A model for the reaction mechanism of α-amylase using ^{3}H-labeled maltopentaose (a,b), and maltotriosyltrehalose (c,d). Nonreducing-end [^{3}H]-labeled maltopentaose (a) and maltotriosyltrehalose (c) were incubated with α-amylase from KM1. The hydrolysate was chromatographed on a TLC plate. Radioactivity of mono- or oligosaccharides fractions were measured [21]. S1, monosaccharide; S2, disaccharide; S3, trisaccharide; S4, tetrasaccharide; S5, pentasaccharide. Symbols were the same as described in Fig. 2 legend (b,d).

3.2. α-Amylase

In order to clarify the mode of action of α-amylase toward glycosyltrehaloses and maltooligosaccharides, the reaction products and hy-

Table 4
Kinetic parameters of glycosyltransferase from KM1 for maltooligosaccharides

Substrate	K_m (mM)	k_0 (s^{-1})
Maltotriose	228	48.3
Maltotetraose	21.8	211
Maltopentaose	5.0	247
Maltohexaose	2.7	204
Maltoheptaose	1.2	181

Table 5
Kinetic parameters of α-amylase from KM1

Substrate	K_m (mM)	k_0 (s^{-1})
Maltosyltrehalose	22.4	339
Maltotriosyltrehalose	5.8	747
Maltotetraosyltrehalose	5.6	866
Maltopentaosyltrehalose	4.7	756

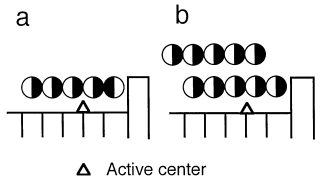

Fig. 4. Model for reaction mechanism of α-amylase on maltotriosyltrehalose (a) and maltopentaose (b). Symbols were the same as described in Fig. 2 legend.

drolyzing mechanism using nonreducing end [^{3}H] glucose labeled substrates have been analyzed. Based on these results, it was concluded that α-amylase hydrolyzes maltooligosaccharides from the reducing end by one or two glucose residues, and particularly, hydrolyzes glycosyltrehaloses from the side of the reducing end only by cleavage of the α-1,4 glucosidic bond adjacent to the α-1,1 glucosidic bond to liberate trehalose (Fig. 3) [21].

The α-amylase hydrolyzed glycosyltrehaloses in preference to the corresponding chain length of maltooligosaccharides (maltotetraosyltrehalose has 15.6 times greater reactivity than maltohexaose).

Because the enzyme has endo-type amylase activity, it should be considered to be an α-amylase. However, substrate specificity and the mode of hydrolysis of the enzyme were quite different from those of α-amylases from other sources. They could not liberate trehalose from glycosyltrehalose (data not shown).

As shown in Table 5, the enzyme had a lower K_m and higher k_0 with a longer chain-length of glycosyltrehalose. Kinetic parameters toward maltooligosaccharides were also calculated to clarify the differences in the relative reaction rates between glycosyltrehaloses and maltooligosaccharides.

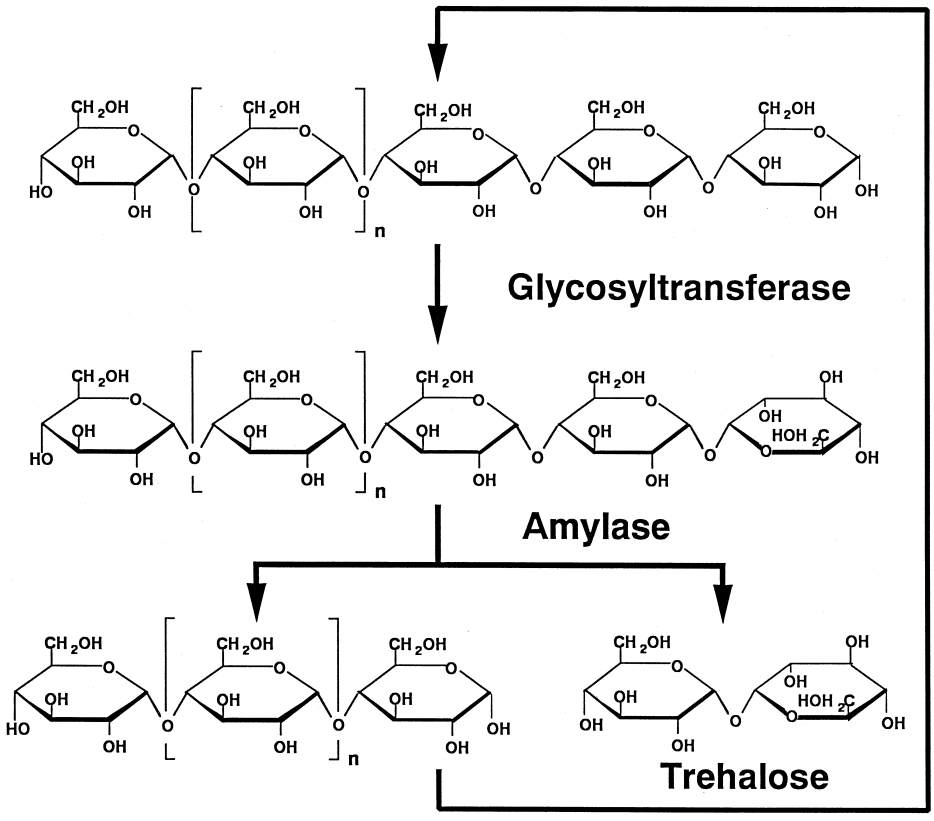

Fig. 5. A new pathway of enzymatic trehalose production from *S. solfataricus* KM1.

The values of apparent K_m toward maltooligosaccharides indicated a similar tendency with regard to chain length as for glycosyltrehalose, while the apparent k_0 was low, so that

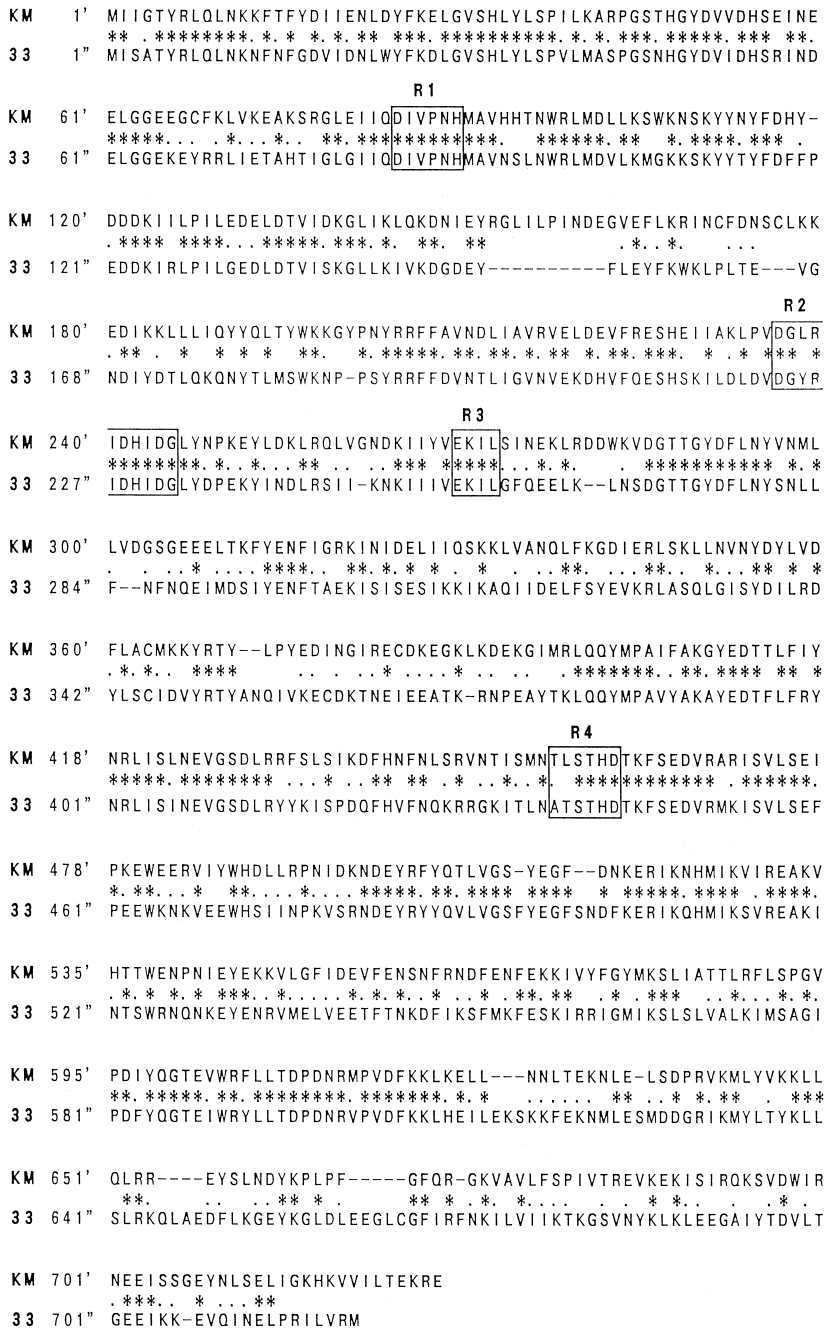

Fig. 6. Comparison of amino acid sequences of glycosyltransferase from *S. solfataricus* KM1 and *S. acidocaldarius* ATCC33909. Upper lane is *S. solfataricus* KM1, and lower lane is *S. acidocaldarius* ATCC33909. (∗) indicates identical amino acids, and (·) indicates similar amino acids. The analysis was done with GENETEX (Software Development). R1, R2, R3, R4 indicate regions that are conserved in the α-amylase family.

the k_0 would affect the relative reaction rate among glycosyltrehaloses and maltooligosaccharides (data not shown).

As shown in Fig. 3 and Table 3, the α-amylase did not produce trisaccharides, such as glucosyltrehalose or maltotriose from the side of the reducing end.

Based on these results, the α-amylase should have a subsite structure, so as to situate the active center at the second α-1,4 glucosidic linkage, numbered from the side of the reducing end, and steric hindrance for the substrate may be situated close to the side of the reducing end of the substrate. The reaction model is summarized in Fig. 4 [21].

Moreover, Nakada et al. [17] reported that MTHase (maltooligosyl trehalose trehalohydrolase) from *S. acidocardarius*, which corresponds to α-amylase, was inactive against maltooligosaccharide and starch. It seemed that there are differences in some properties between the MTHase and α-amylase.

From our investigation of trehalose-producing enzymes, a new enzymatic pathway for trehalose production was thus elucidated as a glycosyltransferase and an α-amylase working in concert (Fig. 5).

First, the glycosyltransferase converts maltooligosaccharide to glycosyltrehalose. Next, the α-amylase hydrolyzes the produced glycosyltre-

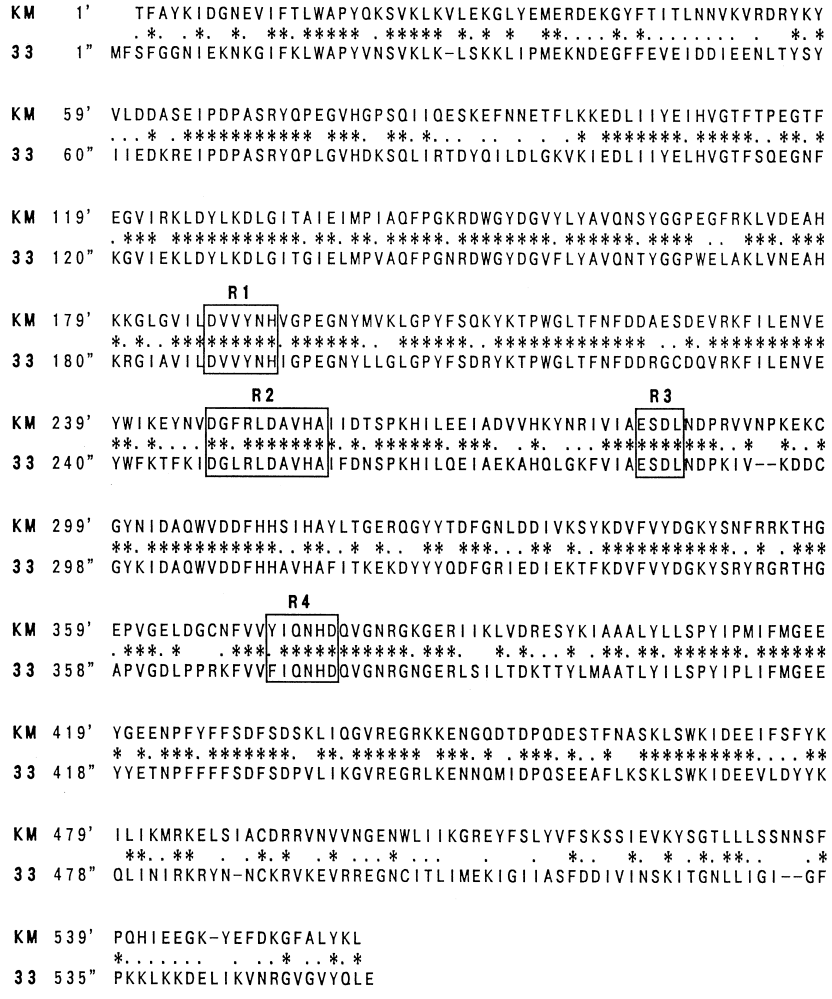

Fig. 7. Comparison of amino acid sequences of the α-amylases from *S. solfataricus* KM1 and *S. acidocaldarius* ATCC33909. Upper lane is *S. solfataricus* KM1, and lower lane is *S. acidocaldarius* ATCC33909. Symbols are the same as those in Fig. 6.

halose only by cleavage of the α-1,4 glucosidic bond adjacent to the α-1,1 glucosidic bond in order to liberate trehalose. These enzymatic reactions produce a high yield of trehalose.

4. Gene analysis

The nucleotide sequences of the genes of the glycosyltransferase and α-amylase indicated proteins with 728 and 558 amino acids and calculated molecular masses of 86 kDa and 65 kDa, respectively (Figs. 6 and 7). These values correspond well with those obtained from the enzymes purified by SDS-PAGE.

Sequence analysis revealed that the N-terminal amino acid of α-amylase is threonine, however, it is estimated from the gene analysis that the start codon is ATG [23].

Although the total amino acid sequences of these trehalose-producing enzymes are different from those of the other enzymes included in the α-amylase family, regions of highly conserved sequence in the α-amylase family [24] exist in their amino acid sequences (Fig. 8) [23]. Therefore, these trehalose-producing enzymes probably belong to the α-amylase family, and have an $(\alpha/\beta)_8$ barrel catalytic domain [25].

MTSase and MTHase from *S. acidocardarius*, which were reported by Nakada et al. [16,17], are considered to catalyze similar reactions to the glycosyltransferase and α-amylase from *S. solfataricus* KM1, respectively.

Therefore, we expected that the similarity of amino acid sequences between the corresponding enzymes is high. However, unexpectedly, gene analysis showed that they were not so high, 49% (glycosyltransferase and MTSase) and 59% (α-amylase and MTHase), respectively.

Although the low homology of amino acid sequences of the whole protein, several highly conserved regions were observed. It was considered that such regions may be important for the enzyme action.

5. Practical production of trehalose

Optimal conditions for trehalose production from starch were examined using the recombinant enzymes produced by *Escherichia coli* JM109 which contain the genes of glycosyltransferase (pKT22) or alpha-amylase (pKA2), respectively [23]. The recombinant enzymes were purified with heat treatment at 70°C for 1 h and ionexchanger column chromatography. Throughout the examination, the conditions were tested using 30 U of glycosyltransferase and 25 U of α-amylase per gram of starch. When soluble starch was treated without addition of a debranching enzyme, the yield of trehalose was

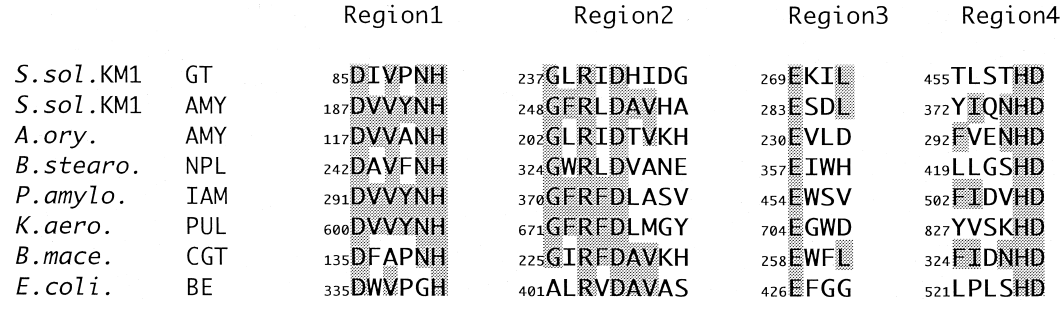

Fig. 8. Alignments of sequences showing sequence similarity to the glycosyltransferase and α-amylase from *S. solfataricus* KM1 with portions of regions 1 to 4 of several amylases including the α-amylase family. GT, glycosyltransferase from *S. solfataricus* KM1; AMY, α-amylase from *S. solfataricus* KM1; NPL, neopullulanase; IAM, isoamylase; PUL, pullulanase; CGT, cyclomaltodextrin glucanotransferase; BE, 1,4-α-branching enzyme. S. sol., *S. solfataricus*; A. ory., *Aspergillus oryzae*; B. stearo., *Bacillus stearothermophilus*; P. amylo., *Pseudomonas amyloderamosa*; K. aero., *Klebsiella aerogenes*; B. mace., *Bacillus. macerans*. The conserved amino acid residues are shown as shaded boxes.

Table 6
Production of trehalose from various kinds of starch and amylose

Substrate	Concentration (%)	DE (%)	Trehalose yield (%, dry substance base)
Amylose DP 17	10	ND	83.5[a]
Soluble starch	10	ND	40.9[a]
	10	ND	76.2
Corn starch	10	0.4	80.7
	10	0.7	80.2
	20	0.7	79.1
	20	4.5	76.0
	20	5.7	75.0
	25	0.7	78.6
Waxy corn starch	10	0.3	81.5
	10	0.5	80.8
	10	1.0	79.3

Each substrate was incubated with 30 U/g-substrate of glycosyltransferase, 30 U/g-substrate of α-amylase, and 10 U/g-substrate of the 'Debranching Enzyme Amano' at 60°C for 72 h followed by 5 U/g-substrate of glucoamylase treatment at 40°C overnight. Corn starch and waxy corn starch were partially liquefied by Termamyl 120L. DE means dextrose equivalent. DE was calculated from the amount of liberated reducing sugar by the Termamyl 120L treatment, reducing power of glucose as 100%.
[a]No treatment with debranching enzyme; ND, not determined.

low (40.9%) because these enzymes could not hydrolyze the α-1,6 bonds (Table 6) [26].

Addition to the reaction mixture of at least 5 U of thermostable debranching enzyme ('Debranching Enzyme Amano,' Amano Pharmaceutical) per gram of starch was found to give a high yield of trehalose. The yield was improved to 76.2% when the this enzyme was added together with the glycosyltransferase and α-amylase.

Furthermore, the conditions were optimized using corn starch (Sanwa Denpun) partially hydrolyzed by the liquefying α-amylase Termamyl 120L (Novo Nordisk). The yield was 80.7% for 10% corn starch at 0.4 dextrose equivalent (DE), and 81.5% for waxy corn starch at 0.3 DE (Nihon Syokuhin Kako) which contains more α-1,6 bonds than corn starch. For the large-scale practical process, 20–25% of starch with 4–5 DE was tested and its yield recorded in 75%.

Although the yield increased when starch with low DE was used, it was difficult to handle the starch solution because of its high viscosity and the formation of insoluble starch by retrogradation. Since this problem is more pronounced at low temperature, an effective solution seems to be to carry out the reaction at a high temperature using thermostable enzymes from KM1.

6. Conclusion

In this study, trehalose was produced directly from starch using the novel glycosyltransferase and α-amylase from KM1. The enzymatic pathway for trehalose production was elucidated as a glycosyltransferase, which converts maltooligosaccharide to glycosyltrehalose, and an α-amylase, which hydrolyzes the produced glycosyltrehalose only by cleavage of the α-1,4 glucosidic bond adjacent to the α-1,1 glucosidic bond. The glycosyltransferase and α-amylase from KM1 are considered to be similar to MTSase and MTHase from *Sulfolobus acidocardarius*, which were reported by Nakada et. al. However, it seemed that there are differences in some properties between the enzymes of both origins as follows: glycosyltransferase from KM1 has a intermolecular transglycosylation activity, while MTSase does not have (unpublished data); α-amylase from KM1 is active against maltooligosaccharide and starch, while

MTHase is inactive; similarity of amino acid sequences between the corresponding enzymes is not so high (49% and 59%, respectively).

For the practical production of trehalose from starch, optimal condition was determined. The maximum yield of trehalose was over 80%. On account of their thermostability, the process can be achieved under conditions of high starch concentration and high temperature, reducing the likelihood of retrogradation of starch or contamination of the reaction mixture by microorganisms.

It is expected that trehalose can be conveniently produced using this process because it is similar to the process of production of sugars, such as glucose and maltose. The yield of maltose from starch by β-amylase is approximately 90%. The yield of trehalose production could be improved if a glycosyltransferase, which has a low activity of hydrolysis of glycosyltrehalose or an α-amylase which has a low activity of hydrolysis of maltooligosaccharide, were generated by protein engineering.

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