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Investigations of a Useful α-Glycosidase for the Enzymatic Synthesis of Rare Sugar Oligosaccharides

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Abstract: Construction of various rare sugar oligosaccharides by glycosidase-catalyzed transglycosylation reaction may require α-glycosidases that possess unique glycon specificity. In order to obtain such α-glycosidase, we carried out two studies to: 1) investigate unknown glycon specificities of several α-glycosidases using various types of rare sugar containing glycosides as substrates, and 2) change the glycon specificities of the α-glucosidase from *Geobacillus stearothermophilus* by site-specific mutagenesis. Through the former studies, several α-glycosidases were found to possess hydrolytic activities towards specific glycon monodeoxy analogs of *p*-nitrophenyl (*p*NP) α-D-glycopyranosides. Using *Aspergillus niger* α-glucosidase that showed activity towards 2-deoxy glucoside and jack bean α-mannosidase that showed activity towards 6-deoxy mannoside (α-D-rhamnoside), the glycon 2-deoxy derivative of isomaltoside (ethyl 2-deoxy-α-D-*arabino*-hexopyranosyl-1,6-β-D-thioglucopyranoside) and α-D-rhamnodisaccharide derivative (ethyl α-D-rhamnopyranosyl-1,2-α-D-thiorhamnopyranoside) were prepared by their transglycosylation reaction in good yields. For the latter studies, fifteen mutant enzymes of *Geobacillus stearothermophilus* α-glucosidase were prepared and their hydrolytic activities towards the maltose, eight diastereomers of *p*NP α-D-aldohexopyranoside, and possible monodeoxy- and mono-*O*-methyl analogs of *p*NP α-D-gluco, -manno and -galactopyranosides were elucidated. For these mutant enzymes, there were differences between the specificities for *p*NP α-D-glucopyranoside and those for maltose, while significant changes were not confirmed in the specificity for other *p*NP α-D-aldohexopyranosides or the partially modified analogs of *p*NP α-D-glycopyranosides.

Key words: α-glycosidase, glycon specificity, oligosaccharide synthesis, rare sugar, site-specific mutagenesis

Numerous substances that contain oligosaccharides in their structures are known to possess various physiological functions in living organisms. In many cases, these oligosaccharides may contain uncommon monosaccharides, which are typically designated as rare sugars. From a physiological viewpoint, many researchers have attempted the synthesis of these oligosaccharides which contain rare sugars. Methodologies for oligosaccharide synthesis often require complicated processes such as regioselective protection and deprotection of the hydroxyl groups and/or separation of the α- and β-anomers of the glycosylation products. In recent synthetic studies of these oligosaccharides, considerable attention has been given to the use of enzymes-specifically, *exo*-type carbohydrate hydrolases, such as glycosidases, due to their availability. In contrast to chemical reactions, enzymatic reactions are simple, involve mild conditions, and possess precise positional and anomeric selectivity in the formation of the glycosidic linkages. Several types of glycosidases have demonstrated efficient catalytic activities in the synthesis of oligosaccharides using donor glycosides and acceptor sugars via transglycosylation. In general, glycosidases are known to possess strict specificities for the glycon structures of the glycosidic substrates, and accordingly, various types of glycosidases are classified based on these specificities. Among the many α- and β-glycosidases that have been isolated from plants, animals, insects, and microorganisms, most show activities towards glycosides that consist of common sugars, which exist in large quantities as components of the biomass. Consequently, the type of oligosaccharides that can be constructed by the glycosidase-catalyzed transglycosylation is rather limited. To employ the glycosidase-catalyzed transglycosylation in the construction of oligosaccharides that consist of rare sugars, we have undertaken the development of α-glycosidases that show broad or unique glycon specificity by recognizing various types of sugars as substrates. For this purpose, we undertook the following studies: 1) investigations of unknown glycon specificity of the enzymes by using various types of rare sugar containing glycosides as substrates, 2) studies in the change of glycon specificity of the enzymes by site-specific mutagenesis, 3) from microorganisms in nature, a search for new α-glycosidases that possess unique and desirable glycon specificities. Herein, we report the results of the first two studies.

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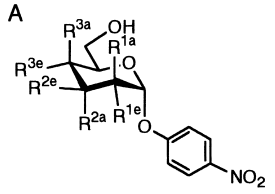
A



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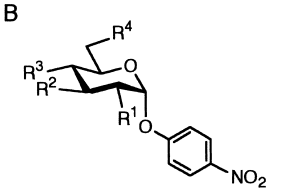
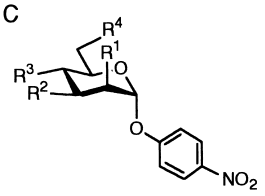
2	OH	H	H	OH	H	OH
3	H	OH	OH	H	H	OH
4	H	OH	H	OH	OH	H
5	OH	H	OH	H	H	OH
6	OH	H	H	OH	OH	H
7	H	OH	OH	H	OH	H
8	OH	H	OH	H	OH	H

B



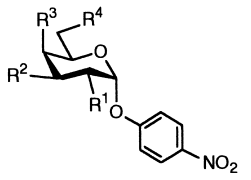
No.	R^1	R^2	R^3	R^4
9	H	OH	OH	OH
10	OH	H	OH	OH
11	OH	OH	H	OH
12	OH	OH	OH	H
13	OMe	OH	OH	OH
14	OH	OMe	OH	OH
15	OH	OH	OMe	OH
16	OH	OH	OH	OMe

C



No.	R^1	R^2	R^3	R^4
9	H	OH	OH	OH
17	OH	H	OH	OH
18	OH	OH	H	OH
19	OH	OH	OH	H
20	OMe	OH	OH	OH
21	OH	OMe	OH	OH
22	OH	OH	OMe	OH
23	OH	OH	OH	OMe

D



No.	R^1	R^2	R^3	R^4
24	H	OH	OH	OH
25	OH	H	OH	OH
11	OH	OH	H	OH
26	OH	OH	OH	H
27	OMe	OH	OH	OH
28	OH	OMe	OH	OH
29	OH	OH	OMe	OH
30	OH	OH	OH	OMe

Fig. 1. Substrates used for the investigation of glycon specificities of various α-glycosidases.

A: diastereomers of *p*NP α-D-aldohexopyranoside; *p*NP α-D-glucopyranoside (**1**), -mannopyranoside (**2**), -allopyranoside (**3**), -galactopyranoside (**4**), altropyranoside (**5**), -talopyranoside (**6**), -gulopyranoside (**7**) and -idopyranoside (**8**); B: 2-,3-,4- and 6-monodeoxy analogs (**9, 10, 11** and **12**, respectively) and 2-,3-,4- and 6-mono-*O*-methyl analogs (**13, 14, 15** and **16**, respectively) of **1**; C: 2-,3-,4- and 6-monodeoxy analogs (**9, 17, 18** and **19**, respectively) and 2-,3-,4- and 6-mono-*O*-methyl analogs (**20, 21, 22** and **23**, respectively) of **2**; D: 2-,3-,4- and 6-monodeoxy analogs (**24, 25, 11** and **26**, respectively) and 2-,3-,4- and 6-mono-*O*-methyl analogs (**27, 28, 29** and **30** respectively) of **4**.

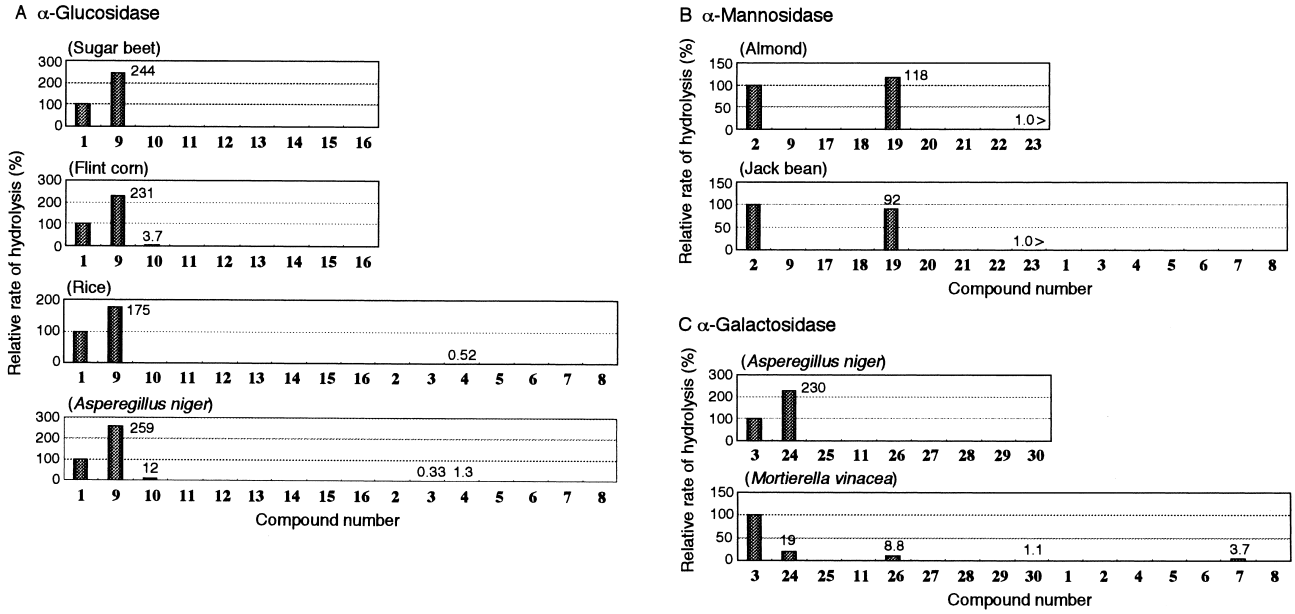


Fig. 2. Relative rates of hydrolytic activity of α-glycosidases towards various *p*NP α-D-glycopyranosides.

A: hydrolytic activity of each α-glucosidase was assayed under the following conditions; sugar beet and flint corn, in 50 mM sodium acetate buffer containing 0.05% Triton X-100 (pH 4.5) at 37°C; rice and *A. niger*, in 50 mM sodium acetate buffer (pH 4.0) at 37°C. B: hydrolytic activities of α-mannosidases from almond and jack bean were assayed in 100 mM sodium citrate buffer (pH 4.5) at 25°C. C: hydrolytic activity of each α-galactosidase was assayed under the following conditions. *A. niger*, in 50 mM sodium acetate buffer (pH 4.0) at 25°C; *M. vinacea*, in 50 mM sodium phosphate buffer (pH 5.9) at 40°C. The amount of *p*-nitrophenol liberated by the enzyme reaction was measured spectrophotometrically at 405 nm. Relative rates of hydrolytic activity of each α-glycosidase towards various glucosides were expressed as a ratio of the amounts of *p*-nitrophenol that was released from the corresponding α-D-glycopyranoside during a reaction time of 30 min, which was taken as 100%.

Investigation of unknown glycon specificities of α-glycosidases.

We investigated the hydrolytic activities of α-glycosidases, specifically α-glucosidases (EC 3.2.1.20), α-mannosidases (EC 3.2.1.24) and α-galactosidases (EC 3.2.1.22) from various sources, towards the diastereomers of *p*-nitrophenyl (*p*NP) α-D-aldohexopyranoside (Fig. 1A), monodeoxy- and mono-*O*-methyl analogs of the corresponding *p*NP α-D-glycopyranoside, *p*NP α-D-glucopyranoside (α-D-Glc*p*-*O*-*p*NP, **1**), -mannopyranoside (α-D-Man*p*-*O*-*p*NP, **2**) and -galactopyranoside (α-D-Gal*p*-*O*-*p*NP, **4**) (Fig. 1B, C and D, respectively). As a note, the above glycosides (except for **1, 2**, and **4**) are not commercially available, and therefore, were synthesized in our laboratories.[1–5] The hydrolytic activities of the above α-glycosidases were investigated by measuring the amount of *p*-nitrophenol released from these glycosides during the reaction under optimum reaction conditions (see caption in Fig. 2). Because different reaction conditions were used for each enzyme, it is important to note that our findings only allow for rough comparisons among the activities of the enzymes. The enzymatic reactions confirmed that fam-

ily 31 α-glucosidases from rice, sugar beet, flint corn, and mold *Aspergillus niger* and family 27 α-galactosidases from *A. niger* possess high hydrolytic activities towards the 2-deoxy analogs (2D-α-D-Glc*p*-*O*-*p*NP, **9** and 2D-α-D-Gal*p*-*O*-*p*NP, **24**) (Fig. 2A and C, respectively).[2–5] In contrast, α-mannosidases from jack bean (family 38) and almond revealed high hydrolytic activities against 6-deoxy analog (*p*NP α-D-rhamnopyranoside, α-D-Rha*p*-*O*-*p*NP, **19**) (Fig. 2B).[1,4] These results indicate that either α-mannosidase can dually function as α-D-mannosidase or α-D-rhamnosidase. None of the α-glycosidases revealed significant hydrolytic activities towards the diastereomers or mono-*O*-methyl analogs of the corresponding α-D-glycopyranoside.

Construction of oligosaccharide derivatives that contain rare sugar using the newly found glycon specificities and transglycosylation reaction of α-glycosidases.

In order to extend the scope of the glycosidase-catalyzed oligosaccharide synthesis, the syntheses of deoxyoligosaccharides were carried out utilizing the newly found specificities of *A. niger* α-glucosidase and jack bean α-mannosidase toward the glycon monodeoxy substrates and their transglycosylation reaction.

2-Deoxy sugars are frequently found as components of oligosaccharides in various naturally-occurring bioactive compounds, including antibiotics (erythromycins and orthomycins), antitumor drugs (anthracyclines, aureolic acids, calycheamycin, esperamycin and olivomycin A), cardiac glycosides (digoxin, digitoxin, kijanimycin and lanatosides) and antiparasitic agents (avermectins). To gain insight into the roles of oligosaccharides that contain 2-deoxy sugars, practical glycosylation reactions of 2-deoxy sugars using various sugar derivatives and chemical catalysts were investigated. Synthetic methodologies of 2-deoxyglycosides using chemical reactions have been recently reviewed.[6–9] In general, glycoside formations of 2-deoxy sugars are typically more difficult than those of normal sugars. In normal sugars, the OH-2 group of the donor sugar plays an important role in the anomer selectivity; furthermore, the OH-2 group of the glycon moiety of the synthesized glycoside can contribute to the stability of its glycosidic linkage. Accordingly, the yield of glycosylation product from 2-deoxy sugar is usually lower for reactions using Lewis acids, which are common activators for chemical glycosylation. In our case, using the specificity of *A. niger* α-glucosidase toward 2-deoxy α-glucoside, the enzymatic glycosylation of 2-deoxy sugar using 2D-α-D-Glc*p*-*O*-*p*NP and β-D-Glc*p*-S-Et as the glycosyl donor and glycosyl acceptor, respectively, were carried out under mild reaction conditions. Because the anomeric thioalkyl group can be readily converted to a hydroxyl group, and furthermore, because the alkyl 1-thio-sugar can be useful as a donor sugar for the chemical glycosylation in the subsequent elongation of the oligosaccharide chain, thioglycosides were chosen as the glycosyl acceptors for the enzymatic transglycosylation. In order to dissolve the donor and six equivalents of the acceptor sugar derivatives, a mixture consisting of MeCN and 50 mM acetate buffer (1:1, v/v) was used as the reaction solvent, in which the transglycosylation reaction using *A. niger* α-glucosidase proceeded efficiently. The resulting two transglycosylation products were purified using silica gel column chromatography; characterization using ^{1}H and ^{13}C NMR and FABMS revealed the compounds as the 2-deoxy analogs of ethyl β-thiomaltoside and ethyl β-thioisomaltoside which include a 2-deoxy glucose moiety at their glycon portions-specifically, ethyl 2-deoxy-α-D-*arabino*-hexopyranosyl-(1,4)-β-D-thioglucopyranoside (2D-β-Mal-*S*-Et) and ethyl 2-deoxy-α-D-*arabino*-hexopyranosyl-(1,6)-β-D-thioglucopyranoside (2D-β-IsoMal-*S*-Et), respectively. The isolated yields of 2D-β-Mal-*S*-Et and 2D-β-IsoMal-*S*-Et after a reaction time of 12 h were 6.72 and 46.6% (based on donor substrate), respectively (Fig. 3).[10] Hence, the *A. niger* α-glucosidase can function as an efficient transglycosylation catalyst for a feasible and practical method to afford the α-anomer of 2-deoxy D-glucose.

D-Rhamnopyranose (Rha), 6-deoxy derivative of D-mannopyranose, is a rare sugar in nature, and has been found in the *O*-specific polysaccharide fractions from the antigenic lipopolysaccharides of *Pseudomonas cepacia, P. syringaee* and *P. aeruginosa*. The repeating units in these

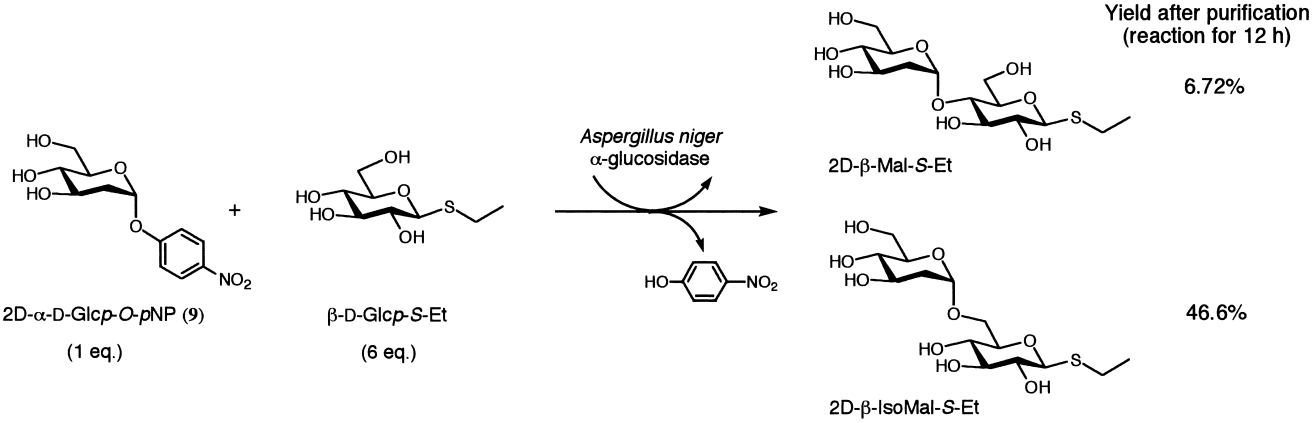


Fig. 3. Schematic representation of the transglycosylation reaction between 2D-α-D-Glc*p*-*O*-*p*NP (**9**) and β-D-Glc*p*-S-Et.

Compound **9** (200 mg, 0.70 mmol) and β-D-Glc*p*-S-Et (944 mg, 4.21 mmol) were dissolved in 50 mM sodium acetate buffer (pH 4.0) / MeCN (5:7, v/v; 3 mL). Following the addition of the enzyme solution (0.5 mL), the mixture was magnetically stirred at 37°C. After stirring of the reaction mixture for 12 h, the products were purified using silica gel column chromatography. Structures of the purified compounds were characterized using ^{1}H and ^{13}C NMR and positive ion FABMS spectra.

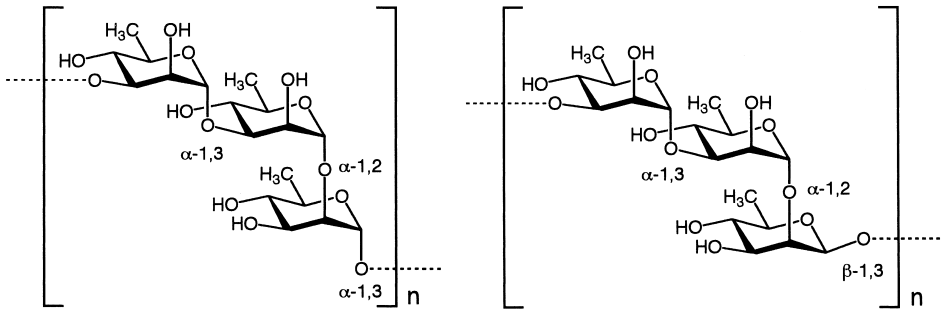


Fig. 4. Structures of D-rhamnotrisaccharide units in *O*-specific polysaccharide of the antigenic bacterial lipopolysaccharides from some strains of *Pseudomonas.*

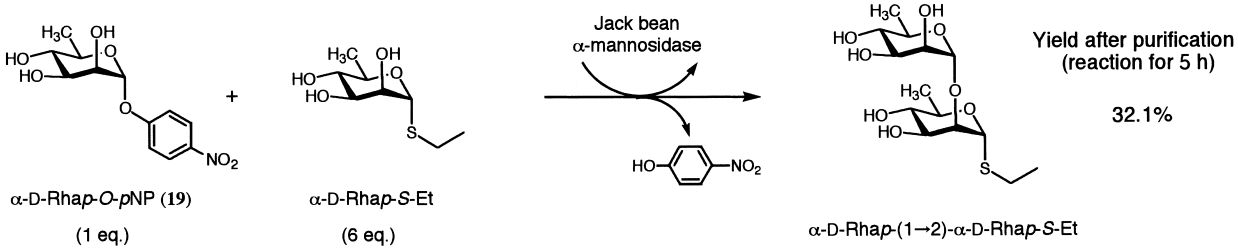


Fig. 5. Schematic representation of the transglycosylation reaction between α-D-Rha*p*-*O*-*p*NP (**19**) and α-D-Rha*p*-S-Et.

Compound **19** (228.2 mg, 0.80 mmol) and α-D-Rha*p*-S-Et (999.7 mg, 4.8 mmol) were dissolved in 100 mM sodium citrate buffer (pH 4.5) / MeCN (3:4, v/v; 3.5 mL). Following the addition of the enzyme solution (0.5 mL), the mixture was magnetically stirred at 25°C. After stirring of the reaction mixture for 5 h, the product was purified using silica gel column chromatography. Structure of the purified compound was characterized using ^{1}H and ^{13}C NMR and positive ion FABMS spectra.

polysaccharides have the following structures: →3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-β-D-Rha*p*-(1→ and →3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→ (Fig. 4).[11–15] The chemical synthesis of these trisaccharide units has been previously undertaken.[16] In the case of α-D-rhamnooligosaccharide, the use of α-D-rhamnosidase for the enzymatic transglycosylation reaction seems appropriate; however, α-D-rhamnosidase is rarely found in nature. Consequently, using the α-D-rhamnosidase activity of Jack bean α-mannosidase, the enzymatic construction of the above oligosaccharide units via the transglycosylation reaction were carried out using α-D-Rha*p*-*O*-*p*NP and α-D-Rha*p*-*S*-Et as the glycosyl donor and glycosyl acceptor, respectively. Effective solvation of the donor and six equivalents of the acceptor for transglycosylation using jack bean α-mannosidase required a mixture of MeCN and 0.1 M sodium citrate buffer (1:1, v/v). The transglycosylation reaction proceeded efficiently at 25°C to yield only one product, which was purified using silica gel column chromatography. Characterization using ^{1}H and ^{13}C NMR and FABMS confirmed the structure as ethyl α-D-rhamnopyranosyl-(1,2)-α-D-thiorhamnopyranoside, α-D-Rha*p*-(1→2)-α-D-Rha*p*-*S*-Et. The isolated yield of this disaccharide derivative after a reaction time of 5 h was 32.1% (based on donor substrate) (Fig. 5).[17] These results demonstrate the feasible and practical application of the jack bean α-mannosidase as a transglycosylation catalyst for the preparation of D-rhamnodisaccharide derivatives having an α-1,2-glycosidic linkage. For the construction of repeating D-rhamnotrisaccharide units in *O*-specific polysaccharides of antigenic bacterial lipopolysaccharides from *Pseudomonas*, this rhamnodisaccharide derivative can be useful as a common building block.

Based on these results, investigations of unknown glycon specificities of glycosidases using glycosides that consist of various types of sugars as substrates can be effective in the development of enzyme-catalyzed construction of rare sugar containing oligosaccharides.

Conversion of glycon specificity of α-glucosidase by site-specific mutagenesis.

To obtain mutant glycosidases that have broad or unique glycon specificity, it was necessary to construct mutant glycosidases by site-specific mutagenesis. For this purpose, family 13 α-glucosidase from *Geobacillus stearothermophilus* (GSGase) was chosen as a parent enzyme because: 1) gene cloning with this enzyme protein had already been accomplished, and 2) this enzyme has excellent thermostability and comparatively low molecular weight.[18,19] In contrast to the family 31 α-glucosidases from rice, sugar beet, flint corn, and *A. niger*, GSGase does not show significant hydrolyzing activity towards the monodeoxy analogs of α-D-Glc*p*-*O*-*p*NP.

Construction of the mutant glycosidase involves the substitution of various amino acids that form the active site of GSGase with Ala or Asn. To select the amino acids, however, three-dimensional (3D) structural information about the active site of the enzyme was necessary; unfortunately, the 3D structure of this enzyme has yet to be established. The amino acid sequence of GSGase (GenBank accession no. D84648) showed high homology with that of *Bacillus cereus* oligo-1,6-α-glucosidase (BCGase) (GenBank accession no. X53507),[20] which has a well-defined 3D structure (PDB accession no. 1 UOK).[21,22] Using the structure of BCGase as a template, the 3D structure of GSGase was constructed by homology modeling using software Swiss-PdbViewer,[23] and digitally rendered by macromolecule visualizing software Py-

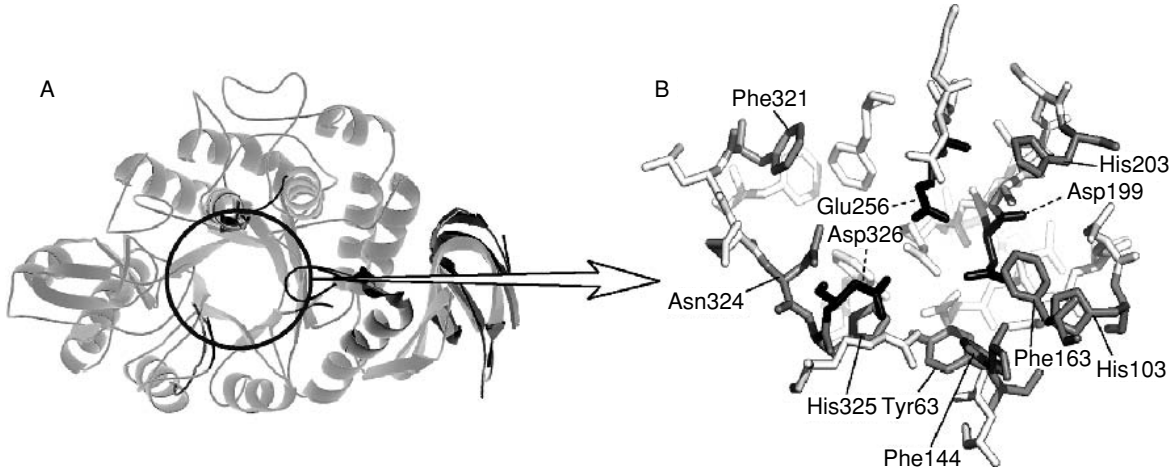


Fig. 6. A modeled 3D structure including the proposed active site amino acids of GSGase.

A: An overlay of the modeled 3D structure of GSGase (gray ribbon) on the 3D structure model of BCGase (black ribbon). B: stereoview of the proposed active site amino acids of GSGase–Asp199, Glu256 and Asp326 are thought to be catalytic amino acids. Tyr63, His103, Phe 144, Phe163, His203, Phe321, Asn324 and His325 are the amino acids for substitution by site-specific mutagenesis.

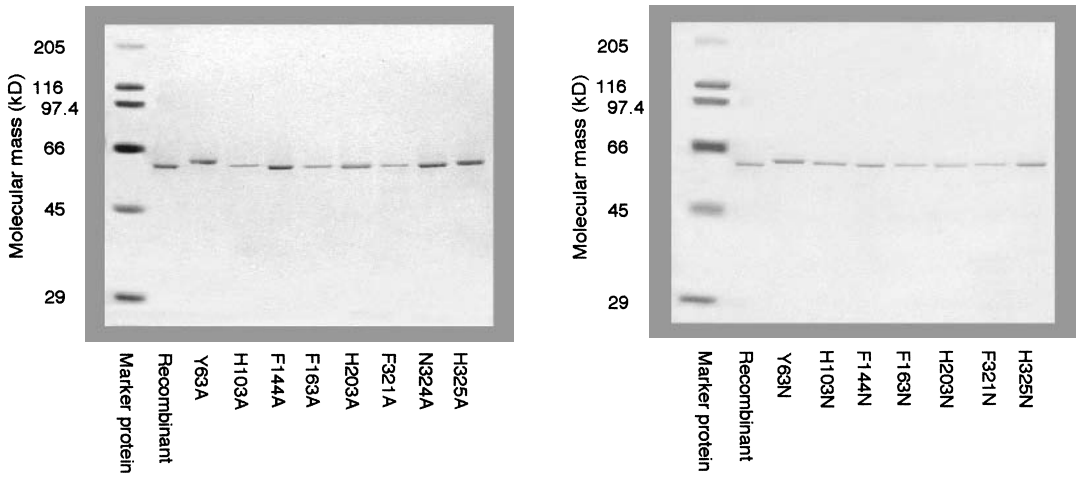


Fig. 7. SDS-PAGE of recombinant GSGase and its mutants.

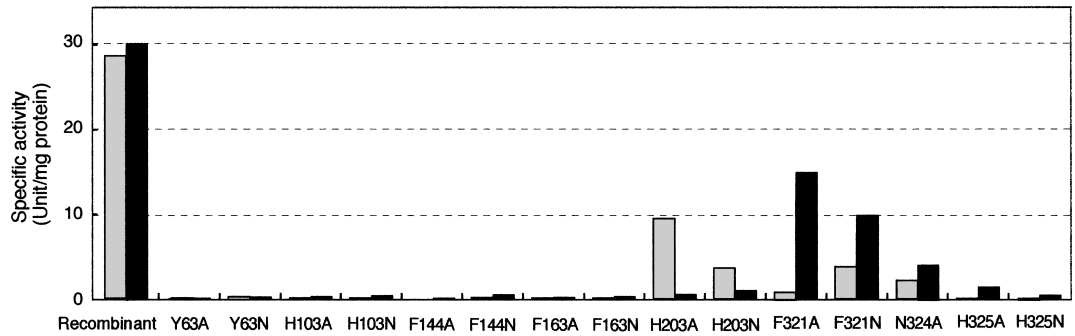


Fig. 8. Specific activities of recombinant GSGase and its mutants for α-D-Glc*p*-*O*-*p*NP and maltose.

Hydrolytic activities of recombinant GSGase and its mutants towards α-D-Glc*p*-*O*-*p*NP and maltose were assayed in 50 mM sodium phosphate buffer (pH 7.0) at 37°C. When α-D-Glc*p*-*O*-*p*NP was used as a substrate (□), the amount of *p*-nitrophenol liberated by the enzyme reaction was measured spectrophotometrically at 405 nm. One unit of the enzyme activity was defined as the amount of enzyme required to liberate 1 mmol of *p*-nitrophenol/min. In the case using maltose (■), the amount of glucose produced by the enzyme reaction was measured by the glucose oxidase method using a commercially available assay kit, Glucose CII-Test (Wako Pure Chemical Ind.). One unit of the enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of maltose/min. The amount of protein in enzyme solution was measured by the method of Lowry, using bovine serum albumin as a standard.

MOL.[24] The results showed good agreement between the structure of GSGase and that of BCGase (Fig. 6), in which several amino acids of the GSGase active site were almost identical to those of BCGase. Based on the modeled 3D structure of GSGase, mutant enzymes were prepared by substituting each of the seven active site amino acids (Tyr63, His103, Phe144, Phe163, His203, Phe321 and H325) with Ala or Asn. In addition, Asn324 of the enzyme was also replaced with Ala. The structural gene of GSGase was cloned from the chromosomal DNA of *G.*

Table 1. Relative rate of hydrolysis of various *p*NP α-D-glycopyranosides by recombinant GSGase and its mutants.

Enzyme	Relative rate of hydrolysis (%)[a]																													
	Compound number																													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
Recombinant	100	—	—	—	0.12	—	—	—	0.10	—	0.07	0.20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Mutant																														
Y63A	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H103A	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F144A	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F163A	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H203A	100	—	—	—	0.26	—	—	—	0.21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F321A	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N324A	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H325A	100	—	—	—	0.08	—	—	—	3.69	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Y63N	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H103N	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F144N	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F163N	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H203N	100	—	—	—	0.60	—	—	—	0.84	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F321N	100	—	—	—	0.25	—	—	—	0.13	—	—	0.09	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H325N	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Hydrolytic activity of each enzyme was assayed in 50 mM sodium phosphate buffer (pH 7.0) at 37°C. The enzyme was added to a solution of substrate (1.0 mM) with the final volume 0.5 mL. The amount of *p*-nitorophenol liberated by the reaction in each mixture was measured spectrophotometrically at 405 nm. [a]Relative rate of substrate hydrolysis by the enzyme was expressed as a ratio of the amount of *p*-nitorophenol that was released from α-D-Glc*p*-*O*-*p*NP after the reaction for 30 min, which was taken as 100%. —, activity was too low to be measured.

stearothermophilus ATCC12016 by nested PCR, and ligated into the vector. Plasmids for mutant enzyme production were made by the non-PCR temperature cycling method or mega-primer method using plasmids that contain the structural gene of GSGase as the template. Recombinant GSGase and its mutants were produced in *Escherichia coli* cells harboring the plasmids, then electrophoretically purified by column chromatography on ion-exchange resins and hydroxylapatite (Fig. 7).

The specific hydrolytic activities of the recombinant GSGase and the 15 mutants towards α-D-Glc*p*-*O*-*p*NP and maltose are shown in Fig. 8. Although the activities of every mutant enzyme towards either substrate were lower than that of the recombinant GSGase, some mutants showed a certain amount of activity for α-D-Glc*p*-*O*-*p*NP (H203A, H203N, F321N and N324A) and maltose (F321 A, F321N and N324A). Consequently, the recombinant and mutant enzymes were reacted with diastereomers of *p*NP α-D-aldohexopyranosides (**1**–**8**, Fig. 1A), monodeoxy- and mono-*O*-methyl analogs of α-D-Glc*p*-*O*-*p*NP (**9**–**16**, Fig. 1B), α-D-Man*p*-*O*-*p*NP (**10**–**16**, Fig. 1C) and α-D-Gal*p*-*O*-*p*NP (**24, 25, 26**–**30**, Fig. 1D). The relative rate of the hydrolytic activities of recombinant GSGase and its mutants towards these glycosidic substrates are shown in Table 1. Although the recombinant enzyme and H325A mutant exhibited slight changes in their activity towards 2D-α-D-Glc*p*-*O*-*p*NP, significant differences in glycon specificity were not observed for the other mutant enzymes.

In an attempt to obtain α-glycosidases that possess unique glycon specificity, we are currently preparing additional mutant enzymes of GSGase using the method of random mutagenesis.

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希少糖からなるオリゴ糖の酵素合成に有用なα-グリコシダーゼに関する研究

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天然界には，多彩な生物機能を有する様々な希少糖から成るオリゴ糖が存在している．われわれは，これらのオリゴ糖をグリコシダーゼの糖転移反応を利用して合成することを目的として研究を行っている．そのためには，構造を異にする多くの糖分子に対応できるような多様な基質特異性を有するグリコシダーゼがあると大変便利である．このような酵素を得るため，われわれは，1）種々のα-グリコシダーゼの潜在性未知基質特異性の探索，および2）α-グルコシダーゼの研究室内進化による基質特異性の改変の二つの研究を行っている．1）の研究では，*p*-ニトロフェニルグリコシドの様々な誘導体を合成し，それらに対する種々の生物由来のα-グリコシダーゼの加水分解活性を調べた．その結果，多くの酵素が特定の部分デオキシ糖基質に対して高い活性を示すことがわかった．そこで，*Aspergillus niger* のα-グルコシダーゼおよびナタ豆のα-マンノシダーゼについてみられた2-デオキシグルコシドおよび6-デオキシマンノシド（D-ラムノシド）に対する特異性と，それらの糖転移作用を利用してオリゴ糖合成を行ったところ，イソマルトースのグリコン2-デオキシ誘導体と，*Pseudomonasu* 属細菌の抗原性リポ多糖糖鎖中の繰返し三糖の部分構造であるα-1,2結合ラムノ二糖を，それぞれ好収率で合成することに成功した．また，2）の研究では，*Geobacillus stearothermophilus* の活性部位を形成すると推定されるいくつかのアミノ酸を，遺伝子工学的手法によりアラニンとアスパラギンに置換して変異酵素を作成し，それらの基質特異性の変化を種々の*p*-ニトロフェニルグリコシド誘導体を基質として用い調べた．その結果，特定の変異酵素について基質特異性に関する若干の変化はみられたが，多くの変異酵素について有意な変化はみられなかった．

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〔質　問〕　中部大　谷口

1）基質に使用したデオキシ糖やジアステレオマー糖の濃度はどれくらいですか．

2）溶解度の点から高濃度基質の使用は難しいようですが，もう少し高濃度で基質を用いれば違った活性プロファイルが得られるのではと思います．

〔答〕

1）通常の活性測定では，1 mM ないし 2 mM です．また，デオキシ基質を用いてカイネティックスを行う場合は，最高でも 5 mM 程度です．デオキシ基質は水に大変溶けにくく，このぐらいの濃度が上限となってしまいます．

2）可能性はあると思います．ただし，活性に関してそんなに大きな違いはみられないのではないかと思います．できれば，それぞれの基質に対し K_m 付近で活性を調べたいのですが，さきほども申し上げましたように，基質の溶解度が低いため，そのようなことができません．ここでは，2 mM ぐらいの基質濃度でも高い活性を示すかどうかということも含めて，基質認識に多様性を示す酵素をさがしております．

〔質　問〕　横浜市大　梶原

2-デオキシおよび6-デオキシ化した *p*NP グリコシドは，グリコシダーゼの種類によっては糖転移の基質にならないものもありました．転移を示さない酵素の場合，2-デオキシおよび6-デオキシ誘導体は酵素に結合しているのでしょうか．基質認識に OH-2，OH-6 が重要な場合は，それらの化合物は酵素に結合できず，転移活性を示さなかったのではないでしょうか．

〔答〕

酵素−基質複合体形成に関する直接的な実験は行っておりませんので明言できませんが，デオキシ基質を阻害剤として用いた酵素阻害実験から，分解されないデオキシ基質は酵素に結合していない可能性があります．また，結合していたとしても，分解されないデオキシ基質は正常な状態で酵素に結合していない可能性があります．いずれに致しましても，部分的な糖水酸基の欠如は酵素−基質複合体形成に大きく影響しているものと思われます．ただし，その様子は酵素の種類によって異なるようです．今回の実験結果は，その違いを示したものでもあります．