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Synthesis of New Glycosides by Transglycosylation of *N*-Acetylhexosaminidase from *Serratia marcescens* YS-1

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Serratia marcescens YS-1, a chitin-degrading microorganism, produced mainly *N*-acetylhexosaminidase. The purified enzyme had an optimal pH of ∼8−9 and remained stable at 40 °C for 60 min at pH 6−8. The optimum temperature was around 50 °C, and enzyme activity was relatively stable below 50 °C. YS-1 *N*-acetylhexosaminidase hydrolyzed *p*-nitrophenyl β-*N*-acetylgalactosamide by 28.1% relative to *p*-nitrophenyl β-*N*-acetylglucosamide. The *N*-acetylchitooligosaccharides were hydrolyzed more rapidly, but the cellobiose and chitobiose of disaccharides that had the same β-1,4 glycosidic bond as di-*N*-acetylchitobiose were not hydrolyzed. YS-1 *N*-acetylhexosaminidase efficiently transferred the *N*-acetylglucosamine residue from di-*N*-acetylchitobiose (substrate) to alcohols (acceptor). The ratio of transfer to methanol increased to 86% in a reaction with 32% methanol. *N*-Acetylglucosamine was transferred to the hydroxyl group at C1 of monoalcohols. A dialcohol was used as an acceptor when the carbon number was more than 4 and a hydroxyl group existed on each of the two outside carbons. Sugar alcohols with hydroxyl groups in all carbon positions were not proper acceptors.

KEYWORDS: ***Serratia*; *N*-acetylhexosaminidase; chitin; transglycosylation; *N*-acetylchitooligosaccharide; oligosaccharide**

INTRODUCTION

Chitin, a polymer of *N*-acetylglucosamine, is abundant in nature as well as cellulosic materials, being attractive as food materials (*1*). In fact, it is used in health foods and medical supplies. Its physiological effect is improved by hydrolysis to *N*-acetylchitooligosaccharides due to their increased solubility in water. Enzymatic hydrolysis by chitinase and *N*-acetylhexosaminidase is critical for the degradation of chitin because of the substrate specificity and the occurrence of the reaction under mild conditions. *N*-Acetylhexosaminidase is well-known as an exo-type enzyme (*2*−*8*). Some *N*-acetylhexosaminidases not only hydrolyze *N*-acetylchitooligosaccharides to GlcNAc (*N*-acetylglucosamine) but also transfer the GlcNAc residue to the hydroxyl group of given acceptors. For example, *Aspergillus oryzae N*-acetylhexosaminidase synthesized phenyl-GlcNAc-β-(4,1)-GlcNAc and phenyl-GlcNAc-β(6,1)-GlcNAc from the phenyl-GlcNAc of the substrate through transglycosylation (*9*). In contrast, the enzyme from *Norcardia orientalis* IFO 12806 produced a β-(1,6) linked disaccharide of GlcNAc and tri-*N*-acetylchitotriose from the di-*N*-acetylchitobiose of the substrate (*4*).

Some oligosaccharides, such as fructooligosaccharides, have been synthesized by transglycosylation and used as sweeteners with health-promoting functions (*10*). Oligosaccharides or glycosides containing GlcNAc are also expected to be functional new sweeteners. *Serratia marcescens* YS-1, producing *N*-acetylhexosaminidase, was isolated from soil. The YS-1 strain produced little chitinase, in contrast to some *Serratia* strains which mainly produced chitinase (*11*−*13*), but had a high yield of intracellular *N*-acetylhexosaminidase.

In this study, the transglycosylation action by *N*-acetylhexosaminidase produced from *S. marcescens* YS-1 was investigated and the synthesis of new glycosides with GlcNAc was examined using acceptors, including alcohols and sugar alcohols.

MATERIALS AND METHODS

Materials. All *N*-acetylchitooligosaccharides and *p*-nitrophenyl derivatives were obtained commercially from Seikagaku Kougyo Ltd. and Sigma Ltd., respectively. All other chemicals were reagent grade. Colloidal chitin was prepared from commercial chitin (Wako Pure Chemical Industries Ltd.) by a treatment with concentrated HCl, in which 10 g of chitin was dissolved in 400 mL of concentrated HCl and dialyzed against water after neutralization with 5 N NaOH.

Production of *N*-Acetylhexosaminidase by Liquid Culture. *S. marcescens* YS-1 was isolated from soil using plate culture (medium of 0.2% colloidal chitin and 1.5% agar) at 30 °C for 3 days. The YS-1 strain was cultivated in 5 mL of liquid medium containing 1% colloidal chitin, 1% yeast extract, and 0.2% $Na_2HPO_4 \cdot 12H_2O$ in a 180 mm × 18 mm test tube at 30 °C for 20 h. Three milliliters of the precultured

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broth was inoculated into 100 mL of the same liquid medium in a 500 mL baffled flask, and cultivated at 30 °C and 220 rpm for 48 h. The cultured broths (1.2−1.3 units/mL of enzyme) of seven identical cultures were collected, and the grown strain containing intracellular *N*-acetylhexosaminidase was separated by centrifugation at 10 000 rpm (12080*g*) for 10 min. *N*-Acetylhexosaminidase was released from the strain by lysis with lysozyme.

Purification of *N*-Acetylhexosaminidase. *N*-Acetylhexosaminidase was purified by the following column chromatography conditions at 4 °C.

Step 1. The released enzyme solution (13.6 units) was subjected to anion-exchange chromatography on a 50 cm × 1.0 cm Super Q Toyopearl column (Toso Ltd.) pre-equilibrated with 10 mM sodium acetate buffer (pH 6). The proteins were eluted with a linear gradient from 0 to 0.6 M NaCl at a flow rate of 0.2 mL/min, and fractions were collected every 30 min.

Step 2. Fractions with *N*-acetylhexosaminidase activity were pooled, and the aliquot (2 mL) was subjected to gel filtration on a 60 cm × 2.15 cm TSKgel G3000SW column (Toso Ltd.) pre-equilibrated with 50 mM phosphate buffer (pH 6.8) containing 0.3 M NaCl. The proteins were eluted at a flow rate of 2.0 mL/min, and fractions were collected every 1 min.

Determination of *N*-Acetylhexosaminidase Activity and Protein Concentration. *p*-Nitrophenyl *N*-acetylglucosaminide (1 mM) was incubated with an enzyme sample (0.1 mL) in 0.1 M phosphate−citrate buffer (pH 6) at 40 °C for 10 min (working volume, 1 mL). The reaction was stopped by adding 0.5 mL of 1 M Na_2CO_3, and the absorbance of the released *p*-nitrophenol was determined at 400 nm. One unit was defined as the amount of enzyme that could produce 1 μmol of *p*-nitrophenol/min.

The protein concentration was determined by the method of Bradford (*14*) using crystalline bovine albumin as the standard.

Transglycosylation. The mixture of 4% (w/v) di-*N*-acetylchitobiose and 8% acceptor (v/v in alcohols and w/v in sugar alcohols) was incubated with 0.2 unit/mL *N*-acetylhexosaminidase at pH 6 and 40 °C for 24 h. In general, a higher concentration of substrate is appropriate for transglycosylation because hydrolysis is inhibited by a decrease in water content. The reaction was stopped by boiling for 10 min, and the solution was subjected to HPLC analysis after dilution with water and filtration through a 0.22 μm membrane filter.

Analysis of Sugars. *N*-Acetylchitooligosaccharides or transfer products were analyzed by high-performance liquid chromatography (HPLC) under the following conditions: flow rate of 1.0 mL/min with a Hitachi model L-3300 differential refractive index monitor detector with a (A) 250 mm × 4.6 mm NH2P-50 column (Asahi Chemical Industry Co. Ltd.) at 25 °C with an acetonitrile/water mixture [from 70:30 to 75:25 (v/v)] as the mobile phase or (B) 250 mm × 7 mm GLC610 column (Hitachikasei Ltd.) at 60 °C with water as the mobile phase.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz with model AT 300K (Nihon Bruker Ltd.). The sample (1%) was dissolved in deuterium oxide, and the chemical shifts were calibrated with a signal of acetone used as an internal standard.

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed with an Atto model AE-6400 apparatus using 7.5% polyacrylamide gels (pH 8.8). SDS molecular weight markers (Sigma Ltd.) consisting of carbonic anhydrase (29 000), egg albumin (45 000), bovine albumin (66 000), phosphorylase *b* (97 400), and β-galactosidase (116 000) were used as the standard proteins.

Amino Acid Sequencing. The protein band of *N*-acetylhexosaminidase on SDS−PAGE was transblotted onto a PVDF (polyvinylidene difluoride) membrane, and the band stained with amido black was excised and subjected to amino acid sequencing. The N-terminal amino acid sequence was analyzed by automatic Edman degradation using a 491 protein sequencing system (Applied Biosystems Ltd.).

RESULTS AND DISCUSSION

Purification of YS-1 *N*-Acetylhexosaminidase and Some of Its Properties. *N*-Acetylhexosaminidase was released from cells grown in liquid culture by lysis with lysozyme, and purified as shown in **Table 1**. The enzyme was eluted at 0.065 M NaCl via anion-exchange chromatography on a Toyopearl Super Q column and then purified by gel chromatography on a TSKgel G3000SW column. The molecular weight was estimated to be 95 000 by SDS−PAGE as shown in **Figure 1**. The N-terminal amino acid sequence of YS-1 *N*-acetylhexosaminidase was QQLVDQLSQLKL. This N-terminal amino acid sequence and molecular weight were similar to those reported by Tews et al. (*15*). However, the enzyme had an optimal pH of ∼8−9, and it remained stable at 40 °C for 60 min at pH 6−8. The alkaline optimum pH is unique in contrast with those from many other origins that have an optimum pH between 5 and 7. The optimum temperature was around 50 °C, and the enzyme activity was relatively stable below 50 °C in an incubation at pH 5 for 30 min. These enzymatic properties are different from those of other strains of *S. marcescens* that have previously been reported (*16*).

Table 1. Purification of YS-1 *N*-Acetylhexosaminidase from the Crude Enzyme

purification step	total activity (units)	specific activity (units/mg)	yield (%)	purification (*x*-fold)
free enzyme (16 mL)	13.6	0.38	100	1.00
Super Q Toyopearl	9.01	2.04	66.3	5.37
TSKgel-G3000SW	8.38	4.36	61.6	11.5

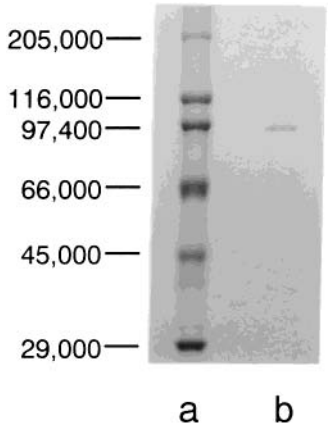

Figure 1. SDS−PAGE of purified YS-1 *N*-acetylhexosaminidase: (a) standard and (b) YS-1 *N*-acetylhexosaminidase.

Table 2. Activities of YS-1 *N*-Acetylhexosaminidase for Some Substrates[a]

substrate	activity (units/mL)	relative activity (%)
p-nitrophenyl-β-GlcNAc	0.135	100
p-nitrophenyl-α-GlcNAc	0.003	2.2
p-nitrophenyl-β-GalNAc	0.038	28.1
p-nitrophenyl-α-GalNAc	0.002	1.5

[a] Each substrate (1 mM) was incubated with the enzyme at pH 6 and 40 °C for 10 min.

Reactivity of YS-1 *N*-Acetylhexosaminidase for Some Substrates. The reactivities of purified YS-1 *N*-acetylhexosaminidase for four types of *p*-nitrophenyl glycosides were measured, in which the substrate was used at the low concentration of 1 mM. As shown in **Table 2**, YS-1 *N*-acetylhexosaminidase had hydrolyzing activities for *p*-nitrophenyl-β-GlcNAc and *p*-nitrophenyl-β-GalNAc, and the specificity for *p*-nitrophenyl-β-GlcNAc was relatively high, compared with

Table 3. Hydrolysis of Some Oligosaccharides by YS-1 *N*-Acetylhexosaminidase[a]

oligosaccharide	relative hydrolysis rate (%)
di-*N*-acetylchitobiose	100
tri-*N*-acetylchitotriose	54
tetra-*N*-acetylchitotetraose	19
cellobiose	0
chitobiose	0

[a] Each substrate (5.6 mg/mL) was incubated with 0.03 unit/mL enzyme at pH 6 and 40 °C for 10–60 min.

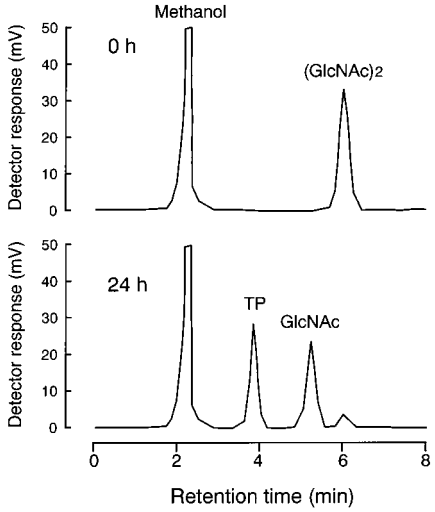

Figure 2. HPLC analysis of the reaction mixture of di-*N*-acetylchitobiose and methanol. The mixture of 4% di-*N*-acetylchitobiose (substrate) and 8% acceptor was incubated with 0.2 unit/mL enzyme at pH 6 and 40 °C for 24 h. The NH2P-50 HPLC column was used. GlcNAc, *N*-acetylglucosamine; $(GlcNAC)_2$, di-*N*-acetylchitobiose; TP, transfer product.

those from other origins (*8*). YS-1 *N*-acetylhexosaminidase hydrolyzed *p*-nitrophenyl-β-GalNAc by 28.1% relative to *p*-nitrophenyl-β-GlcNAc, which is similar to the enzyme from *Bacillus stearothermophilus* (*5*). In contrast with this finding, *S. marcescens* has two types of *N*-acetylhexosaminidase which are different from that of the YS-1 strain: the optimum pHs were 6.5 and 5.0, and the degradation degrees for 4-methylumbelliferyl-GalNAc relative to 4-methylumbelliferyl-GlcNAc were 23 and 45%, respectively (*13*).

YS-1 *N*-acetylhexosaminidase (0.1 unit/mL) was incubated with various polysaccharides (0.5%), such as colloidal chitin, colloidal chitosan, soluble chitosan, carboxymethyl cellulose (CMC), and crystalline cellulose (Funacel, Funakoshi Ltd.), at pH 6 at 40 °C for 24 h, but their polysaccharides were not hydrolyzed. Furthermore, the enzyme did not adsorb onto these polysaccharides (data not shown).

Next, some oligosaccharides were used as the substrate, as shown in **Table 3**. The YS-1 *N*-acetylhexosaminidase was a typical exo-type enzyme, hydrolyzing *N*-acetylchitooligosaccharides more rapidly, as the molecular size was small. *N*-Acetylhexosaminidase hydrolyzed di-*N*-acetylchitobiose rapidly, but could not hydrolyze the cellobiose and chitobiose of disaccharides with the same β-1,4 glycosidic bond as di-*N*-acetylchitobiose. This suggests that YS-1 *N*-acetylhexosaminidase had strict specificity for the acetamido group in C2 of the *N*-acetylglucosamine residue.

Transglycosylation. Methanol as an acceptor was mixed with di-*N*-acetylchitobiose as the substrate. **Figure 2** presents the HPLC chart of the reaction mixture at 24 h. *N*-Acetylglucosamine (GlcNAc) was produced by the hydrolysis of di-*N*-acetylchitobiose, and the peak of the transfer product was detected between the methanol and GlcNAc peaks. The product was identified as methyl *N*-acetylglucosamide by NMR analysis [GlcNAc residue, ^{1}H βH1 4.43 ppm (d, J = 8.41 Hz), ^{13}C C1 101.53 ppm, C2 55.02 ppm, C3 73.53 ppm, C4 69.48 ppm, C5 75.46 ppm, C6 60.28 ppm; methyl group from methanol, ^{13}C 55.64 ppm], in which the GlcNAc residue was transferred to the methanol. Tri-*N*-acetylchitotriose, the product formed when the GlcNAc residue was transferred to the substrate, was also detected by HPLC with the GLC610 column. **Figure 3** presents the change in the concentration of each sugar in the reaction mixture as a function of the reaction time. The concentration of methyl *N*-acetylglucosamide was increased as the reaction progressed, and the ratio of transfer to methanol reached 47% in 24 h. The transfer ratio is the ratio of the transferred *N*-acetylglucosaminyl residue in the degradation of di-*N*-acetylchitobiose. The one *N*-acetylglucosaminyl residue produced by the degradation of di-*N*-acetylchitobiose was free (hydrolysis) or was transferred to the acceptor (transglycosylation). The optimum pH of this transglycosylation was around pH 6.5 (data not shown). As shown in **Figure 4**, when the initial concentration of methanol was increased, the transfer ratio at 24 h reached 86% in 32% methanol, where little transfer product with respect to the substrate was detected (transfer ratio of 0.64%). During this reaction, deactivation of the enzyme was not observed.

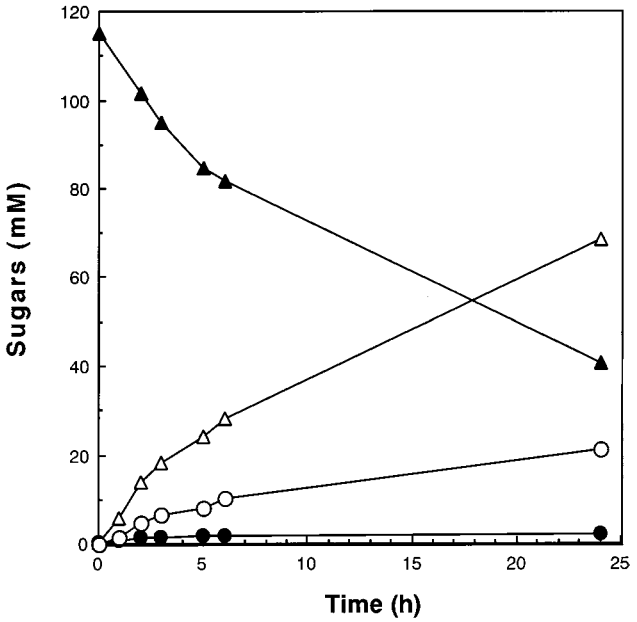

Figure 3. Relationship between the concentration of each sugar and the reaction time. The mixture of 4% di-*N*-acetylchitobiose (substrate) and 8% methanol (acceptor) was incubated with 0.2 unit/mL enzyme at pH 6 and 40 °C: (△) *N*-acetylglucosamine, (▲) di-*N*-acetylchitobiose, (●) tri-*N*-acetylchitotriose, and (○) methyl *N*-acetylglucosamide.

Transglycosylation of *N*-acetylhexosaminidase has been shown in *Penicillium oxalicum*, *Vibrio* sp., *Norcardia orientalis*, and Jack beans, but their acceptor specificities were not investigated in detail (*4*, *6*, *17*, *18*). **Table 4** shows the transglycosylation to some alcohols by YS-1 *N*-acetylhexosaminidase. The ratios of transfer to substrates and alcohols were calculated from the initial concentration of the di-*N*-acetylchitobiose and the concentrations of *N*-acetylglucosamine, di-*N*-acetylchitobiose, and tri-*N*-acetylchitotriose after the reaction (*19*). The *N*-acetylglucosamine (GlcNAc) residue was

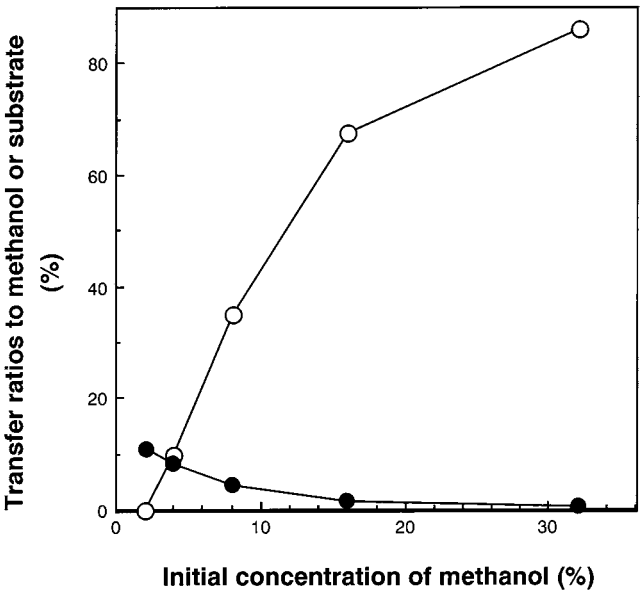

Figure 4. Relationship between the transfer ratio and the concentration of methanol. The mixture of 4% di-*N*-acetylchitobiose (substrate) and 2–32% methanol (acceptor) was incubated with 0.2 unit/mL enzyme at pH 6 and 40 °C for 24 h: (○) ratio of transfer to methanol and (●) ratio of transfer to substrate.

Table 4. Acceptor Specificity of YS-1 *N*-Acetylhexosaminidase in Transglycosylation[a]

acceptor	transfer ratio (%) to substrate	transfer ratio (%) to acceptor	degradation of substrate (%)
none	10.7	–	58.9
methanol	2.74	65.1	74.6
ethanol	5.10	55.2	75.4
1-propanol	5.65	65.3	78.9
2-propanol	11.3	28.6	65.6
1-butanol	7.54	60.7	72.7
2-butanol	9.13	43.2	69.1
2-methyl-1-propanol	7.73	55.9	72.3
2-methyl-2-propanol	13.3	24.3	56.7
1,2-ethanediol	6.16	45.2	65.8
1,2-propanediol	6.90	42.0	64.6
1,3-propanediol	13.4	35.0	32.0
1,2-butanediol	8.87	15.3	62.7
1,3-butanediol	26.7	0.0	14.7
1,4-butanediol	2.93	65.2	79.5
2,3-butanediol	10.2	0.7	59.7
1,2,4-butanetriol	14.3	0.0	38.4
1,5-pentanediol	2.20	65.9	84.3
1,6-hexanediol	2.70	63.4	82.2

[a] The mixture of 4% di-*N*-acetylchitobiose (substrate) and 8% acceptor was incubated with 0.2 unit/mL enzyme at pH 6 and 40 °C for 24 h.

transferred to the hydroxyl group at C1 of monoalcohols. For propanol, butanol, and methylpropanol, C1 is in a more favorable position for transglycosylation than C2. For dialcohols, the transfer ratios were smaller than those for monoalcohols except for 1,4-butanediol. The degradation of di-*N*-acetylchitobiose (substrate) was more inhibited by 1,3-butanediol, and the product of the transfer to 1,2,4-butanetriol was not detected at all. The extent of transglycosylation to alcohol was decreased with increasing numbers of hydroxyl groups, and the hydroxyl group at C1 of alcohols was preferentially selected as a binding point. 1,5-Pentanediol and 1,6-hexanediol were also good acceptors for the transglycosylation, similar to 1,4-butanediol. This suggests that a dialcohol is used as an acceptor when the carbon number is more than 4 and a hydroxyl group exists on each of the two outside carbons.

Table 5. Acceptor Specificity of YS-1 *N*-Acetylhexosaminidase in Transglycosylation[a]

acceptor	transfer ratio (%) to substrate	transfer ratio (%) to acceptor	degradation of substrate (%)
xylitol	9.67	7.65	59.2
arabitol	9.17	3.63	58.6
sorbitol	8.80	9.22	57.5
mannitol	9.16	11.0	59.3
dulcitol	10.4	0.0	56.2
palatinitol	9.99	1.62	56.6
lactitol	9.26	5.74	59.0
myoinositol	8.18	6.24	59.5

[a] The mixture of 4% di-*N*-acetylchitobiose (substrate) and 8% acceptor was incubated with 0.2 unit/mL enzyme at pH 6 and 40 °C for 24 h.

Table 5 shows the transglycosylation to some sugar alcohols. Although di-*N*-acetylchitobiose as the substrate was consumed by 56−59%, the ratios of transfer to sugar alcohols were very small (less than 11%). These sugar alcohols having a hydroxyl group at all of the carbon atoms should not be suitable acceptors, since the increase in the number of hydroxyl groups is not favorable for the transglycosylation by the YS-1 enzyme, as described in the Results for the alcohols. However, the ratio of transfer to sugar alcohols would be improved by increasing the initial concentration of the sugar alcohol in the reaction mixture. In molarity, the initial concentration of the sugar alcohols was much lower than that of the alcohols.

The three-dimensional structure of *N*-acetylhexosaminidase from *S. marcescens* has already been analyzed (*20*, *21*). The enzyme belongs to family 20 of the glycosyl hydrolases and has a conserved amino acid pair (Asp539 and Glu540) in the catalytic site, in which Asp539 restrains the acetamido group of the substrate by hydrogen bonding (*21*). In our data of acceptor specificity in the transglycosylation, monoalcohols, and 1,4-butanediol, 1,5-pentanediol, and 1,6-hexanediol among the dialcohols, were good acceptors. This finding may be explained if the hydrophobic methylene line (DP > 2) does not interact with Asp539, which would result in a decrease in the substrate specificity. It is necessary to analyze the three-dimensional structure of the enzyme in a complex with the acceptor.

S. marcescens YS-1 *N*-acetylhexosaminidase had some properties different from those of the enzymes of other microorganisms, especially in the high transglycosylation activity. Some new glycosides were synthesized by transglycosylation by YS-1 *N*-acetylhexosaminidase using alcohols and sugar alcohols as acceptors. These glycosides may be expected to be new food materials such as functional oligosaccharides.

ABBREVIATIONS USED

GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

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