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Characterization of β-Galactosidase from a *Bacillus* sp. with High Catalytic Efficiency for Transgalactosylation

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Abstract A β-galactosidase with high transgalactosylic activity was purified from a *Bacillus* species, registered as KFCC10855. The enzyme preparation showed a single protein band corresponding to a molecular mass of 150 kDa on SDS-PAGE and gave a single peak with the estimated molecular mass of 250 kDa on Sephacryl S-300 gel filtration, suggesting that the enzyme is a homodimeric protein. The amino acid and sugar analyses revealed that the enzyme is a glycoprotein, containing 19.2 weight percent of sugar moieties, and is much more abundant in hydrophilic amino acid residues than in hydrophobic residues, the mole ratio being about 2:1. The pI and optimum pH were determined to be 5.0 and 6.0, respectively. Having a temperature optimum at 70°C for the hydrolysis of lactose, the enzyme showed good thermal stability. The activity of the enzyme preparation was markedly increased by the presence of exogenous Mg (II) and was decreased by the addition of EDTA. Among the metal ions examined, the most severely inhibitory effect was seen with Ag (I) and Hg (II). Further, results of protein modification by various chemical reagents implied that 1 cysteine, 1 histidine, and 2 methionine residues occur in certain critical sites of the enzyme, most likely including the active site. Enzyme kinetic parameters, measured for both hydrolysis and transgalactosylation of lactose, indicated that the enzyme has an excellent catalytic efficiency for formation of the transgalactosylic products in reaction mixtures containing high concentrations of the substrate.

Key words: *Bacillus* sp., β-galactosidase, transgalactosylation

β-Galactosidase (β-D-galactoside galactosylhydrolase; EC 3.2.1.23) catalyzes not only the hydrolytic cleavage of β-galactoside linkage but also the galactose-transfer reaction, in which the galactose residue forming the glycon part of the substrate is transferred to some hydroxylic acceptors, such as alcohols and sugars [12, 26, 33, 38, 39].

Since lactose can serve as both a galactosyl donor and an acceptor, it yields a number of oligosaccharides, whose amounts and structures depend largely on the sources of the enzyme. These transgalactosylic products, known as galacto-oligosaccharides (GOS), are thought to contribute a share to the symptoms of lactose intolerance due to the inability of intestinal β-galactosidase to hydrolyze them. Much effort has therefore been made to minimize the formation of GOS during the enzymatic processes of milk and whey from a nutritional point of view [39]. However, the finding that GOS can be a growth-promoting factor with a preference for the genus *Bifidobacterium*, intestinal bacteria that play important roles in maintaining human health [18, 35], has led workers in the related fields to take cognizance of its significance. Consequently, the industrial production of GOS has recently become of increasing interest from the same nutritional viewpoint.

Previously, we have reported the isolation from soils of a *Bacillus* strain, which produces β-galactosidase with high transgalactosylic activity [9], and the preparation of one of its mutants, registered as *Bacillus* sp. KFCC10855, which shows a markedly increased productivity of the enzyme, by random chemical mutagenesis followed by selection [8]. In the present study, a β-galactosidase which is significantly different from already known β-galactosidases in many aspects, was purified from this mutant strain and its molecular and enzymatic characterization was performed.

Materials and Methods

Bacterial Cultivation

Bacillus sp. KFCC10855 was grown aerobically in a suspension culture. The medium contained, per liter, 2 g

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of glucose, 3 g of lactose, 5 g of galactose, 5 g of $(NH_4)_2SO_4$, 5 g of enzymatically hydrolyzed vegetable protein (EHVP), 1 g of K_2HPO_4, 1 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of NaCl, and 0.01 g of $FeCl_3 \cdot 6H_2O$, adjusted to pH 7.0, prior to autoclaving. The preculture was carried out in 500-ml Erlenmeyer flasks at 37°C for 24 h on a rotary shaker at 150 rpm. The main cultivation for the production of enzyme was done for 48 h under the same conditions as the preculture.

Chemicals

DEAE-Sephacel, molecular weight marker proteins for gel filtration, materials for isoelectric focusing, and *o*-nitrophenol-β-D-galactopyranoside (ONPG) were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Sephacryl S-300 and hydroxyapatite were the products of Pharmacia Fine Chemicals (Uppsala, Sweden) and Bio-Rad Laboratories (Hercules, Ca, U.S.A.), respectively. Molecular weight markers for SDS-PAGE were supplied by Novel Experimental Technology (San Diego, Ca, U.S.A.). EHVP was obtained from Fuji Oil (Osaka, Japan).

Enzyme Purification

The procedures consisted of four major steps involving ammonium sulfate fractionation, DEAE-Sephacel chromatography, hydroxyapatite chromatography, and Sephacryl S-300 chromatography.

Ammonium sulfate fractionation. After removing cells from the fermented broth by centrifugation, extracellular protein fractions were precipitated by ammonium sulfate at 35~70% saturation. The pellets showing β-galactosidase activity were dissolved in 0.05 M potassium phosphate buffer (pH 6.0) containing 0.001 M dithiothreitol (DTT) and dialyzed against the same buffer for 24 h. This buffer is hereafter referred to as the standard buffer.

DEAE-Sephacel chromatography. The enzyme solution was loaded onto a DEAE-Sephacel column (1.7× 16 cm) equilibrated with the standard buffer, and washed with 6 column volumes of the same buffer. Elution was done with a linear gradient of KCl (0~0.4 M) in the standard buffer at the flow rate of 50 ml/h. Active fractions were pooled and dialyzed against 0.025 M potassium phosphate buffer (pH 6.0) containing 0.001 M DTT.

Hydroxyapatite chromatography. The dialyzed protein solution was loaded onto a hydroxyapatite column (1.0× 9 cm), equilibrated with 0.025 M potassium phosphate buffer (pH 6.0) containing 0.001 M DTT, and washed with 4 column volumes of the same buffer. The bound proteins were eluted with a linear gradient of potassium phosphate (0.05~0.3 M, pH 6.0) containing 0.001 M DTT. The flow rate was 50 ml/h. The fractions showing the enzyme activity were pooled and concentrated using cold solid sugar.

Sephacryl S-300 chromatography. The concentrated protein solution was subjected to gel permeation chromatography (GPC), using a Sephacryl S-300 column (1.7× 120 cm) equilibrated with the standard buffer. The column was developed at 30 ml/h. The fractions containing β-galactosidase (5 ml each) were pooled and concentrated using cold solid sugar.

Enzyme Activity Assays

Enzyme assays during the purification of β-galactosidase were done by spectrophotometrically measuring *o*-nitrophenol released from ONPG, as described by Miller [17].

The rates of the enzyme-catalyzed hydrolytic and transgalactosylic reactions of lactose were determined by following the production of galactose, a product of lactose hydrolysis, and galactosyllactose, a trisaccharide which was virtually the only transgalactosylic product during the early stage of the galactose-transfer reaction, respectively. Reaction mixtures containing the enzyme and lactose (0.001~0.01 M for the hydrolysis and 0.1~1.5 M for the transgalactosylation) were incubated with the standard buffer at 50°C, the reactions were stopped at every 3 min by briefly boiling the mixtures in a microwave oven, and the products were analyzed by HPLC using a Sugar-Pak column and a refractive index detector (Waters, Miliford, Ma, U.S.A.). The initial rates of reactions were determined from the respective time-courses of reactions.

Protein was measured by the Bradford method [1], using bovine serum albumin as the standard. For kinetic experiments in which the enzyme concentration should be quantitatively defined, a measured amount of the enzyme preparation was thoroughly dialyzed against glass-distilled water and lyophilized, and then the dried enzyme was weighed so as to calibrate the concentrations of enzyme contained in reaction mixtures.

Determination of Molecular Mass

The molecular mass of the enzyme was estimated by GPC, using a Sephacryl S-300 column equilibrated with the standard buffer containing 0.05 M KCl. The subunit analysis was done by SDS-PAGE, using commercially available gradient (4~20%) gel calibrated with standard proteins.

Amino Acid and Sugar Analyses

The enzyme was hydrolyzed in 6 M HCl at 110°C for 24 h in a nitrogen atmosphere and then subjected to HPLC for amino acid analysis. In the case of tryptophan analysis, a colorimetric method was employed, as described by Dalby and Tsai [3]. The neutral sugar content of the enzyme was determined by the anthrone method with mannose as the standard, as in Spiro [32], while amino

sugar was analyzed using glucosamine as the standard, as in Johnson [10].

Modification of Amino Acid Residues

The following amino acid residues or functional groups were modified by incubating the enzyme (ca. 0.5 μg/ml) with suitable modifying reagents at 25°C in the respective buffers according to the procedures described in the literature, as indicated in brackets. Cysteine, [N-ethylmaleimide in 0.1 M Na-phosphate buffer (pH 7.0), [27]]; methionine, [chloramine T in 0.1 M Na-phosphate buffer (pH 7.0), [30]]; histidine, [diethylpyrocarbonate in 0.1 M Na-phosphate buffer (pH 7.0), [36]]; tryptophan, [N-bromosuccimide in 0.05 M Na-acetate buffer (pH 4.0), [11]]; tyrosine, [N-acetylimidazole in 0.05 M Na-phosphate buffer (pH 7.5), [28]]; arginine, [phenylglyoxal in 0.1 M Na-bicarbonate buffer (pH 8.0), [31]]; amino group, [trinitrobenzenesulfonic acid in 0.05 M Na-phosphate buffer (pH 6.0), [4]]; and carboxyl group, [(1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide in 0.1 M Na-acetate buffer (pH 4.5), [15]].

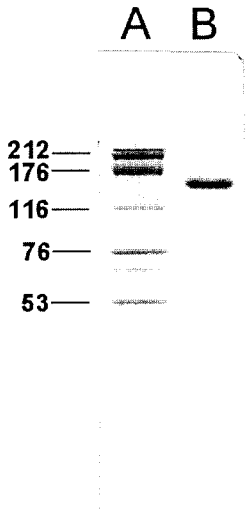

Fig. 1. SDS-PAGE of β-galactosidase from *Bacillus* sp. KFCC10855.
Lane A, molecular weight markers in kDa; Lane B, β-galactosidase.

RESULTS

Purification and General Properties

The overall scheme of purification is compiled in Table 1. The enzyme, purified about 7-fold with a yield of 43.4%, gave a single peak with the estimated molecular mass of 250 kDa on GPC and a single band on SDS-PAGE corresponding to a molecular mass of 150 kDa (Fig. 1), indicating that the enzyme is homodimeric.

The effect of pH on the enzyme activity was examined by monitoring the production of galactose from lactose in three different buffer systems, 0.05 M sodium citrate (pH 3-5.5), 0.05 M potassium phosphate (pH 6-7) and 0.05 M Tris-HCl (pH 8-9). As it turned out, the optimum pH was 6.0. However, the enzyme remained highly active in the range of pH 5.0-8.7, retaining more than 85% of the maximum activity. The isoelectric point was 5.0, as determined by isoelectric focusing.

Measurements of the enzyme activity for lactose hydrolysis from 25 to 80°C revealed the temperature optimum to be 70°C. Incubation of the enzyme in 0.05 M phosphate buffer (pH 6.0) at 50°C for 1 h did not result in any decrease in the activity.

Table 1. Purification of β-galactosidase from *Bacillus* sp. KFCC 10855.

Step	Protein (mg)	Activity (unit)	Specific activity (unit/mg protein)	Yield (%)
Crude enzyme	456	5977	13.1	100.0
$(NH_4)_2SO_4$ fractionation	428	5893	13.8	98.6
DEAE-Sephacel	106	4791	45.2	80.2
Hydroxyapatite	74	4303	58.0	72.0
Sephacryl S-300	28	2594	93.6	43.4

Amino Acid Composition and Sugar Content

The amino acid composition was determined and the results are: Arg (2.5), Asx (18.8), Glx (21.4), His (0.9), Lys (6.7), Ser (3.5), Thr (5.4), Gly (4.5), Ala (6.4), Cys (1/2) (0.7), Ile (3.1), Leu (4.6), Met (1.0), Phe (2.6), Pro (7.5), Trp (0.7), Tyr (3.4), and Val (6.4), with the values in parentheses in mole percent.

As it turned out, the enzyme was much more abundant in hydrophilic residues than in hydrophobic residues, the ratio being about 2:1. This is distinctive from the reported results with other β-galactosidases from different microorganisms, such as *Bacillus magneterum* [25], *Escherichia coli* [2], *Aspergillus oryzae* [21], and *Kluyveromyces fragilis* [14], in which the hydrophilic/hydrophobic residue ratios are in the range of 1.2:1 to 0.9:1.

The enzyme was found to be a glycoprotein containing sugar moieties of 19.2 weight percent in both neutral (10.8%) and amino sugar (8.4%) forms.

Effects of Metal Ions

By incubating the enzyme with various metal ions at 0.1 M, its hydrolytic activity was measured with ONPG as the substrate (Table 2). Several cations exerted stimulatory action on the enzyme; the most remarkable stimulation was seen in the presence of Mg (II), with the others showing inhibitory effects to various degrees. Worthwhile to note was that the enzyme-inactivating

Table 2. Effects of metal ions and EDTA on β-galactosidase activity*.

Ion source	Relative activity	Ion source	Relative activity
None	1.00		
$MgCl_2 \cdot 6H_2O$	2.57	Hg_2Cl_2	0.92
$CaCl_2$	1.21	$HgCl_2$	0.13
$BaCl_2$	1.24	$AgNO_3$	0.11
$CoCl_2 \cdot 6H_2O$	1.34	$CuCl_2 \cdot 2H_2O$	0.45
$CrCl_3$	1.34	CuCl	0.71
$NiCl_2 \cdot 6H_2O$	1.00	$FeCl_2 \cdot 4H_2O$	0.55
$ZnCl_2$	0.95	$FeCl_3 \cdot 3H_2O$	0.52
$MnCl_2 \cdot 4H_2O$	0.94	EDTA	0.44

*The enzyme was incubated with either 0.1 M metal ions or 0.1 M EDTA in 0.02 M phosphate buffer (pH 6.0) at 25°C for 20 min prior to the assay. Data are averages of duplicate determinations; the relative value of spread was not greater than 11%.

cations were either sulfhydryl-reactive metal ions, such as Ag (I) and Hg (II), or redox-active metal ions, such as those of iron and copper. Possible involvement of a certain metal ion in maintaining structural, functional integrity of the enzyme was examined by checking the effect of EDTA. The presence of the chelating agent caused a significant decrease in the enzyme activity.

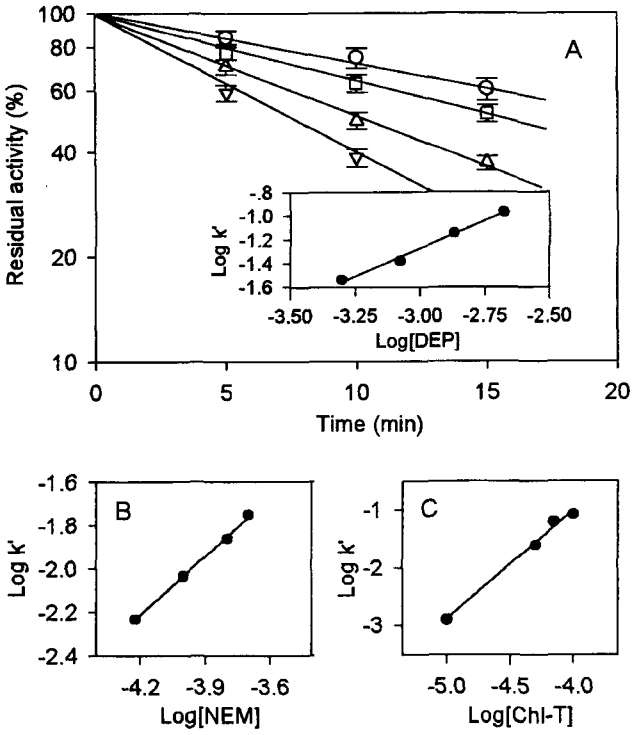

Fig. 2. Kinetics of inactivation of β-galactosidase caused by modification of amino acid residues. Results are means of three separate experiments.

Panel A: Time courses of inactivation of the enzyme by diethylpyrocarbonate (DEP) at varied concentrations (O, 5.0×10^{-4} M; □, 8.4×10^{-4} M; △, 1.34×10^{-3} M; and ▽, 2.1×10^{-3} M). Inset shows the logarithmic relationship between pseudo-first-order rate constant (k') in min^{-1} and concentration of DEP in M. Panel B: The logarithmic relationship between k' in min^{-1} and concentration of N-ethylmaleimide (NEM) in M. Panel C: The logarithmic relationship between k' in min^{-1} and concentration of Chloramine T (Chl-T) in M.

Effect of Chemical Modification

Functional amino acid residues of the enzyme were subjected to modification by appropriate chemical reagents of excessive amounts and the respective time courses of the modification-induced enzyme inactivation were measured. The resulting pseudo first order kinetics of inactivation was observed in only three cases where histidine, cysteine, and methionine residues were the targets for modification (Fig. 2): the modification of the other functional residues did not give rise to any significant change in the activity of the enzyme (data not shown). Since the pseudo rate constant (k') is dependent on the concentration of a modifying agent (M), such as $k' \propto [M]^n$ where n is the number of M reacting with one molecule of enzyme to cause total loss of its activity, plotting log k' versus log [M] produces a straight line whose slope gives n [13]. The values of n turned out to be 0.96 for the modification of histidine residue, 0.91 for that of cysteine residue and 1.88 for that of methionine residue, indicating that 1 histidine, 1 cysteine and 2 methionine residues occur in certain critical sites of the enzyme.

Substrate Specificity and High-substrate Inhibition

The substrate specificity of the enzyme for the reaction of galactose transfer from lactose to a number of sugars was studied with 4 monosaccharides, 6 disaccharides, 1 trisaccharide, and 1 tetrasaccharide. Results of HPLC analysis of the transgalactosylic products showed that glucose, maltose, cellobiose, raffinose, and stachyose also

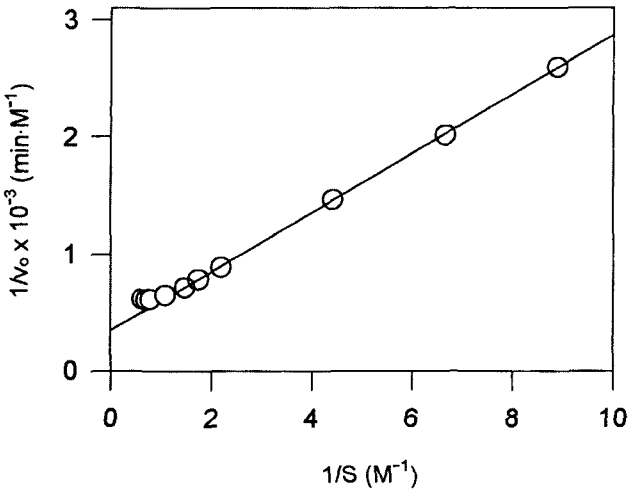

Fig. 3. The double-reciprocal plot for the transgalactosylation reaction of lactose catalyzed by β-galactosidase from *Bacillus* sp. KFCC10855.

served as the galactosyl acceptors. It was interesting to note that lactulose, which has the same galactosyl β(1-4) linkage as lactose, failed to function not only as a galactosyl acceptor but also as a substrate for the hydrolytic reaction (data not shown).

The enzyme was inhibited by excess substrate, as indicated by slight positive deviations in the Lineweaver-Burk plot for the transgalactosylation reaction of lactose at concentrations larger than 0.7 M (Fig. 3).

Kinetic Studies

The apparent Michaelis constant (K_m) and turnover number (k_{cat}) of the enzyme were determined for the transgalactosylation of lactose in 0.05 M K-phosphate buffer (pH 6.0) at 50°C, using the double-reciprocal plot (Table 3). From a comparative standpoint, the kinetic parameters of several commercially available β-galactosidases from different microorganisms were concurrently measured under the following optimal conditions as suggested by the suppliers, because no information was available on the parameters for lactose transgalactosylation by those β-galactosidases: 0.05 M Na-citrate buffer (pH 4.5) at 50°C for β-galactosidase from *Aspergillus oryzae*, 0.05 M K-phosphate buffer (pH 6.0) at 40°C for those from both *Kluyveromyces lactis* and *Bacillus circulans*, and 0.05 M K-phosphate buffer (pH 6.5) at 45°C for that from *Kluyveromyces fragilis*. As it turned out, the enzyme from *Bacillus* sp. KFCC 10855 showed rather low K_m and high k_{cat}, giving the largest value of k_{cat}/K_m for the transgalactosylation reaction among β-galactosidases subjected to the kinetic measurements.

Table 3. Kinetic parameters for the transgalactosylation reaction of lactose by β-galactosidase from various sources.

Enzyme source* (supplier)	K_m (M)	k_{cat} ($msec^{-1}$)	k_{cat}/k_m ($msec^{-1} \cdot M^{-1}$)
Bacillus sp. KFCC10855 (this work)	0.85 (±0.02)	7.01 (±0.10)	8.25 (±0.23)
Aspergillus oryzae (Sigma Chem.)	2.99 (±0.26)	0.35 (±0.03)	0.12 (±0.01)
Kluyveromyces lactis (Gist-Brocade)	2.01 (±0.14)	0.94 (±0.07)	0.47 (±0.05)
Kluyveromyces fragilis (Novo-Nordisk)	15.57 (±1.29)	5.02 (±0.38)	0.32 (±0.04)
Bacillus circulans (Daiwa Kasei)			
isozyme 1	0.34 (±0.02)	0.16 (±0.01)	0.47 (±0.04)
isozyme 2	0.22 (±0.01)	0.13 (±0.00)	0.59 (±0.03)

*The enzymes from the respective suppliers were purified by Sephacryl S-300 column chromatography and preparative electrophoresis prior to use. Data are presented as means (±SD); $n = 5$.

Table 4. Kinetic parameters for the hydrolysis of lactose by β-galactosidase from various sources.

Enzyme source* (supplier)	K_m (M)	k_{cat} ($msec^{-1}$)	k_{cat}/k_m ($msec^{-1} \cdot M^{-1}$)
Bacillus sp. KFCC10855 (this work)	0.005 (±0.000)	0.38 (±0.02)	76 (±0.40)
Aspergillus oryzae (Sigma Chem.)	0.049 (±0.005)	0.36 (±0.03)	7.3 (±0.97)
Kluyveromyces lactis (Gist-Brocade)	0.50 (±0.01)	2.80 (±0.06)	5.6 (±0.16)
Kluyveromyces fragilis (Novo-Nordisk)	0.35 (±0.02)	3.12 (±0.16)	8.9 (±0.68)
Bacillus circulans (Daiwa Kasei) †			
isozyme 1	0.016	0.20	12
isozyme 2	0.050	0.21	4.3

*The enzymes from the respective suppliers were purified by Sephacryl S-300 column chromatography and preparative electrophoresis prior to use. Data are presented as means (±SD); $n = 5$.
† Taken from Mazaffer *et al.* [20].

The kinetic parameters for lactose hydrolysis were also determined under the same conditions as above (Table 4), with the only exception that for the minor isozyme of β-galactosidase (isozyme 2) from *Bacillus circulans*, in every enzyme assayed, the factor k_{cat}/K_m for the hydrolytic reaction was found to be markedly (ca. 10~60 times) larger than that for the transgalactosylic reaction. This indicates that lactose at very low concentrations would convert almost exclusively *via* hydrolysis, as consistent with observation.

DISCUSSION

The results of the present investigation demonstrate that the extracelluar β-galactosidase from *Bacillus* sp. KFCC10855 is significantly different from already known β-galactosidases from other sources in many aspects. Besides its amino acid composition with a high hydrophilic/hydrophobic residue ratio, the subunit composition of this enzyme also differs from those of the reported β-galactosidases with comparable molecular masses from the following bacterial strains: *Bacillus macerans* [homotetrameric enzyme of 320 kDa, [19]], *Bacillus circulans* [two monomeric isozymes of 240 kDa and 160 kDa, [20]], *Bacillus stearothermophilus* [monomeric enzyme of 215 kDa, [6]], and *Bacteroides polypragmatus* [heterotrimeric enzyme of 210 kDa, [24]]. There are several homodimeric β-galactosidases described, such as those from *Theromomyces lanuginosus* [5], *Cryptococcus laurentii* [22], and *Strigmatomyces elviae* [23]. However, they have much smaller subunits and noticeably different properties.

With regard to the amino acid residues occurring at the active site, tyrosine and glutamate residues, methionine residue, and tryptophan residue are known for β-galactosidases from *Escherichia coli* [7], *Aspergillus oryzae* [16], and *Macrophomina phaseolis* [29], respectively. However, the enzyme of our preparation seems to involve at least some of, if not all of, 1 cysteine, 1 histidine, and 2 methionine residues at or near its active site, for the modification of these residues leads to a complete inactivation of the enzyme. Further, considering that these residues are vulnerable to oxidation [34], the observed sensitivity of the enzyme to redox-active metal ions appears consistent with this proposition. In the case of the cysteine residue, its presence at a certain critical site, presumably at the active site, is corroborated by the severely inhibitory effect of Ag (I) and Hg (II) ions. In this respect, it is tempting to speculate that β-galactosidase from KFCC10855 may work via a mechanism hypothesized for β-galactosidase action by Wallenfels and Malhotra [37], in that sulfhydryl and imidazole groups function as the proton donor and the acceptor during the breakdown of the enzyme reaction intermediate.

If a β-galactosidase has a high transgalactosylic activity, it should convert substrate mostly to the transgalactosylic products rather than the hydrolytic products when the substrate concentrations are high. This point was checked with the enzyme of KFCC10855 by calculating $v/[E]_0 = k_{cat}[S]/(K_m + [S])$, that gives the number of substrate molecules converted in a unit time on a single enzyme molecule. From the values of $v/[E]_0$ calculated at [lactose] = 0.7 M for both the hydrolytic and transgalactosylic reactions, 89% of lactose molecules are estimated to take part in the trangalactosylation reaction under the given conditions. Among other β-galactosidases assayed, only the minor isozyme of β-galactosidase from *Bacillus circulans*, which has been reported to have high transgalactosylic activity [20], gives a comparable degree of the estimated transgalactosylic efficiency (83% at 0.7 M lactose). However, the $v/[E]_0$ value for the transgalactosylation by this isozyme is substantially smaller than that by the enzyme of KFCC10855 (970 sec^{-1} vs. 3160 sec^{-1}). Transgalactosylic efficiencies of other β-galactosidases are estimated to be 9~35% at 0.7 M lactose, indicating a poor applicability in the industrial production of GOS.

β-galactosidase from *Bacillus* sp. KFCC10855 is fairly stable at relatively high temperatures, which is comparable to such thermally stable β-galactosidases from *Bacillus stearothermophilus* (M. W. Griffiths *et al.* 1982. *US Patent* 4,332,895) and *Bacillus subtilis* (H. Hirata *et al.* 1989. *US Patent* 4,861,718). Although the enzyme suffers inhibition by high concentrations of lactose, this would not cause a practical problem in its use for industrial production of GOS, because the substrate concentration at which the inhibition begins to occur is close to the saturated concentration, and thus the degree of inhibition should be insignificantly low even in reaction mixtures saturated with lactose.

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