In-gel digestion and MALDI-TOF MS

In-gel trypsin digestion of CBB-stained bands and MALDI-TOF MS analyses were performed as described (Yasukawa *et al.*, 2005).

Affinity purification of IgG

The 30-kDa gp-enriched fraction was applied to the Protein G-Sepharose column. The column was washed with 10 mM Tris–HCl (pH 8.0) containing 0.15 M NaCl and eluted with 50 mM diethylamine–HCl (pH 11.5). The eluted solution was quickly neutralized with 1 M Tris–HCl (pH 8.0).

Affinity purification of plasminogen

L-lysine monohydrochloride (15 mg; Wako Pure Chemical) was coupled with 5 mL of CNBr-activated Sepharose 4B, according to the manufacturer's instructions. Normal mouse sera (6.2 mL) were applied to the non-coupled Sepharose 4B (Amersham Biosciences, Piscataway, NJ), which was equilibrated with 100 mM phosphate buffer (PB; pH 7.4). The pass-through fraction was re-applied to a lysine-coupled Sepharose column, which was equilibrated with 100 mM PB (pH 7.4). The column was washed with 100 mM PB (pH 7.4), 300 mM PB (pH 7.4), and eluted with 100 mM PB (pH 7.4) containing 0.1 M 6-aminohexanoic acid (Sigma).

Affinity purification of vitronectin

Vitronectin was purified from mouse sera, according to the previously described method (Yatohgo *et al.*, 1988; Kitagaki-Ogawa *et al.*, 1990). In brief, serum was passed through a heparin-Sepharose column and supplemented with a final concentration of 8 M urea. The serum was charged again on a heparin-Sepharose affinity column in the presence of 8 M urea. Vitronectin was specifically bound to the column and eluted with 0.5 M NaCl in the presence of 8 M urea.

Fluorescent C_7/C_9 analysis and mild acid hydrolysis–DMB derivatization followed by anion-exchange chromatography

Fluorescent C_7/C_9 analyses were performed as described earlier (Sato, Inoue, *et al.*, 1998). Mild acid hydrolysis–DMB derivatization followed by anion-exchange chromatography was conducted as described previously (Sato *et al.*, 1999).

Hepatectomy of rat and purification of vitronectin from serum

Vitronectins from non-operated, sham-operated, and partially hepatectomized rat plasma were purified as described previously (Uchibori-Iwaki *et al.*, 2000).

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Conflict of interest statement

None declared.

Abbreviations

BSA, bovine serum albumin; diSia, disialic acid; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DTT, dithiothreitol; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PB, phosphate buffer; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Novel carbohydrate-binding activity of bovine liver β-glucuronidase toward lactose/*N*-acetyllactosamine sequences

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β-Glucuronidase is a lysosomal enzyme that plays an essential role in normal turnover of glycosaminoglycans and remodeling of the extracellular matrix components in both physiological and inflammatory states. The regulation mechanisms of enzyme activity and protein targeting of β-glucuronidase have implications for the development of a variety of therapeutics. In this study, the effectiveness of various carbohydrate-immobilized adsorbents for the isolation of bovine liver β-glucuronidase (BLG) from other glycosidases was tested. β-Glucuronidase and contaminating glycosidases in commercial BLG preparations bound to and were coeluted from adsorbents immobilized with the substrate or an inhibitor of β-glucuronidase, whereas β-glucuronidase was found to bind exclusively with lactamyl–Sepharose among the adsorbents tested and to be effectively separated from other enzymes. Binding and elution studies demonstrated that the interaction of β-glucuronidase with lactamyl–Sepharose is pH dependent and carbohydrate specific. BLG was purified to homogeneity by lactamyl affinity chromatography and subsequent anion-exchange high-performance liquid chromatography (HPLC). Lactose was found to activate β-glucuronidase noncompetitively, indicating that the lactose-binding site is different from the substrate-binding site. Binding studies with biotinyl glycoproteins, lipids, and synthetic sugar probes revealed that β-glucuronidase binds to *N*-acetyllactosamine/lactose-containing glycoconjugates at neutral pH. The results indicated the presence of *N*-acetyllactosamine/lactose-binding activity in BLG and provided an effective purification method utilizing the novel carbohydrate binding activity. The biological significance of the carbohydrate–specific interaction of β-glucuronidase, which is different from the substrate recognition, is discussed.

Key words: affinity purification/glycoprobe/lactose and *N*-acetyllactosamine-binding/lysosomal enzyme/zymography/β-glucuronidase

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Introduction

β-Glucuronidase is an exoglycosidase that cleaves β-glucuronic acid linkages from the nonreducing termini of glycosaminoglycans such as chondroitin sulfate, heparan sulfate, and hyaluronic acid. It is present in animals, plants, and bacteria as an essential enzyme for the normal degradation and turnover of components of the extracellular matrix (Paigen, 1989). In animals, β-glucuronidase is widely distributed in tissues, and high activity is detected in spleen, liver, and kidney; it also serves in the incorporation of β-glucuronides of steroid hormones into tissues. Bovine liver β-glucuronidase (BLG) [EC 3. 2. 1. 31] has been reported to be a homotetramer of 290 kDa (Himeno *et al.*, 1974) and to have *N*-linked oligosaccharides with mannose 6-phosphate (Kaplan and Achord, 1977) (Natwicz *et al.*, 1982), which is a targeting signal to lysosomes recognized by specific Golgi receptors to segregate BLG into transport vesicles via a glycan-specific sorting mechanism (Kornfeld, 1987).

β-Glucuronidase from bovine liver is commercially available from several manufacturers. However, most β-glucuronidase preparations are contaminated with many other proteins including several glycosidases, such as *N*-acetyl-β-hexosaminidases and β-galactosidase. It is difficult to separate β-glucuronidase from the lysosomal protein mixtures, and multiple purification steps have been required for β-glucuronidase preparation, that is, a combination of several methods using serial fractionations with ammonium sulfate, ethanol, and organic solvents, gel filtration chromatography, ion-exchange chromatography, and isoelectric chromatofocusing (Himeno *et al.*, 1974; Ho, 1991). In this study, we were trying to isolate BLG from commercial preparations, and while searching for a suitable affinity adsorbent among those immobilized with various carbohydrates including its inhibitor and substrates, we unexpectedly discovered that BLG has binding activity toward lactamyl–Sepharose. Interaction of BLG with glycoconjugates and the effect of carbohydrates on the enzyme activity provide new insights into the biological functions of the carbohydrate binding of β-glucuronidase.

Materials and methods

Materials

Commercial BLG was purchased from Worthington Biochemical Co. (Lakewood, NJ) in most experiments as a crude enzyme preparation and from P-L Biochemicals (present Pharmacia P-L Biochemicals Inc., Milwaukee, WI) or from Sigma-Aldrich Co. (St. Louis, MO) for comparison. p-Nitrophenyl-β-D-glucuronide, 5-bromo-4-chloro-3-indolyl

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(X-) β-D-glucuronide, ceramide (bovine brain), and galactosylceramide (bovine spinal cord) were purchased from Wako Pure Chemicals (Osaka, Japan). *p*-Nitrophenyl-β-D-galactoside was purchased from Nakalai Tesque Inc. (Kyoto, Japan). *p*-Nitrophenyl-β-D-galactoside, transferrin, fetuin, ovalbumin, bovine submaxillary gland mucin (BSM), bovine lactosylceramide, human spleen glucosylceramide, galactosylceramide, ceramide, bovine brain sulfatide, and streptavidin-biotinylated horseradish peroxidase complex (ABC-HRP) were purchased from Sigma-Aldrich Co. Saccharo-1,4-lactone and β-galactosidase (from jack bean) were purchased from Seikagaku Kogyo (Tokyo, Japan). All biotinylated glycoprotein probes and their deglycosylated derivatives were prepared in our laboratory. Biotinylation was performed using EZ-link™sulfo-NHS-biotin (Pierce, Rockford, IL) according to the instruction manual. Asialoglycoproteins, asialo-agalactoglycoproteins, and ahexosamino-asialoagalactoglycoproteins were prepared from biotinylated glycoproteins by sequential glycosidase treatments with *Vibrio cholerae* neuraminidase (Roche Diagnostics, Basel, Switzerland) (0.1 units/mg glycoprotein) in 20 mM sodium acetate-buffered saline (pH 5.5), jack bean β-galactosidase (Seikagaku Kogyo) (0.14 units/mg glycoprotein) in 50 mM sodium citrate buffer (pH 3.5) and then β-N-acetylhexosaminidase (1.43 units/mg glycoprotein) in 50 mM sodium citrate buffer (pH 5.0), each at 37°C overnight. Biotinyl polymer (BP) sugar probes were purchased from GlycoTech Co. (Gaithersburg, MD).

Preparation of affinity adsorbents

Affinity adsorbents containing various carbohydrates as ligands were prepared using Sepharose 4B gel (Pharmacia, Uppsala, Sweden) in our laboratory. Lactose, maltose, melibiose, glucose, galactose, *N*-acetylchitotriose and *N*-acetylchondrosine were immobilized to amino Sepharose by reductive amination (Matsumoto *et al.*, 1981) Saccharo-1,4-lactone was immobilized to amino Sepharose with the aid of *N*-ethyl-*N*′-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (Harris *et al.*, 1973). *p*-Aminophenyl β-glucuronide was immobilized to formyl Sepharose by reductive amination (Ito *et al.*, 1985). The immobilized carbohydrate concentrations of the adsorbents (μmol per g wet gel) were 45 for saccharo-1,4-lactone-, 35 for *N*-acetylchondrosine-, and 30–35 for other neutral saccharides coupled by reductive amination; lactamyl, maltamyl, melibiamyl, glucamyl, galactamyl and *N*-acetylchitotriamyl Sepharose. Those of *p*-aminophenylglucuronide- and *N*-acetylchitotriose-Sepharose were not determined.

Preparation of 1-deoxy-4-O-β-D-galactopyranosyl-1-([2-hydroxyethyl]amino)-D-glucitol and analysis by nuclear magnetic resonance spectroscopy

1-Deoxy-4-*o*-β-D-galactopyranosyl-1-([2-hydroxyethyl]amino)-D-glucitol (GHAG) was prepared by coupling 2-aminoethanol (366 μL) with lactose (0.3 M solution, 7.5 mL) by reductive amination with the pH adjusted to 7.5 with acetic acid. After preincubation for 2 h at 40°C, 300 mg of $NaBH_3CN$ was added and incubated for 2 h at 90°C; progress of the coupling reaction was confirmed by TLC (Holmes and O'Brien, 1979). The reaction mixture was diluted to 100 mL with water and applied to a charcoal column (2 × 35 cm), followed by washing with water and elution with a 0–20% gradient of ethanol. For determination of the structure, ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were obtained by using an ECA-800 spectrometer (JEOL, Tokyo) with a probe temperature of 60°C. The purified product was dried over P_2O_5 under vacuum and dissolved in $(CD_3)_2SO$ (Me_2SO-d_6). Chemical shifts were referenced to tetramethylsilane. Spectral parameters of double quantum-filtered correlated spectroscopy (DQF-COSY), total correlated spectroscopy (TOCSY), heteronuclear multiple-quantum coherence (HMQC), and heteronuclear multiple-bond correlation (HMBC) spectra were as described previously (Iida-Tanaka *et al.*, 2002).

Binding of β-glucuronidase with various carbohydrate-immobilized Sepharose gels

Interactions between various adsorbents and BLG were studied by affinity chromatography on a small column (0.75 × 4.5 cm, Vt=2 mL). Commercial BLG (6 mg from Pharmacia P-L Biochemicals) was dissolved in 1 mL of 2 mM sodium acetate buffer–5 mM NaCl (pH 7.0) (buffer A) and applied to each column. The column was washed with buffer A and then sequentially eluted with 5 mM sodium acetate buffer–5 mM NaCl (pH 5.0) (buffer B), 10 mM sodium acetate buffer–5 mM NaCl (pH 5.0) (buffer C), 25 mM sodium acetate buffer–5 mM NaCl (pH 5.0) (buffer D), 50 mM sodium acetate buffer–5 mM NaCl (pH 5.0) (buffer E), 50 mM sodium acetate buffer–50 mM NaCl (pH 5.0) (buffer F), and 0.2 M sodium acetate buffer–0.2 M NaCl (pH 5.0) (buffer G). The eluted fractions (2 mL/fraction) were measured for enzyme activities, and proteins were detected by absorbance at 280 nm.

Binding and elution of BLG to lactamyl Sepharose

Lactamyl Sepharose 4B (0.1 g) and 1 mg of BLG (from Worthington) were incubated at 4°C for 4 h in a 1.5-mL tube with 0.3 mL of various buffers: 2 mM sodium acetate buffer (pH 5–6), ammonium acetate (pH 6), or Tris–HCl (pH 6–9), each containing 5 mM NaCl. After centrifugation at 900 *g* for 5 min, the supernatant was removed, and the gel was washed three times with the same buffer; then the bound proteins were eluted with buffer G for 4 h at 4°C, and the enzyme activity of the eluted fraction was measured. To study the elution conditions from lactamyl Sepharose gel, we incubated BLG with lactamyl Sepharose gel in 0.2 mL of 2 mM sodium acetate buffer–5 mM NaCl (pH 6.0) (buffer A′). After centrifugation, the supernatant was removed, the gel was washed three times with buffer A′, then incubated in each solution for 4 h at 4°C, and the enzyme activity of the eluted fraction was measured.

Purification of BLG on a lactamyl–Sepharose column and anion-exchange high-performance liquid chromatography

For large-scale purification, the binding of BLG to lactamyl–Sepharose was performed by a batch-wise method to improve the binding capacity. BLG (Worthington or Sigma, 15 mg) was mixed with 13 mL of lactamyl–Sepharose 4B in buffer A′ and incubated for 5 h at 4°C with

gentle shaking, and then, the gel was poured into a column (1.5 × 8 cm). After extensive washing with buffer A′, BLG was successively eluted with buffer C, 25 mM sodium acetate buffer–25 mM NaCl (pH 5.0) (buffer E′), and buffer G. Eluted fractions were monitored by absorbance at 280 nm and measured for enzyme activities. Alternative elution was performed with 0.1 M GHAG after 1 mg of BLG was applied onto a lactamyl–Sepharose column (0.75 × 4 cm). The peak fractions were pooled and concentrated with a Microcon YM-10 Filter Unit (Millipore, Billerica, MA).

For further purification, the buffer C-eluted fraction from the lactamyl column was applied to ion-exchange high-performance liquid chromatography (HPLC) on a DEAE-5PW column (21.5 × 150 mm, Tosoh Corp., Tokyo, Japan). Elution was performed at a flow rate of 1.0 mL/min at room temperature using two solvents, 10 mM Tris–HCl (pH 7.5) (TBS) containing 1 M NaCl. After injection of the sample, the NaCl concentration was increased linearly from 0 to 0.4 M, and 0.4–1.0 M in 100 min. Each peak was collected and concentrated to measure enzyme activities and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and binding studies.

Polyacrylamide gel electrophoresis and zymography

Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was carried out according to the method of Laemmli (1970) under nonreducing conditions using a 9.5 or 7.5% acrylamide separation gel, and native PAGE was performed in the absence of SDS (Davis, 1964). Protein bands were detected with Coomassie Brilliant Blue (CBB) or silver staining (Atto Corp., Tokyo, Japan). For zymography, the gel was incubated in 2.5% Triton X-100 for 1 h after SDS–PAGE to remove SDS or used directly after native-PAGE. The gels were washed and incubated in each substrate solution for several hours at 37°C with gentle shaking. Substrate solutions contained 1 mM X-β-D-glucuronide in 0.2 M sodium acetate buffer (pH 5.0)-50 mM NaCl for BLG.

Solid phase binding assay

Purified BLG fractions eluted from ion-exchange HPLC (each 100 μL) were serially diluted with TBS, placed in the wells of an Immulon I plate (Dynatech Laboratories, Chantilly, VA), and immobilized for 4 h at room temperature. The binding with biotinyl glycoproteins or BP sugar probes (10 μg/mL in TBS) was demonstrated by ELISA using ABC-HRP as described previously (Ueda *et al.*, 1999). Various concentrations of lipids in MeOH (100 μL/well) were dried at 37°C. After the wells were blocked with 3% bovine serum albumin (BSA) in TBS, 100 μL of biotinylated BLG (10 μg/mL) was added to each well, followed by incubation for 2 h. The wells were washed and measured by ELISA as described above.

Measurement of enzyme activity

Enzyme activities were measured in a test tube using a 50 μL aliquot of the sample. For BLG activity, 0.7 mL of 7 × 10^{-5} M *p*-nitrophenyl-β-D-glucuronide in 0.2 M sodium acetate buffer (pH 5.0)–50 mM NaCl was added to the sample according to the method previously described (Harris *et al.*, 1973). After incubation at 37°C for 1 h, 0.25 mL of 2 M glycine-NaOH (pH 10.4) was added to stop the reaction, and the liberated chromogen, *p*-nitrophenol, was measured at 400 nm. For *N*-acetyl β-galactosaminidase and *N*-acetyl-β-glucosaminidase, *p*-nitrophenyl *N*-acetyl-β-galactosaminide and p-nitrophenyl-*N*-acetyl-β-glucosaminide, respectively, were used as substrates according to the method described previously (Kawai and Anno, 1971). After adding 0.2 mL of 0.7 mM substrate dissolved in 0.1 M sodium acetate buffer (pH 5.0), the test tube was incubated at 37°C for 15 min. Then the reaction was stopped with 1 mL of 0.2 M Na_2CO_3. For β-galactosidase, 0.3 mL of 2 mM *p*-nitrophenyl β-galactoside in 0.05 M sodium citrate buffer (pH 4.0) was added to 30 μL of the sample and incubated at 37°C for 30 min (Li and Li, 1972). The reaction was stopped with 0.9 mL of 0.2 M $Na_2B_4O_7$ (pH 9.8). Arylsulfatase activity was measured under the same conditions as BLG but using potassium *p*-nitrophenylsulfate as a substrate. The concentrations of *p*-nitrophenol were calculated from the absorbance at 400 nm by using free *p*-nitrophenol as a standard (ε = 17,600 mol^{-1}). The specific activity was defined as the amount in μmol of *p*-nitrophenol liberated per minute per mg of protein at 37°C. The protein concentration was determined using a DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard.

Kinetic studies were carried out by a microscale assay. The affinity-purified sample (12.5 μL, 10 μg/mL as protein) was added to 175 μL of 0.12 mM to 1.4 mM *p*-nitrophenyl-β-D-glucuronide dissolved in 200 mM sodium acetate buffer–50 mM NaCl (pH 5.0), in the presence or absence of various saccharides (1 μM to 130 mM), in the wells of a plastic microtiter plate at 4°C and mixed well. After incubation for 1 h at 37°C, 62.5 μL of 2 M glycine-NaOH (pH 10.0) was added to each well and mixed, and the absorbance at 405 nm was measured using a microplate reader.

Results

Affinity chromatography of BLG on various carbohydrate-immobilized Sepharose gels

Binding of BLG with various carbohydrate-immobilized gels was studied by affinity chromatography using short columns of various adsorbents, and the activities of the eluted BLG and other enzymes were measured. As shown in Figure 1A, when BLG was bound to an immobilized saccharo-1,4-lactone column, a competitive inhibitor (Kurtin and Schwesinger, 1985), 85% of the activity was eluted with buffer C, and the rest was eluted with buffer D. Surprisingly, *N*-acetyl-β-D-glucosaminidase (β-D-GlcNAcase) and *N*-acetyl β-D-galactosaminidase (β-D-GalNAcase) also bound to and were eluted from the saccharo-1,4-lactone column together with BLG by buffers C and D (Figure 1A). Similar chromatographic behavior of the enzymes was observed on a *p*-aminophenyl β-glucuronide-immobilized column, a substrate for BLG, as shown in Figure 1B, and more than 60% of the activity was eluted with buffer C, together with the other two enzymes. The specific activity and increase in BLG activity and recovery of activities of each enzyme in the fractions eluted from each adsorbent are summarized in Table I. Because of the unexpected chromatographic behavior of

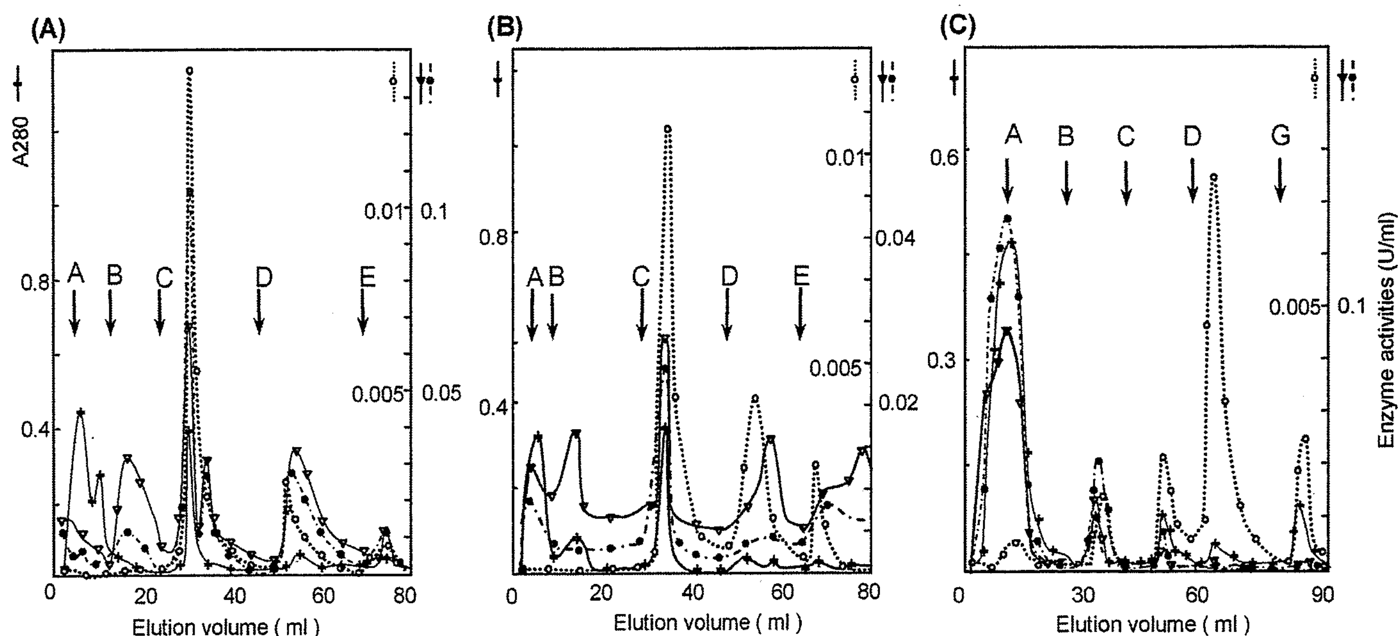


Fig. 1. Affinity chromatography of bovine liver β-glucuronidase (BLG) on a saccharo-1,4-lactone- (**A**), *p*-aminophenyl β-glucuronide- (**B**), and lactamyl- (**C**) Sepharose column. Commercial β-glucuronidase from bovine liver (6 mg from Worthington) was dissolved in buffer A (2 mM sodium acetate buffer [pH 7.0]–5 mM NaCl) and applied to each column (0.75 × 4.5 cm, Vt = 2 ml) at 4°C. The column was washed with buffer A and then sequentially eluted with buffer B (5 mM sodium acetate buffer, [pH 5.0]–5 mM NaCl), buffer C (10 mM sodium acetate buffer [pH 5.0]–5 mM NaCl), buffer D (25 mM sodium acetate buffer [pH 5.0]–5 mM NaCl), buffer E (50 mM sodium acetate buffer [pH 5.0]–5 mM NaCl), buffer G (0.2 M sodium acetate buffer [pH 7.0]–0.2 M NaCl), and 0.1 M sodium borate buffer (pH 7.0). The eluted fractions (2 ml each) were measured at Abs. 280 nm (+), β-D-glucuronidase (O), *N*-acetyl-β-D-glucosaminidase (β-D-GlcNAcase) (●), and *N*-acetyl β-D-galactosaminidase (β-D-GalNAcase) (▼).

the enzymes on these adsorbents, seven other kinds of carbohydrate adsorbents were compared for the ability to separate BLG.

As shown in Figure 1C, lactamyl–Sepharose gave the best separation of BLG from β-D-GlcNAcase and β-D-GalNAcase. BLG bound to the lactamyl–Sepharose column, and 70% of the bound BLG was eluted by the buffer D. In the fractions eluted with buffer D (fraction D), activities of β-D-GlcNAcase and β-D-GalNAcase drastically decreased, and activities of β-D-galactosidase and arylsulfatase were not detected (data not shown). Specific activity of BLG in fraction D increased by 20-fold (Table I). From the other adsorbents summarized in Table I, most BLG activity was eluted by buffer C together with β-D-GlcNAcase and β-D-GalNAcase presenting to various extents. Comparison of the ligand structures of lactamyl– and melibiamyl–Sepharose indicated that β-linked-D-galactopyranosyl residue may contribute to the strong binding and separation of BLG from other enzymes. Based on these observations, the lactamyl–Sepharose column was used for further examination.

Binding characteristics of BLG to lactamyl-Sepharose

The characteristics of BLG binding to lactamyl–Sepharose were studied by microtube assays. As shown in Figure 2A, binding of BLG to lactamyl–Sepharose was maximum at pH 6 and decreased at acidic and basic pH. The amount of bound BLG at pH6 was twice as that at pH 7 in sodium acetate buffer. Based on this result, the binding procedure was hereafter performed at pH 6 (buffer A′) instead of pH 7 (buffer A) to increase the binding capacity.

The abilities of various reagents to elute the bound BLG from lactamyl–Sepharose are summarized in Figure 2B. Buffer G and 5 mM EDTA eluted BLG almost quantitatively, indicating that electrostatic interaction contributes to the binding. Despite the effect of EDTA, addition of Ca^{2+} to the incubation buffer inhibited the binding (data not shown), suggesting that the binding is not Ca^{2+}-dependent and that elution with EDTA may be because of removal of other metal cations or an electrostatic effect. Various sugars including 0.1 M lactose and galactose did not elute more than 20% of the bound BLG from lactamyl–Sepharose (Figure 2B). To examine whether the binding of BLG to lactamyl–Sepharose is a ligand-specific interaction, GHAG, a lactose derivative that is analogous to the ligand structure of lactamyl–Sepharose, was synthesized and studied for ability to elute BLG.

Structural analysis of GHAG and its BLG elution activity

The structure of GHAG was confirmed by NMR. As summarized in Table II, ^{1}H- and ^{13}C-chemical shifts and the coupling constants (data not shown) of the galactosyl residue were in good agreement with typical ones obtained in Me_2SO-d_6 (Iida-Tanaka and Ishizuka, 2000). The chemical shifts of the C-1 carbon in the glucosyl residue, however, did not resonate in the anomeric region, but at 52.7 p.p.m. in the region of the carbon bound by an amino group, -NH-. In addition to the observation of geminal H1 protons, H1a

Table I. Affinity chromatography of β-glucuronidase on various carbohydrate-immobilized Sepharose columns

Adsorbent	Structures of immobilized saccharides	Eluent	Specific activity (mU/mg)	Increase (fold)	Recovery of activity (%) 0 50 100
Saccharo 1,4-lactone-	O=C—NH(CH$_2$)$_3$—NH(CH$_2$)$_3$—NHCH$_2$—CH(OH)—CH$_2$—O—	C	78	12	
		D	48	7	
p-Aminopheny-glucuronide-	—NH(CH$_2$)$_3$—NHCH$_2$—CH(OH)—CH$_2$—O—	C	82	12	
		D	138	20	
		E	79	11	
Lactamyl-	CH$_2$—NH—CH$_2$—CH(OH)—CH$_2$—O—	C	19	3	
		D	138	20	
		G	40	6	
Maltamyl-	CH$_2$—NH—CH$_2$—CH(OH)—CH$_2$—O—	C	80	12	
Melibiamyl-	CH$_2$—NH—CH$_2$—CH(OH)—CH$_2$—O—	C	93	14	
Glucamyl-	CH$_2$—NH—CH$_2$—CH(OH)—CH$_2$—O—	C	58	8	
		D	51	8	
Galactamyl-	CH$_2$—NH—CH$_2$—CH(OH)—CH$_2$—O—	C	45	7	
		D	58	8	
N-Acetylchondrosine-	CH$_2$—NH—CH$_2$—CH(OH)—CH$_2$—O—	C	114	17	
		D	57	8	
Tri*N*-Acetychitotriamyl-	CH$_2$—NH—CH$_2$—CH(OH)—CH$_2$—O—	C	44	7	
		F	50	5	

Commercial bovine liver β-glucuronidase (BLG) (6 mg) was dissolved in 1 mL of 2 mM sodium acetate buffer (pH 7.0)-5 mM NaCl (buffer A) and applied to each column (0.75 × 4.5 cm, Vt = 2 ml). The columns were washed with buffer A and then sequentially eluted with 5 mM sodium acetate (pH 5.0)-5 mM NaCl (buffer B) and buffer C–G, as described in the text. The eluted fractions (2 mL/fraction) were measured for enzyme activities and absorbance at 280 nm. The specific activity is expressed as mU/mg, and 1 U produces 1 μmol of the chromogen, *p*-nitrophenol, per minute at 37°C and pH 5.0 under the conditions described in the text. Recovery of activity (%) is expressed by bar, BLG (▬), β-D-GlcNAcase (▪ ▪ ▪), or β-D-GalNAcase activities (······).

and b, the above result suggests that the aldehyde group in the glucosyl residue was reductively aminated to produce the structure of -CH$_2$-NH-. Long-range connectivity of ethanol-C1/Glc-H1s and Glc-C1/ethanol-H1s was observed in the HMBC spectrum.

As shown in Figure 2B, 77% of the BLG activity was eluted from lactamyl–Sepharose with 25 mM GHAG, with small amounts of β-galactosidase and β-GlcNAcase, indicating that most of the BLG bound to lactamyl–Sepharose via the ligand-specific interaction and that GHAG eluted it more effectively than lactose and galactose owing to its structural similarity to the ligand of lactamyl–Sepharose.

Large-scale purification by affinity chromatography on a lactamyl–Sepharose column

The binding of BLG and subsequent washing of the column were performed with buffer A′ at pH 6.0, and BLG was eluted by buffers at pH 5.0. As shown in Figure 3A, all the applied BLG bound to the column at pH 6.0, and a major part of the bound BLG activity was eluted with buffer C (10 mM sodium acetate–5 mM NaCl [pH 5.0]) and buffer E′ (25 mM sodium acetate–25 mM NaCl [pH 5.0]), at slightly lower salt concentrations than those required for elution when the binding was performed at pH 7.0 (cf. Figure 1C).The specific activity of the buffer C-eluted fraction (fraction C) showed a 20 times increase, and the contaminating β-galactosidase and β-D-GalNAcase markedly decreased. Increasing the ion concentration to 200 mM sodium acetate buffer–200 mM NaCl (pH5.0) (buffer G) did not elute any proteins (data not shown). As shown in Figure 3B, GHAG eluted BLG as a single peak from a lactamyl–Sepharose affinity chromatography column.

The buffer C- and GHAG-eluted fractions showed similar protein patterns on SDS–PAGE (Figure 3C). Zymography of both the fractions indicated the presence of an active

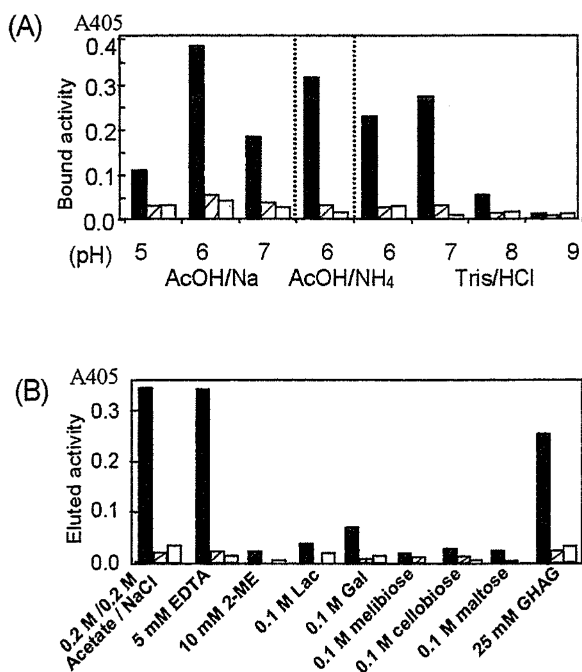
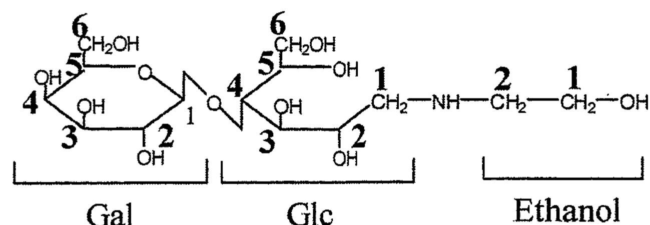


Fig. 2. Optimum binding and elution condition of bovine liver β-glucuronidase (BLG) to lactamyl–Sepharose. (**A**) Lactamyl–Sepharose 4B (0.1 g) and BLG dissolved in 0.3 ml of various buffers (3.3 mg/ml): 2mM sodium acetate buffer (pH 5–6), ammonium acetate buffer (pH 6), or Tris–HCl (pH 6–9), each containing 5 mM NaCl, were incubated in 1.5 ml tube at 4°C for 4 h. After centrifugation, the supernatant was removed, the gel was washed three times with the same buffer, then the bound BLG was eluted with buffer G (0.2 M sodium acetate buffer [pH 5.0]–0.2 M NaCl) for 4 h at 4°C, and measured for its enzyme activity. (**B**) To study the elution from lactamyl–Sepharose gel 0.2 ml of BLG solution in buffer A′ (2 mM sodium acetate buffer [pH 6.0]–5 mM NaCl) at 5 mg/mL concentration was incubated with 0.1 g of lactamyl–Sepharose gel. After centrifugation, the supernatant was removed, the gel was washed three times with the same buffer, and then the bound proteins were eluted with incubation in 0.2 mL of each elution buffer or eluate in buffer A′ for 4 h at 4°C. The enzyme activities of washed and eluted fractions were measured separately.

Table II. ^{1}H- and ^{13}C-chemical shifts (p.p.m.) of 1-deoxy-4-*O*-β-D-galactopyranosyl-1-[(2-hydroxyethyl)amino]-D-glucitol (GHAG)



Residue	^{1}H-chemical shift (p.p.m.)		^{13}C-chemical shift (p.p.m.)	
Ethanol	H1a	3.45	C1	60.1
	H1b	3.48	C2	52.0
	H2a	2.57		
	H2b	2.57		
Glc	H1a	2.40	C1	52.7
	H1b	2.69	C2	69.6
	H2	3.84	C3	71.1
	H3	3.52	C4	81.3
	H4	3.52	C5	71.1
	H5	3.67	C6	62.5
	H6a	3.56		
	H6b	3.59		
Gal	H1	4.21	C1	104.4
	H2	3.30	C2	71.2
	H3	3.26	C3	73.5
	H4	3.59	C4	68.9
	H5	3.33	C5	75.9
	H6a	3.46	C6	61.5
	H6b	3.54		

^{1}H- and ^{13}C-signals were assigned based on the double quantum-filtered correlated spectroscopy (DQF-COSY), total correlated spectroscopy (TOCSY), and heteronuclear multiple-quantum coherence (HMQC) spectra, and the relationships between the residues were confirmed by heteronuclear multiple-bond correlation (HMBC) experiment.

form of BLG at the migration position corresponding to 160 kDa, whereas the 78-kDa subunit monomer was shown to be inactive (Figure 3C, lanes e–f). The reported molecular size of the active BLG in solution, 290 kDa, on sucrose density gradient centrifugation was considered to be a tetramer of the 78-kDa subunit (Himeno *et al.*, 1974). The reason for the discrepancy between the reported and observed molecular mass is yet unclear, but the possibility that the 160-kDa band on SDS–PAGE is a tetramer cannot be denied. Another possibility is that the 160-kDa band corresponded to a dimer whose activity was recovered after removing SDS. In this case, whether active BLG forms a tetramer in gel or remains a dimer is unknown. As shown in Figure 3C, the buffer C eluted fraction more of the active form of BLG than of the monomer, whereas the monomer is predominant in the GHAG-eluted fraction. The BLG activity in fraction E′ in Figure 3A was lost after PAGE (data not shown) possibly because a factor that stabilizes the active form may have been removed in fraction E′ and the activity could not be restored after PAGE. Based on these observations, fraction C was subjected to further purification because it still contained a few other protein bands. BLG purchased from Sigma gave essentially the same elution profile on lactamyl–Sepharose column and protein bands on SDS–PAGE as those of BLG obtained from Worthington (data not shown).

Ion exchange HPLC of BLG on DEAE-5PW

Fraction C was separated into five fractions by NaCl gradient on ion-exchange HPLC on a DEAE-5PW column, as shown in Figure 4A. Peak b exhibited β-glucuronidase activity with trace activity of other enzymes (Figure 4B), and a single protein band corresponding to the monomeric BLG on SDS–PAGE under reducing conditions (Figure 4C). β-Galactosidase activity was detected mainly in peak c. In the course of the study, gel filtration HPLC was also tried for further purification of fraction C. In that case, β-galactosidase was separated from the

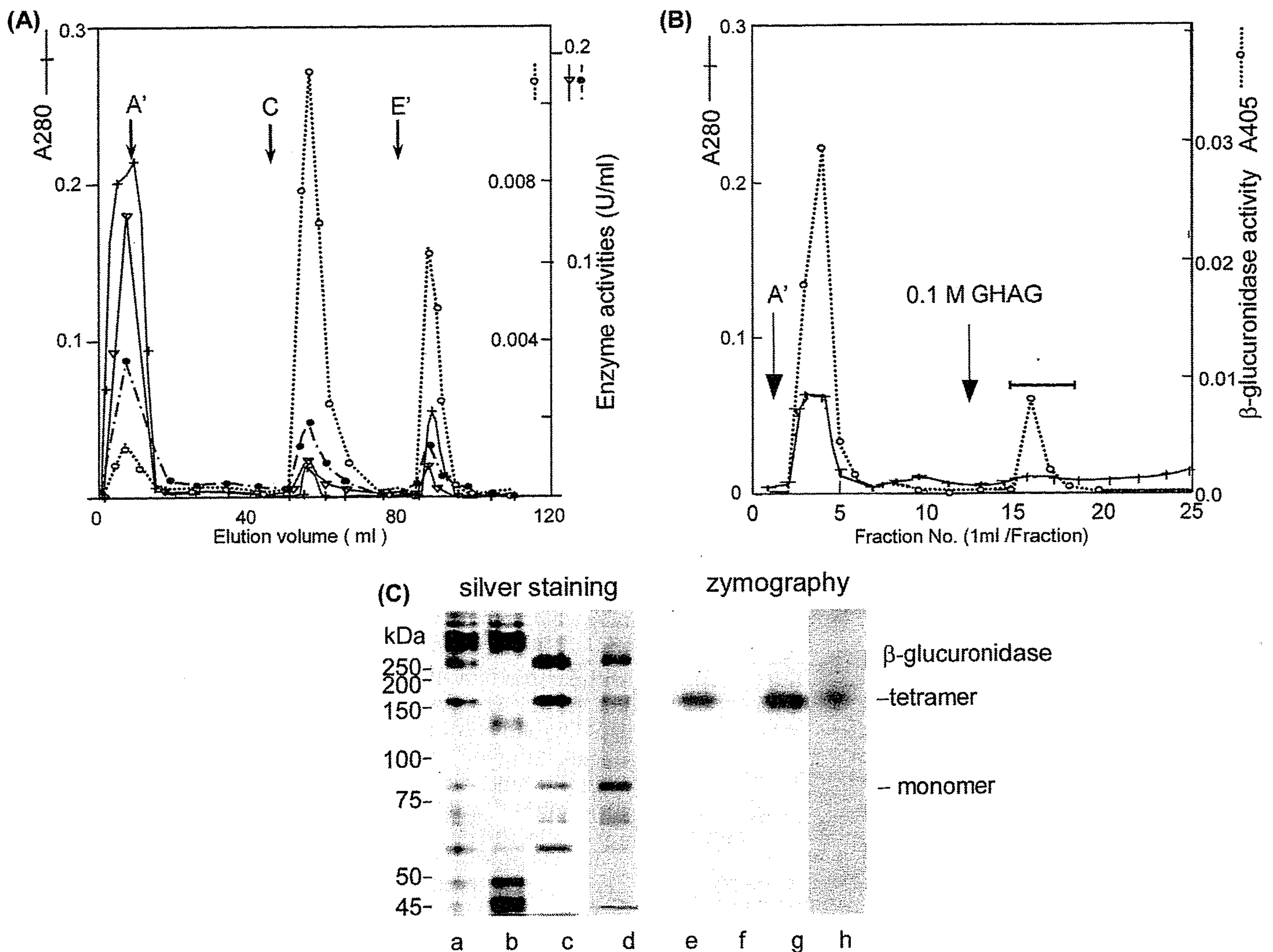


Fig. 3. Affinity chromatography of bovine liver β-glucuronidase (BLG) on a lactamyl–Sepharose column. (**A**) BLG (Worthington, 15 mg) was mixed with 13 ml of lactamyl-Sepharose 4B in buffer A′ and incubated for 5 h at 4°C with gentle shaking; then the gel was poured into a column (1.5 × 8 cm) and washed with buffer A′. BLG was successively eluted with buffer C and buffer E′. (**B**) BLG (sigma, 1mg) in buffer A′ was applied onto a lactamyl column (0.75 × 4 cm) and washed with buffer A′. BLG was eluted with 0.1 M 1-deoxy-4-*o*-β-D-galactopyranosyl-1-[(2-hydroxyethyl)amino]-D-glucitol (GHAG). Eluted fractions were monitored at 280 nm and by enzyme activity. (**C**) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) patterns of fractions eluted from the lactamyl–Sepharose column. Silver staining (lanes a–d) and zymography for detecting BLG activity (lanes e–h). Lanes a and e, crude BLG; lanes b and f, the washed fraction (fraction A′); lanes c and g, fraction C; lanes d and h, the 0.1 M GHAG-eluted fraction.

BLG peak, but β-glucosaminidase and β-galactosaminidase activities remained in the BLG peak (data not shown).

Effect of free saccharides on BLG activity

BLG activity was measured using fraction C from lactamyl affinity chromatography as BLG in the presence or absence of various saccharides (1 μM to 130 mM) at 0.12 mM to 1.4 mM substrate concentrations. The results are summarized in Table III, and examples of the double reciprocal of Lineweaver–Burk plots are shown in Figure 5. According to the Lineweaver–Burk plot analyses, BLG is dependent on the lactose concentration, as shown in Figure 5A. At 1 mM lactose, the interception of the *x*-axis (1/Km) of the plot was unchanged from that of the control, indicating that lactose binds to both the free enzyme and the enzyme–substrate complex. The result suggests that the lactose-binding site of BLG is different from the catalytic site. On the contrary, at lactose concentrations higher than 13 mM, the plot showed a tendency toward competitive inhibition with the interception with the *y*-axis (1/Vmax) unchanged. In contrast, saccharo-1,4-lactone (1 μM) and D-glucuronic acid (1 mM) showed a typical competitive inhibition, as shown in Figure 5B. Cellobiose showed a very weak tendency toward noncompetitive activation. On the contrary, other tested saccharides, maltose (Figure 5C), D-glucose and D-galactose mannose-6-phosphate, *N*-acetylglucosamine, and *N*-acetylneuraminic acid (1 mM), did not have a significant or integrative effect on the enzyme activity, although BLG bound to the affinity adsorbents of some of these saccharides.

Binding studies of BLG with glycoproteins and lipids

The binding activities of purified BLG to biotinyl glycoprobes were studied at pH 7.5 and pH 5.0 by solid-phase binding assay. At pH 5, BLG did not bind to any of tested

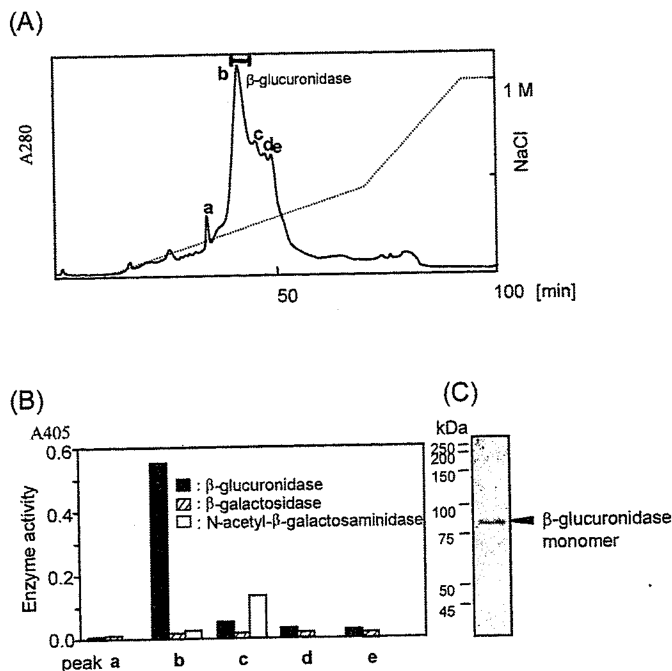


Fig. 4. Purification of fraction C by ion exchange high-performance liquid chromatography (HPLC). (**A**) The buffer C-eluted fraction from lactamyl affinity chromatography in Figure 3A was applied to ion-exchange HPLC on a DEAE-5PW column (21.5 × 150 mm, Tosoh Corp.). Elution was performed at a flow rate of 1.0 mL/min at room temperature with 10 mM TB (pH 7.5). After injection of the sample, the NaCl concentration was increased from 0 to 0.4 M, and then 0.4–1.0 M in 100 min. (**B**) Enzyme activities of each peak fraction. The peak fractions were pooled and measured for enzyme activity. Bars represent the activities of bovine liver β-glucuronidase (BLG) (■), β-D-galactosidase (▨), or *N*-acetyl-β-D-glucosaminidase (β-D-GlcNAcase) activities (□). (**C**) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of peak b under reducing conditions.

glycoproteins and lipids (data not shown). As shown in Figure 6A, immobilized BLG bound to asialofetuin better than fetuin and to asialoagalactofetuin but not to transferrin, asialotransferrin, ovalbumin, or BSM at pH7.5. It suggested that BLG recognized the exposed galactose residues of triantennary complex-type *N*-glycans but not the biantennary complex-type of asialotransferrin or the high Man or hybrid type of ovalbumin. BLG bound to lactosyl ceramide but not to Glc-cer, Gal-cer, ceramide, or sulfatide (Figure 6B). The direct binding of BLG to the sugar residues was demonstrated using sugar-BP probes. As shown in Figure 6C), BLG bound to *N*-acetyllactosamine-BP better than to the Lac-BP probe. Taken together, BLG was shown to bind to the *N*-acetyllactosamine or lactosyl sequence of glycoconjugates at neutral pH but not at pH 5.

Discussion

In this study, BLG was demonstrated for the first time to have binding activity toward lactose and *N*-acetyllactosamine sequences. BLG was effectively separated from contaminating glycosidases by affinity chromatography on a lactamyl–Sepharose column, and the specific activity was increased by 20-fold during one-step affinity chromatography (Table I). Non- or uncompetitive regulation of BLG with lactose indicated that the lactose-binding site is different from the substrate-binding site (Table III). Purified BLG bound best to the glycoconjugates possessing a nonreducing terminal *N*-acetyllactosamine/lactose such as asialofetuin and lactosylceramide (Figure 6), which is attributable to the carbohydrate-binding activity of BLG toward lactose/lactosamine structures.

Preparation of BLG has required multiple purification steps including heat denaturation of proteins (Ho, 1991), or fractionation with organic solvents (Himeno *et al.*, 1974), in combination with several chromatography steps to dissociate complex of BLG with other lysosomal proteins. This study provided convenient protocol to isolate BLG from other contaminating enzymes under mild conditions and

Table III. Effect of various sugars on bovine liver β-glucuronidase activity

Enzyme	Saccharide	Vmax	Kmapp	Mode of effect
Control		1.2×10^2	0.6	
Lactose	1 mM	2.0×10^2	0.6	Noncompetitive activation
	13 mM	1.5×10^2	1.1	Competitive inhibition
	130 mM	1.2×10^2	0.8	
Cellobiose	1 mM	1.3×10^2	0.6	
	10 mM	1.3×10^2	0.6	
	100 mM	1.4×10^2	0.6	
Melibiose	1 mM	1.4×10^2	0.6	
	13 mM	0.8×10^2	0.3	
	130 mM	0.8×10^2	0.4	
Saccharo-1,4-lactone	1 μM	1.2×10^2	2.0	Competitive inhibition
D-Glucuronic acid	1 mM	1.2×10^2	0.9	Competitive inhibition
Glc, Gal, GlcNAc, Man, maltose, Man-6P, NeuAc	No effect			

β-Glucuronidase activity was measured in the presence of free saccharide substrate concentrations (pH 5.0) of 0.1–1 mM and analyzed by a double reciprocal Lineweaver–Burk plot. Examples of the plots are shown in Figure 5.

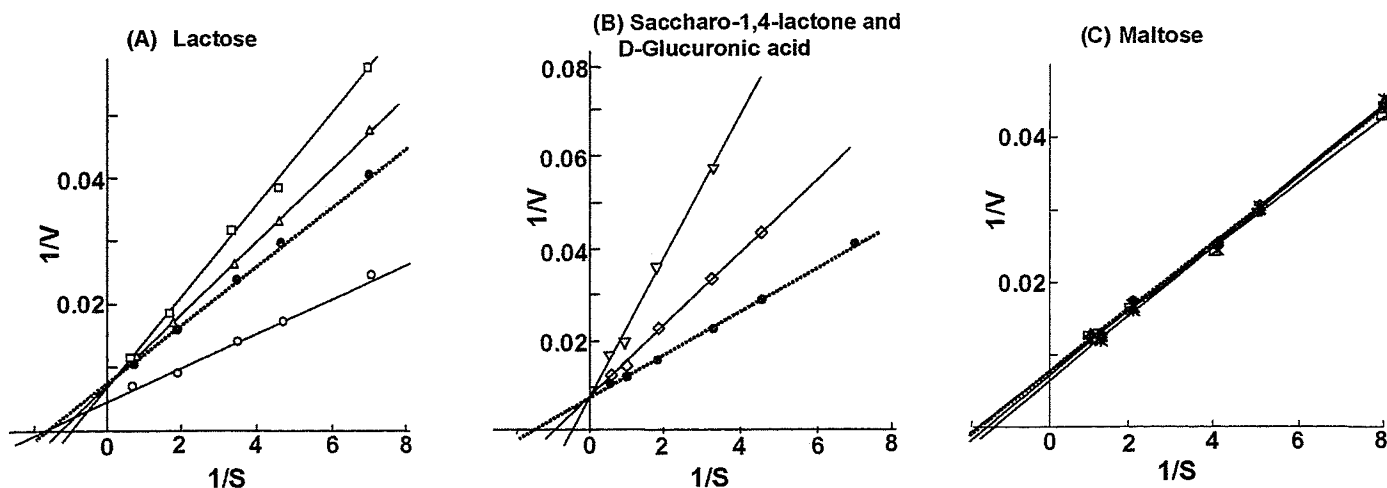


Fig. 5. Effect of free sugars on bovine liver β-glucuronidase (BLG) activity. Activity of BLG was measured using *p*-nitrophenyl-β-D-glucuronide as the substrate in the presence of various concentrations of sugars. (A) D-Lactose at (○) 1 mM, (□) 13 mM, and (△) 130 mM; . (B) (▽) 1 μM Saccharo-1,4-lactone, or (◇) 1 mM D-glucuronic acid; (C) D-maltose at (□) 1 mM, (▲) 10 mM, (*) 100 mM, and (●) control without sugars.

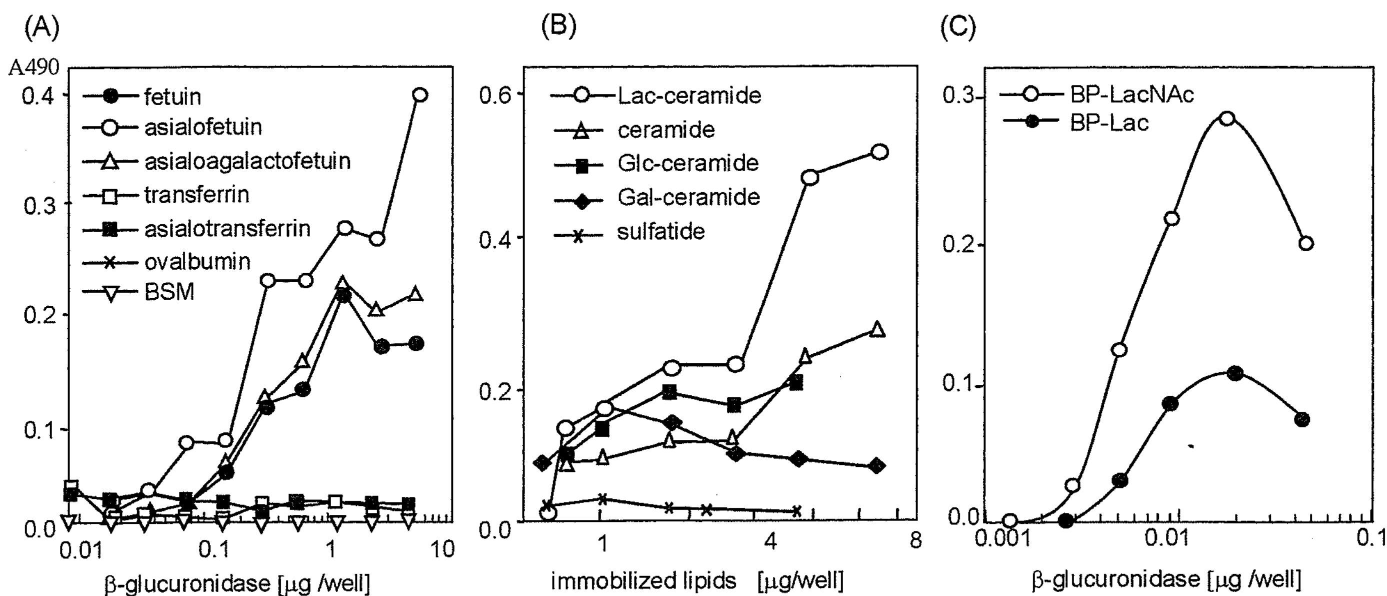


Fig. 6. Interaction of bovine liver β-glucuronidase (BLG) with biotinyl glycoproteins (A) and lipids (B) at pH7.5. (A) A solution of BLG was serially diluted in 10 mM Tris–HCl–150 mM NaCl (pH 7.5) (TBS) and immobilized on microtiter plates. Biotinyl glycoprotein probes were added to the wells, and a solid phase assay was performed as described in the text. Glycoproteins used were fetuin (—●—), asialofetuin (—○—), asialogalactofetuin (—△—), transferrin (—□—), asialotransferrin (—■—), ovalbumin (—✕—) and BSM (—▽—). (B) Binding assay of biotinylated BLG to immobilized glycolipids. Lactosyl-ceramide (—○—), galactosyl-ceramide (—◆—), glucosyl-ceramide (—■—), ceramide (—△—), and sulfatide (—✕—) were immobilized on microtiter plates with serial dilutions in methanol, respectively. Biotinylated BLG was added to the each well, and bound BLG was detected with ABC-HRP as described in the text. (C) BLG was immobilized at various concentrations on a microtiter plate, biotinyl polymer (BP)-sugar probes were added to the wells, and a solid phase assay was performed as described in the text. BP-LacNAc (—○—) and BP-Lac probes (—●—).

furthermore opens new insights into the biological functions of the carbohydrate-specific interaction of β-glucuronidase. The observations that BLG was eluted more effectively with GHAG than lactose and galactose from lactamyl–Sepharose column (Figure 2B), and BLG bound to *N*-acetyllactosamine-BP better than to the Lac-BP probe (Figure 6C) suggest the significance of *N*-acetyllactosamine structure present in the glycoconjugates as the biological ligand for BLG.

Interaction of BLG with carbohydrate ligands in lysosome

Although the lactamyl-binding activity of BLG was maximal at pH 6–7 and weakened to one-third at pH 5, the physiological pH of lysosomes (Figure 2), lactose noncompetitively activated BLG at 1 mM at pH 5, indicating that the carbohydrate-binding activity is exhibited even at pH 5. Therefore, the lactose binding may contribute to regulation of the enzyme activity in lysosomes. As a candidate for lysosomal ligands other than free lactose, BLG is supposed

to interact with lysosome-associated membrane glycoproteins 1 and 2 (Lamp1 and 2), major carriers for poly-*N*-acetyllactosamines (Laferte and Dennis, 1989), because the repeating *N*-acetyllactosamine sequence may enhance the affinity for BLG by a multivalent effect. However, we did not detect the binding of BLG to polylactosaminoglycans of human erythrocyte Band 3 glycoprotein (Fukuda *et al.*, 1984) at pH 5 (data not shown) by solid phase assay, while BLG did bind to it at pH 7 (Figure 6 and our unpublished data). Whether interaction between BLG and the glycoconjugates in lysosomes is possible is unknown at this point.

Biological function of the carbohydrate binding of BLG

Alternatively, the lactose/*N*-acetyllactosamine-binding activity may play a specific role at neutral pH in the endoplasmic reticulum (ER) and Golgi apparatus during glycoprotein maturation and in extracellular matrices after secretion. One possibility is that the lactose/*N*-acetyllactosamine-binding activity of BLG may be involved in the formation of the active tetrameric form because BLG contains a considerable amount of complex-type asialoglycans (Himeno *et al.*, 1974), and a wild-type BLG produced in the presence of tunicamycin was inactive (Shipley *et al.*, 1993).

In the ER and Golgi apparatus, various glycoconjugates are involved in the biosynthetic and lysosome-sorting processes of BLG. For example, phosphodiester α-GlcNAcase, which catalyzes the second step of attachment of the Man6P signal on BLG, is one of the *N*-glycosylated glycoproteins. The active site and the recognition motif of phosphotransferase have been elucidated on human β-glucuronidase (Jain *et al.*, 1996), but recognition motif of α-GlcNAcase has not yet been clarified. Because only a limited number of the α-GlcNAc phosphodiesters that are attached by GlcNAc-1-phosphotransferase are hydrolyzed by α-GlcNAcase to generate Man6P monoester (Natowicz *et al.*, 1982), the carbohydrate-binding activity of BLG could play a role in accessing phosphodiester α-GlcNAcase.

A carbohydrate-binding activity of a macromolecule-degrading enzyme might help localize the enzyme on an appropriate scaffold to exhibit catalytic action efficiently and stably *in vivo*. Lysosomal enzymes, including BLG, are released by the fusion of whole lysosomes with the plasma membrane into the synovial fluid in inflammatory joint diseases and in the invasion by metastatic tumor cells to focal dissolution of the extracellular matrix of surrounding tissues or penetration of endothelial membranes. (Sloane *et al.*, 1986; Andrei *et al.*, 2004). The observation that highly metastasizing tumor cells express more poly-*N*-acetyllactosamine in lysosomes than do their normal and poorly metastasizing counterparts (Dennis *et al.*, 1999, Chakraborty and Pawelek, 2003) supports the idea that the interaction of BLG with poly-*N*-acetyllactosamines may well be involved in concentrating the hydrolases and catalyzing the substrate hydrolysis efficiently at the cell surface when secreted outside the cell. A benefit for BLG of anchoring to poly-*N*-acetyllactosamines is cooperation with other hydrolases because the carbohydrate-binding activity is shared among several other lysosomal exoglycosidases, as shown in this study. β-GalNAcase and β-GlcNAcase exhibited considerable binding activity to affinity adsorbents immobilized with saccharides other than their substrates, such as galactose and tri*N*-acetylchitotriose (Table I). Several extracellular matrix glycoproteins that have polylactosaminoglycans, such as laminin, integrin, and neuronal glycoproteins, could provide a scaffold for lysosomal glycosidases secreted from tumor cells, so that the enzymes can act on substrates cooperatively. Such a hypothetical tie-up of glycan-degrading exoglycosidases on the poly-*N*-acetyllactosaminoglycan chain will increase the efficiency of the degradation of their common substrates. Those possibilities are under investigation in our laboratory.

Conflict of interest statement

None declared.

Abbreviations

BLG, bovine liver β-glucuronidase; BP, biotinyl polymer; GHAG, 1-deoxy-4-*o*-β-D-galactopyranosyl-1-[(2-hydroxyethyl)amino]-D-glucitol; HPLC, high-performance liquid chromatography; $Me_2SO\text{-}d_6$, $^2[CH_3]_2SO$; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TBS, 10 mM Tris–HCl–150 mM NaCl (pH 7.5); X-, 5-bromo-4-chloro-3-indolyl-; β-D-GalNAcase, *N*-acetyl β-D-galactosaminidase; β-D-GlcNAcase, *N*-acetyl-β-D-glucosaminidase.

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Changing Distribution of Norovirus Genotypes and Genetic Analysis of Recombinant GIIb Among Infants and Children With Diarrhea in Japan

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A total of 402 fecal specimens collected during July 2003–June 2004 from infants and children with acute gastroenteritis, encompassing five localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) of Japan, were tested for the presence of norovirus by RT-PCR. It was found that 58 (14.4%) fecal specimens were positive for norovirus. Norovirus infection was detected throughout the year with the highest prevalence in December. Norovirus GII was the most predominant genogroup (98.3%; 57 of 58). The genotypes detected in this study were GI/4, GII/2, GII/3, GII/4, and GII/6. Of these, NoV GII/3 (known as the Arg320 virus cluster) was the most predominant genotype (43.9%), followed by NoV GII/4 (the Lordsdale virus cluster; 35.1%) and others. Two norovirus strains clustered with a "new variant designated GIIb" and a "new variant of GII/4" were found circulating in Japan for the first time. It was interesting to note that NoV GIIb and NoV GII/3 appeared to be the recombinant strains and the recombination site was demonstrated at the overlap of ORF1 and ORF2. The majority (96%) of the dominant norovirus strains were identified as the recombination of GII/3 capsid and GII/12 polymerase. The recombination in the NoV GIIb capsid gene at the breakpoint located at P1 domain was also identified. Obviously, NoV GIIb isolate in Japan had double recombination. This is the first report demonstrating the existence of different "new variants" co-circulating in Japanese infants and children with acute gastroenteritis. ***J. Med. Virol. 78:971–978, 2006.***
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KEY WORDS: PCR; norovirus; recombination; Japan

INTRODUCTION

Norovirus (NoV) is recognized as a significant global enteropathogen, being a major cause of sporadic cases as well as of outbreaks of acute gastroenteritis in humans in various epidemiological settings, such as restaurants, schools, day-care centers, hospitals, nursing homes, and cruise ships [Chiba et al., 1979; McEvoy et al., 1996; Vinje et al., 1997; McIntyre et al., 2000]. The virus can be transmitted by food-borne, water-borne, air-borne, person-to-person spread by close contact and there might be some other unknown modes [Matson, 1994; Bon et al., 1999; Marks et al., 2000; Lopman et al., 2002; Oh et al., 2003]. NoV is highly infectious and spreads by ingestion of contaminated food such as oysters and water. These characteristics make NoV a major public health concern [Kageyama et al., 2004]. NoV is the distinct genus within the family *Caliciviridae*. The prototype strain of NoV is the Norwalk virus (Hu/NoV/Norwalk virus/1968/US), which was originally discovered from an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio, USA in 1968. NoV possesses a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The NoV genome contains three open reading frames (ORFs). ORF1

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encodes the non-structural proteins, including the RNA-dependent RNA polymerase (RdRp) while ORF 2 encodes the capsid protein (VP1) and ORF3 encodes a small capsid protein (VP2). Based on the sequence analysis of the capsid gene, NoV is divided into genogroups I and II, both known to infect humans. A recent study indicated that NoV GI and NoV GII could be classified into 14 and 17 genotypes, respectively [Kageyama et al., 2004]. The first naturally occurring recombinant NoV was the prototype Snow Mountain virus [Hardy et al., 1997]. Later several recombinant NoV strains causing sporadic cases and outbreaks of acute gastroenteritis were reported [Jiang et al., 1999a; Schreier et al., 2000; Katayama et al., 2002]. RNA recombination is one of the major driving forces of viral evolution [Worobey and Holmes, 1999]. To date, NoV is still uncultivable by standard culture methods with different cell lines. However, either VP1 alone or both VP1 and VP 2 could be expressed using recombinant baculovirus forming virus-like particles (VLPs) that are similar morphologically and antigenically to the native virion [Jiang et al., 1995]. Seroepidemiologic studies indicated a worldwide distribution of NoV. Moreover, it was found that serum antibody level to NoV was lowest in the first year of life and then rising after 2 years of age [Lopman et al., 2002; Dai et al., 2004; Peasey et al., 2004].

The objectives of this study were: to determine the incidence of NoV infections in infants and children with acute gastroenteritis in five different localities of Japan during 2003 and 2004; to characterize the genogroup and genotype of the detected NoV; and to describe the genetic diversity among them. Additionally, the age-related and seasonal distributions of NoV infection were determined.

MATERIALS AND METHODS

Fecal Specimens

A total of 402 fecal specimens were collected from infants and children with acute gastroenteritis, encompassing five different localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) of Japan during the period of July 2003–June 2004. Of these, 19 specimens were from Osaka, 22 from Sapporo, 22 from Tokyo, 45 from Saga, and 294 from Maizuru. The ages of the subjects were ranged from 2 months to 11 years with the median of 2.5 years (29 months). The majority (75%) of the affected children were aged less than 36 months and about half (54%) were male. The 10% fecal suspension was prepared in distilled water and clarified by centrifugation at 10,000*g* for 10 min. The supernatant was collected and stored at −30°C until use.

Extraction of Viral Genome

The viral genomes were extracted from 140 µl of 10% fecal suspensions using the QIAamp viral RNA Mini Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions.

Reverse Transcription (RT)

For reverse transcription (RT), 7.5 µl of extracted viral genome was added to the reaction mixture containing 2.05 µl of 5× first strand buffer (Invitrogen, Carlsbad, CA), 0.75 µl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.75 µl of 10 mM DTT (Invitrogen), 0.75 µl (200 U/µl) of superscript reverse transcriptase III (Invitrogen), 0.375 µl (1 µg/µl) of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 µl (33 U/µl) of RNase inhibitor (Toyobo, Osaka, Japan), and 2.325 µl MilliQ water. The total volume of reaction mixture was 15 µl [Yan et al., 2003]. The RT step was carried out at 50°C for 1 hr, followed by 99°C for 5 min and then held at 4°C.

Polymerase Chain Reaction (PCR)

The NoV genogroups were identified by PCR method using specific primers as described [Yan et al., 2003]. Two pairs of specific primers G1SKF (CTGCCCGAATTYG-TAAATGA) and G1SKR (CCAACCCARCCATTRTACA), and COG2F (CARGARBCNATGTTYAGRTGGATGAG) and G2SKR (CCRCCNGCATRHCCRTTRTACAT) [where B was C, G or T; H was A, C or T; N was any base; R was A or G, and Y was C or T] that amplify capsid gene of NoV were used to detect NoV GI and NoV GII, respectively. These primers were specifically generated two different sizes of amplicons of 330 and 387 bp for NoV GI and NoV GII, respectively. The RNA polymerase gene of NoV was also amplified to identify the recombinant strain of NoV using the primers as described [Jiang et al., 1999b; White et al., 2002]. The PCR was carried out with 2.5 µl of cDNA in 22.5 µl of the reaction mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI), dNTPs (2.5 mM/µl), primers (33 µM), Taq DNA polymerase (5 U/µl) (Promega, Madison, WI) and MilliQ water. The PCR was performed at 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 7 min, and then held at 4°C. The full length of capsid and polymerase regions were amplified with a newly designed specific primer NVPOLR/A (GAT GAG GTT CTG ATG AGA) and the specific primers reported by Vinje et al. [2000] and Kawamoto et al. [2001]. The PCR was performed at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and a final extension at 72°C for 7 min, and then held at 4°C.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min and then visualized under ultraviolet (UV) light. The results were recorded by photography.

Nucleotide Sequencing and Phylogenetic Analysis

The nucleotide sequences of PCR products (DNA) positive for NoV were determined using the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster

City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). Phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment data sets were generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). SimPlot software (Version 1.3) was used to compare the recombinant NoV sequences [Lole et al., 1999]. The nucleotide sequences of NoV strains 5424/03/Saga/JP and 5017/04/Maizuru/JP had been submitted to the DDBJ DNA/GenBank database and the assigned accession numbers were AB242256 and AB242257, respectively. Reference NoV strains and accession numbers used in this study were as follows: Manchester (X86560), Saitama T53GII/02/JP (AB112260), Girllington (AJ277606), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Hillington (AJ277607), Alphatron (AF195847), Toronto (U02030), Seacroft (AJ277620), Leeds (AJ277608), Lordsdale (X86557), Idaho Falls/96/US (AY054299), Fayetteville/1998/US (AY113106), Erfurt/546/00/DE (AF42118), M7/99/US (AY130761), Saitama U1 (AB039775), Camberwell (AF145896), Snow Mountain (U70059), Paris Island/2003/USA (AY652979), Oberhausen 455/01/DE (AF539440), C14/2002/AU (AY845056), Herzberg 385/01/DE (AF539439), Arg320 (AF190817), VannesL169/2000/France (AY773210), Amsterdam (AF195848), White River/94/US (AF414423), Mexico (U22498), MD145 (AY032605), Mora/97/SE (AY081134), SaitamaT29GII/01/JP (AB112221), SaitamaKU80aGII/99/JP (AB058582), Mc37 (AY237415), Stockholm/IV4348/01/SE (AJ626633), and Pont de Roide 673/04/France (AY682549).

RESULTS

Epidemiology of Norovirus Infections

A total of 402 fecal specimens collected from infants and children with acute gastroenteritis from five different localities of Japan during July 2003 and June 2004 were examined for the presence of NoV. NoV was detected in 58 out of 402 (14.4%) specimens tested. The highest prevalence of NoV was found in infants and children with the age range of 12–23 months (36.2%). No case of NoV infection was identified among infants aged less than 6 months. It was also found that infants and children younger than 3 years old had a high rate of NoV infection (79.3%). NoV was detected throughout the period of 9 months starting from October 2003 to June 2004. However, none of NoV was detected from July to September 2003. The NoV incidence was found highest in December (27.5%), followed by November (19%), and January (12.1%). The lowest NoV detection rate fell into October (5.2%).

Nucleotide Sequence and Phylogenetic Analyses of NoV Genotypes

The partial nucleotide sequences of capsid gene of NoV detected in this study were compared to each other as well as to those of NoV reference strains available in the DDBJ DNA/GenBank database by BLAST. A total of 58 NoV nucleotide sequences, including 1 of NoV GI and 57 of NoV GII were analyzed by phylogenetic grouping based on the recent NoV capsid region classification schemes described by Kageyama et al. [2004]. It was found that the NoV GI sequence (5226/04/Maizuru/JP) clustered into one distinct group with GI/4, which was represented by the Chiba/87/JP virus cluster. It was of interest to note that the 5226/04/Maizuru/JP strain showed the genetic relationship with the NoV Mie2001-U94/JP, which was previously isolated from oyster in Japan, with the nucleotide sequence identity to as high as 98%. Additionally, the 5226/04/Maizuru/JP strain revealed 90%–97% nucleotide sequence identities with those of other human NoV reference strains.

Fig. 1. Phylogenetic tree of nucleotide sequences of Japanese NoV GII. The tree was constructed from partial nucleotide sequences of capsid region of NoV GII isolates detected in Japan. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Japanese NoV was highlighted in italic. MC strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values if 100% is given. *, Genotype contains Japanese NoV detected in the study.

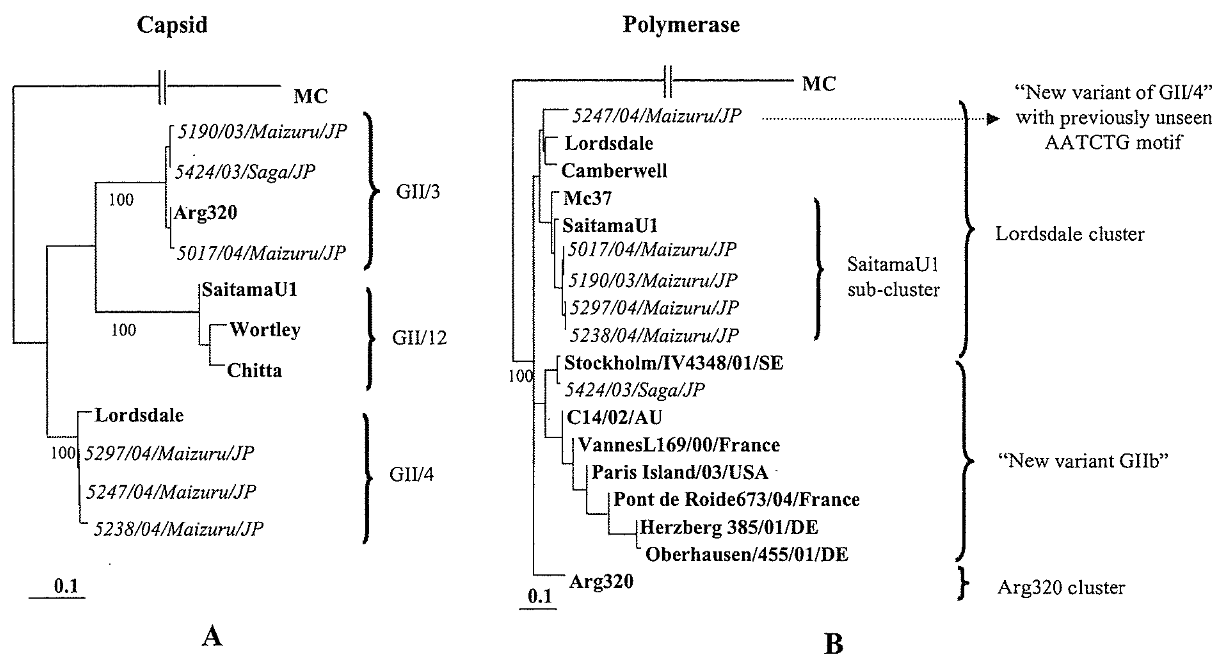

Fig. 2. Observation of changes of NoV genotypes on the basis of phylogenetic trees of nucleotide sequences. The trees were constructed from partial nucleotide sequences of capsid and polymerase regions of the Japanese representative isolates of NoV GII. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Japanese NoV was highlighted in italic. MC strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values if 100% is given.

Of 57 NoV GII sequences, four distinct genotypes, GII/2, GII/3, GII4, and GII/6 had been identified (Fig. 1). Of these, the GII/3 (known as the Arg320 virus cluster) was the most predominant genotype with the prevalent rate of 43.9%, followed by 35.1% of GII/4 (the Lordsdale virus cluster), 14% of GII/2 (the Melksham virus cluster), and 7% of GII/6 (the Seacroft virus cluster). Considering the genotype distribution by localities, GII/3 was also the most predominant in all localities, except for Osaka where none of GII/3 was identified and only one GII/4 was detected in Osaka. The nucleotide sequence identities were ranged from 58% to 99% when NoV GII strains detected in this study were compared with those the reference strains previously registered in the DDBJ DNA/GenBank database.

Nucleotide Sequence and Genetic Analyses of NoV RNA Polymerase Gene

To verify the changing epidemiology of NoV genotypes, the RNA polymerase genes of all NoV GII/3 and

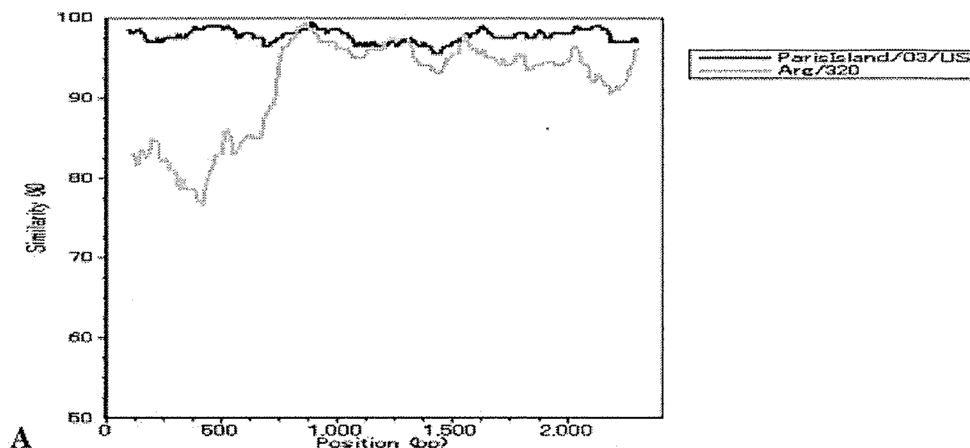

Fig. 3. Genetic characterization of recombinant NoV "new variant with polymerase GIIb." **A**: The Simplot analysis of the 5424/03/Saga/JP, the Paris Island/03/USA, and the Arg320. **B**: Evidence of recombination in NoV capsid gene.

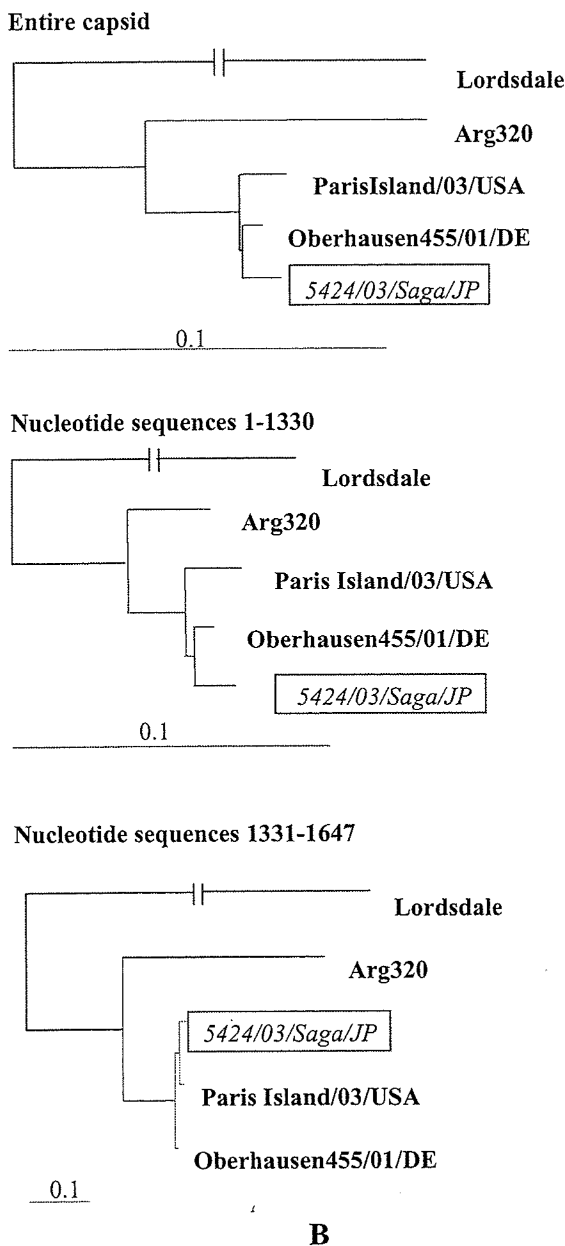

Fig. 3. (*Continued*)

NoV GII/4 were additionally amplified and sequenced. Of 25 NoV isolates with GII/3 capsid, 24 were classified into the SaitamaU1 sub-cluster (known as GII/12) but not into the Arg320 cluster when polymerase-based grouping was performed. The findings suggested that these 24 isolates were all recombinant viruses with GII/3 capsid and GII/12 polymerase. Interestingly, another NoV isolate, the 5424/03/Saga/JP, was grouped with NoV reference "new variants," which were designated as a GIIb in European countries (Fig. 2B). Taken together, the results indicated that the 5424/03/Saga/JP was also the recombinant strain.

In contrast, 20 NoV isolates belonging to GII/4 (the Lordsdale virus cluster), the genotype remained the same no matter the polymerase or capsid regions, was analyzed. Of these, 19 isolates shared significantly high identity of polymerase nucleotide sequences ranging from 98% to 100%. However, they shared only 93% sequence identity with those of the 5247/04/Maizuru/JP. It should be noted that the 5247/04/Maizuru/JP contained the previously unseen AATCTG motif starting at position 4,820 in the polymerase region (referring to the Norwalk virus, M87661). Obviously, this isolate was recognized as a "new variant of GII/4" according to the definition of Lopman et al. [2004]. Furthermore, as shown in Figure 2A, the majority (77.6%, 45 of 58) of NoV isolates were classified into GII/3 and GII/4 based on the partial capsid region, however, they were grouped into a SaitamaU1 sub-cluster based on the partial polymerase region (Fig. 2B).

Genetic Characterization of Recombinant Strain With GIIb Polymerase

To localize the potential recombination site and to understand a possible recombination mechanism of the "new variant" GIIb, the full-length nucleotide sequences of capsid and polymerase regions were determined and analyzed. When the nucleotide sequence of the 5424/03/Saga/JP was compared with those of the Arg320 and the Paris Island/03/US using the SimPlot software, region of genetic recombination was found between nucleotides 1,514 and 1,533 (the overlap of ORF1 and ORF2) (Fig. 3A). Up stream to this junction the nucleotide homology was notably different, and the SimPlot analysis showed a sudden drop in the nucleotide identity for the Arg320 but not for the 5424/03/Saga/JP and the Paris Island/03/US. The results demonstrated that the nucleotide sequences of capsid genes among these three strains were almost identical, but the polymerase sequences of the 5424/03/Saga/JP and the Paris Island/03/US were distinctly different from that of the Arg320.

Within the 5424/03/Saga/JP capsid sequence, the recombination at the breakpoint located at the beginning of P1 domain (position 1,330 nt in the capsid region) was identified. The capsid sequence of the recombinant 5424/03/Saga/JP showed alternate identities to the Oberhausen455/01/DE (nucleotides 1–1,330) and the Paris Island/03/USA (nucleotides 1,331–1,647) (Fig. 3B). The Oberhausen455/01/DE was detected in 2001 in Germany, whereas the Paris Island/03/USA was detected in 2003 in the United States. Quite possibly, the Paris Island/03/USA and the Oberhausen455/01/DE were putative parental strains of the 5424/03/Saga/JP. Taken together, the findings indicated that the 5424/03/Saga/JP had a double recombination.

Genetic Characterization of Recombinant Strain With SaitamaU1 Polymerase

As mentioned above, 24 isolates of GII/3 had high homology (98%–100%) at the nucleotide level of capsid

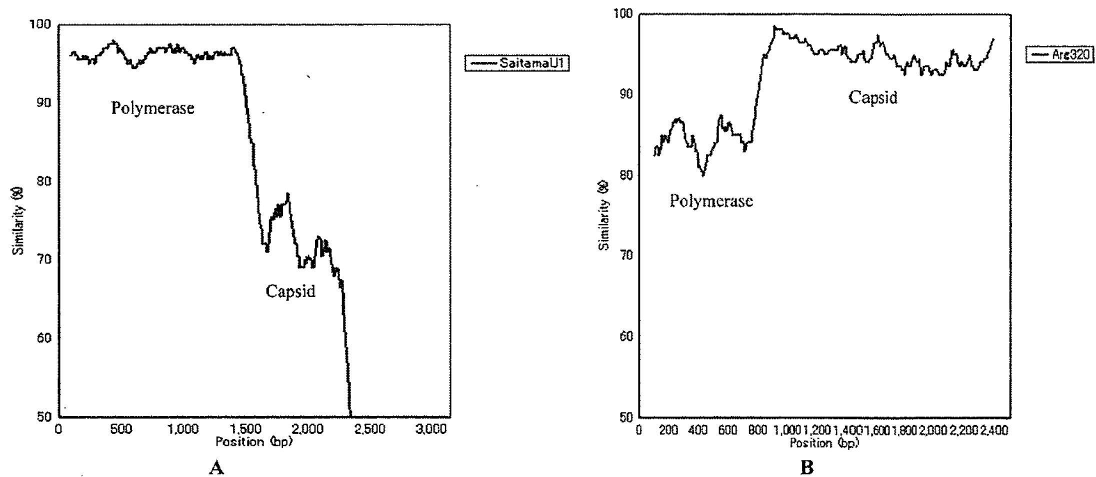

Fig. 4. Genetic characterization of recombinant virus with GII/3 capsid. **A**: The Simplot analysis of the NoV representative isolate, the 5017/04/Maizuru/JP, and the reference strain SaitamaU1. The high and low homologies with the polymerase and capsid regions among them, respectively, were found. **B**: The Simplot analysis of the 5017/04/Maizuru/JP and the reference strain Arg320. The low and high homologies with the polymerase and capsid regions among them, respectively, were found.

and polymerase. The findings demonstrated that they came from the same source of infection and represented the same strain. Furthermore, they were also suspected to be recombinant NoV based on their partial capsid and polymerase sequence. To further analyze this finding, the complete nucleotide sequences of the capsid and polymerase regions of one representative isolate, the 5017/04/Maizuru/JP, were determined. The 5017/04/Maizuru/JP shared a consistently low level of sequence identity (84%) in the RNA polymerase region but consistently high identity (95%) in the capsid region with the Arg320. In contrast, the 5017/04/Maizuru/JP shared consistently high level of sequence identity (96%) in the polymerase region and consistently low identity (70%) in the capsid region with those of the SaitamaU1. A recombinant site was also observed at the overlap of ORF1 and ORF2 (Fig. 4).

DISCUSSION

In this study, the prevalence of NoV infection among infants and children with acute gastroenteritis in five different localities of Japan was reported. Overall, the prevalence rate was 14.4% in all age groups of the subjects included in this study. However, the prevalence rate was increased up to 79.3% in infants and young children with the ages of less than 3 years old. These results were consistent with previous reports on NoV epidemiology worldwide in which the prevalence was ranged from 10% to 60% or more [Marks et al., 2000; Iritani et al., 2002; Lopman et al., 2002; Oh et al., 2003; Phan et al., 2004]. The findings suggested that approximately 14.4% of the etiologic agents of acute gastroenteritis cases in infants and children in Japan might be due to NoV and 85.6% might be responsible by other pathogens. The result also confirmed that NoV is one of the important enteropathogens responsible for viral gastroenteritis among infants and children in Japan. In some reports, NoV was prevalent in cold season, and several studies did not find a seasonal correlation [Vinje et al., 1997; Lopman et al., 2002; Phan et al., 2004]. The findings in this study are in agreement with the surveillance on pediatric cases of viral gastroenteritis in Japan, which demonstrated that the main peak of NoV infection was in the period of November, December, and January [Iritani et al., 2003; Inouye et al., 2000].

The results of this study showed that all Japanese NoV isolates belonged to two distinct genogroups, GI and GII, and these represented 1.7% and 98.3%, respectively. The results indicated that NoV GII was the dominant group causing acute gastroenteritis among Japanese pediatric population. It was interesting to note that the NoV GI 5226/04/Maizuru/JP, which was recovered from one 6-year-old-female patient with diarrhea, was closer genetically to the NoV Mie2001-U94/JP isolated from Japanese oyster than to human NoV reference strains available in the DDBJ DNA/GenBank database. This finding supported a view of possible NoV transmission to human through the contaminated oyster, which known as a reservoir of NoV. According to other reports published by different groups of investigators, NoV belonging to the Lordsdale cluster (GII/4) represented the most predominant genotype detected in sporadic gastroenteritis among infants and children not only in Japan but also in many other countries who run NoV surveillance [Chiba et al., 1979; McEvoy et al., 1996; Vinje et al., 1997; McIntyre et al., 2000]. However, it is surprising to note that in the present study NoV GII/3 was the most predominant, followed by NoV GII/4, NoV GII/2, and NoV GII/6. To verify this unusual observation, the polymerase regions of all NoV GII/3 and NoV GII/4 were further characterized. Remarkably, all NoV GII/3 except the 5424/04/Saga/JP were identified as the recombinant viruses that