We have isolated an endo-β-galactosidase designated E-ABase from *Clostridium perfringens* ATCC 10543 capable of liberating both the A trisaccharide (A-Tri; GalNAc1→3(Fucα1→2)Gal) and B trisaccharide (B-Tri; Ga1α1→3(Fucα1→2)Gal) from glycoconjugates containing blood group A and B glycoproteins, respectively. We have subsequently cloned the gene (eabC) that encodes E-ABase from this organism. This gene was found to be identical to the CPE0329 gene of *C. perfringens* strain 13, whose product was labeled as a hypothetical protein (Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yang, Q., Higuchi, K., and Hayashi, H. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 996–1001). Since the amino acid sequence of E-ABase does not bear detectable similarity to any of the 97 existing families of glycoside hydrolases, we have proposed to assign this unusual enzyme to a new family, GH98. We also expressed proposed to assign this unusual enzyme to a new family, GH98. We also expressed eabC in Escherichia coli BL21(DE3) and obtained 27 mg of fully active recombinant E-ABase from 1 liter of culture. Recombinant E-ABase not only destroyed the blood group A and B antigenicity of human type A and B erythrocytes, but also released A-Tri and B-Tri from group A- and B-containing glycoconjugates. The structures of A-Tri and B-Tri liberated from A* porcine gastric mucin and B* human ovarian cyst glycoprotein were established by NMR spectroscopy. The unique specificity of E-ABase should make it useful for studying the structure and function of blood group A- and B-containing glycoconjugates as well as for identifying other glycosidases belonging to the new GH98 family.

*Clostridium perfringens*, commonly found in sewage, soil, and the gastrointestinal tracts of higher animals, is known to cause gas gangrene (clostridial myonecrosis) and food poisoning in man (1, 2). It is also responsible for such animal diseases as lamb dysentery, ovine enterotoxemia, and pulpy kidney disease in sheep (1–5). The pathogenesis of *C. perfringens* infection has been attributed, in part, to the large number of extracellular toxins and hydrolytic enzymes (6–8) capable of destroying host tissues (9). Among them, sialidase is one of the most extensively studied extracellular enzymes of this organism (10–12).

Although commercially available clostridial sialidase preparations have been widely used for studying the structure and function of sialylglycoconjugates (12), they have been shown to contain proteolytic (13–15), glycosidic (14), and cytotoxic/hemolitic (16–18) activities. We have reported previously that the commercial sialidase prepared from *C. perfringens* ATCC 10543 is contaminated with an unusual endo-β-galactosidase capable of releasing a specific disaccharide glycopote, GlcNAc1→4Gal, from blood group A* porcine gastric mucin (A* -PGM) (19). From the same preparation, we have detected another unique endo-β-galactosidase capable of releasing both the blood group A trisaccharide (A-Tri; GalNAc1→3(Fucα1→2)Gal) and B trisaccharide (B-Tri; Ga1α1→3(Fucα1→2)Gal) glycoproteins from blood group A- and B-containing glycoconjugates, respectively. The unique specificity and the potential usefulness of this enzyme for glycoconjugate research prompted us to carry out the isolation of the native enzyme as well as the cloning and characterization of the recombinant enzyme. Based on the substrate specificity, we propose to name this enzyme E-ABase for blood group A- and B-cleaving endo-β-galactosidase. We found that the gene (eabC) that encodes E-ABase is identical to the CPE0329 gene of *C. perfringens* strain 13, whose product was labeled as a hypothetical protein (20). Sequence comparison of E-ABase with other glycosidases belonging to any of the 97 established families of glycoside hydrolases revealed no significant se-
sequence homology. Thus, we proposed to assign this unusual endo-β-galactosidase to a new glycosidase family, GH98.

**EXPERIMENTAL PROCEDURES**

**Enzyme Assays**

A'-PGM (type II, Sigma) that had been dialyzed exhaustively against distilled water was used as substrate for assaying E-ABase activity by TLC. A 10-μl reaction mixture containing 25 μg of A'-PGM and an appropriate amount of E-ABase in 25 mM sodium acetate buffer (pH 6.0) was incubated at 37 °C for a predetermined time. The reaction was stopped by the addition of 2 μl of glacial acetic acid, and the entire reaction mixture was spotted onto a silica gel-coated TLC plate (EMD Chemicals, Inc.). The plate was developed with 1-butanol/acetic acid/water (2:1.1, v/v/v), and glycoconjugates were revealed by the diphenylboric acid phenylhydrazone reagent (21). The intensity of the glycoconjugate bands was quantified by scanning the plates as described previously (19). One unit of enzyme activity is defined as the amount that releases 1 nmol of A-Tri from A'-PGM/min at 37 °C.

**Purification of Native E-ABase (nE-ABase)**

Unless indicated otherwise, all operations were performed at 0–5 °C, and protein solutions were concentrated by ultrafiltration using an Amicon stirred cell with a PM-10 membrane (Millipore Corp.). Centrifugations were routinely carried out at 8000 × g for 20–30 min using a Sorvall RC5C refrigerated centrifuge.

**Step 1: Preparation of the Crude Enzyme**—C. perfringens ATCC 10543 was cultured for 20 h at 37 °C in 15 liters of culture medium as described previously (22). The culture supernatant was brought to 85% saturation with solid ammonium sulfate and left standing overnight.

The precipitate thus formed was collected, dissolved in a small amount of water, and dialyzed exhaustively against water for 48 h. The dialyzed sample was centrifuged, and the supernatant was lyophilized to yield 11.2 g of crude enzyme powder.

**Step 2: Sephacryl S-200 Gel Filtration**—The crude enzyme powder from Step 1 was dissolved in 70 ml of 0.1 M ammonium acetate buffer (pH 5.8) and applied to a Sephacryl S-200 HR column (5 × 90 cm; Amersham Biosciences) that had been equilibrated with the same buffer. The column was also eluted with the same buffer at 96 ml/h, and 20-ml fractions were collected. A 10-μl aliquot from every other fraction was assayed for enzyme activity using A'-PGM as substrate. Fractions containing nE-ABase activity were pooled, concentrated, and dialyzed overnight against 10 mM Tris-HCl buffer (pH 7.5) (Buffer A).

**Step 3: Fractogel DEAE Chromatography**—The preparation from Step 2 was applied to a Fractogel DEAE-650M column (1.5 × 10.5 cm; EMD Chemicals, Inc.) equilibrated with Buffer A. After removing the unadsorbed proteins with Buffer A, nE-ABase was eluted with 10 mM sodium acetate buffer (pH 4.0) at 1 ml/min. Fractions of 2 ml were collected. The fractions containing nE-ABase were pooled, concentrated, and dialyzed overnight against 10 mM sodium acetate buffer (pH 5.5) (Buffer B).

**Step 4: Fractogel SP Chromatography**—The preparation from Step 3 was applied to a Fractogel SP-650M column (1.0 × 7.5 cm; EMD Chemicals, Inc.) equilibrated with Buffer B, and the column was eluted with the same buffer at 1 ml/min. Fractions of 1 ml were collected. The majority of nE-ABase activity was recovered in the unadsorbed fractions, whereas the bulk of the contaminants was eluted with Buffer B containing 0.4 M NaCl. Fractions with the highest nE-ABase activity were pooled and dialyzed overnight against 25 mM sodium phosphate buffer (pH 7.0) (Buffer C).

**Step 5: Concanavalin A (ConA)-Sepharose Chromatography**—The preparation from Step 4 was concentrated to 0.3–0.5 ml and applied to a ConA-Sepharose column (1.0 × 45 cm; Amersham Biosciences) equilibrated with Buffer C. nE-ABase was eluted in the early breakthrough fractions with Buffer C. Fractions containing nE-ABase activity were pooled, concentrated, and dialyzed against Buffer A.

**Step 6: Mono Q Chromatography**—The enzyme preparation from Step 5 was applied to a Mono Q HR 5/5 column (0.5 × 5.5 cm; Amersham Biosciences) equilibrated with Buffer A at 0.5 ml/min using an Amersham Biosciences AKTA FPLC system. After washing with the same buffer, a linear gradient of NaCl was introduced with the following gradients: 0–0.06 M NaCl for 5 column volumes, 0.06 M NaCl for 5 column volumes, 0.06 M NaCl for 20 column volumes, 0.1 M NaCl for 20 column volumes, 0.1–0.12 M NaCl for 5 column volumes, 0.12 M NaCl for 5 column volumes, and 0.12–0.3 M NaCl for 5 column volumes. Fractions of 1 ml were collected. Fractions containing nE-ABase activity were eluted as a sharp peak at 0.1 M NaCl. They were pooled, dialyzed against 5 mM sodium phosphate buffer (pH 6.0), and used in subsequent experiments. This final enzyme preparation was >95% pure as judged by SDS-PAGE (see Fig. 1). Table I summarizes the purification of nE-ABase from 15 liters of culture medium.

**Amino Acid Sequences of CNBr Peptides Derived from nE-ABase**

CNBr cleavage products were generated from 200 μg of purified nE-ABase according to Charbonneau (23). Peptides were separated by 10% SDS-PAGE and blotted onto a polyvinylidene difluoride membrane using a Trans-Blot semidry transfer cell (Bio-Rad) at 20 V for 1.25 h. The membrane was briefly stained with 0.04% Coomassie Brilliant Blue R-250 and the most prominent bands were excised. The three CNBr peptides (P1–P3), as well as the N-terminal region of the native enzyme, were sequenced by the Core Facility of the Louisiana State University Health Sciences Center (New Orleans, LA). The sequences of these peptides are shown in Fig. 2.

**Generation of a 1.5-Kilobase Pair (kbp) PCR Product (Probe A)**

Genomic DNA from C. perfringens ATCC 10543 was prepared as described (24). We initially designed a sense primer, F1, and an antisense primer, R2 (see Table II), based on the N-terminal sequence (LEESRDYLSDDLWLNTHGGDGXX) and the CNBr P2 peptide (MRATKTKSLLYG), respectively. A 1.5-kbp product was generated by PCR using these two primers and the C. perfringens genomic DNA as template. PCR was run with Taq DNA polymerase (InviGen) for 30 cycles consisting of 45 s of denaturation at 94 °C, 45 s of annealing at 54 °C, and 2 min of extension at 72 °C. The 1.5-kbp fragment (Probe A) was sequenced and subcloned into the pGEM-T Easy vector (Promega). Probe A was later used to screen the first genomic DNA library.

**Cloning of the E-ABase Gene (eabC)**

XL1-Blue MRF' and SOLR cells (Strategen) were grown in LB medium supplemented with 0.2% maltose and 10 mM MgSO4. Construction of the first genomic library in the LZAPII vector (Strategen) and subsequent screenings were performed as described previously (25). PCR was run with Taq DNA polymerase (InviGen) for 30 cycles consisting of 45 s of denaturation at 94 °C, 45 s of annealing at 54 °C, and 2 min of extension at 72 °C. One 1.5-kbp fragment (Probe A) was sequenced and subcloned into the pGEM-T Easy vector (Promega). The enzyme preparation from Table I was used as a probe (pEAB4E) and was found to contain two-thirds of the expected gene sequence from the 5′-end.

To identify the 3′-end of the E-ABase gene, a second genomic library was constructed in Escherichia coli JM109. Genomic DNA (15 μg) from C. perfringens was digested with 100 units of XbaI. Purified DNA fragment was ligated into a pBluescript SK−/− vector and transformed into E. coli JM109. One positive clone (pEABX1), identified by PCR using primers EAB4.1F and EAB4.5R (see Table II), was found to contain the full-length open reading frame of eabC as shown in Fig. 2.

**Construction of the Expression Plasmid**

An expression plasmid (pEABHNB3) with a C-terminal His6 tag was constructed in the pET-15b vector. eabC without the sequence for the N-terminal 35-amino acid signal peptide was amplified by PCR using Taq DNA polymerase with pEABX1 as template and primers EX1 and EX6 (see Table II). The 2.3-kbp PCR product was purified, digested with NotI and BamHI, ligated into the pET-15b vector, sequenced, and given the name pEABHNB3.

**Expression and Purification of Recombinant E-ABase (rE-ABase)**

rE-ABase was expressed in E. coli BL21(DE3) cultured at 37 °C in LB medium containing 100 μg/ml ampicillin. rE-ABase was purified under nondenaturing conditions as described by Hoffmann and Roeder (26), but excluded glycerol, dithiothreitol, and Nonidet P-40. Three 1-liter portions of LB/ampicillin medium were each inoculated with 20–30 ml of an overnight culture, and the cells were harvested 4 h after inoculation. The culture was cooled to 4 °C, and 10 ml of lysis buffer (10 mM Tris-HCl (pH 7.9), 0.5 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM imidazole) using a French press. The cell-free extract was heated at 60 °C for 4 min and centrifuged. This heat treatment precipitated ~30% of nonenzymatic proteins without affecting E-ABase activity. The supernatant was then applied to a nickel-nitrilotriacetic acid column (1.5 × 12.5 cm; QiAGEN Inc.) equilibrated with lysis buffer. After extensively washing the column with the
Tri were pooled and lyophilized. The yield of A-Tri was 11.4 mg/g of solvent, acetonitrile/water (3:1, v/v), and fractions containing pure A-Tri were desalted using a Sephadex G-10 column (1.5 cm) and analyzed by fluorescence-activated cell sorting (FACS). The final yield was 11.4 mg of A-PGM. An identical scheme was used for the preparation of 8.6 mg of B-Tri from 260 mg of B-PGM.

**NMR Spectroscopy**

For NMR measurements, each trisaccharide (8–10 mg) was repetitively exchanged with D2O with intermediate lyophilization and then dissolved in 0.6 ml of D2O. The one-dimensional 1H and 13C and two-dimensional 1H-1H (double quantum-filtered COSY, total correlation spectroscopy, and rotating frame Overhauser effect spectroscopy) and 13C-1H (heteronuclear single quantum correlation spectroscopy and heteronuclear multiple bond correlation spectroscopy) spectra (Bruker pulse program library) were recorded at 25 °C using a Bruker Avance DRX 500 spectrometer at frequencies of 500 MHz (1H) and 125.75 MHz (13C). The spectrometer was equipped with a 5-mm Bruker inverse triple resonance probe with an xyz gradient coil and a broadband observe double resonance probe with a z axis gradient coil. The methyl resonance of an external 1% acetone in D2O measured separately under identical conditions was set at 2.225 ppm (1H) and 31.07 ppm (13C) and used as the reference. 1H-1H coupling constants (JuH,i,juH,j) were measured in one-dimensional spectra and from the double quantum-filtered COSY cross-peaks using the FELIX 98 DQF macro (Accelrys Inc., San Diego, CA). 13C-1H coupling constants (JvC,i,juH,j) were extracted from the 13C-Heteronuclear single quantum correlation spectra recorded without decoupling.

**Flow Cytometric Analysis of Blood Group A and B Antigens**

Analysis of antigen expression in human red blood cells (RBC) was performed by flow cytometry with a BD Biosciences FACSCalibur cytometer at a low speed flow rate. Data from 30,000 events were collected and analyzed using CellQuest software (BD Biosciences). To prepare RBC for enzyme treatment, 400 μl of type A or B RBC (2–4% cell suspension; Immucor Inc., Norcross, GA) was centrifuged at 1250 × g for 4 min. The packed RBC were then washed with autoclaved phosphate-buffered saline (PBS; pH 7.4), centrifuged, and resuspended in PBS to give a hematocrit of 8–16%. A 50-μl aliquot of each type of RBC was then treated with 50 μl (533 units) of rE-ABase, and the mixture was rotated end-over-end on a 5.5-cm diameter wheel at 8 rpm for 18 h at 23 °C. After incubation, RBC were washed with 2.0 ml of PBS and resuspended in 20 μl of PBS. A 5-μl aliquot of packed RBC was then added to 100 μl of PBS wash buffer (PBS containing 1% fetal bovine serum). The indirect immunofluorescence method was used to label 1 × 10^7 RBC (from the control and enzyme-treated RBC samples) with murine anti-A or anti-B monoclonal antibody (Immunoc Inc.) as the primary antibody at a dilution of 1:1000 in PBS wash buffer, followed by the addition of fluorescein 5-isothiocyanate (FITC)-labeled goat antimouse IgG (Fab-specific; Sigma) as the secondary antibody. The secondary antibody was used at a dilution of 1:200 in PBS wash buffer. All incubations were performed for 15–20 min at room temperature under rotation. The labeled RBC were washed with PBS wash buffer, resuspended in 1 ml of PBS, and then analyzed by fluorescence-activated cell sorting (FACS).

**Other Methods**

Exoglycosidases were assayed using p-nitrophenyl glycosides in 20 mM sodium citrate buffer (pH 6.0) as described previously (27). Protease activity was determined using Azocoll as substrate (28). Protein concentrations were determined by the method of Lowry et al. (29) using bovine serum albumin as a standard. SDS-PAGE was performed on a 10% polyacrylamide gel (30). Protein bands were visualized with Coomassie Brilliant Blue R-250.

**RESULTS AND DISCUSSION**

**Purification of nE-ABase**—Table I summarizes the results of a typical purification of nE-ABase from 15 liters of C. perfringens culture supernatant. We chose Sephacryl S-200 HR gel filtration as the first step in the purification of nE-ABase because this step could process a large amount of crude enzyme as well as remove >90% of the contaminating proteins. After gel filtration, the enzyme preparation was further subjected to Fractogel DEAE and Fractogel SP chromatographies. The final two steps in the purification scheme involved ConA-Sepharose and fast protein liquid chromatographies using a Mono Q column. Although ConA-Sepharose chromatography separated a large molecular mass (96 kDa) contaminant from nE-ABase, Mono Q/fast protein liquid chromatography was effective in removing nE-ABase from smaller contaminating proteins. By these steps, nE-ABase was purified by >2600-fold with a recovery of ~18%. As shown in Fig. 1, the purified nE-ABase

**TABLE I**

| Step                  | Total protein | Total activity | Specific activity | Yield % | Purification fold |
|-----------------------|---------------|----------------|-------------------|---------|------------------|
| 1. Crude enzyme       | 10,282        | 11,077         | 1.1               | 100     | 1                |
| 2. Sephacryl S-200   | 279.5         | 9084           | 32.5              | 82      | 29.5             |
| 3. Fractogel DEAE     | 79.8          | 7920           | 99.2              | 71.5    | 90.2             |
| 4. Fractogel SP       | 56.9          | 6855           | 121.0             | 62.2    | 110              |
| 5. ConA-Sepharose     | 5.9           | 4636           | 786.1             | 41.9    | 714              |
| 6. Mono Q             | 0.7           | 2026           | 2894              | 18.3    | 2630             |

**FIG. 1. SDS-PAGE analysis of nE-ABase and rE-ABase.** Both nE-ABase and rE-ABase were analyzed by SDS-PAGE using a 10% gel. Protein bands were visualized by Coomassie Brilliant Blue staining.
migrated as one major protein band of ~88 kDa upon SDS-PAGE. nE-ABase was found to be free of protease activity using Azocoll as substrate. It was also free of the following exoglycosidases activities using p-nitrophenyl glycosides as substrates: α-L-fucosidase, α- and β-glucosidases, α- and β-galactosidases, α- and β-mannosidases, α-N-acetylgalactosaminidase, α-arabinosidase, β-xylanidase, and β-hexosaminidase.

**Cloning of the E-ABase Gene (eabC)**—We initiated the cloning of eabC by generating a DNA fragment (Probe A) using genomic DNA from *C. perfringens* ATCC 10543 as template and the degenerate primers F1 and R2 (Table II), designed from the N-terminal peptide (LEESRDVYLSDLDWLNATHG-YG), respectively (see Fig. 2). The deduced amino acid sequence of Probe A contains the N-terminal sequence of nE-ABase and the CNBr P2 peptide (MRAKTKSLXYG), respectively (see Fig. 2). The deduced amino acid sequence of Probe A was the N-terminal sequence of eabC and the two CNBr peptides P1 (MLNEAQSYXNPK) and P2 (MRAKTKSLXYG), indicating that this DNA fragment is part of the gene encoding E-ABase. After screening the first genomic library (EcoR1-digested) with Probe A, we selected one positive cloned phage for in vivo excision to generate a phagemid, pEAB4E. Although this clone contains the third CNBr peptide, F3 (MSQSPAYTXGRXNYP), it still lacks an estimated one-third of the E-ABase gene sequence at the 3'-end. Therefore, a second genomic library (XbaI-digested) was constructed in the pBluescript SK(–) vector. Several colonies were examined by PCR, and one positive clone (pEABX1) was found to contain the entire sequence of eabC.

The open reading frame of the eabC gene and its deduced amino acid sequence are shown in Fig. 2. The eabC gene consists of 2400 bp that encode 800 amino acid residues. As shown in Fig. 2, the N-terminal 24-amino acid peptide of the native enzyme is found in residues 36–59. Residues 10–35 at the N terminus show an extended region of hydrophobicity as well as a predicted cleavage site after residue 35 as determined by the PSORT program, indicating a signal sequence for secretion into the culture medium. The mature protein consists of 765 amino acid residues with a calculated molecular mass of 87 kDa, which is very close to the value of 88 kDa estimated by SDS-PAGE for the NE-ABase (Fig. 1).

**Relationship between the E-ABase Gene (eabC) and the CPE0329 Gene of *C. perfringens* Strain 13**—A BLASTN search of eabC against the genome of *C. perfringens* strain 13 deposited in the GenBank™/EBI Data Bank revealed a 100% match with the CPE0329 gene, whose product was labeled as a hypothetical protein (20). This finding validated the results of our cloning work and also established the product of the CPE0329 gene of *C. perfringens* strain 13 as E-ABase.

**Assignment of E-ABase to a New Glycoside Hydrolase Family, GH98**—To identify proteins with amino acid sequences related to E-ABase, the DNA and protein sequences of E-ABase were used as a query against the FASTA and BLAST Databases. The results of bioinformatics analysis revealed that the amino acid sequence of E-ABase does not bear any detectable similarity to any of the established glycoside hydrolase families. There are 97 public families of carbohydrate-active enzymes (CAZy Database) to date (available at afmb.cnrs-mrs.fr/CAZY). Since the E-ABase sequence is distinct from the established glycoside hydrolase sequences, it would define a novel CAZy family. In consultation with Dr. Bernard Henrissat (Universités d’Aix-Marseille I and II, Marseille, France), who has established a classification system for glycosidases, we have assigned E-ABase to a new glycoside hydrolase family designated as GH98.

It is noteworthy that E-ABase does not share significant sequence homology with other endo-β-galactosidases cloned from *Flavobacterium keratolyticus* (DDBJ/GenBank™/EBI accession number AF083396) (31) and *C. perfringens* (accession numbers AB038772 and AB059351) (25, 32). The fact that the primary sequence of E-ABase does not contain an EDDXXE motif as found in other endo-β-galactosidases (25, 31, 32) indicates that E-ABase may have evolved along a separate evolutionary line.

**Expression of rE-ABase**—Using the expression plasmid pEABHIN3, we expressed rE-ABase as a soluble protein in *E. coli* BL21(DE3) at a level of ~34 mg/liter of culture without isopropyl-β-D-thiogalactopyranoside induction. We were able to purify 27 mg of rE-ABase from 1 liter of culture with the recovery of ~80%. Similar to nE-ABase, the purified rE-ABase moved as a sharp protein band of ~88 kDa upon SDS-PAGE with a very minor contaminant of 67 kDa (Fig. 1). No exoglycosidase or protease activity was detected in the purified rE-ABase preparation. The specific activity of 2667 units/mg of protein for rE-ABase was very close to that of 2894 units/mg of protein for nE-ABase (Table I), indicating that rE-ABase was fully active.

**General Properties of E-ABase**—The following buffers were used at a final concentration of 20 mM to study the effect of pH on E-ABase activity: sodium citrate/citric acid (pH 4.0–6.0), sodium acetate/acetate acid (pH 4.0–6.0), sodium phosphate/NaOH (pH 6.0–8.0), Tris-HCl (pH 7.5–8.9), and glycine/NaOH (pH 9.6–10.0). Using γ- PGM or B-HOCG as substrates, the maximal activity of both rE-ABase and nE-ABase was found to be between pH 5.5 and 6.0, and each enzyme maintained >75% of its activity between pH 5.5 and 8.5. Furthermore, both enzymes were very stable, with little or no loss of activity upon storage at ~20 °C for 2 years.

At 10 mM, Cu²⁺, Co²⁺, Mg²⁺, and Mn²⁺ had little or no effect on E-ABase activity with either A¹-PGM or B¹-HOCG as substrates. Also, β-mercaptoethanol (up to 100 mM) and various sugars (Gal, Glc, Fuc, and GalNAc at 0.15 mM) had little or no effect on the activity of E-ABase.

**Destruction of the Blood Group Antigenicity of Type A and B RBC by rE-ABase**—We have used FACS to analyze the effect of rE-ABase on the antigenicity of type A and B RBC. Under the...
FIG. 2. Nucleotide and deduced amino acid sequences of E-ABase. The underlined sequences indicate the N-terminal sequence (24 amino acid residues) of the mature protein and the three CNBr peptides (P1–P3) obtained from nE-ABase.
conditions described under “Experimental Procedures,” rE-ABase reduced the blood group A antigen expression of type A RBC by 92% (Fig. 3B) and completely removed the blood group B antigen from type B RBC (Fig. 3D). The incomplete destruction of the blood group A antigenicity of type A RBC by rE-ABase is consistent with the heterogeneous nature of the blood group A immunodeterminants on type A RBC (33). It has also been shown that various blood group A glycotopes on type A RBC differ in their susceptibility to α-N-acetylgalactosaminidase (34). Since both A-Tri and B-Tri glycotopes are linked through an endo-β-galactosyl linkage, the ability of rE-ABase to abolish both blood group A and B antigenicity of RBC established this enzyme as a blood group A- and B-cleaving endo-β-galactosidase.

Substrate Specificity of rE-ABase—Like nE-ABase, rE-ABase was able to liberate A-Tri from A-Penta and B-Penta by rE-ABase. The incubation mixture (70 µl) contained 7 µg of A-Penta (○) or B-Penta (□) and 3.5 units of rE-ABase in 25 mM sodium acetate buffer (pH 6.0). Incubations were carried out at 37 °C. Aliquots of 10 µl were taken at 0, 5, 10, 20, 40, 80, and 160 min for analysis by the TLC method as described under “Experimental Procedures.” Each time point represents the average of duplicate experiments.

A Blood Group A- and B-cleaving Endo-β-galactosidase
Table III

| Atom | Form I (54%) | Form II (23%) | Form III (13%) | Form IV (4%) |
|------|--------------|--------------|----------------|--------------|
|      | ¹H          | ¹³C         | ¹H              | ¹³C          | ¹H                  | ¹³C          | ¹H        | ¹³C          | ¹H                  | ¹³C          |
| 1a   | 5.136 (3.7) | 93.14 (171.8) | 5.171 (3.8) | 92.71 (172.6) | 5.073 (3.9) | 99.64 (171.9) | 5.104 (4.0) | 98.13 (171.1) |
| 2a   | 4.229       | 50.92        | 4.240          | 50.85        | 4.186          | 51.08        | 4.162      | 51.21        |
| 3a   | 3.976       | 68.96        | 3.945          | 69.07        | 3.939          | 68.53        | 3.919      | 68.29        |
| 4a   | 3.980       | 69.99        | 3.995          | 69.95        | 4.019          | 69.72        | 4.039      | 69.82        |
| 5a   | 4.402       | 72.13        | 4.257          | 72.35        | 4.050          | 72.94        | 4.050      | 72.83        |
| 6a   | 3.768       | 62.74        | 3.754          | 62.64        | 3.827          | 62.60        | 3.827      | 62.74        |
| NAc  | 2.043       | 23.27        | 2.047          | 23.27        | 2.047          | 23.27        | 2.047      | 23.27        |
| NHCO | 176.02      | 176.10       | 175.86         | 175.86       | 175.86         | 175.86       |           |              |

Values are from double quantum-filtered COSY.
Resonance cannot be unambiguously assigned.

Table IV

| Atom | Form I (55%) | Form II (23%) | Form III (18%) | Form IV (4%) |
|------|--------------|--------------|----------------|--------------|
|      | ¹H          | ¹³C         | ¹H              | ¹³C          | ¹H                  | ¹³C          | ¹H        | ¹³C          | ¹H                  | ¹³C          |
| 1a   | 5.207 (3.8) | 94.61 (170.2) | 5.240 (3.8) | 94.34 (170.9) | 5.112 (4.0) | 100.52 (170.2) | 5.140 (3.8) | 99.22 (170.2) |
| 2a   | 3.858       | 69.94        | 3.868          | 69.42        | 3.821          | 69.50        | 3.821      | 69.43        |
| 3a   | 3.945       | 70.55        | 3.925          | 70.71        | 3.920          | 70.49        | 3.920      | 70.48        |
| 4a   | 3.968       | 70.66        | 3.975          | 70.55        | 3.975          | 70.55        | 3.975      | 70.55        |
| 5a   | 4.387       | 72.00        | 4.239          | 72.32        | 4.032          | 72.80        | 4.130      | 72.75        |
| 6a   | 3.743       | 62.65        | 3.748          | 62.54        | 3.780          | 62.42        | 3.780      | 62.65        |
| 1b   | 5.108 (4.0) | 101.81 (171.0) | 5.255 (4.0) | 100.20 (173.1) | 5.108 (4.0) | 99.07 (170.2) | 5.143 (3.8) | 101.19 (170.9) |
| 2b   | 3.793       | 69.29        | 3.778          | 69.18        | 3.793          | 69.23        | 3.793      | 69.36        |
| 3b   | 3.908       | 70.75        | 3.838          | 70.78        | 3.838          | 70.74        | 3.838      | 70.74        |
| 4b   | 3.814       | 73.90        | 3.797          | 73.29        | 3.813          | 73.02        | 3.813      | 73.24        |
| 5b   | 4.162       | 68.45        | 4.428          | 68.13        | 4.083          | 68.40        | 4.083      | 68.56        |
| 6b   | 1.216       | 16.59        | 1.189          | 16.48        | 1.215          | 16.64        | 1.215      | 16.75        |
| 1c   | 5.368 (3.8) | 93.11 (171.6) | 4.714 (7.7) | 96.44 (162.1) | 5.432 (2.1) | 101.92 (173.8) | 5.368 (4.3) | 96.49 (174.9) |
| 2c   | 3.995       | 75.17        | 3.775          | 75.97        | 4.266          | 86.56        | 4.239      | 83.44        |
| 3c   | 4.163       | 72.48        | 3.954          | 77.24        | 4.290          | 83.22        | 4.474      | 80.11        |
| 4c   | 4.307       | 65.87        | 4.263          | 64.69        | 4.302          | 84.19        | 4.067      | 81.40        |
| 5c   | 4.192       | 71.19        | 3.688          | 75.87        | 3.907          | 72.59        | 3.844      | 73.07        |
| 6c   | 3.754       | 62.38        | 3.706          | 62.22        | 3.708          | 62.89        | 3.708      | 62.81        |

Values are from double quantum-filtered COSY.
Resonance cannot be unambiguously assigned.

Glc (Fig. 4B). In these oligosaccharides, both blood group A and B glycoproteins are linked β1→4 to Glc. As shown in Fig. 5, rE-ABase hydrolyzed A-Penta only slightly faster than B-Penta, indicating that the enzyme does not show a particular preference for the blood group A or B glycoprotein.

Although the commercial A-Tetra substrate contains a small oligosaccharide contaminant (Fig. 4B, lane 2), this contaminant did not interfere with the analysis of the hydrolysis of A-Tetra by rE-ABase (lane 3). Unlike the above-mentioned blood-group-carrying oligosaccharides, A hexasaccharide (A-Hexa; GalNAc1→3[Fuc1→2Galβ1→3GlcNAcβ1→3Galβ1→4Glc], which carries the blood group A glycoprotein on a type 1 chain, was only slowly hydrolyzed (Fig. 4B, lane 9). These results suggest that, although rE-ABase does not have a strict preference for either the blood group A or B glycoprotein, it does recognize the specific core chain by preferentially cleaving the endo-β-galactosyl linkage of the type 2 core chain (Galβ1→4GlcNAc/Glc) over the type 1 core chain (Galβ1→3GlcNAc). rE-ABase also slowly released A-Tri from GalNAc1→3[Fuc1→2]Galβ1→3GalNAc1→Ser/Thr in the glycopeptides prepared from porcine submaxillary mucin (data not shown). The Galil pentasaccharide (Gal1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc), which is devoid of an N-fucose linked α1,2 to the penultimate β-galactosyl residue in the type 2 core structure,
was not hydrolyzed. Thus, the presence of a L-fucose residue on the substrate is essential for E-ABase to carry out its action. Furthermore, rE-ABase did not hydrolyze the Leβ+ or Hα -HOCG blood group substances. Also, glycosaminoglycans such as heparin, heparan sulfate, dermatan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate, and keratan sulfate were not susceptible to rE-ABase. The substrate specificity of the native enzyme (E-ABase) preparation was found to be identical to that of rE-ABase.

Characterization of the Two Trisaccharides Released from A’-PGM and B’-HOCG by rE-ABase Using NMR Spectroscopy—To establish the specificity of E-ABase, we used NMR spectroscopy to characterize the trisaccharides (A-Tri and B-Tri) released by rE-ABase from A’-PGM and B’-HOCG, respectively. The 1H and 13C chemical shifts for the sugar residues in A-Tri and B-Tri are given in Tables III and IV, respectively. It is well known that aldoxoses generate complex tautomeric equilibria in aqueous solution involving cyclic pyranoses and furanoses as well as trace amounts of acyclic hydrates and aldehydes (35–38). Thus, it is not surprising that pyranoses and furanoses as well as trace amounts of acyclic plex tautomeric equilibria in aqueous solution involving cyclic respectively. It is well known that aldohexoses generate complex tautomeric equilibria in aqueous solution involving cyclic pyranoses and furanoses as well as trace amounts of acyclic hydrates and aldehydes (35–38). Thus, it is not surprising that pyranoses and furanoses as well as trace amounts of acyclic plex tautomeric equilibria in aqueous solution involving cyclic

It is well known that blood group antigens coat the surface of cells exposed to the external environment, including the mouth, lung, urogenital tract, and gastrointestinal tract (33, 45). Through the degradation of blood group A and B antigens of cell-surface glycoconjugates, E-ABase may enhance the infectivity and virulence of C. perfringens. This unique endo-β-galactosidase should become useful for studying the structure and function of glycoconjugates as well as for identifying other glycosidases belonging to the new GH98 family.

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A Clostridial Endo-\(\beta\)-galactosidase That Cleaves Both Blood Group A and B Glycotopes: THE FIRST MEMBER OF A NEW GLYCOSIDE HYDROLASE FAMILY, GH98

Kimberly M. Anderson, Hisashi Ashida, Karol Maskos, Anne Dell, Su-Chen Li and Yu-Teh Li

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