J. Microbiol. Biotechnol. (2005), **15**(4), 722–727

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Cloning of Human Liver Cytosolic Sialidase from Genomic DNA Using Splicing by Overlap Extension and Its Characterization

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Received: May 11, 2004
Accepted: March 7, 2005

Abstract Cytosolic sialidase (Neu2), a member of the sialidase family that is responsible for hydrolysis of sialic acid from the terminal position of sialoglycoconjugates, is poorly expressed in skeletal muscle and not detected in any other adult tissues. Thus, we isolated Neu2 cDNA using splicing by overlap extension (SOEing). In order to further characterize this enzyme, a His-tagged derivative was expressed in the bacterial expression system and purified by Ni^{2+}-affinity chromatography. A recombinant product of approximately 42 kDa had sialidase activity toward 4-methyl-umbelliferyl-α-D-*N*-acetylneuraminic acid (4MU-NeuAc). The optimal pH and temperature of the recombinant Neu2 for 4MU-NeuAc was 6.0 and 37.5°C, respectively. The metal ions, such as Cu^{2+} and Cd^{2+}, showed strong inhibitory effect on the activity of the enzyme. The enzyme efficiently hydrolyzed the gangliosides GM3 and GD3 and had relatively low activities on ganglioside GD1a and GD1b, α2-3 sialyllactose, and sialylated glycoproteins such as fetuin, transferrin, and orsomucoid, but had hardly any activities on α2-6 sialyllactose and ganglioside GM1 and GM2. We concluded that the recombinant Neu2 has a sialidase activity toward glycoproteins as well as gangliosides.

Key words: Cytosolic sialidase, Chang liver cell, splicing by overlap extension, 4MU-NeuAc, ganglioside, glycolipid, glycoprotein

Sialic acids exist mostly in the terminal positions of biomolecules (such as glycoproteins, glycolipids, and gangliosides) and cell membranes, and are involved in a wide variety of physiological processes including cell differentiation, neuronal development, and immune functions [3, 28]. Neuraminidase or sialidase (E.C.3.2.1.18) belongs to a family of glycohydrolytic enzymes that remove *N*-acetyl neuraminic acid residues from various substrates such as glycolipids and glycoproteins [1]. In microorganisms, neuraminidases are involved in nutrition and pathogenesis [5]. In mammals, sialidases are suggested to play a role in such diverse cellular phenomena as molecular transport, antigen masking, proliferation, differentiation, and membrane function [27]. In human tissues, four types of endogenous sialidases have been cloned so far, and they have been classified on the basis of their subcellular localization such as lysosomal (Neu1), cytosolic (Neu2), plasma membrane (Neu3), and inner cell membranes (Neu4) sialidases [24, 25].

The mammalian cytosolic sialidase activity was determined in homogenates from skeletal muscle, brain, and liver [18, 20, 21]. More detailed study on the molecular features of mammalian sialidases has been limited by factors such as low cellular content and instability during the purification procedures [30, 26]. However, since 1993, several mammalian sialidases have been cloned from the corresponding cDNAs, prompting a rapid acceleration of studies on their structural and kinetic properties [1, 24]. In recent years, cytosolic sialidases in rat, mouse, and hamster have been cloned and characterized [19, 8, 7]. Monti *et al.* [22] have cloned cytosolic sialidase from the human genomic library, and the nucleotide sequence analysis of the gene revealed only one intron and two exons. In addition, Nothern blot analysis showed that this gene was poorly expressed in skeletal muscle and not detected in any other adult tissues. Although the expression of cytosolic sialidase in rat liver has been demonstrated [10], the expression and characterization of cytosolic sialidase in human liver has not yet been reported.

In this study, we used gene SOEing methods to clone *Neu2* cDNA from the genomic DNA of liver cells. In order to

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further characterize this enzyme, we cloned the cDNA into the pRSETc bacterial expression vector, which was tagged with His. The His-tagged Neu2 protein was expressed in *E. coli*, purified by Ni^{2+}-affinity chromatography, and characterized.

MATERIALS AND METHODS

Cell Culture and Genomic DNA Isolation

Human liver Chang cells were purchased from American Type Culture Collection (ATCC, U.S.A.) and cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Gibco-BRL, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco-BRL), 100 U/ml penicillin (Sigma, U.S.A.), and 100 mg/ml streptomycin (Sigma) [15, 16]. The cells were cultured at 37°C and 5% CO_2-air atmosphere, and subconfluent grown cells were washed twice with phosphate-buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and trypsinized with 0.05% trypsin and 0.02% EDTA in Puck's saline A (Gibco BRL). The cells were collected by centrifugation at 500 ×*g* for 5 min and washed with PBS. The supernatant was discarded and cell sediment was used for the genomic DNA isolation with Wizard Genomic DNA Purification System (Promega, U.S.A.).

Splicing by Overlap Extension (SOEing) by the Polymerase Chain Reaction

For the construction of full-length *Neu2* cDNA, two flanking primers (P1 and P4) and two overlapped primers (P2 and P3) were used as follows: primer P1 (5'-ATA GAT CTA TGG CGT CCC TTC CTG TCC T-3'), primer P2 (5'-TCC TGA GCT TGC CAC CTG AAC CTG GTG GGT GGG T-3'), primer P3 (5'-CCA CCA GGT TCA GGT GGC AAG CTC AGG AGG TGG T-3'), and primer P4 (5'-ATG AAT TCT CAC TGA GGC AGG TAC TCA G-3'). The overlapped primers were designed to have additional bases complementary to the other exon (indicated by underline). The genomic fragment of *Neu2*, consisting of two exons and one intron, was amplified by PCR with P1 and P4 primers using genomic DNA from Chang liver cells as templates. Two exons with short overlapped oligonucleotides were produced using P1 and P2 for amplifying exon 1, and P3 and P4 for exon 2. The reaction mixture in 50 μl of final volume contained the above-mentioned primers (1 μM each), together with 150 ng of the extracted DNA, 100 μM dNTP, 1 U of LA Taq DNA polymerase (Takara co., Japan), and 10× PCR buffer (10 mM Tris-HCl at pH 9.0, 50 mM KCl, 0.1% Triton X-100, and 2.5 mM $MgCl_2$). A thermal step program for the genes was used, including the following parameters: incubation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 55–58°C for 1 min, 72°C for 1 min (except in the last cycle, which was for 10 min). Then, the gel-purified products of two exons were mixed, and SOE reaction was carried out under the same conditions as above. For the next time, the spliced product was amplified by an additional PCR step with P1 and P4 primers for producing a large amount of *Neu2* cDNA.

Cloning, Expression, and Purification of Neu3

The gel-purified *Neu2* cDNA was subcloned in-frame into the *Bgl*II and *Eco*RI sites of the pRSETc bacterial expression vector (Invitrogen, U.S.A.), which was named pRSETc-Neu2. Clones with the expected insert were identified by *Bgl*II, *Eco*RI, and *Sac*I restriction analysis, and sequenced from the T7 and T3 promoter regions of pRSETc vector by the dideoxy chain termination method [4]. Competent *E. coli* BL21(DE3) cells were transformed by pRSETc and pRSETc-Neu2 plasmids. The control BL21(DE3) cells and two transformants were cultured overnight at 37°C in LB medium (Life Technologies, U.S.A.) supplemented without (for control) or with 100 mg/l ampicillin. A 0.5 ml aliquot of the overnight culture was mixed with 3 ml of LB medium and incubated at 37°C with shaking at 250 rpm to OD_{600}=0.5. The cell suspension was adjusted to 25°C and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. The cells were then harvested and lysed by sonication in PBS, and an aliquot of cells was lysed in binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). After removal of cell debris by centrifugation, the cell extracts were loaded onto a Ni^{2+}-NTA column (Novagen, Germany) and washed first with binding buffer and then with wash buffer (30 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). Proteins were eluted in 500 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9, followed by desalting on a PD10 column (Amersham Biosciences, U.S.A.). The crude and purified proteins were stored at −20°C as aliquots.

SDS-PAGE and Immunoblotting of Total Protein Extracts

The total cell homogenates and purified proteins were resolved by SDS-PAGE with 10% resolving and 4.5% stacking gels. The gels were stained with Coomassie brilliant blue (Bio-Rad, U.S.A.). An immunoblotting analysis was performed as previously described [22] with some modifications. Briefly, the proteins were resolved by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore, U.S.A.) by using the trans-blot apparatus (Amersham Biosciences) at 30 V in 25 mM Tris buffer (pH 8.3) for 16 h. The membrane was blocked by TBS, containing 0.1% (v/v) Tween 20 (TBS-T) and 5% (w/v) skim milk, for 30 min. Then, the membrane was incubated with rabbit anti-His polyclonal antibody (Chemicon, U.S.A.) as the primary antibody and goat anti-rabbit IgG (Chemicon), which was conjugated with horseradish peroxidase, as the secondary antibody. The signals were visualized with ECL (Amersham Biosciences) and exposed to X-ray film for 30 sec.

Fluorimetric Methods for Sialidase Activity

The sialidase activities of total cell homogenates were assayed in the presence of 400 μg of BSA and 0.2 mM 4MU-NeuAc [14]. The mixture was incubated at 37°C for 25 min, and the reactions were stopped by the addition of 1.5 ml of 0.2 M glycine/NaOH (pH 10.2). For determining the effects of pH, temperature, and metal ions on the activity of the purified enzyme, incubation conditions were modified as indicated in the legend section of the figures. Fluorescence emission was measured on a Jasco FP-770 fluorometer with excitation at 365 nm and emission at 445 nm, with the use of 4-methylumbelliferone (4-MU, Sigma) to obtain a standard curve.

Colorimetric Methods for Sialidase Activity

The enzymatic activities of recombinant Neu2 toward various substrates were determined using Warren's thiobarbituric acid method [31]. The substrates were purchased from Sigma. All reactions in a final volume of 100 μl were carried out in triplicate with appropriate concentration of the substrates, 25 μg of protein, and 12.5 mM sodium acetate buffer at pH 3.5–7.5 for optimal pH assay and at pH 6.0 for substrate specificity. After incubation at 37°C for 60 min, the reaction was stopped by immediate freezing. The released sialic acid was determined by measuring absorbance at 532 and 549 nm.

Results and Discussion

Preparation of Neu2 from Genomic DNA Using SOEing

Among the different members of the gene family, human *NEU2* is transcribed at a rather poor level. The low level of transcription was detected only in skeletal muscle, but not detectable in any other adult tissues [23]. The low expression level of *NEU2* was confirmed by the absence of ESTs corresponding to the transcript in dbEST [2], thus the low level hampering the identification of cDNA clones. Consequently, we designed the strategy of cloning the Neu2 gene from genomic DNA by combining two exons, however, there was no useful restriction enzyme site in the rejoining parts. To solve this problem, we used the gene splicing by overlap extension (SOEing) method to produce full-length Neu2 cDNA. Gene SOEing is a technique for recombining DNA molecules at precise junctions, irrespective of nucleotide sequences at the recombination site and without the use of restriction endonucleases or ligase [11, 12]. This technique is especially useful in complicated constructions that require precise recombinant points, such as joining two coding sequences in-frame to produce hybrid genes, and it also provides a straightforward way of performing site-directed mutagenesis [11, 13].

An 1,159 bp DNA fragment of the cytosolic sialidase (Neu2) with restriction enzyme site was obtained by the SOEing method described in Fig. 1. The genomic fragment of *Neu2*, consisting of 2 exons and 1 intron, was amplified by PCR using genomic DNA from Chang liver cells as a template, and this genomic fragment was used in the next stage of the reaction. The two exons with short overlapped sequences were amplified by PCR using P1 and P2 for exon 1, and P3 and P4 for exon 2 (Fig. 2A). The products

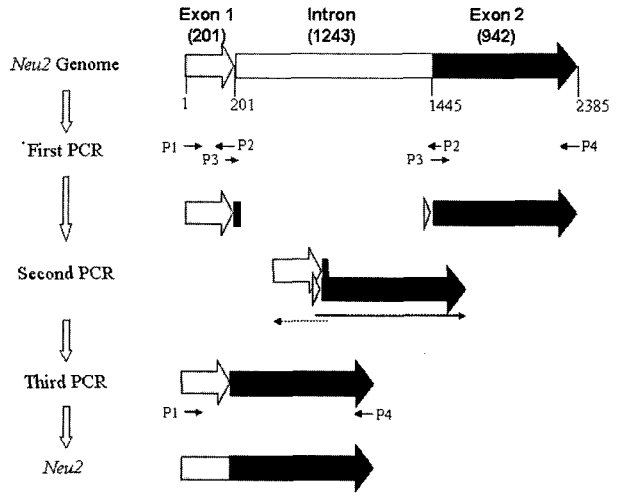

Fig. 1. Strategy for cloning Neu2 from genomic DNA using SOEing.

The SOEing used in the present studies was involved in three separate PCRs. Four primers were required for each construct: two flanking primers (P1 and P4), and two overlapped primers (P2 and P3). The overlapped primers were designed to have additional bases complementary to the other exon. The genomic fragment of *Neu2* consisting of two exons and one intron, which was amplified by PCR using genomic DNA from Chang liver cells, was used as templates in the first stage reactions. The two exons with short overlapped oligonucleotides were produced using P1 and P2 for amplifying exon 1, and P3 and P4 for exon 2. When these PCR products are mixed, denatured, and re-annealed, the strands having the matching sequences at their 3' ends overlap and act as primers for each other. Extension of this overlap by DNA polymerase produces a molecule in which the original sequences are spliced together. This spliced product was amplified by PCR with P1 and P4 primers for producing a large amount of *Neu2* cDNA. An arrowhead indicates the 5'-to-3' direction of each strand of the primers and PCR products.

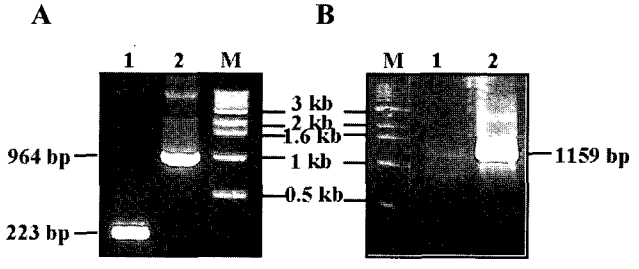

Fig. 2. The products amplified from SOEing. The products amplified from each PCR stage was electrophoresed on a 1% agarose gel and stained by ETBR.

A. The products of exon 1 (lane 1) and exon 2 (lane 2) amplified by first PCR. **B.** The products amplified by second (lane 1) and third (lane 2) PCR.

were mixed in the SOE reaction, and the 3' end of the exons overlapped with each other. This allowed them to act as primers on one another to make a giant "primer dimmer," and extension of this overlap by DNA polymerase created the 1,159 bp of *Neu2* which consisted of only two exons (Fig. 2B. lane 1). To obtain sufficient amount of *Neu2* cDNA for subcloning, this spliced product was amplified by additional PCR with P1 and P4 primers (Fig. 2B, lane 2).

Cloning and Identification of Neu2

The *Neu2* cDNA was subcloned in-frame into the *Bgl*II and *Eco*RI sites of a bacterial expression vector, pRSETc (Figs. 3A and 3B). Clones with expected insert were identified by *Bgl*II, *Eco*RI, and *Sac*I restriction analysis (Fig. 3C) and sequenced from the T7 and T3 promoter regions of pRSETc by the dideoxy chain termination method. Comparison of the sequence of the cloned cDNA with the sequence of the skeletal muscle cDNA [23] revealed 99% homology at the nucleotide level (data not shown). The sequences were analyzed by the Swiss-prot database (accession number Q9Y3R4). Translation of this cDNA generated a protein of 380 amino acids with a molecular weight of 42,230 Da. The sequence contained a Tyr(Phe)-Arg-Ile-Pro sequence and two Asp-boxes, which were highly conserved in all of the sialidase enzymes described so far [1]. When the sequence of *Salmonella typhimurium* sialidase [6] was compared based on the mode of Milner *et al.* [17], the protein had six potential active site amino acid residues.

Expression of Neu2 in *E. coli*

The pRSETc-Neu2 expression vector coding His-tagged Neu2 and pRSETc empty vector were transformed into competent *E. coli* BL21(DE3) cells. The expression of Neu2 was achieved by lowering the temperature to 25°C, indicating the entrapment of sialidase in inclusion bodies after induction at 37°C. The extracts of control and transformed cells were analyzed by 10% SDS-PAGE and

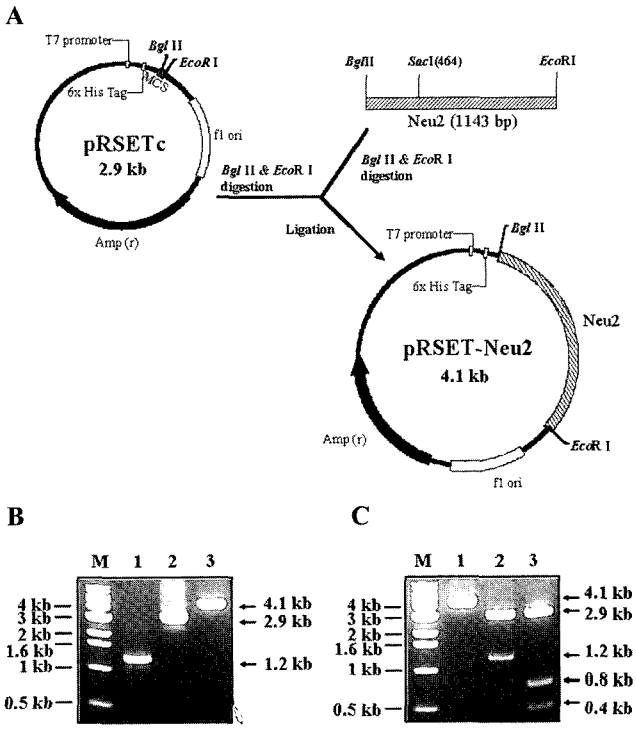

Fig. 3. Construction of the pRSET-Neu2 expression vector.
A. A 1,159 bp Neu2 cDNA subcloned into the *Bgl*II and *Eco*RI sites of pRSETc vector. **B.** ETBR-stained 1% agarose gel electrophoresis of Neu2 (lane 1), pRSETc vector cut with *Bgl*II and *Eco*RI (lane 2), pRSETc-Neu2 cut with *Bgl*II (lane 3). **C.** ETBR-stained 1% agarose gel electrophoresis of pRSETc-Neu2 digested with *Eco*RI (lane 1), *Bgl*II and EcoRI (lane 2), *Bgl*II, *Eco*RI, and *Sac*I (lane 3).

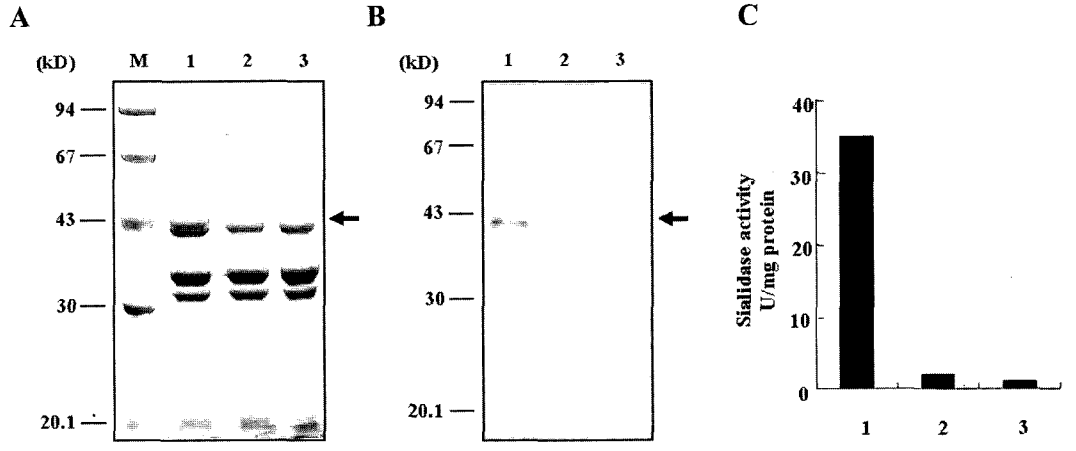

Fig. 4. Expression of His-tagged Neu2 in *E. coli*.
A. Extracts of transformed cells were analyzed by 10% SDS-PAGE and stained with Coomassie brilliant blue. **B.** Immunoblot analysis of extracts of transformed cells using a rabbit anti-His polyclonal antibody. **C.** Sialidase activities of transformed cell extracts. Lanes in (A), (B), and (C) are as follows: protein size marker (lane M), bacteria containing pRSETc-N3 (lane 1), pRSETc (lane 2), and no vector plasmid (lane 3).

Western blot analysis using a rabbit anti-His polyclonal antibody. A protein band of 42 kDa was observed by SDS-PAGE and Western blot analysis (Figs. 4A, 4B) In addition, sialidase activities of extracts from control and transformed cells were analyzed by spectroflurometric measurement of 4-MU released at pH 4.5. The sialidase activity of extracts from pRSETc-Neu2-harboring cells was about 15-fold higher than that of pRSETc or no vector-containing cells (Fig. 4C). The enzyme has been expressed in mammalian cell lines such COS-7 and in *E. coli* [23]; however, detailed characteristics of Neu2 have not been fully elucidated. The expression of Neu2 in the *E. coli* system may provide a good model for characterization and inhibitor screening of this enzyme.

Characterization of the Purified Recombinant Neu2

The recombinant Neu2 proteins were purified by Ni^{2+}-affinity column, and the purified proteins were analyzed by 10% SDS-PAGE (Fig. 5A). Aliquots of the purified proteins were used to determine optimal pH and substrate specificity. The optimal pH and temperature of the purified recombinant Neu2 was approximately pH 6.0 and 37.5°C, respectively, in sodium acetate buffer using 4MU-NeuAc as a substrate (Figs. 5B, 5C). In addition, the activity of the enzyme was not affected by addition of Na^+, K^+, Mg^{2+}, and Ca^{2+}, but Cu^{2+} and Fe^{2+} showed strong inhibitory effects on the enzyme activity (Table 1). The relative sialidase activities toward various sialoconjugates were analyzed by determining sialic acid released, using Warren's thiobarbituric acid methods [31]. The enzyme efficiently hydrolyzed the gangliosides GM3 and GD3, but hardly had any activities on α2-6 sialyllactose and ganglioside GM1 and GM2. In addition, the enzyme had relatively low activities on GD1a and GD1b gangliosides, α2-3 sialyllactose, and sialylated glycoproteins such as fetuin, transferrin, and orsomucoid (Fig. 5D). These data showed that the recombinant enzyme acted preferentially on terminal sialic acid residues of ganglioside, which are linked to the oligosaccharide moiety with both α2→3 and α2→8 sialyl linkages, but not α2→6 sialyl linkage. Unlike other types of sialidase [24, 9], Neu2 has a hydrolytic activity toward sialylated glycoproteins, such as fetuin, transferrin, and orsomucoid. These data are in good agreement with recent results reported by Tringali *et al.* [29].

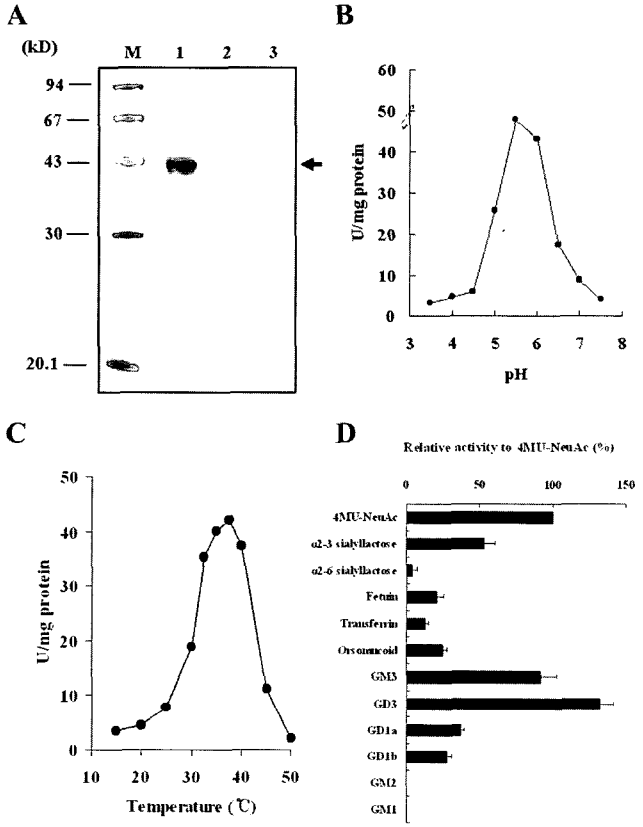

Fig. 5. Purification and characterization of His-tagged Neu2.
A. SDS-PAGE of purified proteins. The purified proteins from bacteria, containing pRSETc-N3 (lane 1), pRSETc (lane 2), and no vector plasmid (lane 3), were analyzed by 10% SDS-PAGE. **B.** Optimal pH of purified recombinant Neu2. Sialidase activity was assayed using 4MU-NeuAc and sodium acetate buffer pH 3.5–7.5. **C.** Effect of temperature on the activity of purified recombinant Neu2. The activity was measured using 4MU-NeuAc at pH 4.5 and different temperatures. **D.** Substrate specificity of purified Neu2. Sialidase activities toward various sialoconjugates were expressed as the percentage of sialic acid release relative to that obtained with 4MU-NeuAc. Values are means±S.D. of three independent duplicated experiments.

Table 1. The effect of metal ions, including Na^+, K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, and Fe^{2+}, on the activity of recombinant Neu2 using 4MU-NeuAc as substrate. Assay conditions were as follows: 0.02 μg of enzyme; 0.5 mM substrate; pH 5.5; incubation time, 15 min; concentration of ions, 1 mM. Three independent results were calculated by percentage of the control (n.d. means not determined.).

Metal ions (1 mM)	Relative activity (% of control)
Na^+	97.4
K^+	101.2
Ca^{2+}	89.2
Mg^{2+}	94.7
Cu^{2+}	n.d.
Fe^{2+}	45.2

Acknowledgment

This work was supported by National Research Laboratory Program, KISTEP, MOST, Korea (No. M10203000024-02J0000-01300 to C. H. Kim).

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