

# Construction of an in vitro trans-sialylation system: surface display of *Corynebacterium diphtheriae* sialidase on *Saccharomyces cerevisiae*

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**Abstract** Sialidases can be used to transfer sialic acids from sialoglycans to asialoglycoconjugates via the transglycosylation reaction mechanism. Some pathogenic bacteria decorate their surfaces with sialic acids which were often scavenged from host sialoglycoconjugates using their surface-localized enzymes. In this study, we constructed an in vitro trans-sialylation system by reconstructing the exogenous sialoglycoconjugate synthesis system of pathogens on the surfaces of yeast cells. The *nanH* gene encoding an extracellular sialidase of *Corynebacterium diphtheriae* was cloned into the yeast

surface display vector pYD1 based on the Aga1p–Aga2p platform to immobilize the enzyme on the surface of the yeast *Saccharomyces cerevisiae*. The surface-displayed recombinant NanH protein was expressed as a fully active sialidase and also transferred sialic acids from pNP- $\alpha$ -sialoside, a sialic acid donor substrate, to human-type asialo-*N*-glycans. Moreover, this system was capable of attaching sialic acids to the glycans of asialofetuin via  $\alpha(2,3)$ - or  $\alpha(2,6)$ -linkage. The cell surface-expressed *C. diphtheriae* sialidase showed its potential as a useful whole cell biocatalyst for the transfer of sialic acid as well as the hydrolysis of *N*-glycans containing  $\alpha(2,3)$ - and  $\alpha(2,6)$ -linked sialic acids for glycoprotein remodeling.

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## Introduction

Sialic acids are nine carbon sugar acids that are typically detected in the terminal sugars of *N*-glycans, *O*-glycans, and glycosphingolipids in animals, protozoa, pathogenic fungi, viruses, and bacteria (Vimr et al. 2004; Varki 2007). In higher eukaryotes, a variety of sialoglycoconjugates on the surfaces of cells play important roles in the regulation of cell–cell and cell–molecule interactions by masking recognition sites and by mediating cell recognition or adhesion processes (Buschiazzo and Alzari 2008). Although the majority of bacteria cannot synthesize sialic acid, some pathogenic bacteria generate sialic acids by scavenging them from animal host cells (Vimr and Lichtensteiger 2002; Buschiazzo and Alzari 2008). Among the more than 40 sialic acid derivatives detected

in both eukaryotic and prokaryotic species, the most frequently detected is *N*-acetylneuraminic acid (Neu5Ac), which is  $\alpha(2,3)$ - or  $\alpha(2,6)$ -linked to galactose, *N*-acetyl-galactosamine, and *N*-acetylglucosamine or  $\alpha(2,8)$ -linked to another *N*-acetylneuraminic acid unit at the terminal position of the oligosaccharide (Vimr et al. 2004). Recently, enzymatic synthesis using sialyltransferases or sialidases has been highlighted as a promising method to overcome the limitations of classical chemical synthesis techniques, as they can selectively carry out regio- and stereo-specific bond formation under mild conditions without the need for any elaborate procedures, such as protection and deprotection steps (Crout and Vic 1998; Johnson 1999; Izumi and Wong 2001). However, the use of sialyltransferase requires an expensive nucleotide-sugar, CMP-sialic acid, as a sialic acid donor. In contrast, sialidase is capable of synthesizing sialoglycoconjugates using cheaper glycosides, via the condensation and trans-glycosylation reaction (Ajisaka et al. 1994; Crout and Vic 1998; Schmidt et al. 2000). The trans-sialylation mechanism occurs naturally in certain pathogenic bacteria and protozoa. They decorate their cell surfaces with sialic acids scavenged from the sialoglycoconjugates of host cells using their sialidases or trans-sialidases in order to evade the host's immune system and to interact with host cells (Vimr and Lichtensteiger 2002). The trans-sialidase activities from certain clinically isolated *Corynebacterium diphtheriae* strains and several *Trypanosome* strains have been previously reported (Mattos-Guaraldi et al. 1998; Vimr and Lichtensteiger 2002; Vimr et al. 2004).

The enzyme immobilization on the cell surface is an attractive technique in the development of novel biocatalysts and bioprocesses as a cell factory (Schreuder et al. 1996; Ueda and Tanaka 2000a, b; Kondo and Ueda 2004). The cell surface display systems have several advantages, including the lack of a requirement for enzyme purification, ease of separation of the product and enzyme after bioconversion reactions, and the possibility of enzyme reuse (Schreuder et al. 1996; Ueda and Tanaka 2000a, b; Kondo and Ueda 2004). Moreover, the charged, insoluble, hydrophobic, or macromolecule substrates have low mass-transfer efficiency for an intracellular enzyme reaction. However, the enzyme immobilized on the cell surface, which enables direct access to the substrates in the reaction media without any transportation into cells, overcomes these substrate transport problems into the insides of cells (Murai et al. 1997; Kondo and Ueda 2004). The yeast *Saccharomyces cerevisiae*, which has well-established surface display systems, lacks the enzymes and transporters involved in sialic acid metabolism (Berninsone et al. 1997; Callewaert et al. 2005). The lack of the sialic acid metabolism in this yeast would provide an advantage to the reaction of sialic acid hydrolysis (or condensation), because the cell is unable to utilize the enzymatic reaction

products during the process. The sialidases immobilized on the yeast cell surfaces might also prove useful in the enzymatic analysis of sialic acid-containing glycan structures for  $\alpha(2,3)$ -,  $\alpha(2,6)$ -, and  $\alpha(2,8)$ -linkage analysis. Recently, Contreras et al. introduced a trans-sialidase from *Trypanosoma cruzi*, a pathogenic protozoan, on the yeast surfaces for the production of sialylated oligosaccharides or glycoproteins via in vitro enzyme reactions (Callewaert et al. 2005; Ryckaert et al. 2005). In addition, the glycoengineered *Pichia pastoris* was developed for the production of sialylated glycoproteins by introducing a whole sialic acid biosynthesis pathway (Hamilton and Gerngross 2007). From the aspect of therapeutic glycoprotein production from recombinant yeast strains, the sialylation technology is an important process since the terminal sialic acids on the *N*-glycan of therapeutic glycoproteins increase the in vivo half-life by protecting them against clearance by the hepatic asialoglycoprotein receptor (Lodish 1991; Farley 2003). The production of homogeneously sialylated glycoproteins from recombinant yeast is still a developing technology to improve the production yield and quality control of the products (Hamilton and Gerngross 2007).

In this study, in an effort to apply *C. diphtheriae* extracellular sialidase (NanH) as a whole cell biocatalyst for the in vitro sialylation reaction toward asialo-*N*-glycan, we constructed *S. cerevisiae* strains expressing NanH on the yeast surface using Aga1p–Aga2p proteins. We also tested the trans-sialylation activity of the immobilized sialidase for the sialylation of human-type *N*-linked asialoglycans and an asialoglycoprotein.

## Materials and methods

### Bacterial strains, plasmid, and culture condition

*Escherichia coli* DH5 $\alpha$  [ $\lambda^-$  supE44  $\Delta$ lacU169 ( $\Phi$  80 lacZ  $\Delta$ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1] was used as a host for the construction and amplification of a recombinant plasmid. *E. coli* cells were grown in LB medium supplied with an appropriate antibiotic at 37 °C. *S. cerevisiae* EBY100 [MATa ura3-52 trp1 leu2 $\Delta$ 1 his3 $\Delta$ 200 pep4::HIS3 prb1 $\Delta$ 1.6R can1 GAL (pIU211::URA3)] was used as a host strain for the expression of recombinant NanH $_{\Delta N}$  on its surface. Yeast cells were grown at 30 °C in YPD (1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose) or YNBD dropout medium (6.7% yeast nitrogen base and 2% glucose) supplemented with amino acids and nucleotide bases as required. The pGEM-nanH and the pYD1 (Invitrogen, Carlsbad, CA, USA) were used as a template for the amplification of the *C. diphtheriae* nanH gene and an expression vector of the truncated nanH gene (nanH $_{\Delta N}$ ) in *S. cerevisiae*, respectively (Kim et al. 2010).

### Construction of the NanH yeast display vector

To construct pYD1-Cdip\_NanH<sub>ΔN</sub>, the *nanH*<sub>ΔN</sub> DNA fragment, encoding the *C. diphtheriae* NanH protein lacking its signal sequence, was amplified from pGEM-nanH (Kim et al. 2010) by polymerase chain reaction (PCR) using the primer pairs NanH<sub>ΔN</sub>\_F (5'-GGG GTA CCT ACC GCA GAA CTG GAA GGA GAA GTG GCT GC-3') and NanH<sub>ΔN</sub>\_R (5'-CCG CTC GAG CCT TAT TCC AGG AAT GTT AAA AG-3'). The PCR products were digested with *KpnI* and *XhoI*, and the resulting 2,095-bp fragment was cloned into the *KpnI*-*XhoI*-digested pYD1 to yield pYD1-Cdip\_NanH<sub>ΔN</sub>. This plasmid harbors the fused gene encoding for Aga2p-Cdip\_NanH<sub>ΔN</sub> followed by a 6×His-tag at the C-terminal region under the control of the inducible *GAL1* promoter. After sequence verification, pYD1-Cdip\_NanH<sub>ΔN</sub> was transformed into *S. cerevisiae* EBY100 by using the lithium acetate method to generate the recombinant strain YSK003 containing pYD1-Cdip\_NanH<sub>ΔN</sub>, which was selected in synthetic complete medium without uracil and tryptophan (SC-Ura-Trp) containing 2% glucose.

### Induction of Aga2p-fused Cdip\_NanH<sub>ΔN</sub> protein

To express the Aga2p-fused Cdip\_NanH<sub>ΔN</sub> protein, a single colony of YSK003 was seeded in SC-Ura-Trp containing 2% glucose. The cells were cultivated for 48 h at 30 °C. The 1/100 volume of seed culture was inoculated into fresh SC-Ura-Trp medium containing 0.1% glucose and cultivated at 30 °C to OD<sub>600</sub>=0.6–0.8. For induction of the protein, 1/10 volume of 20% galactose was added to the culture and then cultivated further at 20 °C. At each time point, the cells were harvested to assess the protein expression and to measure the sialidase activity. *S. cerevisiae* EBY100 containing pYD1 as a negative control was cultivated according to the procedure described above.

### Extraction of cell surface proteins and Western blot analysis

After galactose induction, yeast cells were harvested at each time point up to approximately 10 OD<sub>600</sub> by centrifugation at 1,000×g for 10 min at 4 °C, washed in distilled water, and then resuspended in a buffer [50 mM Tris-HCl, pH 8.0, 1 mM PMSF, containing EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland)] containing 100 mM dithiothreitol (DTT) to dissociate the disulfide bonds between the Aga2p-fused protein and Aga1p. Following 2 h of incubation on ice, the DTT-treated cells were centrifuged for 10 min at 10,000×g at 4 °C, and the supernatants were harvested for Western blot analysis. The proteins released from the yeast cell walls were then separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred from the gels to nitrocellulose

(NC) membranes. The NC membranes were blocked with 5% non-fat milk and incubated with primary mouse anti-Xpress™ antibody (Invitrogen, Carlsbad, CA, USA) for 4 h at room temperature. The NC membrane was subsequently incubated with the secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing the membranes three times with phosphate-buffered saline (PBS) containing 0.1% Tween 80, the proteins were detected with an ECL kit (GE Healthcare, Waukesha, WI, USA) according to the manufacturer's protocols.

### Immunofluorescence microscopy analysis

For confocal microscopic experiments, yeast cells were cultivated in the presence of 2% galactose for 48 h at 20 °C. The harvested cells were then washed three times in PBS and incubated with anti-Xpress™ antibody (1:250) for the detection of Aga2p-fused NanH<sub>ΔN</sub> or bovine serum albumin (BSA) as a negative control for 2 h on ice. After incubation, the yeast cells were washed free of unbound antibody and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG; 1:50; Sigma-Aldrich, St. Louis, MO, USA) for 1 h on ice. After three more washings with PBS, the fluorescent-labeled cells were analyzed using a Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany) with an excitation wavelength of 470 nm and an emission wavelength of 515 nm.

### Flow cytometric analysis of the yeast cells

After galactose induction, cells were incubated with polyclonal anti-His antibody (1:20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-V5 antibody (1:50; Invitrogen), or monoclonal anti-Xpress™ antibody (1:100; Invitrogen) for 1 h at 4 °C. After incubation, the cells were washed free of unbound antibody and incubated with FITC-conjugated goat anti-mouse or anti-rabbit immunoglobulin G (IgG; 1:50; Sigma-Aldrich) as a secondary antibody for 45 min on ice. The cells were washed twice in PBS, supplemented with 1.0 mgmL<sup>-1</sup> BSA, and analyzed using a FACS Calibur™ flow cytometer (BD Sciences, Franklin Lakes, NJ, USA). Estimation of the number of the yeast cell surface-displayed enzyme molecules per cell was carried out by enzyme-linked immunosorbent assay (ELISA) using anti-Xpress™ antibody for quantifying Aga2p-fused NanH<sub>ΔN</sub> protein displayed on *S. cerevisiae* cells (detailed protocols in the [Supplementary Material](#)).

### Measurement of sialidase activity

Cdip\_NanH<sub>ΔN</sub> sialidase activity attached to the yeast cell surface was determined with 2'-(4-methylumbelliferyl)-α-

D-*N*-acetylneuraminic acid (MU-Neu5Ac) as the substrate. The cells harvested at each time point were washed in distilled water then resuspended in 50 mM Tris–HCl buffer (pH 7.5). The enzyme reaction was initiated by the addition of substrate solution. The reaction was conducted in a total volume of 50  $\mu$ l containing 1 mM MU-Neu5Ac. After the enzyme reaction for 60 min at 30 °C, the reaction mixture was centrifuged for 10 min at 20,000 $\times$ *g* at 4 °C. The fluorescence of 4-methylumbelliferone (MU) released from MU-Neu5Ac in the supernatant was measured using an RF5301-PC spectrofluorometer (Shimadzu, Kyoto, Japan) with excitation and emission wavelengths of 365 and 450 nm, respectively. The quantity of released sialic acid was calculated on the basis of the extinction coefficient ( $\epsilon$ ), 3.206 $\times$ 10<sup>6</sup> mM<sup>-1</sup>cm<sup>-1</sup>, for MU. One unit was defined as the amount of enzyme required to release 1  $\mu$ mol of MU from MU-Neu5Ac per minute. Each data was expressed as the mean of three independent experiments.

The sialic acid hydrolysis activity of the cell immobilized C dip\_NanH $\Delta$ <sub>N</sub> toward various sialylated substrates was determined at 30 °C for 1 h in 50 mM Tris–HCl buffer, pH 7.0, in a final volume of 50  $\mu$ l containing 10 mM sialoglycoconjugate or 1 mgmL<sup>-1</sup> glycoprotein and 10 OD<sub>600</sub> of intact yeast cells. The sialoglycoconjugates and glycoproteins used in this study were as follows: sialyl- $\alpha$ (2,3)-lactose, sialyl- $\alpha$ (2,6)-lactose, *p*NP- $\alpha$ -sialoside, MU- $\alpha$ -sialoside, fetuin,  $\alpha$ <sub>1</sub>-acid glycoprotein, transferrin, and bovine submaxillary mucin. Following enzyme reaction, the free sialic acids generated in the reaction product were determined by the periodic acid/thiobarbituric acid-based method (Warren 1959).

Trans-sialylation reaction using PA-labeled asialo-*N*-linked glycans as an acceptor

The *in vitro* trans-sialylation reaction was conducted with C dip\_NanH $\Delta$ <sub>N</sub> immobilized on the surfaces of *S. cerevisiae* cells, using pyridylamino (PA)-labeled asialoglycans (Takara, Otsu, Japan), PA-asialo-biantennary (PA-sugar chain 001), and PA-asialo-triantennary (PA-sugar chain 002, 010) as an acceptor substrate. Enzyme reactions were conducted using 293 and 262 mg of yeast cells (wet weight) containing an empty vector (pYD1) and pYD1-C dip\_NanH $\Delta$ <sub>N</sub>, respectively. The intact cells were incubated for 6 h in 50 mM Tris–HCl (pH 7.0) containing 0.1 nM PA-labeled oligosaccharide as an acceptor and 0.02 nM *p*NP- $\alpha$ -sialoside as a sialic acid donor at 25 °C. After sialylation reaction, the reaction mixture was centrifuged for 10 min at 10,000 $\times$ *g* at 4 °C for the separation of sialylated products from cells.

PA-labeled *N*-linked glycan products were analyzed using a high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) equipped with a Waters 2475 fluorescence detector. After the reactions, the

mixtures were clarified via 10 min of centrifugation at 10,000 $\times$ *g* at 4 °C. The supernatant containing the glycans was analyzed with a Shodex Asahipak NH2P-50 normal phase column (4.6 $\times$ 150 mm; Showa Denko, Kawasaki, Japan) with solvent A (90% acetonitrile and 200 mM acetic acid triethylamine, pH 7.3) and solvent B (10% acetonitrile and 200 mM acetic acid/triethylamine, pH 7.3) at a flow rate of 1.0 mlmin<sup>-1</sup>. The column was then equilibrated for 5 min with 70% solvent A and 30% solvent B. After sample injection, the proportion of solvent B was increased in a linear fashion from 30% to 80% over 60 min. The fluorescence intensity of the PA-conjugated glycans in the eluent was monitored with excitation and emission wavelengths of 310 and 400 nm, respectively.

*In vitro* trans-sialylation reaction using an asialofetuin

The intact yeast cells (453 mg of wet weight), which were cultivated for 48 h in the presence of 2% galactose at 20 °C, were incubated for 60 h with 200  $\mu$ g of asialofetuin as an acceptor and 10  $\mu$ M *p*NP- $\alpha$ -sialoside as a sialic acid donor in 50 mM Tris–HCl (pH 6.8) at 25 °C. The sialylated proteins were analyzed using 8% SDS-PAGE gel and a precast isoelectric focusing gel (IEF)-PAG (pH gradient 3.0–7.0; Koma Biotech, Seoul, South Korea) gel. The reaction products were mixed with IEF sample buffer and applied to a precast IEF-PAG gel in accordance with the manufacturer's recommendations. The proteins separated using SDS-PAGE and IEF gel were transferred to NC membranes. After 1 h of blocking with 5% BSA, the membranes were incubated with 0.5  $\mu$ g mL<sup>-1</sup> of biotin-conjugated *Maackia amurensis* lectin or biotin-conjugated *Sambucus nigra* lectin (EY laboratories, San Mateo, CA) at room temperature for 4 h to detect sialylated glycoproteins containing  $\alpha$ (2,3) or  $\alpha$ (2,6)-linked sialic acid. After washing the membranes six times with PBS containing 0.1% Tween 80, the membranes were incubated for 1 h with 0.2  $\mu$ g mL<sup>-1</sup> HRP-conjugated streptavidin (Sigma-Aldrich) and then washed. Finally, the sialylated proteins were detected using an ECL kit.

## Results

### Construction of a yeast surface display system

In order to express *C. diphtheriae* NanH $\Delta$ <sub>N</sub> (C dip\_NanH $\Delta$ <sub>N</sub>) on the surfaces of *S. cerevisiae* cells, a commercially available **a**-agglutinin-based system was employed: the agglutinin adhesion receptor as a mating factor mediates a cell–cell interaction during the mating time of haploid yeast (Lu et al. 1995; Boder and Wittrup 1997). The **a**-agglutinin receptor consists of two subunits:

Aga1p and Aga2p. Aga1p is secreted and attached to the  $\beta$ -glucan of cell surface. The secreted Aga2p fused to a target protein attaches to Aga1p on the surfaces of yeast cells via disulfide bonds (Fig. 1a). The Aga2p was connected to the catalytic region Glu<sub>34</sub>-Arg<sub>698</sub> of the *C. diphtheriae* NanH protein lacking its signal sequence by a Gly-Ser linker of 17 amino acids (Fig. 1a). For immune detection, the Xpress™ epitope at the N-terminal region and the V5 epitope followed by the 6×histidine (6×His) tag at the C-terminal region of Aga2p-fused Cdicp\_NanH $\Delta$ N were used, respectively. The backbone plasmid, pYD1, is a low copy number vector that contains a CEN6/ARSH4 origin of replication and a *TRP1* auxotroph marker (Fig. 1b). The Aga2p-Cdicp\_NanH $\Delta$ N fusion construct was expressed using the strong inducible *GAL1* promoter. After sequence identification of the construct pYD1-Cdicp\_NanH $\Delta$ N, the expression vector was consecutively transformed into *S. cerevisiae* EBY100 harboring the *AGA1* gene controlled by the *GAL1* promoter for the expression of the recombinant protein on the cell surface; the plasmid-harboring strain was designated as YSK003.

#### Surface expression of *C. diphtheriae* NanH $\Delta$ N sialidase

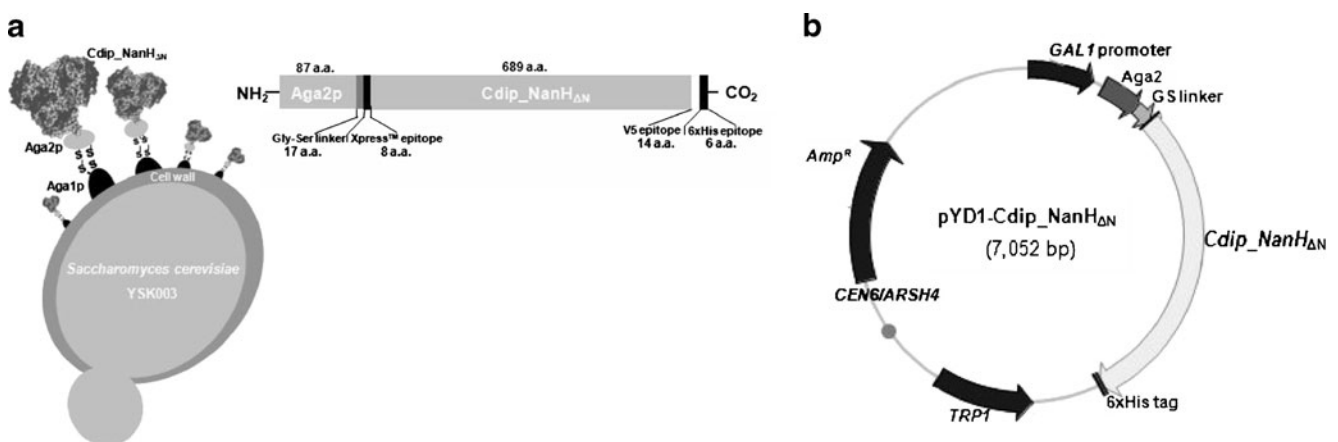
The expression levels of Aga2p-fused Cdicp\_NanH $\Delta$ N protein on the surfaces of YSK003 cells were monitored over the culture period after the galactose induction by a sialidase activity test and immunoblotting analysis. Sialidase activities were assayed using 2'-(4-methylumbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid (MU-Neu5Ac) as a substrate at each culture time point. The fluorescence of the 4-methylumbelliferone (MU) hydrolyzed from MU-Neu5Ac was measured using a fluorospectrophotometer. As shown in Fig. 2, the sialidase activity of YSK003 cells was increased after 2% galactose induction and reached a peak value at 36 h, which corresponded to the early stationary growth

phase. During mid-stationary growth, however, the enzyme activity was slightly reduced, which may result from the shedding of Cdicp\_NanH $\Delta$ N, as sialidase activity was detectable in the cell-free culture supernatant (data not shown). In contrast, the EBY100 cells harboring the empty vector showed no sialidase activity during the same culture times.

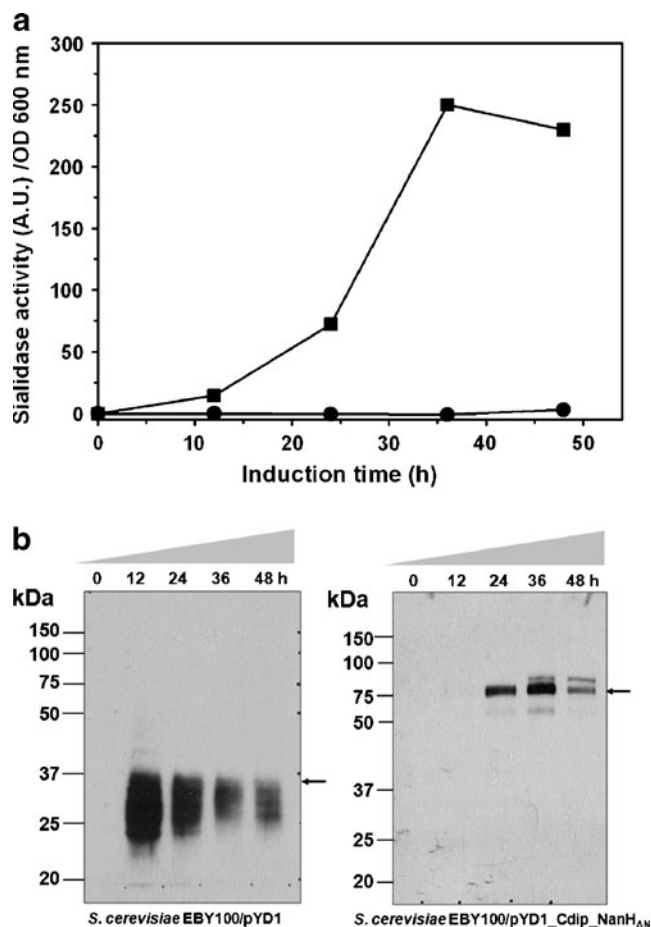
In an effort to further confirm whether the observed activity was derived from the enzyme displayed on the yeast cell surfaces, the surface proteins were dissociated by 1 h of treatment with 100 mM DTT with gentle shaking at 4 °C. The sialidases expressed on the surfaces of the YSK003 cells, harvested at each time point, were analyzed by Western blot analysis using anti-Xpress™ antibody. As shown in Fig. 2b, the Aga2p-Cdicp\_NanH $\Delta$ N was detected on the surface of YSK003 from 24 h of cultivation after galactose induction. However, it was observed that the expression levels of the Aga2p-Cdicp\_NanH $\Delta$ N were lower than those of the Aga2p protein only without NanH fusion. The theoretical molecular weights of the fusion protein Aga2p-Cdicp\_NanH $\Delta$ N and Aga2p lacking the signal sequence were 89,576 and 16,672 Da, respectively. In Western blot analysis, they were detected as several heterogeneous bands within the range of calculated molecular weights (Fig. 2b). It appears that the Aga2p-Cdicp\_NanH $\Delta$ N proteins would be glycosylated in the yeast cells. In addition, Aga2p was also detected as extremely smeared bands, probably due to the high degree of mannosylation.

#### Immunofluorescence detection of surface-displayed *C. diphtheriae* NanH $\Delta$ N sialidase

To confirm the presence of *C. diphtheriae* NanH $\Delta$ N sialidase on the surfaces of the yeast cells, YSK003 cells were analyzed by immunofluorescence microscopy. To detect the recombinant protein, the yeast cells were treated with mouse anti-Xpress™ primary antibody, followed by



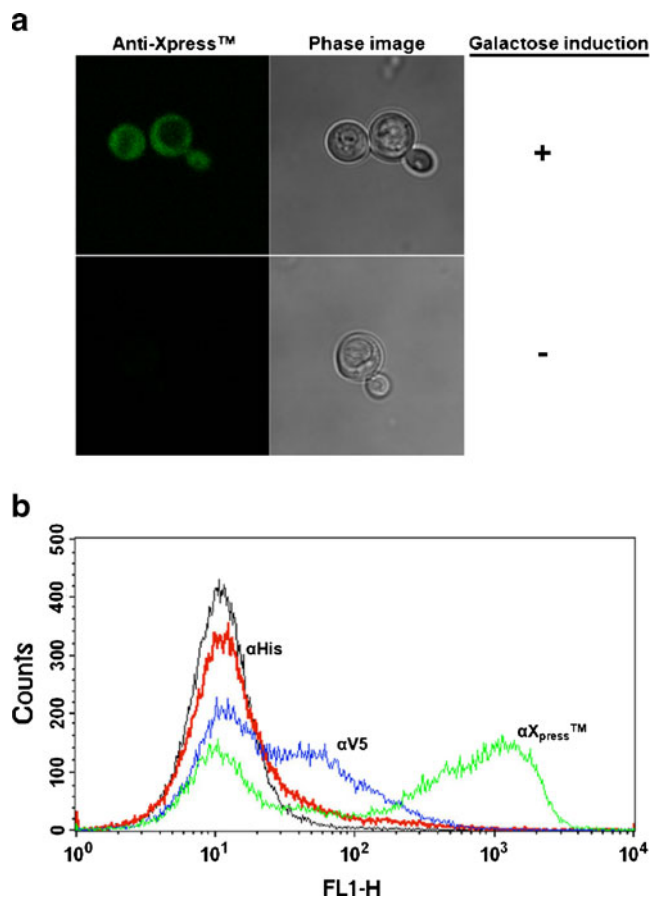
**Fig. 1** Schematic representation of *C. diphtheriae* NanH $\Delta$ N immobilized on yeast cell surface (a) and construction of a plasmid, pYD1-Cdicp\_NanH $\Delta$ N, for expression of the Aga2p-fused Cdicp\_NanH $\Delta$ N protein in *S. cerevisiae* (b)



**Fig. 2** Enzyme activity (**a**) and Western blot analysis (**b**) of *S. cerevisiae* strains harboring pYD1-Cdip\_NanH $\Delta$ N or pYD1 after 2% galactose induction. **a** Time course of enzyme activity of *S. cerevisiae* EBY100 strain harboring an empty vector (filled circle) and YSK003 strain harboring pYD1-Cdip\_NanH $\Delta$ N (filled square). At a galactose induction time of 48 h, optical densities (OD<sub>600</sub>nm) of *S. cerevisiae* EBY100 and YSK003 strains were 5.2 and 4.3, respectively. Sialidase activities were determined with MU-Neu5Ac as the substrate. **b** Western blot analysis of cell surface proteins expressed in *S. cerevisiae* EBY100 harboring pYD1 (left panel) and YSK003 harboring pYD1-Cdip\_NanH $\Delta$ N (right panel). All samples of cell surface proteins were prepared from cells corresponding to 10 OD<sub>600</sub> at each time point. Surface proteins were detected using mouse anti-Xpress<sup>TM</sup> antibody at a dilution of 1:2,000. The arrows indicate the expressed proteins

treatment with FITC-conjugated goat anti-mouse IgG secondary antibody. FITC fluorescence was observed on the cell surface of the yeast grown in SC-Ura-Trp containing 2% galactose (Fig. 3a). On the other hand, the cells cultivated in the medium without galactose did not display any fluorescence (Fig. 3a). The results indicate that galactose-induced Aga2p-Cdip\_NanH $\Delta$  proteins had been successfully localized on the surfaces of YSK003 cells.

In order to quantify the displayed recombinant proteins, fluorescence on the cell surface of YSK003 was measured by flow cytometry using anti-Xpress<sup>TM</sup>, anti-V5, or anti-His antibodies (Fig. 3b). The histograms of a flow cytometry



**Fig. 3** Immunofluorescence (**a**) and flow cytometry (**b**) analysis of the recombinant *S. cerevisiae* YSK003 harboring pYD1-Cdip\_NanH $\Delta$ N. The expression of recombinant Cdip\_NanH $\Delta$ N protein was induced for 48 h with 2% galactose prior to the labeling of the yeast cells. **a** The detection of surface proteins was conducted using mouse anti-Xpress<sup>TM</sup> (αXpress<sup>TM</sup>) and FITC-conjugated goat anti-mouse IgG as a primary and a secondary antibody with dilution ratios of 1:250 and 1:50, respectively. The labeled cells were then analyzed using a LSM510 confocal microscope (Zeiss). **b** The immune staining of the YSK003 cells was conducted using the same procedure for the sample preparation of a confocal microscopy. The cells were incubated with the antibodies anti-V5 (αV5; dilution ratio, 1:50; blue), αXpress<sup>TM</sup> (1:100; green), or anti-His-tag (αHis) (1:20; red) and then detected using FITC-conjugated goat anti-mouse or rabbit IgG antibody at dilution ratio of 1:50 for flow cytometry analysis

using each antibody showed different patterns: The anti-Xpress<sup>TM</sup> antibody targeting the N-terminal part of the Aga2p-Cdip\_NanH $\Delta$ N fusion protein generated the strongest signal. In contrast, the histogram using anti-His antibody staining displayed a population that overlapped almost completely with the negative control population, whereas both anti-Xpress<sup>TM</sup> and anti-V5 antibodies showed two separated populations of expressing and non-expressing cells. It appears that the poly-histidine epitope located at the C-terminal region of the fusion protein might be truncated. Another possibility is that the affinity of the anti-His antibody for the detection of the poly-histidine epitope localized at the C-terminus would be weaker than

those of the other antibodies. When the population detected by the anti-Xpress™ antibody was quantified, 65.4% of YSK003 cells at 48 h cultivation after galactose induction expressed the Aga2p-fused C dip\_NanH $\Delta$ <sub>N</sub> protein on their surfaces. The numbers of surface-displayed C dip\_NanH $\Delta$ <sub>N</sub> protein molecules were approximately estimated as  $3.7 \times 10^8$  molecules per cell based on the ELISA experiment using anti-Xpress™ antibody for quantifying Aga2p-fused C dip\_NanH $\Delta$ <sub>N</sub> protein.

#### Sialidase activity of surface-expressed *C. diphtheriae* NanH $\Delta$ <sub>N</sub>

We attempted to determine whether the Aga2p-fused protein immobilized on YSK003 cells shows sialidase activity. To measure the enzyme activities, yeast cells were employed as a whole cell enzyme, using sialoglycoconjugates or glycoprotein substrates for the hydrolysis reaction. Following 1 h of an enzyme reaction, the free sialic acids in the reaction products were measured using the periodic acid/thiobarbituric acid-based method (Warren 1959). The sialidase immobilized on yeast cell surfaces was fully active for the hydrolysis of sialic acids from sialoglycoconjugates as well as glycoproteins. Moreover, the immobilized sialidase is capable of cleaving both  $\alpha(2,3)$ - and  $\alpha(2,6)$ -sialic acids linked to a galactose moiety of glycoconjugates. In contrast, the cells harboring the empty vector, pYD1, did not exhibit sialidase activities toward the substrates (data not shown). The relative hydrolysis activities toward sialylated *N*-glycan or *O*-glycan are summarized in Table 1.

Trans-sialidase activity toward human-type asialo-*N*-glycans and an asialoglycoprotein

The Aga2p-C dip\_NanH $\Delta$ <sub>N</sub> expressed on the surfaces of YSK003 cells was employed as a catalyst for trans-sialylation toward human-type asialo-*N*-glycans. For the assay, pyridylamino (PA)-labeled asialo-biantennary and asialo-triantennary glycans were used as an acceptor substrate with *p*NP- $\alpha$ -sialoside as a sialic acid donor (acceptor/donor ratio 5:1). In contrast to hydrolysis reaction, the trans-glycosylations, including a case of *Trypanosoma* trans-sialidase, are reported to generally produce higher yields at low temperatures (Ribeirão et al. 1997; Schmidt et al. 2000). Thus, the trans-sialylation reaction using the surface-expressed Aga2p-C dip\_NanH $\Delta$ <sub>N</sub> was conducted for 6 h at 25 °C. After the enzyme reaction, the resulting reaction products were analyzed by HPLC, and the ratios between the sialylated glycan products and the asialo-substrate were calculated from their corresponding peak areas in the HPLC profiles. In order to confirm that the obtained products are sialylated glycans generated from the trans-sialylation reaction by C dip\_NanH $\Delta$ <sub>N</sub>, *Vibrio cholerae* *exo*-sialidase was applied to the reaction mixtures. After treatment with the *V. cholerae* enzyme, the peak corresponding to the enzyme reaction product was reduced, whereas that of the substrates was increased in the HPLC profiles (data not shown). The addition of sialic acid to human-type asialo-*N*-glycans strongly supported the notion that the C dip\_NanH $\Delta$ <sub>N</sub> sialidase immobilized on the surface of YSK003 cells has trans-glycosylation activity. The sialylated products of asialo-

**Table 1** Hydrolysis activity of *C. diphtheriae* NanH $\Delta$ <sub>N</sub> immobilized on yeast cell surface

Substrates	Representative glycan structure	Relative activity <sup>a</sup> (%)	Specific activity ( $\mu\text{Mmin}^{-1}\text{OD}_{600}^{-1}$ )
<i>Natural sialic acid</i>			
Sialyl- $\alpha$ 2,3-lactose	Neu5Ac $\alpha$ 2,3 Gal $\beta$ 1,4Glc	66.6 $\pm$ 5.6	0.23 $\pm$ 0.04
Sialyl- $\alpha$ 2,6-lactose	Neu5Ac $\alpha$ 2,6 Gal $\beta$ 1,4Glc	55.7 $\pm$ 9.5	0.21 $\pm$ 0.04
<i>Unnatural sialic acid</i>			
<i>p</i> NP- $\alpha$ -sialoside	–	69.1 $\pm$ 6.5	0.27 $\pm$ 0.03
MU- $\alpha$ -sialoside	–	68.4 $\pm$ 9.8	0.26 $\pm$ 0.04
<i>Glycoprotein</i>			
Fetuin	Neu5Ac $\alpha$ 2,3(6) Gal $\beta$ 1,4GlcNAc Neu5Ac $\alpha$ 2,3 Gal $\beta$ 1,3GalNAc Neu5Ac $\alpha$ 2,3 Gal $\beta$ 1,4(Neu5Ac $\alpha$ 2,6)GalNAc	33.8 $\pm$ 18.3	0.13 $\pm$ 0.07
$\alpha$ <sub>1</sub> -Acid glycoprotein	(Neu5Ac $\alpha$ 2,3(6)) Gal $\beta$ 1,4 GlcNAc	86.7 $\pm$ 11.9	0.33 $\pm$ 0.04
Transferrin	Neu5Ac $\alpha$ 2,6 Gal $\beta$ 1,4 GlcNAc	88.8 $\pm$ 18.8	0.34 $\pm$ 0.07
Bovine submaxillary mucin	Neu5Ac $\alpha$ 2,6GalNAc Gal $\beta$ 1,3(Neu5Ac $\alpha$ 2,6)GalNAc GlcNAc $\beta$ 1,3(Neu5Ac $\alpha$ 2,6)GalNAc	100.0 $\pm$ 19.5	0.38 $\pm$ 0.07

<sup>a</sup> The hydrolysis activities of the intact yeast cells expressing C dip\_NanH $\Delta$ <sub>N</sub> were determined with 10 mM each of sialylated glycan or 1 mgmL<sup>-1</sup> of glycoprotein. Free sialic acids hydrolyzed from sialoglycoconjugates were determined by the periodic acid/thiobarbituric acid-based method (Warren 1959). The value from the hydrolysis reaction with bovine submaxillary mucin (1 h incubation at 30 °C) was set at 100%

biantennary-*N*-glycan (PA-001), asialo-triantennary *N*-glycan (PA-002), and fucosylated asialo-triantennary-*N*-glycan (PA-010) were generated at yields of 8.7%, 13.7%, and 9.0%, respectively (Table 2). Interestingly, the conversion of triantennary *N*-glycan to sialylated form was shown to be slightly more efficient in reaction with the YSK003 intact cells: asialo-triantennary-*N*-glycan was sialylated approximately 1.5-fold more than the biantennary-*N*-glycan acceptor.

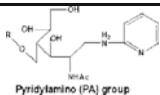
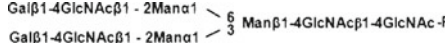

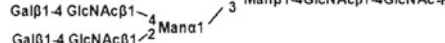

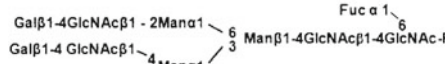
To further evaluate the trans-glycosylation activity of the sialidase observed in YSK003 cells toward an asialoglycoprotein, the trans-sialylation activity was assayed using asialofetuin as an acceptor and *p*NP- $\alpha$ -sialoside as a sialic acid donor. After enzyme reaction, the reaction mixture was applied to 8% SDS-PAGE gel and a precast IEF gel (pH range, 3–7). In order to confirm the sialylation of asialofetuin and the synthesis of sialic acid linkages, proteins in SDS-PAGE were transferred to NC membranes for lectin blot analysis using biotinylated lectins from *M. amurensis* or *S. nigra*, recognizing terminal sialic acid- $\alpha$ (2,3)-galactose (Gal) or sialic acid- $\alpha$ (2,6)-Gal structures, respectively (Shibuya et al. 1987; Wang and Cummings 1988). The lectin blot assay clearly demonstrated that the reacted asialofetuin obtained sialic acids after the enzyme reaction (Fig. 4a). In contrast, no protein bands of unreacted asialofetuin were detected upon lectin blot analysis. The sialylated fetuin was detected as a single band corresponding to the lowest range of the broad fetuin band. This may indicate that not all of the asialofetuin in the reaction mixture could be completely sialylated, and thus a much smaller portion of the sialylated form was generated as compared to native fetuin. Interestingly, the sialylated fetuins were detected by both *M. amurensis* and *S. nigra* lectin. This implied that the glycans of sialylated fetuin could harbor both  $\alpha$ (2,3)- and  $\alpha$ (2,6)-sialic acid. This again indicates that C dip\_NanH $\Delta_N$  is capable of synthesizing  $\alpha$ (2,3)- as well as  $\alpha$ (2,6)-sialic acid linked to acceptor sugar moieties. The

mobility shifting of sialylated fetuin was detected in a range of pI from 5.8 to 5.2 on IEF gel (Fig. 4b). Although asialofetuin were also detected as smearing bands in a similar pI range on IEF gel stained with Coomassie blue (data not shown), only the sialylated proteins were clearly identified via lectin blot analysis. The sialylated fetuin exhibited a ladder pattern in the lectin blot, and the protein mobility was shifted to a negative charge because of the change in the pI value of proteins harboring the negatively charged sugars. Asialofetuin harbors three *N*-glycosylation sites ( $^{99}$ NCS $^{101}$ ,  $^{156}$ NDS $^{158}$ , and  $^{176}$ NGS $^{178}$ ) and three *O*-linked glycosylation sites ( $^{271}$ S,  $^{280}$ T, and  $^{282}$ S). The partially sialylated fetuins would contain heterogeneously sialylated glycans at each of the glycosylation sites. The sialylated proteins generated from the C dip\_NanH $\Delta_N$  reaction did not fall below pI 5 in the IEF gel, unlike the fetuin used as a positive control, thereby indicating incomplete sialylation. Some of the asialofetuin were still detected near the pI value of 6.0 on IEF gel, even after 60 h of enzyme reaction (data not shown). In an effort to improve the sialylation efficiency in the enzyme reaction, we added 30% (v/v) of dimethyl sulfoxide as an organic cosolvent to the reaction mixture, but the mobility of the protein was not changed profoundly. The sialylation efficiency of asialoglycoprotein appears to be similar to that of the free asialoglycans, with a conversion ratio of less than 15% (Table 2) in the reaction mediated by the intact yeast cells displaying C dip\_NanH $\Delta_N$  on their surfaces. These results demonstrate that the yeast surface-displayed C dip\_NanH $\Delta_N$  is capable of transferring a sialic acid to a variety of asialoglycan acceptors, including glycoproteins.

## Discussion

*C. diphtheriae* KCTC3075 NanH is a secreted sialidase. It can catalyze the hydrolysis of  $\alpha$ (2,3)- and  $\alpha$ (2,6)-linked

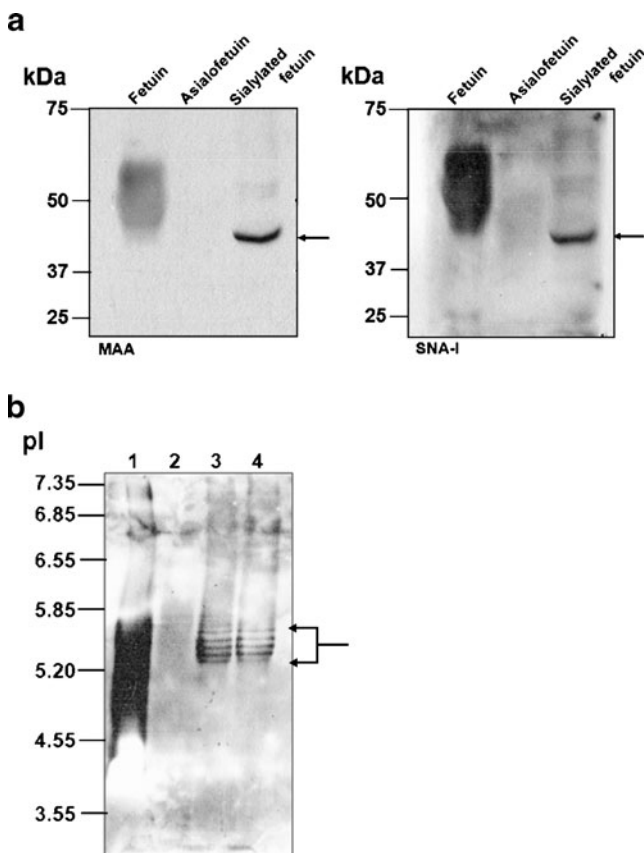
**Table 2** Sialylation of pyridylamino (PA)-labeled asialoglycans by C dip\_NanH $\Delta_N$  immobilized on yeast cell surface

Acceptor substrate	Retention time (t <sub>r</sub> , min)		Specific production rate (nM h <sup>-1</sup> g-wet cell <sup>-1</sup> )	Conversion (%) <sup>c</sup>	
	substrate <sup>a</sup>	product <sup>b</sup>			
 Galβ1-4GlcNAcβ1-2Manα1 Galβ1-4GlcNAcβ1-2Manα1	 Manβ1-4GlcNAcβ1-4GlcNAc-R	6.58	23.71	29.2	8.7
 Galβ1-4GlcNAcβ1-2Manα1 Galβ1-4GlcNAcβ1-4Manα1 Galβ1-4GlcNAcβ1-2Manα1	 Manβ1-4GlcNAcβ1-4GlcNAc-R	7.25	37.59	50.5	13.7
 Galβ1-4GlcNAcβ1-2Manα1 Galβ1-4GlcNAcβ1-4Manα1 Galβ1-4GlcNAcβ1-2Manα1	 Manβ1-4GlcNAcβ1-4GlcNAc-R Fucα1-6	7.38	40.09	33.1	9.0

<sup>a</sup> Substrate indicates PA-oligosaccharides used as an acceptor sugar

<sup>b</sup> Product indicates sialylated PA-oligosaccharides produced by the enzyme reaction

<sup>c</sup> Conversion ratio (percent) was calculated by the following formula: Conversion(%) = 100 × [product]<sub>t</sub> / ([substrate]<sub>t</sub> + [product]<sub>t</sub>)



**Fig. 4** Lectin blot analysis for sialylation of asialofetuin by C<sub>dip</sub>\_NanH<sub>ΔN</sub> immobilized on yeast cell surface. **a** Sialylated fetuins separated on 8% SDS-PAGE gel were detected by *M. amurensis* and *S. nigra* lectins. Fetuin and asialofetuin were used as positive and negative controls, respectively. Sialylated fetuin was a product generated by reaction with the intact YSK003 cells in 50 mM Tris-HCl buffer (pH6.8). **b** Sialylated proteins, separated on IEF gel (pH gradient, 3–7), were detected by biotinylated *M. amurensis* lectin with dilution rate 1:1,000. Lane 1, fetuin; lane 2, asialofetuin; lane 3, sialylated fetuin reacted in 50 mM Tris-HCl buffer (pH 6.8) containing 30% (v/v) dimethyl sulfoxide (DMSO) as a cosolvent; lane 4, sialylated fetuin reacted in the same buffer without any cosolvent. The arrows indicate the sialylated proteins

sialic acids, as well as the transfer of sialic acid from  $\alpha$ -sialosides to a galactose moiety of oligosaccharides (Kim et al. 2010). Our previous study demonstrated that the purified mature NanH sialidase (NanH<sub>ΔN</sub>) protein was capable of transferring sialic acids to several asialoglycans *via* the trans-glycosylation reaction using  $\alpha(2,3)$ - and  $\alpha(2,6)$ -linked sialic acids as donor substrates. In this study, we constructed the yeast surface-displayed *C. diphtheriae* NanH<sub>ΔN</sub> system using an Aga2p-fusion tag to exploit the sialylation reaction mediated by the trans-sialylation of the sialidase. The Aga2p-fused C<sub>dip</sub>\_NanH<sub>ΔN</sub> was expressed successfully on the yeast cell surface as a fully active form to hydrolyze sialic acid containing  $\alpha(2,3)$ - and  $\alpha(2,6)$ -linked sialic acid from free sialoglycans as well as glycoproteins (Table 1). In addition, immobilized proteins could be reused

several times for *in vitro* trans-glycosylation reaction. The C<sub>dip</sub>\_NanH<sub>ΔN</sub> displayed on yeast cell surface had kept more than 90% of its activity even after ten times of recycled uses in the enzyme reaction (Supplementary Material Fig. S1). However, the expression level of the fusion protein in the yeast cells was shown to be less than that of Aga2p alone (Fig. 2b). The gene encoding for NanH<sub>ΔN</sub> derived from *C. diphtheriae* contains relatively high G+C contents of 47.7%. The genomic GC content of *C. diphtheriae* is even higher as 53.5%, and it is reflected in the codon usage of *C. diphtheriae*, which favors the CGN<sub>arg</sub> and CCC<sub>pro</sub>/CGC<sub>pro</sub>-encoded arginine and proline, respectively. In contrast, *S. cerevisiae*, which harbors high A+T content (average GC content, 39.8%), uses these codons only rarely and rather prefers AGA<sub>arg</sub>/AGG<sub>arg</sub> and CCA<sub>pro</sub>/CCU<sub>pro</sub> for the amino acids. Thus, the difference of codon usage preference would result in the low-level expression of Aga2p-fused *C. diphtheriae* protein in *S. cerevisiae*.

In addition, Western blot analysis demonstrated the heterogeneity of the proteins expressed in *S. cerevisiae*, probably as the result of glycosylation. The Aga2p-fused C<sub>dip</sub>\_NanH<sub>ΔN</sub> protein contains eight putative *O*-glycosylation sites as well as one *N*-glycosylation sequon, <sup>462</sup>NXS<sup>464</sup>, on its amino acid sequence. Glycosylation increases the molecular weight of the protein. In addition, the expressed glycoproteins often show heterogeneity in Western blot analysis due to heterogeneity of glycans attached or different occupancies between each glycosylation sites. In fact, after PNGase F treatment to remove *N*-glycan and  $\beta$ -elimination to discard *O*-glycosylation in the Aga2p-fused C<sub>dip</sub>\_NanH<sub>ΔN</sub> protein, the protein detached from yeast cells showed an identical size of approximately 80 kDa (data not shown). However, several bands were still observed between approximately 37 and 50 kDa, thereby implying that this immobilized protein might be degraded when cells were cultivated at 30 °C. The amino acid sequence of the C<sub>dip</sub>\_NanH<sub>ΔN</sub> contains several putative endogenous aspartic protease cleavage sites, K<sub>145</sub>R<sub>146</sub>, K<sub>230</sub>R<sub>231</sub>, K<sub>379</sub>R<sub>380</sub>K<sub>381</sub>, R<sub>552</sub>K<sub>553</sub>, and R<sub>673</sub>R<sub>674</sub> (Kim et al. 2010). Further, the protein contains a putative coiled-coil structure containing the alanine-rich linker domain (Asp<sub>515</sub>–Gln<sub>733</sub>) as well as a putative transmembrane domain of 23 amino acids at the C-terminal region (Gly<sub>696</sub>–Phe<sub>709</sub>), which might be unstable in a heterologous expression system. These might explain why anti-His antibody to recognize the C-terminal epitope of Aga2p-fused C<sub>dip</sub>\_NanH<sub>ΔN</sub> could not bind to the protein immobilized on yeast cell surfaces in FACS analysis, although other antibodies,  $\alpha$ V5 and  $\alpha$ Xpress<sup>TM</sup>, proved effective in the detection of the target protein (Fig. 3b).

*S. cerevisiae* lacks the gene cluster for the metabolism of sialic acid. This microorganism does not synthesize sialic acid, nor does it metabolize its derivatives. Moreover, no

transporters for sialic acid could be detected in *S. cerevisiae* (Berninsone et al. 1997). Thus, the sialic acid hydrolyzed from pNP- $\alpha$ -sialoside by C dip\_NanH $_{\Delta N}$  could be used only for the sialylation of asialoglycan. However, the enzyme did not transfer sialic acids to all galactose moieties of *N*-glycan and *N*-acetylgalactosamine moieties of *O*-glycan in asialofetuin. The enzyme reaction reflects the equilibrium between a sialylated product and an asialo-reactant in the trans-sialylation (data not shown). *M. amurensis*- and *S. nigra*- lectin blot analysis showed that C dip\_NanH $_{\Delta N}$  transferred sialic acids to the acceptor protein with  $\alpha(2,3)$ - and  $\alpha(2,6)$ -linkage without regioselectivity. The non-regioselective catalytic properties of C dip\_NanH $_{\Delta N}$  might be advantageous in some circumstances for in vitro sialylation reaction of proteins containing heterogeneous asialo-*N*-glycans and *O*-glycans (van der Boog et al. 2005; Bork et al. 2009). However, in general, it would be disadvantageous for the synthesis of regioselectively sialylated glycans. Several groups already reported that the regioselectivity of trans-glycosylation reaction using sialidases depends on the acceptor substrates as well as physical reaction conditions (Thiem and Sauerbrei 1991; Ajisaka et al. 1994; Schmidt et al. 2000). To improve the regioselectivity of C dip\_NanH $_{\Delta N}$  reaction, it will be required to test and optimize the sialylation reaction processes using various asialo glycan substrates.

Several parameters, including the reaction temperature, acceptor/donor ratio, reaction time, and so on for enzyme reaction, could influence the efficiency of the sialylation by *C. diphtheriae* NanH $_{\Delta N}$  immobilized on yeast cell surfaces, which has been noted in the case of trans-sialylation reactions by sialidases from *V. cholera*, *Clostridium perfringens*, *Salmonella typhimurium*, or *Arthrobacter ureafaciens* (Thiem and Sauerbrei 1991; Ajisaka et al. 1994; Schmidt et al. 2000). The enzyme preferred a triantennary *N*-glycan as a sialic acid acceptor as compared to biantennary-*N*-glycan and exhibited a conversion ratio of approximately 13%. The optimization of reaction conditions for sialic acid transfer toward free asialoglycans and glycoproteins by *C. diphtheriae* NanH $_{\Delta N}$  is required to improve the trans-sialylation yield. Additionally, an appropriate organic cosolvent in the enzyme reaction mixture would be required to increase the sialylation yield for the synthesis of sialylated glycans and glycoproteins (Thiem and Sauerbrei 1991; Schmidt et al. 2000). It will be necessary, in the future, to screen for an effective cosolvent for the sialylation reaction of the enzyme.

In summary, we expressed *C. diphtheriae* sialidase on the surfaces of yeast cells for the transfer of sialic acid to asialo-*N*-glycans via the trans-sialylation reaction. The immobilized sialidase was efficiently localized on the cell walls using a mating factor, Aga1p–Aga2p. The protein was expressed in fully active form on the yeast cell surfaces, and the bacterial sialidase displayed on the yeast

cell surface was shown to transfer sialic acid from a sialoglycoconjugate donor substrate to human-type asialo-*N*-glycans, as well as to an asialoglycoprotein via the trans-sialylation reaction. The cell surface-expressed *C. diphtheriae* NanH $_{\Delta N}$  sialidase could therefore be usefully developed as a whole cell biocatalysts for the synthesis of sialylated glycoconjugates, as well as the hydrolysis of oligosaccharides containing  $\alpha(2,3)$ - and  $\alpha(2,6)$ -sialic acid for the remodeling of the glycan structures of glycoproteins.

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