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Biotransformation Using Recombinant Cmp Sialic Acid Synthetase and α-2, 6-Sialyltran Sferase: Enzymatic Synthesis of Sialosides

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ABSTRACT

In this research, we successfully expressed recombinant CMP-sialic Acid Synthetase (CSS) from *Neisseria meningitides* and 2,6-Sialyltransferase (SAT) from *Photobacterium damsela* in *E. coli* BL21(DE3) fermented at a scale of up to 8 litres using individual plasmids pIRL-1 and pIRL-4b, respectively. After cell lysis with BugBuster, enzyme levels of 2U and 22U per litre were produced for CSS and SAT, respectively. The enzyme solutions were either used directly as crude preparations or further purified by affinity chromatography. Characterization of the CSS and SAT confirmed that both enzymes had comparable properties to those described in the literature. The production of cytidine 5'-monophosphate N-acetylneuraminic acid (CMP-NeuAc) and CMP-9-azido-NeuAc using crude CSS was successful with >90% conversion at scales from 100 mg to 5 g. Activated sugar purification by ethanol precipitation was optimized. Finally, the CSS and SAT enzymes were applied to a large-scale synthesis of a sialylated lactosamine glycoside via a two-step biotransformation. The initial step employed crude CSS to convert Cytidine Triphosphate (CTP) and 9-azido-NeuAc to CMP-9-azido-NeuAc at a conversion efficiency of 98%. This reaction mixture, after ultrafiltration to remove β-galactosidase activity co-expressed by *E. coli* BL21, was used as the donor substrate for the second step involving SAT. The sialoside 9-azido-sialyl-α-2,6'-lactosamine glycoside was produced with 86% conversion of the starting glycoside. Purification of the product was achieved by chromatography on Diaion HP-20 (a hydrophobic styrenic resin).

Keywords: CMP-Sialic Acid Synthetase, Sialyltransferase, CMP-Neu5Ac, Lactosamine Glycoside, Sialylation

1. INTRODUCTION

Sialic acids are a group of over 50 acidic sugars related to *N*-acetylneuraminic acid (NeuAc). They are found in all higher animals from the echinoderms onwards, but have also been described for some fungi and bacteria (Angata and Varki, 2002; Schauer, 2000). In general, sialic acids are glycosidically linked to the non-reducing end of the carbohydrate residues of glycoconjugates including glycoproteins and glycolipids, to form sialylglycoconjugates (Hakomori and Igarashi, 1995; Paulson, 1989).

Sialylglycoconjugates play an important role in a number of biological processes including cell-cell interactions and -signaling involved in pathological processes such as viral infections, inflammation and tumor metastasis (Blomme *et al*., 2009; Hakomori and Igarashi, 1995; Mehlen and Puisieux, 2006). In addition, numerous responses and processes of the immune system are modulated by glycoconjugates on a cell's surface. Soluble mimics of these structures (sialoside analogues in particular) can be used to modulate these processes and can potentially provide therapeutic benefits.

In vivo models have provided evidence for the involvement of glycoconjugates in the improvement and

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maintenance of gut health in infants by preventing the adhesion of pathogenic microorganisms (e.g., *E. coli* and *Helicobacter pylori*) to the uroepithelial cells and through the promotion of the growth of beneficial bacteria including *bifidobacteria* (Gibson and Roberfroid, 1995; Lasky, 1995; Mysore *et al*., 1999; Sharon and Ofek, 2000). Furthermore, there are indications for their involvement in healthy brain development in infants (Izumi *et al*., 1993; Wang *et al*., 2003). Therefore, sialoside analogues are valuable targets for both pharmaceutical and nutraceutical applications.

The chemical synthesis of sialosides involves multiple steps and requires the introduction and subsequent removal of protecting groups to ensure both regio-and stereo-selectivity. In contrast, sialoside production using biotransformation is achievable in a single step without protecting groups by employing specific sialyltransferases (SAT). These enzymes transfer sialic acid from CMP-sialic acid to a glycosyl acceptor in a regio- and stereoselective manner. CMP-sialic acid is produced (*in situ*) from CTP and sialic acid by employing a CMP-sialic Acid Synthetase (CSS).

The use of purified enzymes has been described for the synthesis of several sialosides using either a 'one-pot' system consisting of two enzymes or two/three-step processes where the biotransformations are carried out in sequential steps (Blixt *et al*., 2008; Blixt and Paulson, 2003; Yu *et al*., 2005; 2006). A recombinant CSS from *N. meningitides* has been extensively utilized for the synthesis of CMP-sialic acid analogues due to its broad specificity in activating a diverse range of sialic acid molecules. Although SATs derived from both mammalian and bacterial sources have been utilised, the recombinant bacterial SAT (particularly from *Photobacterium*) is generally favoured because of its broad specificity in both acceptor and donor substrates.

A 'whole-cell approach' by genetically engineered *Escherichia coli* strain expressing both CSS and SAT enzymes has also been described (Drouillard *et al*., 2010; Endo *et al*., 2001; Fierfort and Samain, 2008). This microbiological process has an economic advantage for large scale production. However, the direct use of genetically modified organisms in the synthesis of nutraceuticals or food ingredients introduces a number of regulatory considerations.

Sialylated lactosamine glycosides are present in the backbone of many glycans. These molecules are important probes in the investigation of the biological functions of the sialylglycoconjugates (Niemela Ritva *et al*., 1998; Ujita *et al*., 1999). The enzymatic synthesis of these sialosides has been of interest to several research groups (Blixt and Paulson, 2003; Endo *et al*., 2001; Mine *et al*., 2010; Yu *et al*., 2006). Yu *et al.* (2005) carried out the sialylation of azido-lactosamine on a 100 mg scale via a 2-step enzymatic process (with CSS and a 2, 6-SAT derived from *P. damsela*). Blixt *et al.* (2008) prepared a sialylated (*N*-carboxybenzyl aminoethyl) lactosamine glycoside from the respective monosaccharides using a galactosyltransferase and a recombinant human 2, 6-SAT (hST6Gal-I; EC2.4.99.1) in a one-pot reaction mixture.

In this study, we describe the production of recombinant CSS from *N. meningitides* and 2, 6-SAT from *P. damsela* and their application in the synthesis of several sialosides including a multi gram scale production of a sialylated lactosamine glycoside. The product can be used to prepare a synthetic ligand for Siglec-2 (CD22) which may have therapeutic benefit (Blixt *et al*., 2008).

2. MATERIALS AND METHODS

2.1. Chemicals and Materials

2.1.1. Chemicals

Fluoresceinyl-lactose (ßGal-(1-4)-ßGlc-$(CH_2)_6$NHC(S) NH-fluorescein) (LacF) was synthesized according to a previously reported method (Limberg *et al*., 1996). *N*-carboxybenzyl-aminoethyl *N*-acetyl-lactosamine glycoside (2-Cbz-aminoethyl ß-D-galactopyranosyl-(1-4)-ß-D-glucopyranoside) (Wang *et al*., 2011) and 9-azido-*N*-NeuAc were obtained from GlycoSyn (Lower Hutt, New Zealand). *N*-Acetyl-lactosaminide was from Toronto Research Chemical (North York, Canada). Sialic acid (95% in purity) was purchased from NZ Pharmaceuticals (Palmerston North, New Zealand) and CTP (>85% in purity) was from Weihua Pharma Co. (Hangzhou, China). All other chemicals were from Sigma and of analytical grade. All solvents were from Merck and of HPLC grade.

Recombinant α-2,6-SAT from *Photobacterium damsela* expressed in *E. coli* BL21 was purchased from Sigma and used to compare with 2,6-SAT enzyme produced in this study. A clone encoding the CSS from *N. meningitides* was kindly provided by Prof. Rita Gerardy-Schahn (Medical School Hannover, Hannover, Germany). Inorganic pyrophosphatase was from Sigma.

The *Strep*-Tactin column (1 mL) and the buffers required for equilibration, elution and storage were purchased from IBA (Göttingen, Germany). Another *Strep*-Tactin column (10 mL) was packed using *Strep*-Tactin resin according to the supplier's Instructions (IBA).

2.2. Instrumentation

The HPLC analysis was conducted on a quaternary pump system (Agilent 1100) with a gradient mixer and an auto-sampler. The system was also equipped with a diode array detector (Agilent) and a fluorescence detector (Shimadzu RF-10A XL).

The liquid-chromatography-mass spectrometry (LC-MS) experiments were performed on a Waters 2795 Separations Module combined with a Q-Tof Premier mass spectrometer (Micromass, UK).

A low pressure chromatography system (ÄKTA Purifier, GE Healthcare) was used to conduct the protein purification.

^{1}H NMR and ^{13}C NMR spectra were recorded in D_2O on a NMR Spectrometer (Bruker Avance III 500) with a HPxw 4400 linux workstation installed with Bruker TopSpin 2.1 (patch level 6) software. The spectra were referenced with tert-butyl alcohol (^{1}H, 1.24 ppm and ^{13}C, 30.29 ppm).

2.3. Protein Analysis

Protein concentration was determined according to the method of Bradford using assay reagents supplied by Bio-Rad and calibrated using bovine serum albumin as the standard. A Bio-Rad DC method was also used to determine the level of protein in the enzyme preparations containing detergent. Protein purity was analyzed by SDS-PAGE on Pre-cast gels (4-12%, Bis Tris, Invitrogen) as described by (Laemmli, 1970). Protein bands were visualized using Simple Blue Safe Stain (Invitrogen) following the suppliers protocol.

2.4. Centrifugation

Centrifugations were performed using either a Sorvall RC 12BP centrifuge (Thermo Scientific (Massachusetts, MA, United States) for large capacity (>1 L) and low spin force (<5000×g) or a Sorvall RC-5B centrifuge with GS-3 rotor for sample capacity less than 1L and low spin force (<9000×g) For ultracentrifugations (>90,000×g) a Beckman Coulter Optima L-100XP centrifuge (Brea CA, USA) was used with a Ti 70 rotor.

2.5. Cloning and Expression of Recombinant CSS and SAT

2.5.1. Construction of Plasmids and Recombinant *E. coli* Strains

One Shot® *E. coli* BL21 (DE3) chemically competent cells were purchased from Life Technologies (CA, USA). CSS expression plasmid pIRL-1 (pStrep-NmB-Syn-His, a kind gift from Professor Rita Gerardy-Schahn MHH, Hannover, Germany) contained the CSS gene from *N. meningitidis* with the DNA sequences of a Strep-tag at its 5' end and a His-tag at the 3' end on the gene expression vector pET-22b(+) (Novagen, Madison, WI, USA). SAT expression plasmid pIRL-4b was constructed with a truncated α-2, 6-SAT (GenBank accession no.: ATCC33539) from *Photobacterium damsela*. Firstly the DNA sequence was amplified with PCR by using primers: ST-F: 5' GGATCC-TGTAATAGTGACAATACC 3' and ST-R: 5' CTCGAG-AGCCCAGAACAGAACATC 3' with a *Bam*HI or *Xho*I site (underlined), respectively. After confirmation of the PCR product by DNA sequencing, the *Bam*HI/*Xho*I digested PCR product was cloned onto *Bam*HI/*Xho*I digested plasmid pStrep-ZfCSS which was also a gift from Professor Rita Gerardy-Schahn. After expression, the encoded recombinant SAT on plasmid pIRL-4b has a Strep- and 6×His-tag at its N- and C-terminus, respectively. Both plasmids were transformed into *E. coli* BL21 (DE3) by heat shock and the final *E. coli* BL21 (DE3) strains IRL-450 (harboring pIRL-1) and IRL-459 (harboring pIRL-4b) were constructed respectively. *E. coli* BL21 (DE3) strains were routinely maintained either on Luria broth agar plates at 4°C or in 15% glycerol at 80°C unless otherwise stated.

2.6. Expression of Recombinant CSS and SAT Enzymes in *E. Coli*

Enzyme production strains IRL-450 and IRL-459 were prepared from 100 mL to 3 L shake flasks, according to the manufacture's manual for *E. coli* BL21 (DE3) from Life Technologies. Briefly, Luria broth containing ampicillin (100 μg mL^{-1}) was inoculated with 1.0% (v/v) overnight seed culture and then incubated at 25°C under gentle shaking (150 rpm) until an OD_{550} of 0.6 was reached. Then IPTG was added to the culture to a final concentration of 0.75 mM and the incubation continued over night. For enzyme production at an 8 L scale, a 15 L BIOSTAT® C-DCU bioreactor (Melsungen, Germany) was used. An 8 L initial broth [yeast extract (5 g L^{-1}), peptone (10 g L^{-1}), NaCl (1 g L^{-1}), glucose (2.5 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.2 g L^{-1}), K_2HPO_4 (4 g L^{-1}), KH_2PO_4 (2 g L^{-1}), $Na_2HPO_4{\cdot}2H_2O$ (7 g L^{-1}), $(NH_4)_2 SO_4$ (2.5g L^{-1}), NH_4Cl (0.2 g L^{-1}), LG 109 (antifoam) (1 g L^{-1}) and ampicillin (100 mL^{-1}) (Li *et al*., 2006)] was inoculated with 1.0% (v/v) over night seed culture and then incubated under the following conditions: aeration: 8 L min^{-1} (1 vvm), agitation: 150 rpm, temperature: 25°C

and pH 7.0 (controlled with 1 M sulphuric acid and 1 M NaOH). When the OD_{600} reached 0.6, the gene expression was induced by the addition of 200 mL sterile induction solution containing 200 g of lactose, 75 g of yeast extract and 5 g of $MgSO_4 \cdot 7H_2O$ (Li *et al*., 2006) to the broth and the cultivation continued under the same conditions until it was stopped after a total incubation time of 21 h.

After the preparation of the *E. coli* cultures, the cells were harvested by centrifugation at 5,311×g for 30 min at 4°C. The harvested *E. coli* cells were suspended in BugBuster® protein extraction reagent (EMD Chemicals, San Diego, CA, USA) (5 mL g^{-1} biomass) containing lysozyme at a final concentration of 1.0 mg mL^{-1}. The cell lysis was performed at room temperature by gentle shaking (100 rpm) for 30 min. After cell lysis, the cell debris was removed by ultracentrifugation at 126,000×g for 1 h at 4°C. The supernatants were collected and stored at 4°C as the crude enzyme or further purified as required.

2.7. CSS

2.7.1. Determination of CSS activity

CSS activity during the purification was determined according to Fujita *et al.* (2005) with small modifications. Briefly, 20 µL of the sample solution were added to 50 µL substrate solution consisting of 1 mM NeuAc and 5 mM CTP in Tris/HCl buffer (0.1 M, pH 9.0 containing 20 mM $MgCl_2$ and 0.1 mM $NaVO_3$). The mixture was incubated for 90 min at 37 µC. Then 50 µL of this reaction mixture were added to 50 µL Tris/HCl buffer (0.125 M, pH 7.9) containing sialic acid lyase (SAL, 1U mL^{-1}), lactate dehydrogenase (LDH, 1U mL^{-1}) and NADH (2 mM). The resulting solution was incubated on a 96 well microtitre well plate at 37°C for 100 min. The decrease of the fluorescence due to the transformation of NADH to NAD^+ was measured at an excitation wavelength (Ex) of 350 nm and an Emission wavelength (Em) of 538 nm using a microtiter plate reader (Fluoroskan Ascent, Thermo Labsystems) supported and controlled by the Ascent Software (version 2.6).

One unit of the CSS activity was defined as the amount of enzyme that consumes 1 µmol of sialic acid per minute under the conditions used. Therefore, in the second step (SAL/LDH assay) a consumption of sialic acid at a rate of 1 µmol min^{-1} is equivalent to the conversion of NADH to NAD^+ at 1 µmoL^{-1} min.

2.8. Purification of Recombinant CSS

Pure CSS was obtained by applying crude cell lysis extract on a Strep-Tactin column. This column contains a modified streptavidin which specifically binds to the octapeptide with the sequence Trp-Ser-His-Pro-Gln-X-Glu-Lys.

The supernatant obtained as described in 2.2.2.(1.0 mL) was applied to a *Strep*-Tactin column (1.0 mL) which had been equilibrated in application buffer (0.1 M Tris/HCl, pH 8, containing 150 mM NaCl and 1 mM EDTA). After washing the column with 10 mL of the application buffer, the bound protein was eluted with 5 mL of elution buffer (application buffer containing 2.5 mM desthiobiotin). Fractions with a volume of 0.5 mL were collected. All chromatographic steps were carried out at a flow rate of 1 mL min^{-1}. The eluant was monitored at 280 nm. The protein concentration and enzyme activity in the fractions were determined as described above.

2.9. Characterization of Purified CSS

A typical incubation mixture contained 20 nmol sialic acid (NeuAc, NeuGc, KDN or 9-azido-NeuAC) and 40 nmol CTP in 90 µL Tris/HCl buffer (0.1 M at pH 7.0 to 9.0 containing 20 mM $MgCl_2$ and 0.1 mM $NaVO_3$). This solution was heated at the respective reaction temperature (25-50°C). The reaction was started by the addition of 10 µL of enzyme preparation. After time periods of 0.5, 1, 2, 4 and 24 h, the reaction was stopped by adding 25 µL of SDS (2.5% in water). The solution was diluted with 125 µL of water and was analyzed by HPLC using an amino-phase column (Luna 5µ NH_2, 250×4.6 mm, Phenomenex) under isocratic elution with 20 mM phosphate buffer pH 2.7/acetonitrile (95:5, v/v). The eluting peaks were detected by UV absorption at 214, 232 and 280 nm.

Reaction conditions were varied by changing the ratio between sialic acid and CTP. In addition, Mg^{2+} was replaced by other divalent cations such as Zn^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} and Ni^{2+}.

2.10. Production of CMP-Sialic Acid

The enzymatic synthesis was performed on various scales, from 5 mg to 3 g of starting material. In a typical reaction solution, sialic acid and CTP were dissolved in diluted crude enzyme preparation (1:1 diluted with Tris/HCl buffer, 0.1 M, pH 8.5 containing 40 mM $MgCl_2$) to give end concentrations of approximately 30 and 18 mM, respectively. After addition of inorganic pyrophosphatase (end concentration 1 mU mL^{-1}), the reaction mixture was incubated at 37°C for one to 4 h with gentle shaking. Aliquots of CTP were added after 1, 2 and 3 h and the pH was monitored and adjusted to pH 8.5 using 2 M NaOH. The reaction mixture was allowed

to cool and was centrifuged for 15 min at 11,900×g to remove any precipitate. The supernatant was subjected to ultra filtration by centrifugation at 2500×g for 30 min using centrifugal ultra-filtration cartridges (MWCO: 10 kDa, Millipore). The filtrate containing CMP-sialic acid was directly used for the sialyltransferase reaction (2.5). The reaction products were analyzed by HPLC as described in 2.3.3.

2.11. Purification of CMP-NeuAc

2.11.1. Optimization of Precipitation Step

Ice cold ethanol (18 mL) was added to the reaction solution (2 mL, as described in 2.3.4) and the mixture was left at 4°C for 2 hs. The precipitate was then collected by centrifugation (10 min at 11,900×g). After removal of the supernatant the pellet was dissolved in water (2 mL) and analyzed by HPLC (2.3.3). The influence of pH on precipitation was investigated by adjusting the pH of the reaction solution to 3, 4, 5 and 6 prior to the addition of ethanol.

2.12. Purification of CMP-NeuAc and CMP-9-azido-NeuAc

NeuAc (100 mg, 0.32 mmol) and CTP (100 mg, 0.19 mmol) in 10 mL reaction buffer (2.3.4) were incubated with CSS (0.1 U) at 37°C for 4 h in the presence of inorganic pyrophosphatase (1 mU). After 1, 2 and 3 hs aliquots of 100 mg CTP were added and samples were taken for HPLC analysis (2.3.3). The reaction mixture was centrifuged and subjected to ultrafiltration (2.3.4). To 5 mL of the filtrate 45 mL cold ethanol were added and the mixture was left at 4°C for 2 h. The precipitate was collected by centrifugation (15 minutes at 11,900×g). After removal of the supernatant, the pellet was dissolved in 5 mL water and the solution was frozen and freeze-dried. The process yielded 92.5 mg (0.15 mmol, 47%) white powder. Quantification and analysis by HPLC indicated the presence of CTP (approx. 9%) and some salt.

9-azido-NeuAc (111 mg, 0.323 mmol) was converted to CMP-9-azido NeuAc as described above. Following the same protocol as for NeuAc, 110 mg product (0.17 mmol, 52% yield) was isolated after precipitation and freeze drying.

2.13. SAT

2.13.1. Determination of Sialyltransferase Activity

The sialyltransferase activity was measured using LacF as an acceptor substrate and CMP-NeuAc as donor substrate (Limberg *et al*., 1996). A typical assay contained 10 µL each of LacF (0.12 mM) and CMP-NeuAc (0.25 mM) and 70 µL of Tris buffer (0.1 M, pH 7.5). The reaction was initiated by adding 10 µL of enzyme solution. After incubating the reaction mixture for 40 min at 37°C under gentle shaking (160 rpm), the mixtures were boiled for 5 min to stop further conversion and centrifuged at 15,000×g for 3 min. An aliquot of 10 µL of the supernatant were diluted with 190 µL of water and analyzed by fluorimetric HPLC. One unit of sialyltransferase activity was defined as the amount of enzyme that was required to transfer 1 µmol of sialic acid per min to LacF.

The sample mixtures were separated on a C18 column (250×4.6 mm, 5µ, Phenomenex) coupled with a guard cartridge and eluted by a linear gradient of phosphate buffer at pH 7.2 in acetonitrile **Table 1**. The elution was monitored by fluorescence at Ex 488 nm and Em 510 nm.

2.14. Purification of Wild-Type 2, 6-SAT

The seeding of *P. damsela* (ATCC33539) was produced according to Yamamoto *et al.* (1996). The cell culture was grown in 3PYBG broth (200 mL) for 8 h at 30°C under gentle shaking (150 rpm). The cells were harvested by centrifugation at 6,000×g for 20 min. The pellet was suspended in 20 mM Mes buffer (pH 6.0 containing 1 M NaCl) and centrifuged at 6,000×g for 20 min. Then this pellet was suspended in 20 mM Mes buffer (pH 6.0 containing 0.2% Triton X-100 and 1 M NaCl) and treated with ultrasound until the OD_{600} dropped to 30% of the value in the original cell culture. The lysate was centrifuged at 6,000×g for 20 min. The resulting supernatant was then centrifuged at 126,000×g for 60 min at 4°C. The salt in the supernatant was removed via overnight dialysis (4°C) against 20 mM Mes buffer (pH 6 containing 0.2% Triton X-100). The crude extract was concentrated by ultrafiltration (10 kDa cut-off, AmiconUltra, Millipore) and used as the wild type SAT preparation.

2.15. Purification of recombinant of SAT

The supernatant (5 mL) obtained as described in 2.2.2 was applied to a Strep-Tactin affinity column (10 mL).

Table 1. Elution program for SAT assay using LacF as an acceptor

Time (min)	A (%)	B (%)
0	10	90
5	10	90
25	75	25
27	98	2
28	98	2
30	10	90
33	10	90

The enzyme was eluted as outlined in section 2.3.2. Fractions (5 mL each) which contained SAT activity and were pure according to SDS PAGE were combined and concentrated by ultrafiltration (3000×g for 30 min) using centrifugal cartridges (10 kDa molecular cut off, AmiconUltra, Millipore). The retentate was diluted with glycerol to approximately 20% and stored at 20°C.

2.16. Characterization on Recombinant SAT

Kinetic analysis of recombinant SAT was conducted using LacF and CMP-NeuAc as acceptor and donor substrates, respectively. Commercial recombinant SAT (Sigma) and wild type SAT (crude extract, see 2.4.2) were studied in parallel. LacF and CMP-NeuAc at concentrations between 13 µM and 524 µM in a total assay volume of 50 µL were incubated for 40 min at 37°C with either the recombinant or wild type SAT (1 mU). The Michaelis Menten constants were determined by using Lucenz version II. The effect of pH were studied in the range of pH 4 to 9 using citric buffer (0.1 M) for pH values between 4 to 5, Mes buffer (0.1 M) for pH values between 5.5 to 6.5 and Tris buffer (0.1 M) for pH values between 7 to 9. The influence of the temperature in the range of 2250°C and the time course up to 23 hs were also studied for the recombinant 2,6-SAT.

The acceptor selectivity of the recombinant 2, 6-SAT was characterized by using lactose, *N*-acetyllactosaminide and the *N*-carboxybenzyl-aminoethyl *N*-acetyl-lactosamine glycoside. The reactions were conducted in 200 µL Tris HCl buffer (50 mM, pH 7.5) containing 0.5 µmol of CMP-NeuAc (95% pure) and 0.25 µmol of the respective acceptor substrate. The reactions were initiated by the addition of pure 2, 6-SAT (0.1 U) and incubated at 30°C for 1 h under shaking (160 rpm). The mixtures were analysed by LC-MS. For the reactions with lactose and lactosamine a HILIC column was used (Luna 3µ, 150×2.0 mm, Phenomenex, Torrance, USA). The compounds were eluted using a linear gradient of 0-40% water in acetonitrile over 20 min. Products of the reaction with *N*-carboxybenzyl-aminoethyl N-acetyl-lactosamine glycoside were separated on a C18 column (Kinetex 2.6 µ, 50×3.0 mm, Phenomenex). After an isocratic elution for 8 min with water: Acetonitrile (95:5, v/v), the column was eluted with a linear gradient to an acetonitrile concentration of 50% (v/v) over 20 min, followed by a linear increase to 80% over the next 5 min. This final concentration was maintained for 3 min.

2.17. Elimination of ß-Galactosidase from the Crude Cell Culture

The elimination of ß-galactosidase in the lysed cell culture containing SAT (see 2.2.2) was achieved via partial purification on a HisTrap Fast Flow column (GE Healthcare, 5 mL). Mops buffer (50 mM, pH 6.5) containing 5 mM imidazole was used to elute non-binding components including ß-galactosidase over 6 column volumes. Then the bound proteins (including SAT) were eluted with Mops buffer (50 mM, pH 6.5) containing 300 mM imidazole (5 column volumes). Fractions in this elution step were combined and dialysed against Mops buffer overnight at 4°C to remove imidazole. The ß-galactosidase activity was assayed according to Torpenholt using *p*-nitrophenyl ß-galactoside (Torpenholt *et al*., 2011).

2.18. Large scale Enzymatic Synthesis of Sialosides

The sialylation of *N*-carboxybenzyl-aminoethyl *N*-acetyl-lactosamine glycoside was conducted in two separate reactions, each containing 4.49 mmol of CMP-azido-NeuAc in 150 mL of filtered reaction mixture obtained as described above (see 2.3.4). The lactosamine glycoside (2.10 g, 3.75 mmol) was dissolved in each solution by shaking at 37°C for 30 min. Partially purified recombinant 2, 6-SAT (see 2.4.5) was used in both reactions. In the first reaction, 100 mL of the enzyme preparation (containing 10.7 U of SAT activity in a total of 180 mg protein) was added. In the second batch, 95 mL of the preparation (containing 15 U of SAT activity in a total of 78 mg of protein) was added to the 150 mL mixture. Both reactions were carried out at 37°C for 5 hs under gentle shaking (180 rpm). After completion of the reaction, the mixtures from two reactions were combined and directly applied to a column of hydrophobic styrenic resin (150 g, Diaion HP-20). Step-wise elution with water: Ethanol mixtures (0-13% ethanol (v/v)) followed by rotary evaporation and subsequent lyophilisation gave the desired product (2-Cbz-aminoethyl 5-acetamido-9-azido-3,5,9-trideoxy-D-glycero-D-galacto-2-nonulopyranosyl-onate-α-(2-6)-*O*-ß-D-galactopyranosyl-(1-4)-2-acetamido-2-deoxy-glucopyranoside) as the sodium salt.

3. RESULTS AND DISCUSSION

3.1. Expression of Recombinant CSS and SAT

The production of recombinant CSS and SAT was carried out at different scales ranging from 100 mL to 8.0 L cell culture (for SAT) as outlined in 2.2.2. In the

case of small scale culture preparation, the OD_{600} ranged between 4.0 and 6.0 after overnight incubation with an equivalent of 7.0-8.0 g wet biomass per L. In comparison, the final OD_{600} for the large scale in the bioreactor was higher (OD_{600} 8.8) while the biomass was 11.5 g wet biomass per L. Since the supply of oxygen and control of pH are the profound factors for the growth of *E. coli* (Studier, 2005), the control of these parameters is crucial for optimal growth conditions. In contrast to the shake flasks used in the small scale application, the conditions in the bioreactor can be controlled resulting in the generation of more biomass in the latter. For the large scale culture preparation, lactose (73.0 mM) was used as the inducer rather than IPTG (0.75 mM) reducing the cost of the culture preparation. This system produced 22.6 U of SAT and 2.1 U of CSS per L of cell culture without further optimization.

3.2. Production of CSS

3.2.1. Purification of CSS

The construct of the recombinant CSS produced in this study contained both a His Tag and a Strep Tag as described in 2.2.1. This allows the single step purification using either a HisTrap or a *Strep*Tactin column, respectively. In this study, the recombinant CSS was purified on the StrepTactin column following the protocol described in 2.3.2. A typical elution profile is shown in **Fig. 1a**. SDS PAGE analysis of fractions 45 and 46 showed a single band with an apparent molecular mass of 24 kDa **Fig. 1b**. In comparison, the break through (fractions 23 to 25) which contained the majority of the protein did not contain this band and had only little CSS activity. The fractions containing CSS were combined. The specific activity in the combined fractions was 875 mU mg^{-1} of protein corresponding to a purification factor of 87.5.

3.3. Characterization and Enzymatic Synthesis

3.3.1. Characterization of CSS

CSS from N. meningitides was first described and characterized by Warren and Blacklow (1962). Since then this enzyme has been successfully expressed in *E. coli* using different expression plasmids (Ganguli *et al*., 1994; Gilbert *et al*., 1997; Karwaski *et al*., 2002; Knorst and Fessner, 2001). Previous characterization included pH and temperature optimum as well as the requirement for divalent cations and reducing agents and substrate specificity. During this study the expressed CSS was tested and the results were correlated with the earlier findings. The results are summarized in **Table 2**.

As in the studies mentioned above, the pH optimum of the CSS was between pH 8.5 and 9. At pH 8 only half of the maximum activity was observed while at pH values lower than 7.5 the activity dropped below 10%. However, in contrast to previous findings the enzyme showed a broad temperature optimum with over 80% of the maximum activity found in a temperature range from 25-40°C. At temperatures over 50°C the activity decreased significantly (data not shown).

Previous studies mentioned above also reported the requirement of Mg^{2+} or Mn^{2+} cations for CSS activity. When the influence of several divalent cations on the enzyme activity was tested, a significant variation in activity regarding the effect of the different cations was observed. While Mg^{2+}, Ca^{2+} and Mn^{2+} cations had a stimulatory effect on the CSS activity, Zn^{2+} and Ni^{2+} ions had only little effect. In contrast, Cu^{2+} ions inhibited the enzyme activity completely, which could be explained by thiol oxidation.

In addition, reaction conditions regarding substrate concentrations and ratio between NeuAc and CTP were tested. Best conversion rates were observed with a NeuAc/CTP ratio of 1:5. Furthermore, the yields were above 90 % for substrate concentrations below 20 mM NeuAc and 40 mM CTP (with CSS activity of 0.2 mU). However, no conversion was observed at CTP concentrations above 50 mM indicating an inhibitory effect of this substrate.

In addition to NeuAc, NeuGc, KDN and 9-azido-NeuAc were tested as substrates for CSS from N. meningitides. Previously Gilbert *et al.* (1997) determined the K_M values for both NeuAc and NeuGc as 0.34 mM and 2.6 mM, respectively. Despite the significant difference in the K_M values, however, both substrates showed similar k_{cat} values (Gilbert *et al*., 1997). When incubating NeuAc, NeuGc and KDN (at concentrations of 5 mM) overnight at 37°C with CSS (0.2 mU), a complete conversion of the sialic acid to its respective activated form was observed confirming that these sialic acids are converted by this enzyme. Furthermore, a complete conversion was found for the reaction with 9-azido-NeuAc though with a slower rate. Other sialic acids modified in the 5 position have also been shown to be accepted as substrates by the CSS from N. meningitides indicating a broad substrate specificity (Knorst and Fessner, 2001).

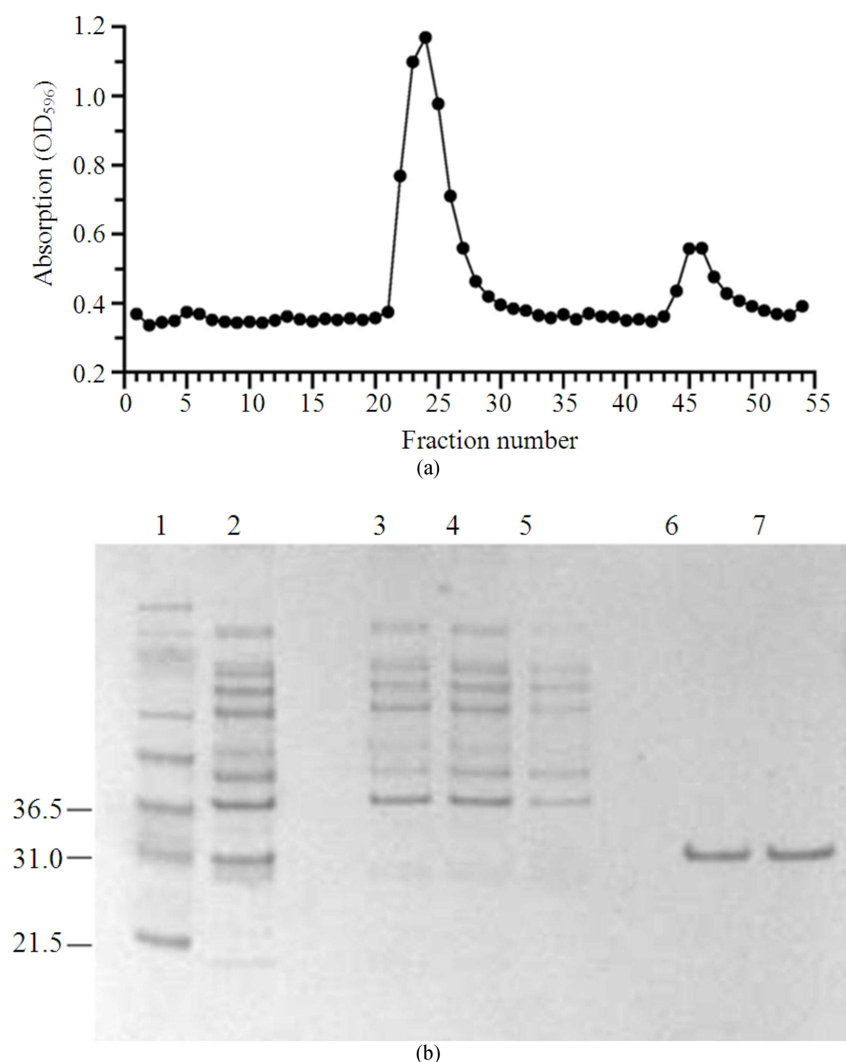

Fig 1. Purification of CSS. (a). Elution profile for CSS purified by Strep-Tactin column (1 mL) under the conditions described in 2.3.2; (b). SDS PAGE analysis with fractions from Strep-Tactin column: Lane 1: Protein standard, lane 2: Crude cell lysate, lane 3: Fraction 23, lane 4: fraction 24, lane 5: fraction 25, lane 6: fraction 45 and lane 7: Fraction 46

Table 2. Characterization of CSS

Characteristics	Optimum
pH	8.5-9.0
Temperature	25-40°C
Time (0.2 mU for 5 mM NeuAc and 10 mM CTP)	Reaction completed after 1 h
Ratio CTP: NeuAc	5:01
Divalent cation (20 mM)	Mg>Ca>>Mn>Ni>>Zn Cu inhibitory
Sialic acids	NeuAc, NeuGc and KDN at same rate 9-azido NeuAc at half rate

3.4. Enzymatic Synthesis of CMP-Sialic Acid

The production of sialosides using the combined reaction of CSS and a suitable sialyltransferase is generally carried out by either a ‘one-pot’-two enzyme-reaction with both enzymes in the same solution (Yu *et al*., 2004) or a reaction performed using two separate consecutive steps (Rauvolfova *et al*., 2008). Considering the difference in pH and temperature optima of the enzymes used in this study, the latter approach was followed. Initially, sialic acid (30 mM) and CTP (17-20 mM) were dissolved in Tris/HCl buffer as described in 2.3.4 and the reaction was initiated by adding crude CSS (prepared according to 2.1.1, 10-30

mU mL^{-1}). CTP concentrations above 50 mM were inhibitory. To overcome this limitation CTP was added in aliquots. HPLC analysis was used to determine the amount of non-reacted CTP and to monitor conversion rates. After 3 h the conversion of sialic acid to CMP-sialic acid was greater than 95% and the reaction was stopped. The yields of different experiments at different scales are shown in **Table 3**.

The biotransformation resulted in a significant acidification of the solution. This effect is especially pronounced at the beginning of the reaction. Without adjustment, the pH dropped from an initial optimum value of pH 8.5 to pH 6.5 within 20 min. The substrates CTP and sialic acid also contributed to this pH drop from a pH value of 8.5 to 7.9.

The reaction mixture was centrifuged to remove the precipitate that formed. The precipitate was most likely magnesium phosphate (Rauvolfova *et al*., 2008). During the reaction, pyrophosphate is formed and broken down to phosphate by pyrophosphatase. The phosphate combines with magnesium, which is added as a co-factor for CSS.

The crude CSS preparation used in the reaction contained other proteins originating from *E. coli* including ß-galactosidase which competes with the SAT reaction by hydrolysing the acceptor substrate. Therefore, ß-galactosidase was removed by ultrafiltration (MWCO: 10 kDa) and the pH of the resulting filtrate was adjusted to pH 7.5 prior to performing the SAT biotransformation.

3.5. CMP-Sialic Acid Purification

The product, CMP-sialic acid was purified by precipitation by the addition of ice cold ethanol. The precipitate was analysed by HPLC. Both CMP-NeuAc and CTP (pK_A 2.5) are negatively charged. Therefore, their solubility is influenced by pH especially on the addition of ethanol. The pH of a reaction mixture containing 77% CMP-NeuAc and 23% CTP was adjusted to pH values ranging from 3 to 6. The results show the influence of pH on yield and purity of CMP-sialic acid **Table 4**. While a complete precipitation of CMP-sialic acid was observed at pH values below 5, only 20% was recovered at pH 6. In contrast, the CMP-sialic acid purity was greatest at pH 5 (approximately 80%) and decreased with decreasing pH.

Considering both yield and purity, pH 5.0 was optimal for CMP-sialic acid recovery. However, limiting the amount of non-reacted CTP in the reaction mixture allowed pH values below 5 to be used for quantitative precipitation of the product.

3.6. Production of SAT

Wild type 2, 6-SAT from *P. damsela* (ATCC33539) was partially purified as described in 2.4.2 yielding a crude enzyme preparation (after ultracentrifugation) with a specific activity of 1.4 mU mL^{-1} and a total activity of 0.1 U/L of cell culture. In comparison the recombinant SAT was produced as a crude enzyme with 2.6 mU mL^{-1} and a total activity of 22.6 U L^{-1} in cell culture when expressed at small scale.

In large scale applications (8 L), IPTG was replaced by lactose as an inducer of the lac promoter and SAT expression. In comparison to the yields obtained in a small scale (1 L), the total enzyme activity dropped by 31% to 15.5 U L^{-1}. It is unclear if this is due to the changed efficiency of the inducer or the change in reaction parameters. However, the use of lactose as an inducer in the larger culture volumes would reduce production costs and compensate for the decreased yield at this scale. This manufacturing procedure requires further optimization to increase the efficiency of production of recombinant 2, 6-SAT.

3.7. Purification of Recombinant 2, 6-SAT

Recombinant 2, 6-SAT was purified using a Strep-Tactin column **Fig. 2a**. SDS-PAGE analysis showed that fractions 13 to 15 contained a single band with an estimated molecular weight of 55 kDa **Fig. 2b**, which is very close to the theoretical molecular weight of 56 kDa for the recombinant 2,6-SAT. The specific SAT activity increased from 26 mU/mg of protein in the crude lysate to 420 mU mg^{-1} of protein for the purified enzyme, which corresponds to a sixteen fold increase in enzyme purity.

The yield of the recombinant 2, 6-SAT produced in this study (22.6 U L^{-1}) was higher than that reported by Sun *et al.* (2008), but substantially lower than that obtained in the study by Yamamoto *et al.* (1998). Possible reasons for this discrepancy are the use of different constructs for the expression of the recombinant enzyme and different acceptor substrates used to determine SAT activity. Furthermore, it has to be considered that the conditions for the expression of SAT in *E. coli* have not been optimized in the current study. The comparison of the three enzymes is shown in **Table 5**.

3.8. Characterization and Enzymatic Synthesis

Although 2,6-SAT has been characterized by other researchers (Sun *et al*., 2008; Yamamoto *et al*., 1996), it is important to characterize the SAT enzyme produced in this research since a different plasmid construct has been used for the enzyme expression. The type of purifications tags and the conditions used for expression and cell growth might have an effect on the enzyme activity.

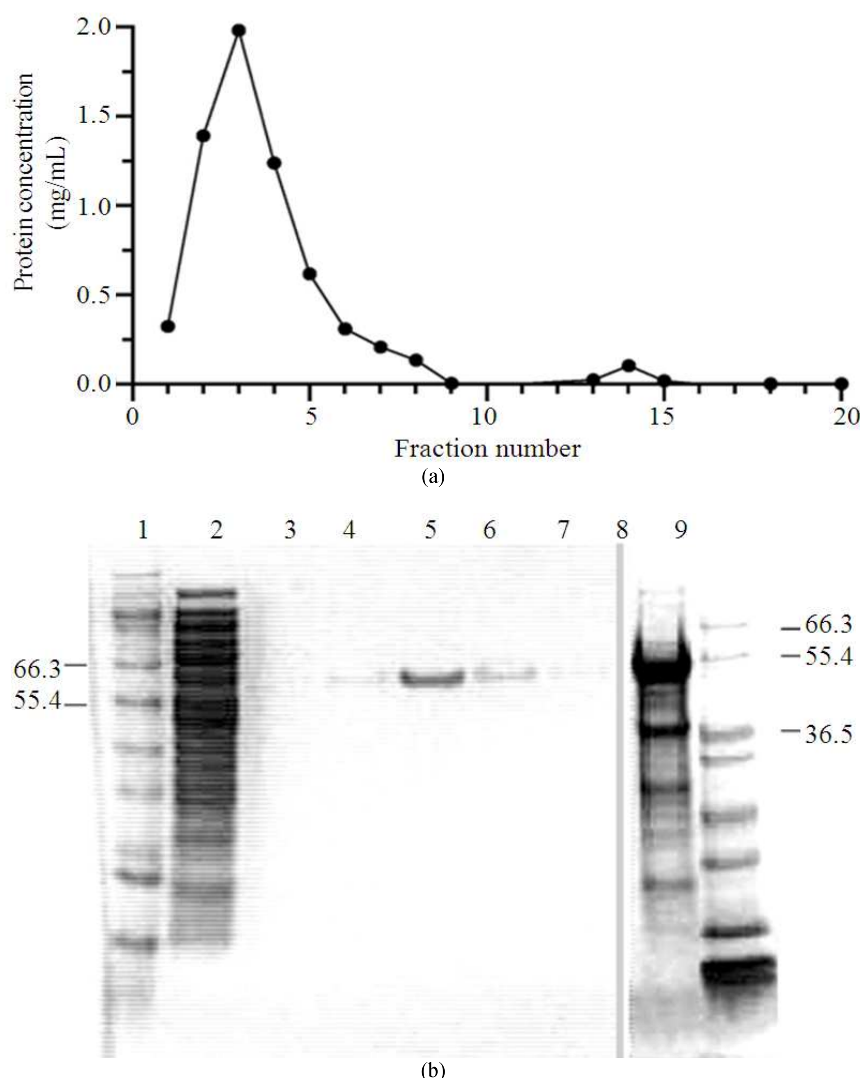

Fig 2. Purification of 2,6-SAT on Strep-Tactin column. (a) Elution profile for SAT using Strep-Tactin column (10 mL) under the conditions described in 2.4.3; (b) SDS-PAGE analysis of the fractions from the Strep-Tactin column; Lane 1&9: protein standards, lane 2: crude cell lysate, lane 3: fraction 12, lane 4: fraction 13, lane 5: fraction 14, lane 6: fraction 15, lane 7: fraction 16 and lane 8: partially purified SAT on HisTrap column

Table 3. Conversion rates of selected reactions; the yield was determined based on the absorption at 280 nm and the ratio between the absorbance of the peaks for CMP-NeuAc and non-reacted CTP

Sialic acid (mg (μmol))	Initial addition Sialic acid (mg (μmol))	Addition in of CTP mg (μmol)	after 1 h after 2 h	Addition after 3 h	Addition (mL)	Vol (mU)	CSS time (h)	Reaction (%)	Yield
NeuAc	99.4 (321)	98.8 (187)	100.3 (190.3)	101.9 (193.3)	93.1 (176.6)	10	100	4.0	>90
NeuAc	300 (969.9)	300.4 (569.9)				30	100	4.5	95
9-azido NeuAc	111.1 (323.4)	100.9 (191.4)	100.0 (189.7)	99.8 (189.3)	101.5 (192.6)	10	100	4.0	>90
9-azido NeuAc	200 (582.2)	198.5 (376.6)	191.5 (363.3)	104.1 (197.5)		20	100	4.0	>90
9-azido NeuAc	3000 (8732.7)	3040 (5767.2)	3180.3 (6033.4)			300	1080	3.5	>95

Table 4. Precipitation of CMP-NeuAc with ethanol

pH of solution	Percentage precipitated	Ratio CMP-NeuAc/CTP
3	100	1.08
4	100	1.70
5	82	4.00
6	20	4.56

Moreover, the kinetic data provide pivotal information in consideration of the application in large scale enzymatic synthesis. Recombinant 2, 6-SAT purified on a Strep-Tactin column (see 2.4.3) was used for the kinetic study. For comparison, wild type 2, 6-SAT and a commercial 2, 6-SAT (Sigma) were run in parallel. The time course of the conversion of LacF to SiaLacF under the conditions outlined in 2.4.4 for all three enzymes is shown in **Fig. 3**. All three SAT enzymes displayed similar characteristics regarding the conversion rates. The initial reaction was linear over the first 2 h, then tailed off to reach equilibrium after 4 h. Longer incubation times did not increase the yield. No significant hydrolysis of the sialylated product was observed.

The results of the study on the temperature and pH effects on the activity of both the wild type and recombinant SAT are summarized in **Table 6** as well as kinetic data for these enzymes. In addition the kinetic data for SAT from earlier studies are listed.

The data for K_m and V_{max} for both the acceptor and donor substrates for the recombinant 2, 6-SAT produced in this research and the Sigma supplied 2,6-SAT are different by a magnitude between 30 and 40 **Table 6**. However, the values for V_{max}/K_m are essentially identical for both acceptor and donor substrate indicating similar conversion efficiency towards these substrates. In addition, these two enzymes had similar pH and temperature optima. The use of lactose as the acceptor in a radiometric assay in Yamamoto's research could account for the differences in the kinetic data when compared to this study.

The conversions of lactose, N-acetyllactosaminide and the N-carboxybenzyl-aminoethyl N-acetyl-lactosamine glycoside to their respective sialosides demonstrated slight differences in the selectivity of the recombinant 2,6-SAT produced in this research towards these acceptor substrates. The specific activities of 2, 6-SAT towards lactose, N-acetyllactosaminide and N-carboxybenzyl-aminoethyl N-acetyl-lactosamine glycoside, as determined by LC-MS, were 0.46, 0.40 and 0.57 U mg^{-1}, respectively. These results indicate a similar broad substrate specificity as described previously by Yamamoto (2010); Yu *et al.* (2006) for recombinant SAT derived from *Photobacterium*. The amount of 2, 6-SAT needed in the large scale synthesis of sialosides was estimated based on these enzyme specific activity data.

3.9. The Elimination of ß-Galactosidase

Preliminary investigation revealed that *E. coli* BL21 co-expressed sufficient ß-galactosidase to hydrolyze galactose-containing substrates. This activity can compete with the sialylation reaction and reduce yields significantly. For a large scale synthesis of sialosides, the use of crude enzyme preparations is preferred due to the increased stability of the enzyme and the decreased production costs. However, the removal of ß-galactosidase from the crude preparation was required for an efficient synthesis of sialosides.

ß-galactosidase is a 464-kDa tetramer protein with a single unit molecular weight of 116 kDa (Fowler and Zabin, 1977). The removal of ß-galactosidase activity in the CSS reaction mixture containing CMP-9-azido-NeuAc by ultrafiltration was effective (see 2.3.4). The ß-galactosidase activity in the filtrate decreased from a rate of 0.107 to 0.001 (Abs 405 nm sec^{-1}), indicating a removal of >99% of ß-galactosidase activity.

A chromatography of the crude SAT lysate extract (see 2.2.2) on a HisTrap column using a two step elution (see 2.4.5) effectively removed ß-galactosidase during the first wash step. The result showed that>98.5% of ß-galactosidase activity in the crude extract was removed. The rate decreased from 0.150 in the crude extract to 0.003 in the fractions eluted in the second elution step. These latter fractions contained partially purified SAT.

Determination of the SAT levels in these fractions demonstrated that 83.6±10.6% of the initial total SAT activity was retained in the eluate. When compared to the purification on a StrepTag column (see 2.4.3) for a SAT enzyme with high purity, the yield was 10 times higher (8.4±0.8%).

SDS-PAGE analysis revealed four major protein bands (**Fig. 2**, lane 8). The band showing the highest density at 55 kDa indicated that approximately 50% of the protein loaded was SAT enzyme.

3.10. Large Scale Two-Step Enzymatic Synthesis of Sialosides

The overall scheme of two-step synthesis of the target sialoside (compound 3) is illustrated in **Fig. 4**. 9-azido-Neu4Ac was converted to the CMP-glycoside with CSS prior to the addition of the 2, 6-SAT and acceptor substrate for the sialylation (see 2.5). The reaction mixture after sialylation was analysed by HPLC and LC-MS. The degree of conversion of the acceptor to the target glycoside was determined as 82.9 and 89.0% for the first and second reaction, respectively.

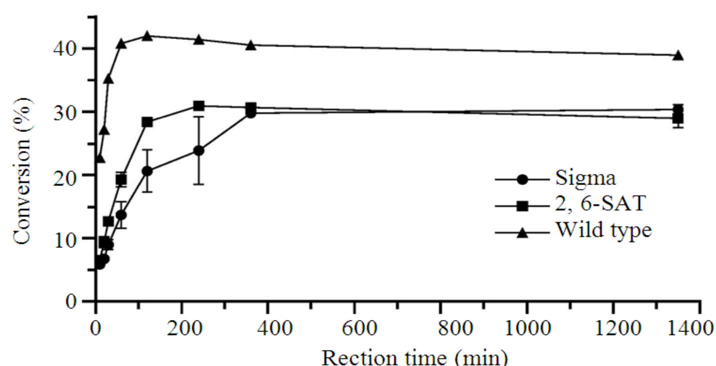

Fig. 3. The reaction time course of LacF and CMP-NANA catalysed by three 2, 6-SAT enzymes. Error bars indicate the difference between the high and low values from the duplicate experiments

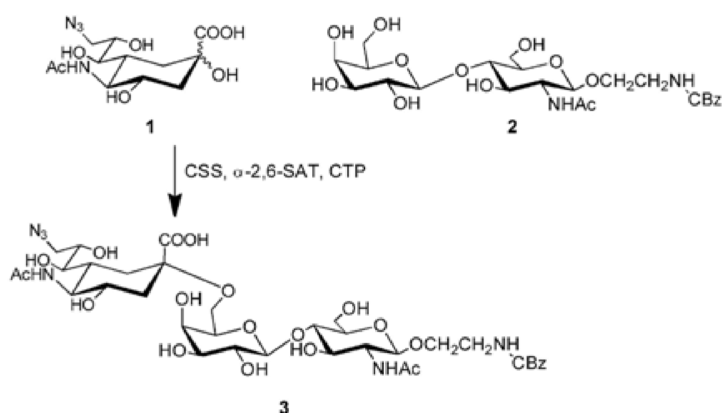

Fig 4. The overall scheme of two-step synthesis of the 9-azido-sialyl-α-2,6'-lactosamine glycoside (compound 3). Compound 1: 9-azido-Neu5Ac; compound 2: *N*-CBz (aminoethyl *N*-acetyl-lactosamine); compound 3: 2-Cbz-aminoethyl 5-acetamido-9-azido-3,5,9-trideoxy-D-glycero-D-galacto-2-nonulopyranosyl-onate-α-(2-6)-O-ß-D-galactopyranosyl-(1-4)-2-acetamido-2-deoxy- lucopyranoside

Table 5. The yield and specific activity of the recombinant 2,6-SAT made in this research in comparison to that from Yamamoto *et al*. (1998) and Sigma (Sun *et al*., 2008)

2,6-SAT sources	This research	Yamamoto *et al*. (1998)	Sigma
MW (kDa)	55.00	54	56.80
Specific activity (U/mg)	0.42	7	0.35
Total RSAT(mg/L)	58.00	32	36.00
Total U/L	22.60	224	12.60

Table 6. Kinetics data of recombinant 2,6-SAT produced in this research and crude wild type in comparison to the data obtained from Yamamoto *et al*. (1996), Yamamoto *et al*. (1998) and Sun *et al*. (2008)

2,6-SAT sources	This research	Sigma[b]	Yamamoto *et al*. (1998)	Wild type SAT(crude)
K_m (CMP-sialic acid) (μM)	29.5±0.3	920	320	85.7±0.1
V_{max} (mU/mg)	94.3±0.1	4200	7700	7.51±0.1
V_{max}/K_m	3.19	4.56	24.1	0.0877
K_m (LacF, lactose) (μM)	231±0.2	830±0.4	6820	1140±0.4
V_{max} (mU/mg)	324±0.2	1590±0.4	6400	4.1±0.5
V_{max}/K_m	1.4	1.9	0.93	0.0036
Optimal pH range	7.0-8.5	7.5-8.5	6.0-8.0	6.5-8.5
Optimal T (°C)	35-45	35-45	25-40	22-50

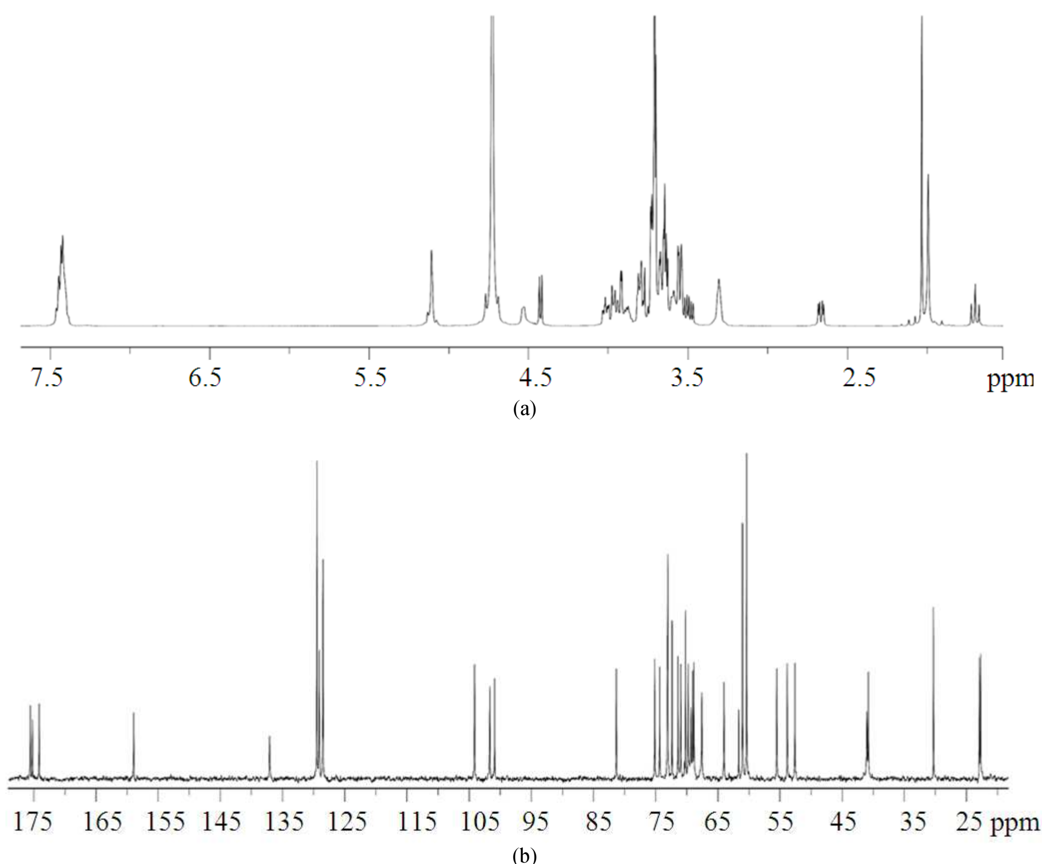

Fig. 5. (a) ^{1}H and (b) ^{13}C NMR spectra of the 9-azido-sialyl-α-2,6'-lactosamine glycoside (compound 3 in **Fig. 4**)

Combining the products of both reaction mixtures, 5.45 g of sialoside were obtained.

In previous studies, the purification of sialosides has generally been achieved by using preparative HPLC, charcoal chromatography, ion-exchange chromatography or gel-permeation chromatography. Each of these methods has its own limitations depending on the sialoside. In our case they were not suitable for further scale-up. In this research, a single step chromatographic separation using HP-20 resin was developed. We expect the method to be fully scalable up to several hundred grams of product. This procedure exploits the differences in the hydrophobic character of the reaction buffers, the starting materials and the product to give the desired separation. Interestingly the sialylated product does not bind as strongly to the hydrophobic resin as the non-sialylated lactosamine acceptor. This is thought to be due to its negative charge and the formation of a salt, whereas the lactosamine acceptor is non-ionic. Less hydrophobic salts and buffers were eluted prior to the sialylated compound. The purity of the isolated material was found to be acceptable as analysed by NMR **Fig. 5a** and **b**).

After chromatographic purification 3.54 g (3.94 mmol) of final product were isolated giving an overall yield of 53%. The ^{1}H and ^{13}C NMR spectra **Fig. 5a** and **b** of the purified product were as reported in the literature (Blixt *et al*., 2008). HRMS calc. $[M\text{-}Na]^{-}$ m/z = 875.3164, found m/z = 875.3152.

As mentioned in the Introduction, Blixt *et al*. (2008) have prepared the sialylated *N*-carboxybenzyl aminoethyl lactosamine glycoside (compound 3 in **Fig. 4**) with 29% overall yield (in total of 0.2 g after purification by size-exclusion chromatography). The lower yield in comparison to the current work (53%) can be explained by the complexity of the reaction system used, since in our case we used fewer enzymes and had the galactosylated-acceptor prepared by chemical synthesis prior to the biotransformation.

4. CONCLUSION

Enzymatic synthesis of sialosides has been at the forefront of sialylglycoconjugate research during the last decade. However, the large scale biotransformation of these sialosides is still in its infancy. In this study, we have developed a large scale production system (up to 8 L) for two of the key recombinant enzymes (CSS and SAT) that are employed for this process. The recombinant enzymes were used for the two-step enzymatic synthesis of a sialylated lactosamine glycoside (a ligand for CD22 (Siglec-2)) on the gram scale. The new strategies developed for the recombinant enzyme expression and final sialoside purification, including the detailed methodology provided here for CMP-sialic acid purification, have the potential to provide an economic benefit in the large scale manufacture of CSS, SAT, CMP-sialic acid and sialosides.

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