Expression and characterization of recombinant human α-3/4-fucosyltransferase III from *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Tn) cells using the baculovirus expression system

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The human α-3/4-fucosyltransferase III (Fuc-TIII) participates in the synthesis of Lewis determinants. The enzyme from human sources is scarce and heterogeneous. In this paper we describe the expression of a secreted form of Fuc-TIII (SFT3) in two insect cell lines, *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Tn), using the baculovirus expression system. The Sf9 cells secreted approx. 0.4 unit/l (1 mg/l) of the enzyme. The Tn cells secreted approx. 3-fold this amount. A large proportion of active protein was accumulated in the two cell lines (50 and 75 % respectively for Sf9 and Tn cells, on the fourth day after infection) indicating a possible limitation not only of the folding machinery, but also a saturation of the secretory pathway. SFT3 was purified by cation-exchange chromatography followed by affinity chromatography. The enzyme from the Tn cell line had a lower global charge, possibly due to post-translational modifications, such as phosphorylation or sulphation. The two glycosylation sites from SFT3 were occupied. SFT3 secreted by Sf9 cells was completely deglycosylated by peptide-*N*-glycanase F, whereas 50 % of SFT3 secreted by Tn cells was resistant to deglycosylation by this enzyme. The apparent kinetic parameters determined with the type I acceptor were $k_{cat} = 0.4\ s^{-1}$ and $K_m = 0.87$ mM for the SFT3 secreted by Tn cells, and $k_{cat} = 0.09\ s^{-1}$ and $K_m = 0.76$ mM for the SFT3 secreted by Sf9 cells, indicating that the enzymes had substrate affinities within the same order of magnitude as their mammalian counterpart. Furthermore, SFT3 secreted by either cell type showed a clear preference for type 1 carbohydrate acceptors, similarly to human Fuc-TIII.

Key words: glycosyltransferase, insect cell, Lewis determinants.

INTRODUCTION

The human α-3/4-fucosyltransferase III (Fuc-TIII; EC 2.4.1.65) catalyses the transfer of Fuc on to type 1 and type 2 core structures from neutral or sialylated carbohydrates, in α-1,4 and α-1,3 linkages, therefore participating in the synthesis of Lewis determinants: Le^a, sialyl-Le^a, Le^b, Le^x, sialyl-Le^x and Le^y [1–3]. *In vitro* the enzyme uses oligosaccharides, glycolipids and glycoproteins as substrate acceptors [2,3]. The human Fuc-TIII exists in digestive tract tissues, such as colon, stomach and jejunum epithelia [4], and is a product of the human Lewis blood group locus on chromosome 19. The enzyme is a transmembrane Golgi protein with two N-glycosylation sites.

The cell-surface fucosylated oligosaccharides mediate important processes, such as cell recognition and adhesion occurring in inflammation and metastases formation (reviewed by Lowe [5]). Foetal development, tumorigenesis and haematopoietic cell differentiation are also processes where fucosylated lactosaminoglycans perform an important role [6].

Biochemical and biophysical characterization of glycosyltransferases, namely Fuc-TIII, are difficult due to the low amounts of enzyme detected in their native tissues and also to the concomitant expression of highly similar isoenzymes therein [2,7–9]. Gene cloning of Fuc-TIII [1] and its expression in a heterologous recombinant system, as described in the present work, constitute a major advance in obtaining high amounts of a homogeneous preparation of the enzyme for further characterization.

Several gene expression systems have been developed, such as bacteria transformed with plasmids and cosmids, yeast strains transfected with yeast artificial chromosomes, retrovirus-infected mammalian cells and baculovirus-infected insect cells [10]. The cultured insect cells associated with the baculovirus expression system are often used to produce foreign proteins. This system has proven to be useful for the production of eukaryotic recombinant proteins, due to the conventionally used polyhedrin promoter, which is strong and gives high levels of transcription during the late phase of infection, and also due to the post-translational modifications, processing and trafficking mechanisms necessary for higher eukaryotic protein expression that are present in this system [11]. Insect cells are capable of performing many of the processes required for the formation of active mammalian heterologous proteins, such as glycosylation, phosphorylation and proteolytic processing. Even if these processes exhibit several differences between mammalian and insect cells, many biologically active recombinant glycoproteins have been produced from insect cells [11–13]. The insect cell lines more widely used are from *Spodoptera frugiperda* (Sf9), the army fallworm, and *Trichoplusia ni* (Tn), the cabbage looper. *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is one of the most used baculoviruses, because it was observed that the expression of foreign proteins using its polyhedrin promoter allows high rates of gene expression [14].

In the present work, we describe the expression of a secreted form of Fuc-TIII (SFT3), containing the catalytic domain of human Fuc-TIII, in Sf9 and Tn cells using the baculovirus expression system. The secreted forms are purified and partially characterized with respect to their activity, specificity and post-translational modifications. The proteins lack any affinity tag,

Abbreviations used: AcMNPV, *Autographa californica* multiple nuclear polyhedrosis virus; BHK, baby hamster kidney; FBS, foetal bovine serum; Fuc-TIII, α-3/4-fucosyltransferase III; MOI, multiplicity of infection; pfu, plaque-forming units; PNGase F, peptide-*N*-glycanase F; Sf9, *Spodoptera frugiperda*; SFT3, secreted form of Fuc-TIII; Tn, *Trichoplusia ni*.

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are similar to the native forms and can therefore be used for further structural studies.

MATERIALS AND METHODS

Materials

The insect cell lines Sf9 and Tn (High Five™), and the homologous recombination cotransfection kit were supplied by Invitrogen, the culture medium Sf900II and the foetal bovine serum (FBS) were obtained from Gibco BRL, the PCR reagents were purchased from Boehringer, and the other chemical reagents were obtained from Sigma. The 8-methoxycarbonyloctyl glycoside acceptor was a gift from Dr Ole Hindsgaul (University of Alberta, Canada). The oligosaccharide acceptors β-linked to $(CH_2)_3NHCO(CH_2)_5NH$-biotin were obtained from Syntesome (Munich, Germany).

Construction of baculovirus vectors expressing SFT3 and Fuc-TIII

The expression vector pBlueBacSFT3 encoded SFT3, where the cytoplasmic and transmembrane domains, and part of the stem region, had been replaced by the signal sequence of human β-trace protein [15]. The construction of the cDNA was made in two PCR steps. PCR was performed using the Expand High Fidelity DNA-polymerase-mixture (Boehringer) according to the manufacturer's protocol under standard conditions with 0.3 μM of each primer and 0.2 mM of each deoxynucleotide. First, a nucleotide sequence corresponding to the β-trace signal sequence and the N-terminus (starting at Ala^{47}) of the catalytic domain of Fuc-TIII was constructed; the template used was the plasmid pMT-βTP [15]; the forward and the reverse primers were 5′-GATCGAATTCGCACACCTGCTCGGCTGCAG-3′ and 5′-CGGGAGGACCCACTGGGTGCTGCCTGTAGGT-C-3′ respectively. PCR consisted of a 2 min denaturation step at 93 °C, followed by 30 cycles of 15 s of denaturation at 93 °C, 20 s of annealing at 52 °C, 2 min of elongation at 68 °C, and a final elongation step for 8 min at 68 °C. The second PCR step used the pCR3.1 vector as the template, containing the full-length form of Fuc-TIII [3]; as forward primer the product from the first PCR reaction was used, and as reverse primer the oligonucleotide 5′-GGCCTCTCAGGTGAACCAAGCCGCTATGCT-3′ was used. PCR conditions were as indicated above with denaturation, annealing and elongation temperatures of 94, 53 and 72 °C respectively. The DNA fragment was cloned into the transfer pBlueBac vector using the baculovirus TA cloning kit (Invitrogen). Mutations were confirmed by automated DNA sequencing.

The expression vector pBlueBacFT3 encoded full-length human Fuc-TIII and was obtained by PCR from the pCR3.1 vector containing the full-length form of Fuc-TIII [3].

Recombinant baculovirus production

Sf9 and Tn cells (4×10^5 cells/ml) were subcultured in suspension flasks with Sf900II medium until they reached a cell density of approx. 2–2.5×10^6 cells/ml. The cells were grown at 27 °C and 90 rev./min. Cell density was determined by haemocytometer counts (Brand, Wertheim/Main, Germany), and cell viability was evaluated by the exclusion of 0.4 % Trypan Blue dye in PBS.

The cotransfection of Sf9 cells with Bac-N-Blue™ DNA and the baculovirus transfer vector containing SFT3 (pBlueBacSFT3) was accomplished by the homologous recombination of the viral DNA sequence with the transfer vector, yielding a recombinant viral DNA, essentially according to the procedure described in the Bac-N-Blue™ transfection kit manual (Invitrogen). For a freshly seeded Sf9 cell monolayer (2×10^6 cells/30 mm plate), the following cotransfection mixture was used: 2 μg of recombinant transfer plasmid (pBlueBacSFT3), 0.5 μg of Bac-N-Blue™ DNA, 0.5 ml of Sf900II medium and 10 μl of Insectin™ liposomes.

Determination of virus titre

The recombinant AcMNPV titre was determined by a plaque assay, with adherent Sf9 cell monolayers on 96-well plates (0.8×10^5 cells/well). Several dilutions of the viral suspension were made in Sf900II medium containing 10 % (v/v) FBS, and 60 μl of each dilution was added per well. The plate was centrifuged at 1000 ***g***, for 1 h at 27 °C, and incubated overnight at 27 °C. The cells were fixed with 200 μl/well of 0.4 % (v/v) formaldehyde and 5.4 % (v/v) glutaraldehyde in PBS for 2 min, and washed twice with 200 μl of PBS/well. The final step consisted of the β-galactosidase assay with 200 μl/well of 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ and 200 μg/ml 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-Gal'). Following incubation for 24 h at 37 °C, the cells infected with recombinant virus were identified as blue plaques, allowing the determination of the viral titre in plaque-forming units (pfu)/ml.

Expression of SFT3

To determine the ideal multiplicity of infection (MOI) for collecting viral supernatant and SFT3, an assay with three different MOIs was performed. Mid-phase exponential Sf9 cells (2×10^6 cells/ml) were seeded in suspension cultures with Sf900II medium in the presence or absence of 10 % (v/v) FBS, and they were infected with three different MOIs: 0.1, 0.5 and 5 pfu/cell. The culture supernatants were monitored for virus production, by plaque assay, and for fucosyltransferase activity.

SFT3 was produced from Sf9 and Tn cells in 250 ml Schott flasks containing 2×10^6 cells/ml in 25 ml of Sf900II medium, in the presence or absence of 10 % (v/v) FBS. The cell infections were carried out with a recombinant virus at an MOI of 5 pfu/cell, and the active SFT3 (determined by fucosyltransferase activity) and total SFT3 (determined by Western-blot analysis) were monitored for seven days after infection in cell extracts and supernatants.

Fucosyltransferase activity

The Fuc-TIII activity in the cell supernatants was determined from the incorporation of radioactive fucose from the GDP-[^{14}C]Fuc donor into the type 1 methoxycarbonyloctyl glycoside or the Galβ3GlcNAcβ-$(CH_2)_3NHCO(CH_2)_5NH$-biotin acceptor as previously described [3]. One unit of enzyme activity was defined as the amount of enzyme catalysing the transfer of 1 μmol of Fuc/min to the 8-methoxycarbonyloctyl glycoside type 1 substrate.

Enzyme kinetics of the recombinant SFT3 were performed in standard reaction mixtures, using a saturating concentration of 0.05 mM GDP-Fuc, where the concentration of the type 1 Galβ1-3GlcNAc-$(CH_2)_3$-NHCO-$(CH_2)_5$NH-biotin substrate varied between 0.056 and 3.6 mM for SFT3 secreted by Tn cells, and 0.056 and 4.5 mM for SFT3 secreted by Sf9 cells. The apparent kinetic parameters were determined from a Michaelis–Menten curve fit to the experimental data using the least-square method with the solver function from the program EXCEL.

Protein analysis

Intracellular SFT3 from cells in 20 mM Mops buffer, pH 6.5 (2×10^5 cells/100 μl of buffer), and secreted SFT3 from the

corresponding 100 μl of culture supernatant were precipitated with ethanol. SDS/PAGE was carried out according to Laemmli [16]. SFT3 was detected by Western-blot analysis and ECL® (Amersham), using an anti-(Fuc-TIII) primary antibody (see below) at 1:1000 dilution.

Peptide-*N*-glycanase F (PNGase F; Glyko, Bicester, Oxon., U.K.) hydrolysis was performed under denaturing conditions as previously described [3].

Anti-(Fuc-TIII) production and purification

A purified anti-(Fuc-TIII) serum was raised by Eurogentec (Seraing, Belgium) against the human Fuc-TIII peptide HH-WDIMSNPKSRLPPSPRPQGQRC (single-letter amino-acid notation) coupled to ovalbumin. The antiserum was then purified by affinity chromatography on a column of Affigel 10 + 15 (Bio-Rad) containing the immobilized peptide. Peptide immobilization was performed according to the supplier's protocol. The antiserum was applied on to the column at 0.5 ml/min, and the column was washed with 20 column vols. of PBS. Elution was performed with 10 column vols. of 0.1 M glycine (pH 2.5). Antibody-containing fractions were immediately neutralized with 1 M Tris.

SFT3 purification

Supernatants from Sf9 and Tn cells expressing SFT3 were concentrated 4-fold at 4 °C in a Nucleopore system (nitrogen pressure = 4 bar) using a membrane (Amicon) with a 10 kDa cut-off. After adjustment to pH 6.0 and centrifugation at 10000 ***g*** for 10 min, the supernatant was applied on to a cation-exchange carboxymethyl-Sepharose column. For SFT3 secreted by Sf9 cells the column was equilibrated with 20 mM Mops/KOH (pH 7.8) containing 25 % (v/v) glycerol, 1 mM dithioerythritol, 0.02 % sodium azide (buffer A) and 50 mM NaCl. Column washing was performed with buffer A containing 50 mM NaCl, and SFT3 was eluted with an NaCl gradient from 50–300 mM in buffer A. For SFT3 secreted by Tn cells the column was equilibrated with buffer A containing 25 mM NaCl, column washing was performed with buffer A containing 25 mM NaCl, and SFT3 was eluted with an NaCl gradient from 25–300 mM in buffer A.The fractions containing Fuc-TIII activity were pooled and applied on to a GDP-Fractogel column [3] equilibrated with buffer A (pH 6.8) containing 50 mM NaCl. Buffer A containing 0.5 M NaCl was used to wash the column, and SFT3 was eluted with an NaCl gradient from 0.5–1.5 M NaCl in buffer A (pH 6.8).

RESULTS AND DISCUSSION

SFT3 and FT3 expression

Construction of transfer vector

SFT3 was constructed using site-directed mutagenesis by adding the signal sequence of a secretory protein, β-trace protein (Met^{1}-Ala^{23}), to the stem region of human Fuc-TIII (Ala^{47}-Thr^{361}). This construct and the full-length form of Fuc-TIII were cloned into the pBlueBac transfer vectors, pBlueBacSFT3 and pBlueBacFT3 respectively. Adherent Sf9 cells were cotransfected with each of the vectors and viral-linearized Bac-N-Blue AcMNPV DNA using liposomes. Virus titre, determined in the SFT3-containing supernatants by plaque-assay using the β-galactosidase method, was found to be 2×10^7 pfu/ml after three successive infections. It was observed that the full-length form of Fuc-TIII, a membrane glycoprotein, expressed in Sf9 cells was active and that a significant amount of enzyme activity was secreted into the medium (0.18 m-unit/1×10^6 cells from adherent cells on the fourth day after infection), however, it accounted for only approx. 67 % of the activity detected for SFT3 under identical conditions. The large-scale production in flasks, purification and characterization were performed with the soluble SFT3, which contained the active catalytic domain of Fuc-TIII.

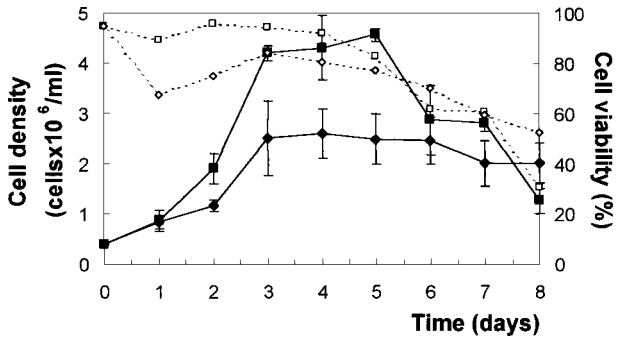

Figure 1 Growth curve of Sf9 (■, □) and Tn (◆, ◇) cells in suspension cultures

A 25 ml suspension culture was seeded with 4×10^5 cells/ml in Sf900II medium. The cell density (continuous line) and the cellular viability (broken line) were monitored up until the eighth day after infection. Results are presented as means ± S.D. ($n = 3$).

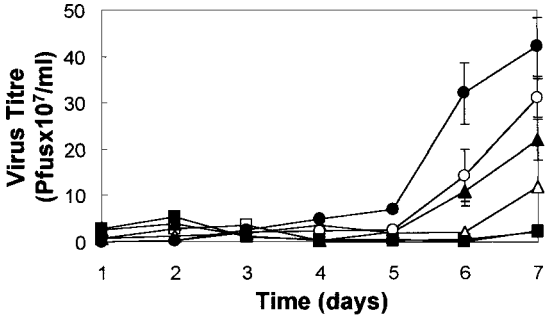

Figure 2 Production of virus at different MOIs

Suspension cultures were infected in their mid-exponential phase of growth (2×10^6 cells/ml) at MOI values of 5 (■, □), 0.5 (▲, △) and 0.1 (●, ○). Infections were performed in the presence (solid symbols) or absence (open symbols) of FBS. Results are presented as means ± S.D. ($n = 3$).

Cell density and MOI

In order to determine the optimum cell densities of infection the growth curves of Sf9 and Tn cells in medium Sf900 II were established (Figure 1). It was observed that the exponential phase of Sf9 and Tn cells varied between 0.9–4.6×10^6 and 0.8–2.6×10^6 cells/ml respectively. Maximal cell density was lower than that observed in spinner flasks (6×10^6 cells/ml) [12], possibly due to aeration limitations [17]. High cell densities have a negative effect on infection efficiency [18]. Cell infections were then performed at a cell density of 2×10^6 cells/ml.

In order to determine the optimum conditions for virus production MOIs of 0.1, 0.5 and 5 pfu/cell were tested (Figure 2). Virus produced with the lowest MOI (0.1 pfu/cell) in the presence of serum had the highest titre (4.2×10^8 pfu/ml on the seventh day after infection). This corresponded to budded virus [19]. Furthermore, virus produced at the highest MOI (5 pfu/cell) showed low titre, both in the presence and absence of

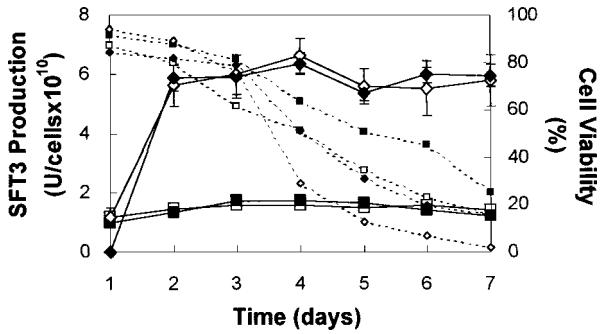

Figure 3 Production of SFT3 by Sf9 and Tn cells

Production of active SFT3 by Sf9 (■, □) and Tn (◆, ◇) cells in the presence (solid symbols, continuous line) or absence (open symbols, continuous line) of FBS. Results are presented as means ± S.D. ($n = 6$). Sf9 (■, □) and Tn (◆, ◇) cell viability in the presence (solid symbols, broken line) or absence (open symbols, broken line) of FBS was determined as the daily percentage of living cells.

serum. The virus produced under these conditions was highly unstable, since it had been obtained after cell lysis [20]. It is possible that under these conditions glycoproteins from the viral envelope, necessary for host cell recognition and infection, were proteolyticaly cleaved. Virus stocks for protein production were performed at MOIs of 0.1 and 0.5 in 10% (v/v) FBS supplemented medium, and were collected on the seventh day after infection.

SFT3 production

In order to determine the best MOI for protein production, values of 0.1, 0.5 and 5 were used in the presence or absence of FBS. An MOI of 5 yielded the highest volumetric and specific productivity of active SFT3 on the fourth day after infection (approx. 0.4 unit/l; 0.2 m-unit/1×10^6 cells; 1 mg/l) both with and without FBS. High MOIs are normally required for recombinant protein expression, in order to guarantee that all cells are infected at the same time and initiate viral protein expression.

Since the amount of protein produced depends on the host cell used, we have compared Sf9 with Tn cells for the production of active and total SFT3 in the presence and absence of FBS at an MOI of 5 (Figure 3). Tn cells produced approximately 3-fold more active SFT3 than Sf9 cells on the fourth day after infection, in the presence and absence of FBS (Figure 3).

SFT3 was collected on the fourth day after infection, prior to extensive cell lysis and protease release into the medium.

SFT3 retention in the Sf9 and Tn cells

The comparison of the total amount of SFT3 from 2×10^5 cells and its corresponding supernatant showed that two days after infection, for both Sf9 and Tn cells, there was a significant amount of protein retained in the cells (Figure 4). From the second to the fourth day after infection the ratio of secreted protein to intracellular protein was essentially constant (Figures 4A–4D). After the fourth day of infection SFT3 increasingly accumulated in the cells, whereas the levels detected extracellularly decreased, probably due to deficient secretion combined

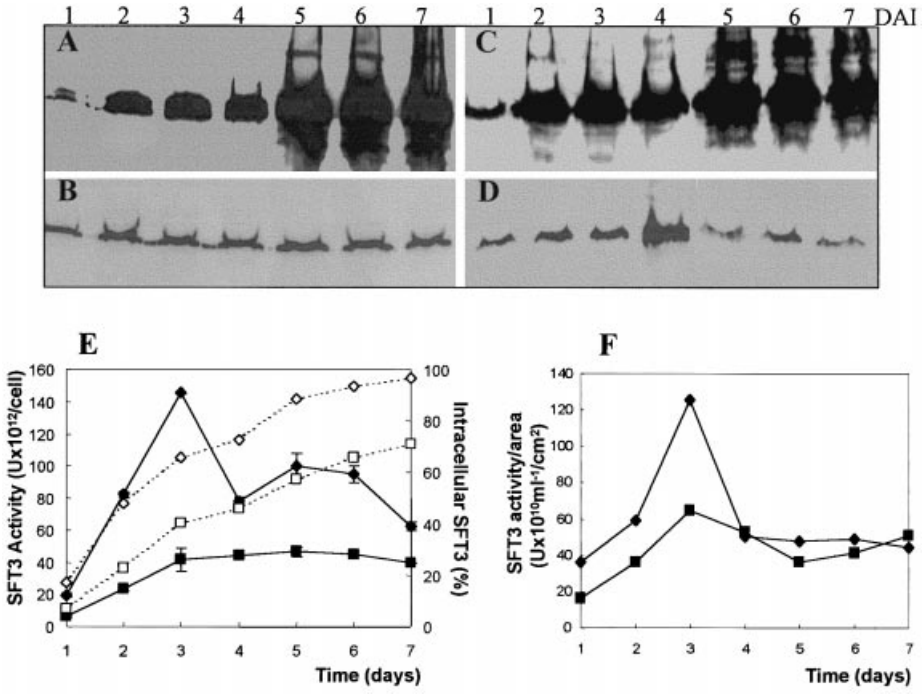

Figure 4 Analysis of cell-associated and secreted SFT3

Sf9 (**A** and **B**) and Tn (**C** and **D**) cells in the absence of FBS were used for Western-blot analysis. Protein was obtained from cell extracts (**A** and **C**) (2×10^5 cells) and the corresponding supernatants (**B** and **D**). All blots were exposed for 5 min according to the ECL® detection method. (**E**) Active intracellular SFT3 in Sf9 (■) and Tn (◆) cell extracts (2×10^5 cells) in the absence of FBS. Results are presented as means ± S.D. ($n = 2$). The percentage of active intracellular SFT3 in Sf9 (□) and Tn cells (◇) was calculated from the ratio of intracellular to total (intra- and extracellular) activity. (**F**) Variation of intracellular active SFT3 to total SFT3 ratio, calculated from the intracellular activity divided by the corresponding band intensity in the Western blot (**A** and **C**) in Sf9 (■) and Tn (◆) cells. DAI, day after infection.

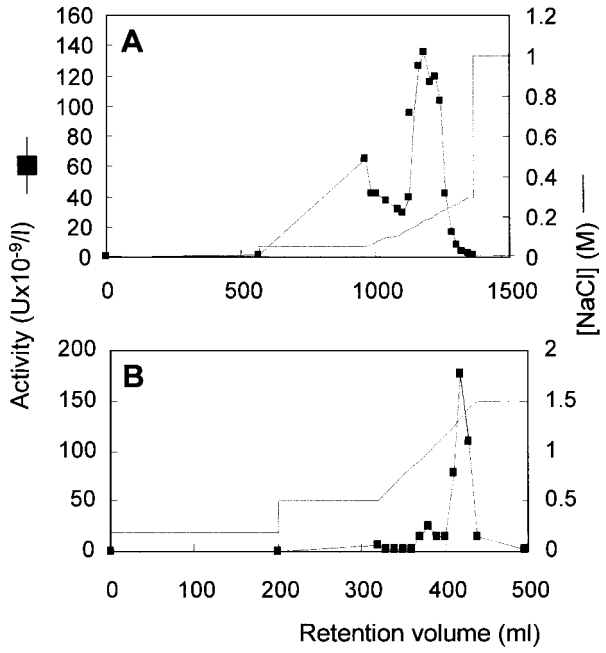

Figure 5 Purification of SFT3 from culture supernatants of Sf9 cells

Elution profiles of cation-exchange chromatography using carboxymethyl-Sepharose (**A**) and of affinity chromatography using GDP-Fractogel (**B**). Activity was measured using Galβ3GlcNAc-$(CH_2)_3$-NHCO-$(CH_2)_5$NH-biotin as substrate. Samples were applied at retention volume zero.

with proteolysis of extracellular SFT3. On the fifth day after infection, approximately 65 % of the Sf9 cells and 90 % of the Tn cells were lysed (Figure 3) and probably released considerable amounts of intracellular proteases into the culture medium. In contrast with the supernatants, where no degradation products were observed for the presented protein concentrations, the cell extracts exhibited bands at lower molecular masses than SFT3, probably consisting of degradation products. Bands detected at higher molecular masses probably consisted of SFT3-containing homo- or hetero-oligomers. In order to investigate whether intracellular SFT3 was active, enzyme activity associated with Sf9 and Tn cells was determined (Figure 4E). The increase in intracellular active SFT3 was more dramatic up to the third day (Figure 4E). It has been observed that much of this intracellular active protein was soluble, since activities were similar in the presence and absence of 1 % (w/v) Triton X-100 (results not shown). After the third day of infection a large proportion of accumulated intracellular protein was not active, since it was observed that the fucosyltransferase activity to band intensity ratio (Figures 4A, 4C and 4F) decreased and stabilized after the third day. These results showed that until the third day after infection the cells were accumulating active protein, indicating limitations of the secretory pathway. After the third day of infection cells were concomitantly accumulating inactive protein, indicating inefficiency of the folding machinery in the endoplasmic reticulum of the cells, as previously suggested [21,22]. This could possibly be overcome by coexpression of the cells with proteins involved in transport from the endoplasmic reticulum to the Golgi/trans-Golgi network to the extracellular medium, such as components of the coat protein ('COP') II complex, and chaperones, such as immunoglobulin heavy-chain binding protein (BiP) or protein disulphide-isomerase, as reviewed by Ailor and Betenbaugh [23].

Purification and characterization of SFT3 from the supernatants of Sf9 and Tn cells

Purification

Supernatants from Sf9 cells collected on the fourth day after infection were concentrated 4-fold in an Amicon system, and were purified by cation-exchange chromatography on a carboxymethyl-Sepharose column, followed by affinity chromatography on a GDP-Fractogel column (Figure 5 and Table 1). Of the initial enzyme activity 13 % did not bind, and was eluted from the column when washed with buffer A containing 50 mM NaCl. Of the initial enzyme activity 42 % was eluted from the column with buffer A containing 180 mM NaCl (Figure 5A). This latter pool was then applied on to a GDP-Fractogel column and 11 % of the initial enzyme activity was eluted with buffer A containing 1.3 M NaCl (Figure 5B). A purification of approx. 5349-fold was observed (Table 1). The active pool was analysed by SDS/PAGE and appeared as a diffuse band (Figure 6).

SFT3 secreted from Tn cells was purified in a similar way. However, it was observed that when the carboxymethyl-Sepharose column was washed with buffer A containing 50 mM NaCl approximately 50 % of the activity did not bind. The column was then washed with buffer A containing 25 mM NaCl and it was observed that 11 % of the initial activity was not bound, whereas 44 % of the initial activity was eluted at 150 mM NaCl concentration (Table 1). This latter pool was then applied

Table 1 Summary of the purification of SFT3 from the culture medium of Sf9 and Tn cells

The amount of enzyme catalysing the transfer of 1 nmol of Fuc/min to Galβ3GlcNAcβ$(CH_2)_3$NHCO$(CH_2)_5$NH-biotin corresponds to 1 m-unit. CM, carboxymethyl.

Cells	Purification step	Volume (ml)	Total protein (mg)	Activity (m-units/ml)	Total activity (m-units)	Specific activity (m-units/mg)	Cumulative recovery (%)	Total purification (fold)
Sf9	Culture medium	1800	3060	0.180	323	< 1	–	1
	Concentrated medium	570	2508	0.416	273	< 1	85	1
	CM-Sepharose	200	38	0.675	135	4	42	34
	GDP-Fractogel	30	< 1	1.145	34	567	11	5349
Tn	Culture medium	2000	3800	0.150	300	< 1	–	1
	Concentrated medium	550	3795	0.528	290	< 1	97	1
	CM-Sepharose	180	25	0.735	132	5	44	67
	GDP-Fractogel	50	< 1	0.175	9	72	3	911

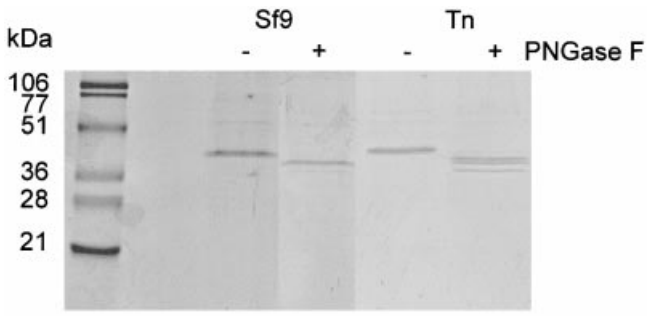

Figure 6 SDS/PAGE analysis and deglycosylation of SFT3 from Sf9 and Tn cells

SFT3 secreted by Sf9 cells and Tn cells was deglycosylated with PNGase F under denaturing conditions. The lower molecular-mass band observed was PNGase F. Gels were stained with Coomassie Brilliant Blue R-250.

on to a GDP-Fractogel column, and only 3% of the initial enzyme activity was eluted with buffer A containing 1.3 M NaCl (Table 1). A purification of approx. 911-fold was observed. The active pool was analysed by SDS/PAGE and appeared as a diffuse band (Figure 6). It migrated with a molecular mass slightly higher than the enzyme secreted from Sf9 cells, due to differential N-terminal processing (see below). SFT3 produced from Tn cells bound the carboxymethyl-Sepharose resin less strongly, indicating that it had a lower isoelectric point. This might be due to post-translational modifications, such as serine phosphorylation or tyrosine sulphation [24].

N-terminal determination of SFT3

The N-terminal sequence of SFT3 secreted from Sf9 cells was found to be ^{55}DTTPTRPTLL (single-letter amino-acid notation), which was different from the engineered cleavage site for signal peptidase. However, it was interesting to observe that the same virus produced an SFT3 with distinct N-terminal processing when infecting the Tn cells. In this case the N-terminal sequence was determined to be ^{47}APSGSXRQ^{55}DT (single-letter amino-acid notation), and it was the expected one.

These results suggest that signal peptidases from Sf9 and Tn cells have different *in vivo* specificities. According to the statistical analysis of substrate specificities from signal peptidases [25] cleavage at Asp^{55} would be tolerated since amino-acid residue 52 is a serine residue. Though unlikely, due to the homogeneity of each N-terminal sequence, it cannot be excluded that Sf9 protease(s) are cleaving the N-terminal of SFT3 during trafficking from the endoplasmic reticulum to the Golgi to the extracellular medium, or in the extracellular medium.

SFT3 deglycosylation with PNGase F

Fuc-TIII has two conserved glycosylation sites. The enzyme from A431 cells is glycosylated [2], and when the recombinant enzyme was expressed in baby hamster kidney (BHK) cells both sites were occupied [3]. SFT3 secreted from Sf9 and Tn cells was totally or partially deglycosylated with PNGase F under denaturing conditions yielding one or two bands, respectively, at lower molecular masses. A band at approx. 35 kDa, corresponding to PNGase F, was also detected (Figure 6). The highest shift in molecular mass was approximately 3–4 kDa, which indicated that SFT3 from the two cell lines had the two glycosylation sites occupied. This was based on the structures of

Table 2 Substrate specificity of SFT3 expressed in Sf9 and Tn insect cell lines with low-molecular-mass acceptors

Activity relative to Galβ1-3GlcNAc-R at 1.7 mM concentration in the presence of 0.05 mM GDP-Fuc; R = -$(CH_2)_3$-NHCO-$(CH_2)_5$-NH-biotin.

Substrate	Relative activity (%)	
	Sf9	Tn
Type 1 acceptors		
Galβ1-3GlcNAc-R	100	100
Fucα1-2Galβ1-3GlcNAc-R	139	116
NeuAcα2-3Galβ1-3GlcNAc-R	48	33
Type 2 acceptors		
Galβ1-4GlcNAc-R	2	< 1
Fucα1-2Galβ1-4GlcNAc-R	3	3
NeuAcα2-3Galβ1-4GlcNAc-R	< 1	< 1

the N-linked glycans from Sf9 and Tn cells previously described (reviewed by Altmann et al. [18]), where the maximum mass found for an insect N-linked glycan was 1884 ($Man_9GlcNAc_2$). SFT3 secreted from Sf9 cells was completely sensitive to PNGase F indicating the presence of oligomannose or complex-type glycans with proximal fucose α6-linked. SFT3 secreted from Tn cells was approx. 50% resistant to PNGase F. This may possibly be due to the presence of complex-type glycans with proximal fucose α3-linked. These results agree with previous reports from other authors. The difucosylated structure Manα3[Manα6]Manβ4GlcNAcβ4[Fucα6][Fucα3]GlcNAc has been described before [26] as constituting 18% of the N-glycans from a recombinant secreted IgG from Tn cells. Proximal α3-fucosylation has been observed to a lesser extent in membrane glycoproteins of Sf9 [27], however, when human interferon ω1 was expressed in Sf9 cells no proximal α3-fucosylation was observed [28].

Activity and specificity of SFT3 from Sf9 and Tn cells

The apparent kinetic parameters determined with the type 1 acceptor, using saturating concentrations of GDP-Fuc, were $k_{cat} = 0.4\ s^{-1}$ and $K_m = 0.87$ mM for SFT3 secreted by Tn cells, and $k_{cat} = 0.09\ s^{-1}$ and $K_m = 0.76$ mM for SFT3 secreted by Sf9 cells. The apparent K_m of SFT3 secreted by Sf9 cells for the type 1 acceptor was slightly lower compared with the K_m of SFT3 secreted by Tn cells, which indicated a higher affinity of SFT3 secreted by Sf9 cells for this type of acceptor. This difference could be due to the differences in post-translational modifications that occur in these two insect cell lines, such as phosphorylation, sulphation or glycosylation. The K_m value obtained for the recombinant S2FT3T2 expressed in BHK-21 cells [3] was even lower (0.54 mM), and was closer to that obtained for the soluble enzyme purified from the culture supernatant of the A431 human carcinoma cell line (0.3 mM) [2]. This higher affinity for type 1 structures could be due to differences in patterns of glycosylation, or other post-translational modifications from the host cell lines, since the mammalian cell line BHK-21 has properties more closely related to human cells. In order to further characterize the enzyme activity, fucosyltransferase assays were performed using a number of different oligosaccharides as acceptor substrates (Table 2), linked to a hydrophobic spacer arm conjugated to biotin. The two enzymes showed a specificity pattern similar to that given by the Fuc-TIII purified from the medium of the A431 cell line [2]. In general, the two enzymes exhibited a clear preference for transfer

of fucose to type 1 acceptors (Table 2). The corresponding type 2 acceptors were very poor substrates of both enzymes. Substitution of type 1 disaccharide with α2-linked fucose (Table 2; Fucα1-2Galβ1-3GlcNAc-R), slightly enhanced the activity, in contrast with substitution with α2,3-linked sialic acid (Table 2; NeuAcα2-3Galβ1-3GlcNAc-R), which caused a decrease in enzyme activity.

CONCLUSIONS

Structural studies on fucosyltransferases have been hindered by the difficulty in obtaining large amounts of active homogeneous preparations of these enzymes. Recombinant glycosyltransferases have been expressed in *Saccharomyces cerevisae* or *Pichia pastoris*, however, they showed significant molecular-mass heterogeneity, possibly due to glycosylation microheterogeneity [29,30]. In the present work, we have described the expression in the baculovirus insect-cell system of SFT3, containing the catalytic domain of human Fuc-TIII without any affinity tag (1 mg/l; 0.4 unit/l). This value is approximately 5-fold higher than the values previously described for the expression of a recombinant human glycosyltransferase using this system [31]. Recombinant SFT3 from both Sf9 and Tn cells has kinetic parameters within the same order of magnitude as their mammalian counterpart and show similar substrate specificities. Furthermore, they appear as a single band when resolved by SDS/PAGE, following purification. Therefore they are suitable for further structural studies.

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