Human fucosyltransferase IX: Production, purification and characterisation

Dissertation

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submitted by
Christina Stacke
from Jena

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Für meinen Mann und meine Eltern. Ohne euch hätte ich nie die Kraft oder die Ausdauer gehabt das alles durchzustehen.

"Summing up, it is clear the future holds great opportunities. It also holds pitfalls. The trick will be to avoid the pitfalls, seize the opportunities, and get back home by six o'clock." (Allen, 1980)

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Abbreviations

E. coli	*Escherichia coli*
AIEX	anion exchange chromatography
AP	alkaline phosphatase
APS	ammonium persulfate
BSA	bovine serum albumin
C/P	citrate/phosphate
CD	cluster of differentiation antigen
cpm	counts per minute
CV	column volume
DC-SIGN	dendritic cell-specific ICAM-3 grabbing nonintegrin
dNTP	desoxyribonucleosidtriphosphate
ER	endoplasmic reticulum
F9H	C-terminal hexa-histidine tagged N-terminally truncated human fucosyltransferase (FucT) IX starting from serine 39
FRET	Förster resonance energy transfer
FucT	fucosyltransferase
GDP-Fuc	GDP-β-L-fucose
HA	hemagglutinin
HCMV	human cytomegalovirus
HF9	N-terminal deca-histidine tagged hS39F9
HIV	human immunodeficiency virus
hP40F9	N-terminally truncated human FucT IX starting from proline 40
hS39F9	N-terminally truncated human FucT IX starting from serine 39
ICAM-3	intercellular adhesion molecule-3
IMAC	immobilised metal ion affinity chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
LacNAc	N-acetyllactosamine
Le^x	Lewis x epitope
LPS	lipopolysaccharide

MBP	maltose binding protein
MCS	multiple cloning site
MES	2-(N-morpholino)ethanesulfonic acid
NEM	N-ethylmaleinimide
Ni-NTA	nickel-nitrilotriacetic acid
OPIE	*Orgyia pseudotsugata* multicapsid nucleopolyhedrosis virus immediate-early
PH	polyhedrin
PVDF	polyvinylidene fluoride
SDS-PA	SDS polyacrylamid
SEA	soluble egg antigen
Sf9	*Spodoptera frugiperda*
SSEA	stage-specific embryonic antigen
SV40	simian vacuolating virus 40
TEMED	N N N'N'-tetramethylethylenediamine

Abstract

Human fucosyltransferase (FucT) IX belongs to the α-1,3 FucT family. In contrast to other members of this family, FucT IX is highly conserved among mice, rat, hamster and human. It fucosylates distinct acceptors with high specificity, by catalysing the transfer of activated fucose to a sugar acceptor, generating a Lewis x epitope (Le^x). This epitope is involved in intercellular recognition, *e.g.* autoaggregation of teratocarcinoma cells or viral infections. Hence, inhibitors directed against the enzymatic activity of human FucT IX could be useful for clinical applications. The design of inhibitors would be greatly facilitated by a better comprehension of enzyme kinetics and structural information. For these analyses considerable amounts of protein are necessary. Thus, in this work it was attempted to recombinantly overproduce, purify and characterise a soluble truncated variant hS39F9 – missing 38 N-terminal amino acids, starting with serine 39 if compared to wild type FucT IX. *Escherichia coli* (*E. coli*) and insect cell systems were tested to evaluate the most suitable recombinant expression system for the FucT IX.

In *E. coli* production of hS39F9 fused to maltose binding protein yielded soluble, but inactive protein. Constitutive protein production in insect cells yielded enzymatically active hS39F9 fusion protein in the cell culture supernatant. A His-tag was fused to hS39F9 to facilitate protein purification. Tagging the C-terminus resulted in inactive protein, whereas N-terminally tagged hS39F9 was active with a maximum yield of ∼240 mU/l in the culture supernatant. The production of hS39F9 fusion protein using baculoviral infected insect cells gave the highest amount of protein with a yield of ∼500 mU/l culture supernatant.

The culture supernatant of baculoviral infected cells was concentrated by cross flow filtration and the buffer was exchanged simultaneously. The processed supernatant was used for immobilised metal ion affinity chromatography

(IMAC). The IMAC-fractions were dialysed and further purified by anion exchange chromatography (AIEX).

The temperature optimum of the hS39F9 fusion protein was 37 °C and the pH optimum ranged from pH 5.5 to 9.0. A dependency on divalent cations, which is known for other FucTs, could not be detected. As expected, the hS39F9 fusion protein exhibited Michaelis Menten kinetic, with the kinetic constant K_m=1.6 µM for the donor sugar GDP-β-L-fucose (GDP-Fuc) and K_m=0.70 mM for the acceptor sugar N-acetyllactosamine (LacNAc). The specificity constants for GDP-Fuc and LacNAc were k_{cat}/K_m=485.2×10^2 $M^{-1}s^{-1}$ and 1.1×10^2 $M^{-1}s^{-1}$, respectively. The determined catalytic constant was k_{cat}=4.6 min^{-1}.

This results presented in this thesis laid the basis to further characterise the catalytic mechanism or structure of hS39F9. Thus, it will be possible to understand the functionality of the enzyme and develop specific inhibitors to treat pathological processes associated with excess fucosylation.

Zusammenfassung

Humane Fucosyltransferase (FucT) IX gehört zur Familie der α-1,3 FucTs. Sie ist hochkonserviert zwischen Mäusen, Ratten, Hamstern und Menschen. FucT IX fucosyliert verschiedene Akzeptoren mit hoher Spezifität, indem sie den Transfer von aktiviertem Donator-Zucker auf einen Zuckerakzeptor katalysiert. Bei dieser Reaktion entsteht das Lewis x Epitop. Dieses Epitop ist an interzellulären Erkennungsprozessen beteiligt, weswegen FucT IX Inhibitoren für klinische Anwendungen bedeutend sein könnten. Eine mögliche Anwendung liegt beispielsweise in der Unterdrückung der Tumormetastasierung. Ein genaueres Verständnis der Enzymkinetik und Proteinstruktur würde das Design von Inhibitoren stark vereinfachen. Derartige Analysen benötigen größere Mengen gereinigtes Enzym. Das Ziel dieser Arbeit war die Produktion großer Mengen FucT IX, deren Reinigung und grundlegende enzymatische Charakterisierung.

Im Rahmen dieser Arbeit wurde eine N-terminal verkürzte Variante humaner FucT IX (hS39F9) rekombinant in *E. coli* und Insektenzellen produziert. Die Produktion in *E. coli* ergab lösliches, jedoch inaktives Protein. Im Gegensatz dazu ergab die konstitutive Proteinproduktion in Insektenzellen enzymatisch aktive FucT IX. Zur einfacheren Reinigung wurde hS39F9 mit einem His-Tag versehen. Ein C-terminal fusionierter Tag führte zur Produktion eines inaktiven Proteins. Im Gegensatz dazu wurde mittels einer N-terminalen Fusion des His-Tags $\sim$240 mU/l aktives Enzym hergestellt. Zeitgleich erfolgte eine Produktion in baculoviral infizierten Insektenzellen. Hierbei konnte im Zellkulturüberstand eine Enzymaktivität von $\sim$500 mU/l Kultur gemessen werden.

Im Folgenden wurde das Protein aus dem Zellkulturberstand gereinigt. Der erste Schritt der Reinigung des Zellkulturüberstands bestand in dessen Konzentrierung mittels Cross Flow Filtration. Diese Methode diente der Abtrennung kleinerer Proteine und ermöglichte einen Pufferwechsel. Der so behandelte Überstand wurde mit Hilfe eines Histidin-Tag über eine Nickel-Affinitäts-

chromatographie gereinigt. Anschließend erfolgte eine Dialyse um als weiteren Reinigungsschritt eine Anionenaustausch-Chromatographie durchzuführen. Die erhaltenen Elutionsfraktionen enthielten die gereinigte N-terminal verkürzte Variante der FucT IX.

Mit diesen Fraktionen konnten erste Untersuchungen des Enzyms durchgeführt werden. Dabei konnte das Temperaturoptimum mit 37 °C und ein breites pH Optimum von pH 5,5 bis 9 festgestellt werden. Eine Abhängigkeit der Katalysereaktion von zweiwertigen Metallionen, wie sie für andere α-1,3 FucTs bekannt ist, konnte nicht nachgewiesen werden. Wie erwartet, folgt die katalysierte Reaktion einer Michaelis Menten Kinetik. Die kinetische Konstante K_{m} betrug 1,6 µM für den Donator-Zucker GDP-Fuc und 0,70 mM für den Akzeptor-Zucker LacNAc. Die katalytische Konstante k_{cat} hatte einen Wert von 4,6 min^{-1}. Zusätzlich konnte eine katalytische Effizienz von $k_{\mathrm{cat}}/K_{\mathrm{m}}$=485.2×$10^2$ $\mathrm{M}^{-1}\mathrm{s}^{-1}$ für GDP-Fuc und 1.1×10^2 $\mathrm{M}^{-1}\mathrm{s}^{-1}$ für LacNAc ermittelt werden.

Die in dieser Arbeit erfolgreich durchgeführten Versuche bieten die Grundlage zur Aufklärung des Reaktionsmechanismus' und der Struktur von FucT IX. Durch das so gewonnene Verständnis der Funktionsweise des Enzyms wird die Entwicklung spezifischer Inhibitoren möglich. Diese Inhibitoren könnten bei Krankheiten Anwendung finden, die durch übermäßige Fucosylierung hervorgerufen sind.

1 Introduction

1.1 Protein glycosylation

The human genome project unveiled 20,000 – 25,000 protein coding sequences, much less than previously anticiptated (International Human Genome Sequencing Consortium, 2004). In comparison, the genome of the nematode *Caenorhabditis elegans* contains 19,000 protein coding genes but its organism consists of ∼1000 cells where approximately 10^{14} cells exist in the human body. Several elements compensate this apparent lack of complexity in the human protein coding genes. First, a large number of non-coding RNAs adds a supplementary regulatory layer, which increases complexity (reviewed by Costa, 2005). Second, human mRNAs appear to be heavily alternatively spliced. Thus, several proteins can be produced from one single gene (reviewed by Hui, 2009). Third, human proteins are subjected to posttranslational modifications, which affect their physico-chemical properties. These modifications permit a greater variability than the protein coding amino acids can offer (reviewed by Eisenhaber and Eisenhaber, 2010).

The most complex form of posttranslational modifications of proteins are glycosylations. Proteins and DNA are characterised by a linear composition of their building blocks whereas glycans consist of multiple branches. Unlike proteins, glycostructures are not directly encoded by genes but rather by a network of hundreds of glycosyltransferases, glycosidases, transporters, transcription factors, and other proteins (Lauc *et al.*, 2010). Therefore, they are more difficult to study than proteins or nucleic acids and research focused only recently on the role of glycosylations in regulation of cellular processes (Finkelstein, 2007). Protein glycosylations can be grouped in three different types: proteoglycans, O-glycosylation, and N-glycosylation.

Proteoglycans

Proteoglycans consist of glycosaminoglycans covalently linked to proteins (reviewed by Esko *et al.*, 2009). These glycosaminoglycans are long, linearly linked carbohydrates which are bound to the proteins by O-glycosylations. Proteoglycans are mostly part of the extracellular matrix in tissues and provide a hydrated gel to protect from compressive forces.

O-glycosylations

O-glycosylations are the result of the direct transfer of a monosaccharide to the hydroxyl group of serine, threonine, hydroxyproline or -lysine residues of proteins. This transfer takes place within the Golgi apparatus. Hydroxyproline or -lysine O-glycosylations are found for example in collagens (Freeze and Haltiwanger, 2009). Proteins that are heavily O-glycosylated with α-linked N-Acetylgalactosamine at serines or threonines are named mucins (reviewed by Brockhausen *et al.*, 2009). Those proteins are ubiquitous in mucous secretions on cell surfaces and in body fluids. Nonmucin O-glycans include several different O-linked sugars. At the innermost sugar different sugars are attached to form low-branched structures. These structures have no common core-structure, but several core classes.

N-glycosylation

N-glycosylation starts in the endoplasmic reticulum (ER) with the co-translational transfer of an oligosaccharide precursor from a dolichol-donor. During transport from the ER to the Golgi apparatus mono- or oligosaccharides are attached to, or removed from the precursor. Thus, three general types of N-glycans are generated: oligomannose, complex, and hybrid (Figure 1.1 and reviewed by Stanley *et al.*, 2009). These N-glycans share the common core structure $Man_3GlcNAc_2$ displayed in Figure 1.1. The sugar chains of these N-glycan types are linked to a asparagine in a consensus sequence of the protein which is followed by any amino acid except proline, it ends with a serine or a threonine (Asn-X-Ser/Thr). Not all of these consensus sequences within a protein are glycosylated. Therefore, if this sequence is found in the primary structure of a protein it is only potentially an N-glycosylation site.

N-glycosylations play an important role in protein folding processes by linking the sugar moiety to the nascent protein to protect hydrophobic areas. Through

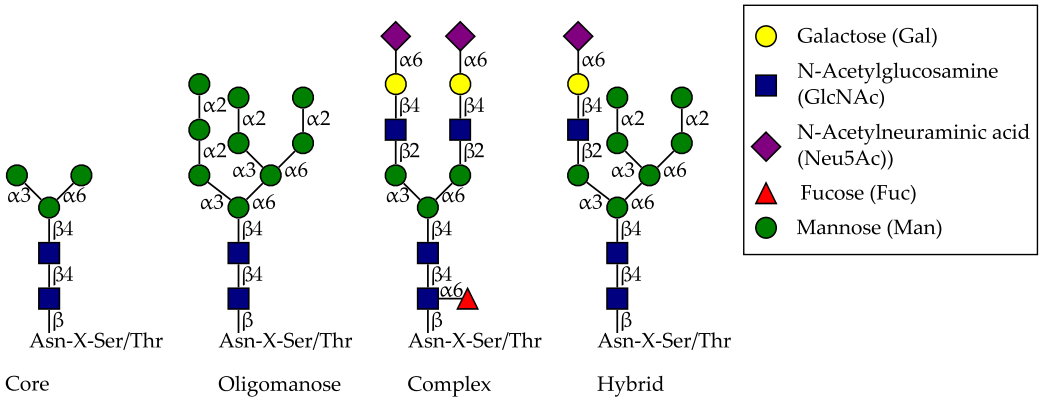

Figure 1.1: Types of N-Glycans. Oligomannose, complex, and hybrid N-Glycans. Figure according to Stanley *et al.* (2009).

this process, solubility of folding intermediates is enhanced and aggregation of incompletely folded proteins is diminished. The folding process in the ER is supported by the specific carbohydrate binding chaperones calnexin and calreticulin. They take part in a glycoprotein specific chaperone cycle to perform a quality control of the nascent glycoproteins. Thus, correctly folded proteins are transmitted to the Golgi apparatus (Schrag *et al.*, 2003; Stronge *et al.*, 2001).

N-glycans have been reported to enhance serum half life of proteins by addition of sialic acid or sulfate (Baenziger *et al.*, 1992). Also intracellular transport and localisation can be regulated by N-glycosylation (Nagai *et al.*, 1997; Martina *et al.*, 1998). Furthermore, cancer progression is facilitated when a change of N-glycans to more complex structures occurs. Conversely, the progression of cancer is retarded if the responsible glycosyltransferases are missing. Accordingly, inhibition of these glycosyltransferases might be an important way to design new anti-cancer therapeutics (reviewed in Varki *et al.*, 2009).

The most versatile roles of N-glycans are their functions as lectin ligands. For example, selectins bind specific carbohydrates during cell-cell contacts. Thus they play a role in blood-clotting, apoptosis, leukocyte extravasation from the blood stream or lymphocyte homing to lymph nodes (Lee, 1992).

1.2 Human fucosyltransferases

Glycosyltransferases catalyse the transfer of a sugar with either inversion or retention of the anomeric stereochemistry with respect to the donor sugar. According to this reaction they are grouped as inverting and retaining glycosyltransferases (Coutinho *et al.*, 2003). Fucosyltransferases (FucTs) are a subgroup of inverting glycosyltransferases. They catalyse the transfer of fucose from GDP-β-L-fucose (GDP-Fuc) to an oligosaccharide, glycoprotein, or glycolipid acceptor molecule (Lairson *et al.*, 2008). FucTs transfer the fucose on a N-acetyllactosamine (LacNAc) unit of these acceptor molecules. By their action they invert the configuration of the anomeric carbon C1 in GDP-Fuc from β to α, and fucosylate LacNAc at a distinct carbon (Figure 1.2). Based on the site of fucose addition to LacNAc units FucTs are classified into the sub-families α-1,2, α-1,3, and α-1,6. α-1,4 FucTs attach fucose in α-1,4 linkage to galactoseβ-1,3 N-acetylglucosamine. Furthermore, there exist O-FucTs which catalyse O-fucosylation of proteins at serine or threonine residues.

In eukaryotic organisms the sub-families α-1,2, α-1,3/4, and α-1,6 FucTs are type II transmembrane Golgi-anchored proteins (for details see section 1.2.2 and Figure 1.4). These FucTs form an α-glycosidic linkage to the sugar acceptor. In humans the proteins are encoded by the FUT genes: FUT1 and FUT2 encode α-1,2 FucTs, FUT3 to FUT7 and FUT9 to FUT11 encode α-1,3/4 FucTs, and FUT8 encodes α-1,6 FucT. By CAZy classification they are categorised into glycosyltransferase family 11, 10, and 23, respectively (Cantarel *et al.*, 2009a).

In contrast O-FucTs are soluble proteins in the endoplasmic reticulum (Luo and Haltiwanger, 2005; Okajima *et al.*, 2005). The human protein O-fucosyltransferase 1 and protein O-fucosyltransferase 2 are categorised into the different glycosyltransferase families 65 and 68, respectively (Cantarel *et al.*, 2009a; Lairson *et al.*, 2008).

1.2.1 Evolutionary conservation of α-1,3 fucosyltransferase IX

To date, eight human α-1,3 fucosyltransferase genes (FUT) have been identified: FUT3 – FUT7 and FUT9 – FUT11. They encode the corresponding α-

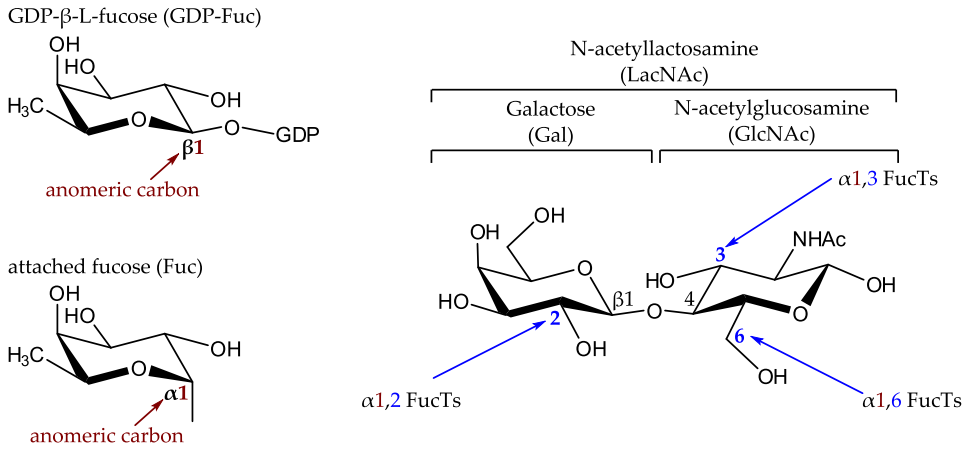

Figure 1.2: Fucosylation sites of α-1,2, α-1,3, and α-1,6 FucTs at LacNAc. During the FucTs catalysed transfer of GDP-Fuc to LacNAc the configuration at the anomeric carbon C1 (red) in GDP-Fuc is inverted from β to α. Depending on the FucTs LacNAc is fucosylated at a specific carbon (blue).

1,3 FucTs. The classical FucTs FUT3 – FUT7 and FUT9 are encoded by monoexonic genes while the recently discovered FUT10 and FUT11 are encoded by polyexonic genes (Mollicone *et al.*, 2009). Based on a sequence alignment, the FUT9 gene shows the lowest identity with the other five monoexonic FUT3 – FUT7 genes. Hence, in 1999 it was supposed by Kaneko *et al.* (1999a) that FUT9 was the first gene to diverge from an ancestral α-1,3 FUT gene. Recently, Mollicone *et al.* (2009) showed that FUT10 and FUT11 genes diverged even earlier from the ancestral α-1,3 FUT gene.

In contrast to FUT3 - FUT7 genes, the FUT9 gene is evolutionary highly conserved between mouse and human. Only three amino acid exchanges were found between the proteins of these two mammals. It is also notable that between mouse and human the number of non-synonymous nucleotide substitutions in the FUT9 gene is in the same range as that of the highly conserved α-actin gene. This indicates the same strong selective pressure on FUT9 as on α-actin gene and demonstrates its importance during mammalian development (Kaneko *et al.*, 1999a; Baboval *et al.*, 2000).

1.2.2 Protein structure of fucosyltransferases

Human α-1,3 FucTs are predicted to have a glycosyltransferase-B fold (Figure 1.3). This fold is characterised by a protein topology consisting of two β/α/β

Rossmann domains, a protein structure motif that binds nucleotides, that face each other and are linked flexible (Lairson *et al.*, 2008). α-1,3 FucTs consist of a short cytoplasmic N-terminus, a single membrane spanning region, a lumenally oriented C-terminus with a stem region, and the large catalytic domain (Figure 1.4). Secreted forms are produced by proteolysis in the Golgi apparatus at multiple protease sensitive sites within the stem region of the protein (Staudacher, 1996).

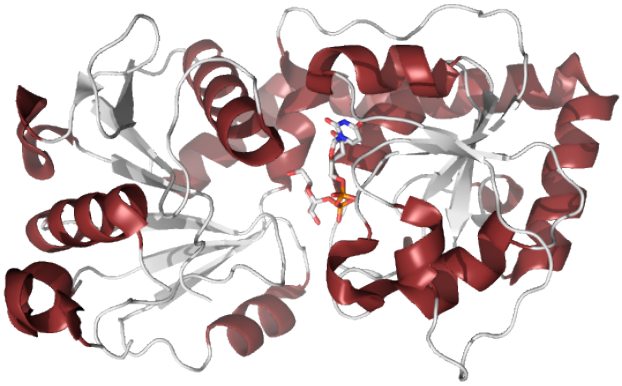

Figure 1.3: Ribbon diagram of a representative glycosyltransferase-B fold. The structure corresponds to T4 phage β-glucosyltransferase (PDB ID 1J39). Secondary structures are displayed in cartoon mode; α-Helices are ruby and β-sheets are grey. Bound nucleotide sugar donor substrate is shown in stick presentation.

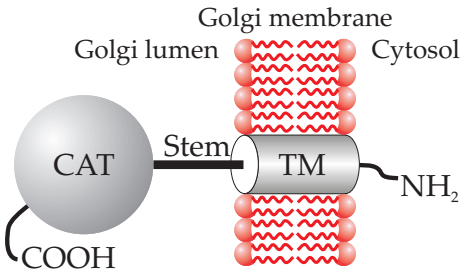

Figure 1.4: Schematic structure of FucTs. Mammalian FucTs are anchored at the Golgi membrane. They consist of a short cytoplasmic N-terminus (NH_2), a single membrane spanning region (TM), a lumenally oriented C-terminus (COOH) with a stem region, and the large catalytic domain (CAT). Adapted from Ma *et al.* (2003).

No crystal structure of a human α-1,3 FucTs has been reported yet. Structures were reported for the bacterial *Helicobacter pylori* (*H. pylori*) α-1,3 FucT (Sun *et al.*, 2007), for the α-1,6 FucT NodZ from nitrogen-fixing symbiotic bacteria (Brzezinski *et al.*, 2007) and for human α-1,6 FucT VIII (Ihara *et al.*,

2007). Despite little sequence identities, the *H. pylori* α-1,3 FucT folds like a glycosyltransferase family B enzyme (Cantarel *et al.*, 2009b). While most regions within human α-1,3 FucTs are different from *H. pylori* α-1,3 FucT, the GDP-Fuc binding site has a certain sequence similarity in both. This region accounts for the same donor substrate specificity of the enzymes (Brzezinski *et al.*, 2007).

1.2.3 Conserved motifs in α-1,3 fucosyltransferases

Monoexonic human α-1,3 FucTs share conserved regions (Figure 1.5). Some of them are also found in *H. pylori* α-1,3 FucT and human multiexonic α-1,3 FucTs. Mollicone *et al.* (2009) renamed the conserved structural motifs of α-1,3 FucTs (Figure 1.5). According to their nomenclature motifs I-III are involved in recognition of the acceptor substrate (Legault *et al.*, 1995) while the recognition and binding of donor substrate GDP-Fuc is located in motif IV and V (Dupuy *et al.*, 2004, 1999).

The functionality of specific amino acids within these motifs is already known. Two histidines in motif II (see label "a" in Figure 1.5) are involved in acceptor binding. They probably interact with GlcNAc of type II acceptors (Sherwood *et al.*, 2002). The amino acid next to this histidines (see label "b" in Figure 1.5) is crucial for H-type I/II specificity, an aromatic residue in this position is required for α-1,4 fucosylation (Dupuy *et al.*, 2004). The next downstream amino acid (label "c" in Figure 1.5) was proposed by Dupuy *et al.* (1999) to be the catalytic base. Holmes *et al.* (1995) investigated the N-ethylmaleinimide sensitivity of different human α-1,3 FucTs and found that a cysteine (label "d" in Figure 1.5) is protected by GDP-Fuc binding. Christensen *et al.* (2000) identified an N-glycosylation site (label "e" in Figure 1.5) in motif IV, which is in connection with another one required for full enzyme activity. Another important conserved sequence is the acidic sequence DxD, which is present in unrelated glycosyltransferase families (Wiggins and Munro, 1998; Breton and Imberty, 1999; Unligil *et al.*, 2000). In human α-1,3 FucT it is located inside motif IV and has the sequence DSD (label "f" in Figure 1.5). In gylcosyltransferases of the glycosyltransferase-A fold this region is essential for their Mn^{2+} dependent activity (Wiggins and Munro,

1998; Zhang *et al.*, 2001). In glycosyltransferases of the glycosyltransferase-B fold divalent cations may act as activators but are not essential for enzymatic activity (reviewed in Breton *et al.*, 2006). By crystallisation of *H. pylori* α-1,3 FucT Sun *et al.* (2007) identified tyrosine, glutamine, and lysine (label "g-i" in Figure 1.5) as specific amino acids responsible for GDP-Fuc binding. The glutamine is supposed to stabilise the developing oxonium. An arginine located between motif IV and V is involved in GDP-Fuc binding and is conserved among *H. pylori* and human α-1,3 FucT.

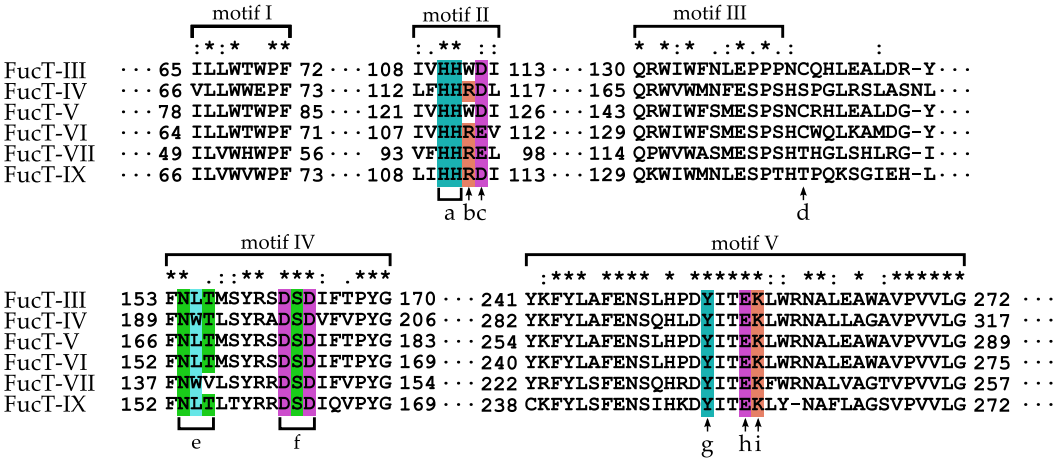

Figure 1.5: Details of an alignment of classical monoexonic human α-1,3 FucTs. The alignment was performed using COBALT (constraint-based alignment tool for multiple protein sequences) (Papadopoulos and Agarwala, 2007) and ClustalX 2.0.12 (Thompson *et al.*, 1997) with accession numbers P21217.1, P22083.3, Q11128.1, P51993.1, Q11130.1, and Q9Y231.1 for FucT III – FucT VII, and FucT IX, respectively. Motif length and numbering was taken from Mollicone *et al.* (2009). The following colorscheme was used: darkblue: Tyr, His, light blue: hydrophobic amino acid, red: Arg, Lys, green: Ser, Thr, Asn, violet: Asp, Glu. The following homology scheme was used: "*" : same amino acid, ":": substitution with closely related amino acid, "." : substitution with related amino acid. The Motifs I-III are responsible for the recognition of the acceptor substrate (Legault *et al.*, 1995), and Motif IV, V for recognition and binding of donor substrate GDP-Fuc (Dupuy *et al.*, 2004, 1999). Lower case letters indicate specific amino acid, where "a" shows histidines involved in acceptor binding (Sherwood *et al.*, 2002), "b" an amino acid for H-type I/II specificity, an aromatic residue in this position is required for α-1,4 fucosylation (Dupuy *et al.*, 2004), "c" indicates the putative catalytic base (Dupuy *et al.*, 1999), "d" is the GDP-Fuc binding cysteine (Holmes *et al.*, 1995), "e" an N-glycosylation site (Christensen *et al.*, 2000), "f" shows the DxD motif responsible for Mn^{2+} binding (Palma *et al.*, 2004), and g, h, i are involved in GDP-Fuc binding (Sun *et al.*, 2007).

1.2.4 Mechanism of catalysis

The members of the α-1,3 FucT family transfer a guanosine diphosphate activated L-fucose to the α-1,3 position of a sugar acceptor (see Figure 1.6 and de Vries *et al.*, 2001a). This yields the Lewis x epitope (Le^x) (see section 1.3), also known as cluster of differentiation antigen (CD) 15 or stage-specific embryonic antigen (SSEA) 1 (Kudo *et al.*, 1998).

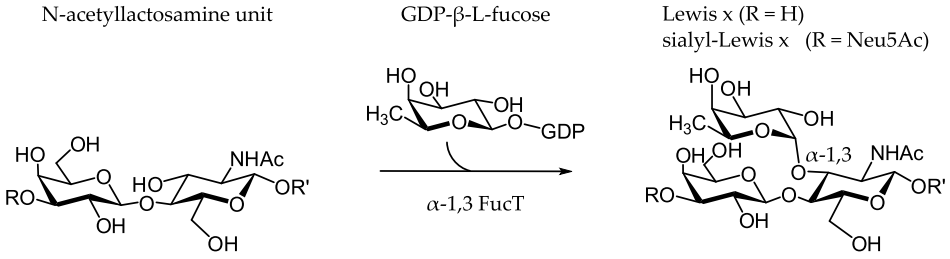

Figure 1.6: α-1,3 FucT catalysed reaction. α-1,3 FucTs transfer an activated GDP-β-L-fucose to the α-3 position of a N-acetyllactosamine unit in a polysaccharide chain. This reaction results in the Lewis x epitope or sialyl-Lewis x epitope. Figure according to Murray *et al.* (1997).

Crystallisation of *H. pylori* α-1,3 FucT and kinetic data from Murray *et al.* (1997) enabled Sun *et al.* (2007) to predict a model for the interaction of FucTs with GDP-Fuc. They suggested an in-line displacement catalytic mechanism (Pedersen *et al.*, 2002) similar to that of human FucT V. Figure 1.7 illustrates the proposed mechanism. LacNAc is bound to the active site pocket in the N-terminal domain of the FucT that reduces the pK_a of the C3-OH group of GlcNAc to increase its nucleophilicity. Upon deprotonation of the C3-OH group by the catalytic base (c, see figures 1.2.4 and 1.7), the acceptor nucleophile can attack the anomeric position of GDP-fucose to form a new glycosidic bond. The side product GDP dissociates at the same time (Sun *et al.*, 2007).

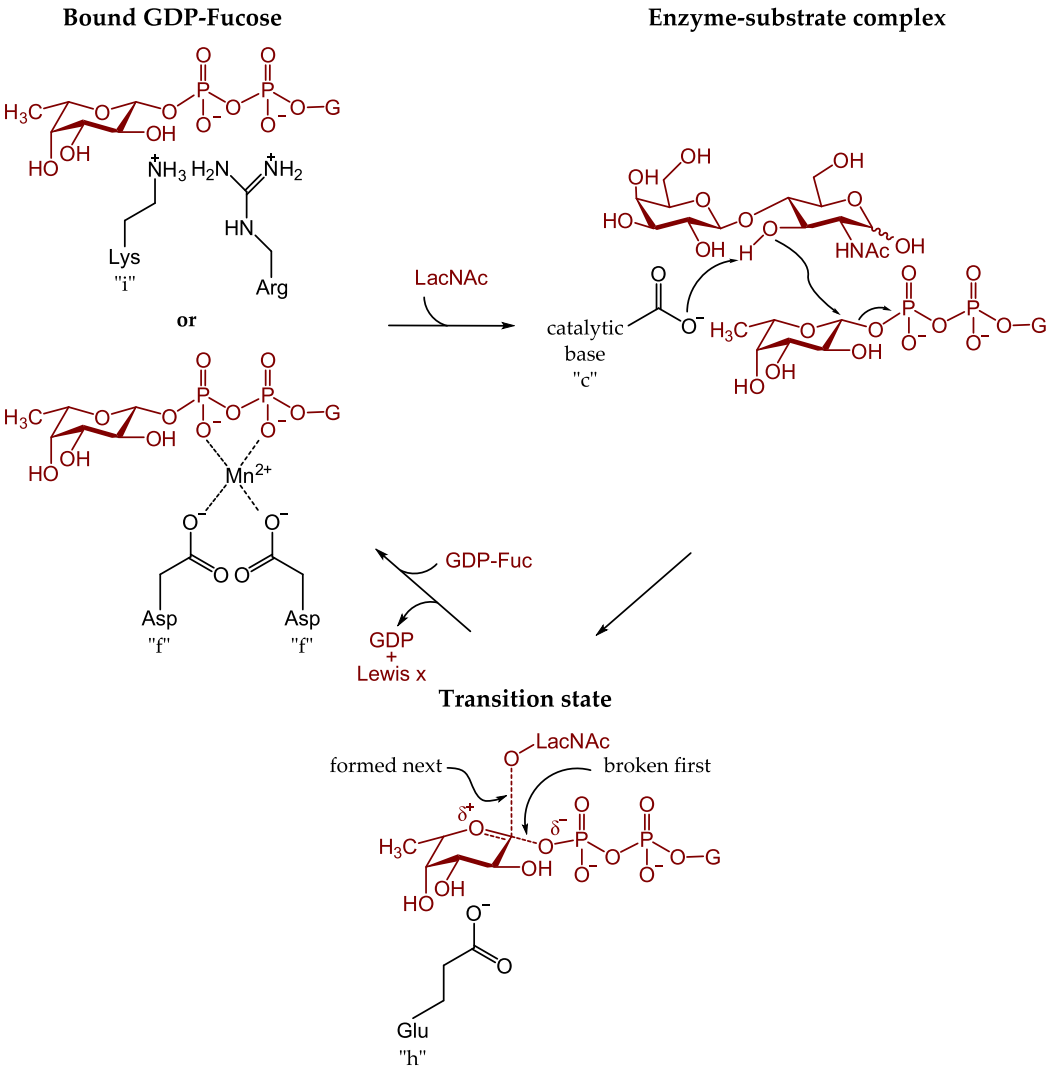

Figure 1.7: α-1,3 FucT active site and proposed reaction mechanism. Proposed catalytic mechanism of α-1,3 FucT where Glu or Asp serve as catalytic base (c). The developing positive charge (δ^+) on fucose is stabilised in part by Glu (h). Enzyme residues are black and substrates are red. Figure according to Sun *et al.* (2007); Murray *et al.* (1997); Palma *et al.* (2004).

1.2.5 Substrate specificity of α-1,3 fucosyltransferase IX

The finding that the FUT9 gene is not closely related to the FUT3 – FUT7 genes (see section 1.2.1) agrees with the fact that FucT IX has a different substrate specificity pattern in comparison to other members of the α-1,3 FucT family. FucT III to FucT VII preferentially fucosylate the inner polylactosamine chain, whereas FucT IX prefers the outer chain as acceptor. This acceptor specificity

pattern enables FucT IX to synthesise the terminal Le^x more efficiently *in vivo* than other FucTs (Nishihara *et al.*, 1999).

Cailleau-Thomas *et al.* (2000) and Toivonen *et al.* (2002) demonstrated a low reactivity of FucT IX towards sialylated LacNAc and type I based acceptors (see Figure 1.8) with a faint ability to synthesise sialyl-Le^x, Le^a, sialyl-Le^a, and Le^b, but good reactivity towards type II based acceptors with efficient synthesis of Le^x and Le^y epitopes (see Figure 1.8).

Toivonen *et al.* (2002) analysed FucT IX specificity towards type II polylactosamine acceptors in detail. The enzyme preferentially fucosylates terminal LacNAc units of neutral chains. Substitution of the polylactosamine chain with terminal Neu5Acα-2,3 shifts the site-specificity of FucT IX to the two innermost LacNAc units.

Brito *et al.* (2008) overproduced FucT IX in HeLa cells and found that it was able to efficiently fucosylate asialo-erythropoietin and bovine asialo-fetuin, but not sialylated erythropoietin. They revealed that FucT IX predominantly monofucosylated type II di-, tri-, and tetraantennary N-glycans carrying proximal α-6 fucose, with and without N-acetylactosamine repeats. Furthermore they found that FucT IX was activated by Mn^{2+} and able to synthesise small amounts of Le^a.

In summary, the differences of the several α-1,3 FucT in the acceptor profiles and in tissue distribution suggests distinct biological roles.

1.2.6 Role of α-1,3 fucosyltransferase IX in the body

The human FUT9 gene is localised at 6q16, the long arm of human chromosome 6 (Kaneko *et al.*, 1999b). The gene is monoexonic and there are two known transcript sizes, which represent a tissue specific distribution. The 2 kb transcript is ubiquitous in embryonic tissues and decreases in adult tissues whereas the 12 kb transcript shows a tissue-specific distribution. The 12 kb transcript is strongly produced in the embryonic brain and decreased in adult brain. Additionally to the low amounts detected in adult brain the 12 kb transcript is detected in kidney, pancreas and placenta (Cailleau-Thomas *et al.*, 2000). Furthermore, Osanai *et al.* (2001) showed an increased expression of FUT9 during neuronal retinoic acid-induced differentiation of PC19 murine

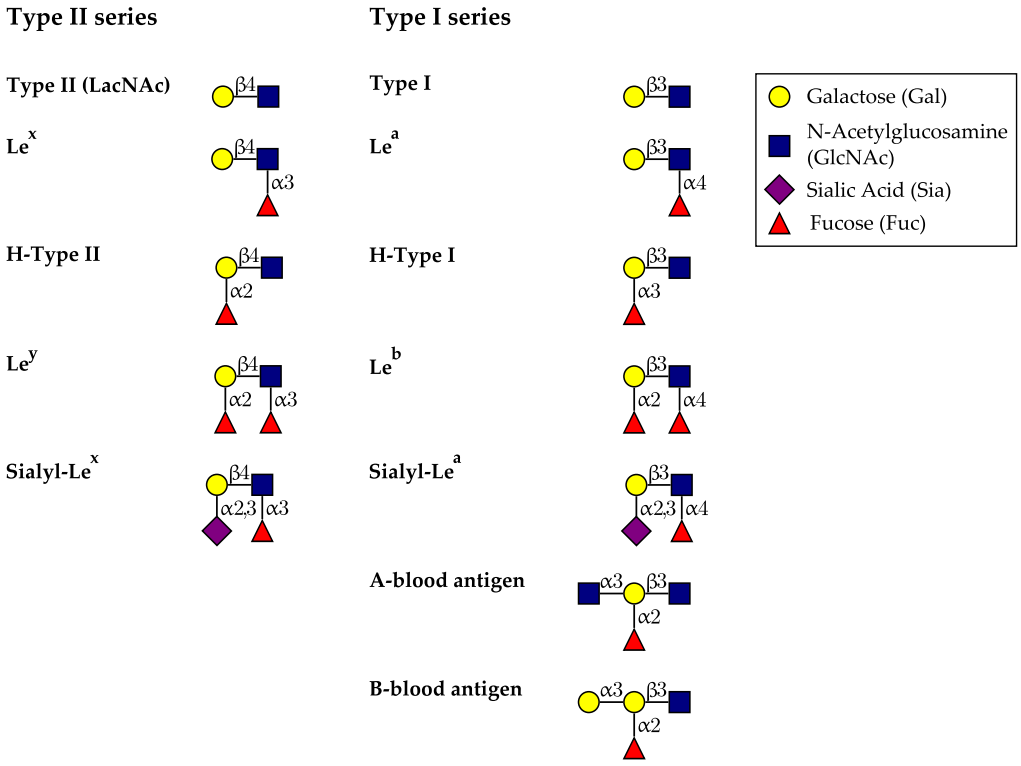

Figure 1.8: Structures of type II and type I acceptor sugars. Adapted from Ma *et al.* (2006).

embryonic carcinoma cells.

FUT9 is mainly expressed in the central nervous system, in both developing and mature brain of human, rat, and mouse (Kaneko *et al.*, 1999a; Baboval *et al.*, 2000; Cailleau-Thomas *et al.*, 2000). The production of FucT IX in rat brain is controlled by Pax6, a transcription factor involved in brain patterning and neurogenesis. By regulating the production of FucT IX Pax6 controls Le^x production in the rat embryonic forebrain (Shimoda *et al.*, 2002). Nishihara *et al.* (2003) and Kudo *et al.* (2007) showed that FucT IX is the α-1,3 FucT, which is responsible for the synthesis of Le^x in the mouse brain. In $FUT9^{-/-}$ double knockout mice no Le^x is produced, accompanied by increased anxiety-like behaviours. This indicates an important role of the carbohydrates synthesised by FucT IX in functional regulation of interneurons in the amygdalar subdivision (Kudo *et al.*, 2007).

Recently, in the genome of a girl with behavioural abnormalities a *de novo*

balanced chromosomal translocation associated with a deletion of ∼1.5 Mb, including FUT9, was found. Due to the findings with the knock out mice, the abnormal behaviour of the girl might also be explained by the deletion of the FUT9 gene (Derwinska *et al.*, 2009).

Human FucT IX is located in the trans-Golgi and the trans-Golgi network. The signal for the intra-Golgi localisation is encoded within the cytoplasmic domain (Brito *et al.*, 2008). It is responsible for synthesis of Le^x in various cell types and tissues. For example FucT IX synthesises Le^x on human granulocytes and peripheral leukocytes (Nakayama *et al.*, 2001). On these cells a class of adhesion molecules of the immunoglobulin superfamily that binds to the dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) lectin on dendritic cells is fucosylated (Bogoevska *et al.*, 2006, 2007).

Taken together, the presented evidence points towards a spatial and temporal regulation of FUT9 expression and a specific role for FucT IX in regulation of cell-cell interactions via the Lewis x epitope.

1.3 Biological relevance of the Lewis x epitope

FucT IX preferentially produces the Le^x (see section 1.2.5 and Nishihara *et al.*, 1999). The trisaccharide Le^x consists of a fucose α-1,3 linked to the N-acetylglucosamine residue of the disaccharide LacNAc. Like type II (LacNAc), H-type II, Le^y, and Sialyl-Le^y, Le^x belongs to the type II series of Lewis blood group antigens. The type I series consists of type I, Le^a, H-type I, Le^b, Sialyl-Le^b, A-blood antigen, and B-blood antigen (type I and II epitopes are summarised in Figure 1.8 Ma *et al.*, 2006).

Known binding partners for the Le^x are Le^x itself, selectins, c-type lectins, and growth factors. Thus, Le^x plays an important role in various recognition processes in health and disease (for review see Ma *et al.*, 2006).

1.3.1 Function of the Lewis x epitope in physiological processes

One of the major roles of Le^x is the regulation of recognition processes in the nervous system. Capela and Temple (2006) found a strict spacial and temporal production of Le^x in mouse embryos. Beginning on day 10, mouse embryos strongly produce the Le^x in several areas of the brain. After birth

the production is diminished and only found on neuronal progenitor cells and no longer on differentiated cells (Capela and Temple, 2006). In development of the nervous system, Le^x regulates *e.g.* cell migration (Gocht *et al.*, 1996), neuronal adhesion (Brito *et al.*, 2007a), and neurite growth (Yoshida-Noro *et al.*, 1999). Calcium dependent Le^x-Le^x interactions mediate contact of gliacells and neurons (Sajdel-Sulkowska, 1998). Neuronal growth factors Wnt-1 and FGF2 are able to bind free Le^x, which is secreted to the extracellular matrix (Capela and Temple, 2006).

Furthermore, both Wnt-1 and Le^x play a role in embryogenesis (Capela and Temple, 2006). Another of the various roles of Le^x in healthy tissues is the regulation of the compaction of the morula during the pre-implantation phase of the embryo and in the development of the blastocyste (Hakomori, 2004; Fenderson *et al.*, 1984).

Le^x is widely distributed in mucosa of normal breast, colon, and mouth. It is co-localised with different mucins. Therefore it is possible that the mucins themselves are the Le^x carriers (Croce *et al.*, 2007).

In the regulation of the immune system, Le^x on intercellular adhesion molecule-3 (ICAM-3) binds to DC-SIGN and mediates the contact of granulocytes and dendritic cells (Bogoevska *et al.*, 2007). Amongst others the SRCL (scavenger receptor c-type lectin) is found on epithelium of blood vessels in the placenta and the liver. By binding to Le^x carrying glycoproteins it captures bacteria and fungi out of the blood to mediate immune responses (Coombs *et al.*, 2005).

1.3.2 Function of the Lewis x epitope in pathological processes

The beneficial functions Le^x plays in physiological processes made it a target for pathogens.

For example *H. pylori*, a bacterial, gastroduodenal pathogen of humans, produces Le^x on its surface lipopolysaccharides (LPSs). This Le^x is a mimicry of host structures and thought to be responsible for immune evasion and gastric adaptation by the bacterium (Appelmelk *et al.*, 2000; Wirth *et al.*, 1997). Recent studies indicate a participation in gastric colonisation and bacterial adhesion through the galectin-3 receptor for polymeric Le^x on the

bacterium. Host immune responses are affected by modulation of Le^x on LPSs which interact with the cellular innate immune receptor DC-SIGN, contributing to changes in T-cell polarisation. Due to the molecular mimicry, autoimmunogenic anti-Le^x antibodies which can react with the gastric mucosa were found after long-term *H. pylori* infections (reviewed by Moran, 2008).

Schistosoma mansoni, a flatworm parasite of humans, produces soluble egg antigens (SEAs). These antigens modulate the host immune response to infection by *S. mansoni*. Human dendritic cells bind SEAs through the c-type lectin DC-SIGN. Binding of DC-SIGN to SEAs was inhibited by monoclonal antibodies against the carbohydrate antigen Le^x. This suggests a role of this glycan in binding to antigens (van Die *et al.*, 2003).

Furthermore, Le^x are also used by viruses to provide a mechanism of infection. For example, human immunodeficiency virus (HIV)-1 infection of T-cells is enhanced by DC-SIGN. It does not allow viral infection but captures HIV-1 at sites of entry, enabling its transport to lymphoid tissues. Despite the HIV-1 gp120 binding site on DC-SIGN being different from that of ICAM-3 (reviewed by Geijtenbeek and van Kooyk, 2003), epithelial mucin MUC1, with Le^x structures on O-linked glycans, can prevent DC-SIGN-mediated transmission of HIV-1 from dendritic cells to CD 4 positive T-cells. Those repetitive units of Le^x on the protein MUC1, which is abundant in milk, might play an important role in inhibiting transmission of HIV-1 from mother to child (Saeland *et al.*, 2009).

Another example is infection with human cytomegalovirus (HCMV). It induces cell surface production of Le^x and upregulation of several glycosyltransferases in differentiated human teratocarcinoma cells and in human diploid fibroblasts. Glycolipids bearing the Le^x epitope were detected in the infected cells but not in the uninfected cells (Andrews *et al.*, 1989).

Lewis antigens are used as a tumour marker for certain cancer types. Increased numbers of those antigens correlate with poor prognosis in colon cancer, due to high incidence of liver metastasis. Transfection with α-1,3 FucTs antisense sequences suppresses the metastatic potential of the parental tumour cells (Weston *et al.*, 1999).

The Le^x epitope also promotes autoaggregation of mouse teratocarcinoma

F9 tumour cells mediated by binding of Le^x to Le^x in the presence of Ca^{2+}. This process mimics the compaction process of a preimplanted mouse embryo (Hakomori, 2004).

1.4 Recombinant protein production

The FucT enzymes available from human sources are scarce and heterogeneous (Morais *et al.*, 2001). Therefore recombinant protein production systems are crucial to study function, kinetic, and structure of human fucosyltransferases. There are different pro- and eukaryotic systems available for recombinant overproduction of proteins.

1.4.1 Protein production in *Escherichia coli*

Escherichia coli (*E. coli*) has the advantages of low costs, high production speed, and an elevated protein yield, in comparison to eukaryotic production systems (Krogsdam *et al.*, 2003). Some disadvantages of the overproduction in *E. coli* are, that proteins may not fold correctly and therefore would need to be refolded *in vitro*. Additionally, *E. coli* is not able to perform eukaryotic posttranslational modifications, like glycosylation which might be important for enzymatic activity (Fernandez and Hoeffler, 1998).

Several mammalian glycosyltransferases have been produced in a soluble and active form with *E. coli* as host. Nakazawa *et al.* (1993) obtained soluble, recombinant β-1,4 galactosyltransferase from *E. coli*, when they transformed *E. coli* with the human cDNA lacking the transmembrane segment. The $V_{\max}$ value of the transferase produced in *E. coli* was low, compared to that of the human transferase. This was probably caused by an instability of the transferase due to the lack of protein glycosylation.

Soluble, active ST6 β-galactosamide α-2,6 sialyltransferase-1, lacking the membrane and cytosolic regions, was produced by Hidari *et al.* (2005) as a fusion protein with the maltose binding protein (MBP). Using low cultivation temperature during isopropyl β-D-1-thiogalactopyranoside (IPTG) induction they were able to improve solubility of the recombinant enzyme.

Pasek *et al.* (2010) established a new fusion partner to help folding of mammalian proteins in *E. coli*, as production in *E. coli* often leads to inactive,

aggregated proteins in inclusion bodies. They used a small soluble lectin, human galectin-1, instead of the commonly used MBP protein, because none of these fusion tags works universally with every partner protein. With their new method they were able to produce soluble folded recombinant human β-1,4 galactosyltransferase-7 in *E. coli*.

Until today, no active human FucT could be produced with *E. coli* as host.

1.4.2 Protein production in insect cells

Protein production in insect cells offers several advantages over *E. coli* production. Mostly, insect cells facilitate proper folding and perform more post-translational modifications, which lead to increased amounts of soluble and functional proteins. The use of insect cells is associated with lower costs and a higher yield per litre cell culture compared to protein production in mammalian cells (Fernandez and Hoeffler, 1998). Until now, several human FucTs have successfully been produced in insect cells, either by constitutive or baculoviral induced production systems. The following two subsections give examples for these systems.

1.4.2.1 Constitutive protein production in insect cells

Soluble secretory form of α-1,3/4 FucT-III was produced by Morais and Costa (2003) in *Spodoptera frugiperda* (Sf9) using a non-lytic vector system. They deleted a part of the stem region and replaced it by the signal sequence of human β-trace protein (Grabenhorst *et al.*, 1995). The construct was produced under the transcriptional control of the constitutive *Orgyia pseudotsugata* multicapsid nucleopolyhedrosis virus immediate-early (OPIE) 2 promoter.

The same strategy was applied by Münster *et al.* (2006) for production of α-1,3 FucT V. The authors truncated the transmembrane region and the cytosolic N-terminal domain of the protein and fused it to the signal sequence of human β-trace protein. They also produced the α-1,3 FucT V fusion protein under OPIE 2 control, but using stably transfected *Trichoplusia ni* insect cells instead of Sf9 as used by Morais and Costa (2003).

Brito *et al.* (2007b) fused a secretory form of α-1,3 FucT IX with the signal sequence of human interleukin-2 for efficient secretion (Smith *et al.*, 1985).

The authors also used an OPIE 2 controlled vector to produce N-terminally truncated human FucT IX starting from proline 40 (hP40F9) in Sf9 insect cells.

1.4.2.2 Baculoviral induced protein production in insect cells

The baculovirus production system provides the means to produce a wide range of recombinant proteins at levels as high as 500 mg per liter cell culture (O'Reilly *et al.*, 1994). Therefore, several human FucTs were prepared using the baculovirus induced production.

Ihara *et al.* (2006) designed a soluble form of α-1,6 FucT VIII by deletion of amino acid residues 1-67. They used the baculoviral signal peptide gp67 for efficient secretion and added a polyhistidine tag at the C-terminus. With this method they could generate enough α-1,6 FucT VIII for structural studies including crystallisation (Ihara *et al.*, 2007)

α-1,3/4 FucT III was produced as a secreted form using the baculovirus production system (Morais *et al.*, 2001). The cytoplasmic and transmembrane domains as well as a part of the stem region were replaced by the signal sequence of human β-trace protein. Through overproduction in Sf9 and *Trichoplusia ni*, considerable amounts of active protein accumulated in the cells. This indicates a possible limitation not only of the folding machinery, but also a saturation of the secretory pathway.

Shinkai *et al.* (1997) prepared α-1,3 FucT VII as secretory fusion protein. They fused the human granulocyte colony-stimulating factor signal peptide, an IgG-binding domain of protein A, a FucT VI derived peptide and the putative catalytic domain of FucT VII to produce the protein.

As a conclusion, human FucTs could be produced as secreted, truncated, and soluble forms of the according FucT with constitutive or baculovirus production systems.

1.5 Aim of the thesis

Human FucT IX belongs to the α-1,3 FucT family. It fucosylates distinct acceptors with high specificity, by catalysing the transfer of activated fucose to a sugar acceptor, generating a Le^x. This epitope is involved in intercellular

recognition, *e.g.* autoaggregation of teratocarcinoma cells or viral infections. Therefore, it would be valuable to gain insights into the mechanism and structure of FucT IX in order to obtain specific and potent inhibitors of the enzyme.

A prerequisite for such studies is the availability of high amounts of pure FucT IX. Protein production in *E. coli* leads to high amounts of protein. However, it was shown in the past for other human FucTs, that production in *E. coli* leads to inactive protein, putatively due to a lack of glycosylation. An alternative to production in bacteria is the production in insect cells, which produce glycosylations similar to mammalian cells. To maximise the yield of active protein, different production systems and secretion signals must be tested. The aim of this thesis was therefore

1. to test the feasibility of producing FucT IX in *E. coli* and insect cells using different secretion signals.
2. to develop a purification procedure for the recombinant protein using a histidine tag.
3. to characterise the kinetic parameters and biochemical properties of the FucT IX catalysed reaction.

2 Material and Methods

2.1 List of suppliers

- Applied Biosystems, Frankfurter Str. 129B, D-64293 Darmstadt
- ASA Spezialenzyme, Am Exer 19c, D-38302 Wolfenbüttel
- BD Biosciences, Tullastr. 8-12, D-69126 Heidelberg
- Bio-Rad, Heidemannstr. 164, D-80901 München
- Carl Roth (Roth), Schoemperlenstr. 1-5, D-76185 Karlsruhe
- Eppendorf, Barkhausenweg 1, D-22339 Hamburg
- Fermentas, Opelstr. 9, D-68789 St. Leon-Rot
- GE Healthcare, Oskar-Schlemmer-Str. 11, D-80807 München
- Hoefer; distributor: Fisher Scientific, Im Heiligen Feld 17, D-58239 Schwerte
- Invitrogen, Frankfurter Str. 129B, D-64293 Darmstadt
- Lonza, Nattermannallee 1, D-50829 Köln
- Macherey-Nagel, Valencienner Str. 11, D-52355 Düren
- Merck (Novagen and Calbiochem), Frankfurter Str. 250, D-64293 Darmstadt
- MP biomedicals (formerly ICN), Parc d'Innovation, BP 50067, F-67402 Illkirch
- New England Biolabs (NEB), Brüningstr. 50, D-65926 Frankfurt am Main
- PAA, Unterm Bornrain 2, D-35091 Cölbe
- PeqLab, Carl-Thiersch-Str. 2b, D-91052 Erlangen
- Perkin Elmer, Ferdinand-Porsche-Ring 17, D-63110 Rodgau
- Qiagen, QIAGEN Strasse 1, D-40724 Hilden
- Süd-Laborbedarf (Süd-Labor), Starnberger Str. 22, D-82131 Gauting
- Santa Cruz Biotechnology (Santa Cruz), 2145 Delaware Avenue, Santa Cruz, CA. 95060 U.S.A.

- Sarstedt, Rommelsdorfer Straße Postfach 1220, D-51582 Nümbrecht
- Sigma-Aldrich, Eschenstrasse 5, D-82024 Taufkirchen bei München
- Solis BioDyne, Riia 185a, EST-51014 Tartu
- Uptima; distributor: VWR, Zum Siegblick 37-39, D-53757 Sankt Augustin Buisdorf

2.2 DNA technologies

2.2.1 *E. coli* strains used for cloning

Table 2.1: *E. coli* strains used for cloning.

Strain	Genotype	Supplier
E. coli TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG	Invitrogen
E. coli DH10Bac	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ (ara, leu) 7697 galU galK λ- rpsL nupG / pMON14272 / pMON7124	Invitrogen

2.2.2 Primers

Table 2.2: Sequencing primers.

Primer	Sequence	Backbone
OpIE2 fw	5' CGC AAC GAT CTG GTA AAC AC 3'	pIB/V5-His
OpIE2 fw (-90)	5' ACA GGA CGC GCC TCC ATA TC 3'	pIB/V5-His
OpIE2 rv	5' GAC AAT ACA AAC TAA GAT TTA GTC AG 3'	pIB/V5-His
pMAL fw	5' GGT CGT CAG ACT GTC GAT GAA GCC 3'	pMAL-c2X
M13 fw (-20)	5' GTA AAA CGA CGG CCA G 3'	universal
M13 rv	5' CAG GAA ACA GCT ATG AC 3'	universal
pFast rv	5' CTA CAA ATG TGG TAT GGC TG	pFastBac
polyhedrin fw	5' AAT GAT AAC CAT CTC GC	pFastBac

Table 2.3: Primers used for cloning.

Primer	Sequence	resulting Vector
CS2	5' tac tct aga TTA ATT CCA AAA CCA TTT CTC 3'	pMALHF9
CS3	5' c CAT CAT CAC CAT CAC CAT TAA tga tca t 3'	pTF9H
CS4	5' ct aga tga tca TTA ATG GTG ATG GTG ATG ATG ggt ac 3'	pTF9H
CS5	5' TCG AAT TAA AAG CTT aat aat GAG CTC GGA TCC ATG 3'	pTF9HA-K
CS6	5' CAT GGA TCC GAG CTC aat aat AAG CTT TTA ATT CGA 3'	pTF9HA-K
CS7	5' act gaa ttc agg cct cat cat cac cat cac cat AGT CCA ATG GAA TCA GCC AGC TCT 3'	pMALHF9
CS10	5' c gac tcg aga AGT CCA ATG GAA TCA GCC AGC 3'	pMHF9
CS11	5' at tac cat atg TTA ATT CCA AAA CCA TTT CTC 3'	pMHF9
CS13	5' tag aag ctt ATG GCT ACT CAT CAC ACG CTG 3'	pTHF9
CS14	5' tac gga tcc GGC TGC CTG CAG GTC G 3'	pTHF9
CS15	5' agct TCT AGA AGG CCT GAA TTC 3'	pTHF9+ESX
CS16	5' agct GAA TTC AGG CCT TCT AGA 3'	pTHF9+ESX
CS17	5' tag aga tct gaa ttc ATG CTA CTA GTA AAT CAG TCA CAC CAA 3'	pFastGHF9
CS18	5' tac gga tcc CGC AAA GGC AGA ATG CGC 3'	pFastGHF9
IO1	5' tgg aat tca gcg ctA GTC CAA TGG AAT CAG CCA GCT CTG TG 3'	pTF9HA
IO2	5' aat cta gaT CAA GCG TAA TCT GGA ACA TCG TAT GGG TA 3'	pTF9HA
PZ1	5' tcg aAA TCG AGG GAA GGG TCG ACT CGA GGA TCG ATA TGG TAC Cgc gg 3'	pMelHis(+)
PZ2	5' GGT ACC ATA TCG ATC CTC GAG TCG ACC CTT CCC TCG ATT 3'	pMelHis(+)
PZ3	5' tcg aCT CGA GAA TTC GCT AGC GGC CGC GAG CTC A 3'	pMelHis(+)
PZ4	5' taT GAG CTC GCG GCC GCT AGC GAA TTC TCG AG 3'	pMelHis(+)

Annealing nucleotides are in upper case letters. Overhangs are in lower case letters.

2.2.3 Construction of Plasmids

Isolation of plasmid DNA from *Escherichia coli* (*E. coli*) TOP10 cells (see Table 2.1) was performed either by column based plasmid minipreparation kit (peqGOLD Plasmid Miniprep Kit I (C-Line), PeqLab; Hi Yield Plasmid

Mini-Kit, Süd-Labor; NucleoSpin Plasmid, Macherey-Nagel) or by alkaline lysis (Birnboim and Doly, 1979). Isolation of bacmid DNA from *E. coli* DH10Bac (see Table 2.1) was performed according to the manufacturers protocol (see section 2.2.3.3 and Invitrogen, 2004). Restriction digestions of plasmids or PCR products (see Table 2.4) were performed according to the manufacturers suggestions for restriction endonucleases (Fermentas or NEB). Isolation of DNA fragments $>$ 200 bp from agarose gels (Sambrook *et al.*, 1989) was accomplished by a column based gel extraction kit (peqGOLD Gel Extraction Kit (C-Line), PeqLab; NucleoSpin Extract II, Macherey-Nagel). DNA fragments $<$ 200 bp were purified from native PAA gels by extraction with TEN-buffer (50 mM Tris-HCl pH 8; 10 mM EDTA; 150 mM NaCl), followed by DNA precipitation (Sambrook *et al.*, 1989) using glycogen as carrier. Ligation of DNA Fragments was realised by using an equimolar ratio of 1:3 vector:insert and buffer conditions according to the manufacturers protocol (Fermentas). *E. coli* cells for transformation by heat shock or electroporation were produced according to Sambrook *et al.* (1989). Either one third of ligation reaction or 10 ng of plasmid were used to transform *E. coli* cells. Plasmids were checked by Sanger sequencing (see Table 2.5) to ensure that they contained the correct sequence.

All plasmid constructs were produced with a N-terminal 38 amino acid truncated version of human fucosyltransferase (FucT) IX, starting with serine 39, further on referred to as hS39F9. The truncation was established in the proteolysis sensitive stem region N-terminal of the catalytic domain. All

Table 2.4: Standard PCR reaction.

final concentration		component
1	×	Buffer BD (Solis BioDyne)
2.5	mM	$MgCl_2$
0.2	mM	dNTPs
1	µM	fw-Primer
1	µM	rv-Primer
1	ng/µl	Template
0.05	U/µl	FIREPol (Solis BioDyne)

Table 2.5: Standard sequencing reaction according to Sanger *et al.* (1977).

final concentration		component
25	ng/µl	Plasmid
0.5	µM	appropriate primer, see Table 2.2
1	×	BigDye sequencing buffer (Applied Biosystems)
1	×	Ready reaction premix (Applied Biosystems)

primers used for cloning can be found in Table 2.3, the ones used for sequencing are listed in Table 2.2.

2.2.3.1 pMAL-c2X based plasmid

With vector pMAL-c2X (see Table 2.6) it is possible to produce maltose binding protein (MBP) fusion proteins under isopropyl β-D-1-thiogalacto-pyranoside (IPTG) control. The protein of interest can be cleaved from MBP with the factor X_a protease cleavage site. For production of hS39F9 in *E. coli*, the vector pMALHF9 encoding the fusion protein of MBP and hS39F9 was used. The vector pMAL-c2X was used for inserting the PCR product generated by the primers CS2 and CS7 (see Table 2.2.2) and the template pEF-BOS-FUT9 (Bogoevska *et al.*, 2006) via EcoRI and XbaI restriction sites. The resulting vector encodes MBPHF9, a fusion of MBP, factor X_a protease cleavage site, the six amino acids Ile, Ser, Glu, Phe, Arg, and Pro from the multiple cloning site (MCS) and a hexa-histidine tag directly followed by hS39F9 (Figure 3.1).

Table 2.6: Plasmids used for cloning.

Plasmid	Supplier
pIB/V5-His	Invitrogen
pMAL-c2X	NEB
pFastBac1	Invitrogen

2.2.3.2 pIB/V5-His based plasmids

The pIB/V5-His (see Table 2.6) vector enabled transient or constitutive production of the protein of interest under *Orgyia pseudotsugata* multicapsid nucleopolyhedrosis virus immediate-early (OPIE) 2 promotor control. For

selection of stable insect cell lines the blasticidin resistance gene was under control of the OPIE 1 promotor. For selection of transformants in *E. coli* the ampicillin resistance gene was under the control of the synthetic EM7 promotor.

For constitutive expression in insect cells several vectors based on pIB/V5-His were designed using the N-terminal signal sequences melittin (Grunwald *et al.*, 2006) or β-trace (Grabenhorst *et al.*, 1995) as well as N- or C-terminal His- or hemagglutinin-tags, respectively.

By courtesy of Ikenna Obi the vector pTF9HA was provided. It encoded for hS39F9 with an N-terminal β-trace secretion signal and a C-terminal hemagglutinin (HA) tag. It was constructed in two steps. The PCR product of the primer IO1 and IO2 and pEF-BOS-FUT9 as template was inserted into pIB/V5-His via EcoRI and XbaI sites. The resulting plasmid was digested with BamHI and Eco47III and ligated with a 74 bp BamHI NaeI fragment of pIB-FUT5 (Münster *et al.*, 2006) to obtain pTF9HA. It features two KpnI sites, the one KpnI site located upstream of hS39F9 was replaced by SspI using site directed mutagenesis with the primers CS05 and CS06 giving pTF9HA-K.

To produce the vector pTF9H, encoding hS39F9 with an N-terminal β-trace signal sequence and a C-terminal histidine tag, a hexa-histidine tag was introduced between the remaining KpnI site and the XbaI site of pTF9HA-K using two annealing primers CS3 and CS4.

The hS39F9 fusion construct pMHF9 employing the N-terminal melittin signal sequence followed by a deca-histidine tag and a factor X_a protease cleavage site was generated using the vector pIB/Mel-opt-H10 (Grunwald *et al.*, 2006), a vector based on pIB/V5-His. From this plasmid Patrick Ziegelmüller constructed the plasmid pMelHis(+) by the following procedures. To create new restriction sites the annealed product of primers PZ1 and PZ2 was introduced into pIB/Mel-opt-H10 between the XhoI and SacII restriction sites. In a second step the annealed product of primers PZ3 and PZ4 was inserted between the SalI and NdeI sites resulting in the vector pMelHis(+). Finally, to obtain the vector pMHF9, the PCR product using the primers CS10 and CS11 with pTF9HA as template was cloned into pMelHis(+) using XhoI and NdeI.

To obtain pTHF9 encoding hS39F9 with a N-terminal β-trace secretion signal, a deca-histidine tag and a factor X_a protease cleavage site (factor X_a site), the β-trace signal sequence was amplified with primers CS13 and CS14 using pTF9HA as template. The PCR product was inserted via HindIII and BamHI into pMHF9.

2.2.3.3 pFastBac1 based plasmids

The Bac-to-Bac Expression System was used for the generation of recombinant baculoviruses. One component of the system was the pFastBac1 (see Table 2.6) vector. In this vector hS39F9 fusion constructs were cloned under the control of a polyhedrin (PH) promotor for high-level expression in insect cells. This expression cassette was flanked by the left and right arms of the bacterial transposon Tn7, and also contained a gentamicin resistance gene and a simian vacuolating virus 40 (SV40) polyadenylation signal to form a mini-Tn7.

The vector pFastBac1 was used to generate the shuttle vectors necessary for bacmid preparation. The pFastTHF9 donor vector encodes a β-trace secretion signal, a deca-histidine tag and a factor X_a site located at the N-terminus of hS39F9. For construction of pFastTHF9 additional restriction sites were introduced in the vector pTHF9 by inserting the annealed product of primers CS15 and CS16 at the HindIII site yielding pTHF9+ESX. Finally, pTHF9+ESX and pFastBac1T (see Table 2.6) were digested with EcoRI and KpnI and ligated to obtain pFastTHF9.

The donor vector pFastGHF9 bearing the baculoviral secretion signal gp67, a deca-histidine tag and a factor X_a site, located N-terminal of hS39F9, was generated by replacing the β-trace signal sequence in pFastTHF9 by the gp67 signal sequence. Therefore, gp67 was amplified with CS17 and CS18 using pAcGP67B (BD Biosciences) as template. The PCR product was inserted via BglII and BamHI into pFastTHF9 digested with BamHI.

Generation of bacmids

The second component of the Bac-to-Bac Expression System was the *E. coli* DH10Bac strain, which was used as the host for the pFastBac1 vector. These cells contain a baculovirus shuttle vector (bacmid bMON14272) with a mini-attTn7 target site and a helper plasmid (pMON7124). The bacmid propagates

in *E. coli* DH10Bac and contains a low-copy number mini-F replicon, kanamycin resistance marker and DNA encoding the LacZα peptide into which the attachment site for the bacterial transposon, Tn7 (miniattTn7) has been inserted. The LacZα peptide can complement a lacZ deletion present on the chromosome of the cells to form colonies that are blue in the presence of X-gal and the inducer IPTG.

Once the *E. coli* DH10Bac cells were transformed with the pFastBac1 shuttle vector, transposition occurred between the mini-Tn7 element on the pFastBac1 vector and the mini-attTn7 target site on the bacmid to generate a recombinant bacmid. This transposition reaction occurred in the presence of the helper plasmid which encoded the transposase and conferred resistance to tetracycline. Upon transposition the LacZα peptide was destroyed. This abolished the ability to form blue colonies in the presence of X-gal and IPTG, and was used for selection of transformants (Invitrogen, 2004). With the method described above the donor plasmids pFastTHF9 and pFastGHF9 were used to generate the bacmids bacmidTHF9 and bacmidGHF9.

Isolation of bacmid DNA

This method was described by Invitrogen (2004), but performed slightly modified. Using a sterile tip, a single, isolated bacterial colony was inoculated in 2 ml of LB medium containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline. The culture was grown at 37 °C over night. 1.5 ml of bacterial culture were transferred to a 1.5 ml tube and centrifuged at 14,000 × g for 1 min to pellet cells. The supernatant was removed by vacuum aspiration and the cell pellet was resuspended in 0.3 ml of Solution I (15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A; filter-sterilised and stored at +4 °C). The cell pellet was resuspended by gentle vortexing or pipeting up and down. To this cell suspension 0.3 ml of Solution II (0.2 N NaOH, 1% SDS; filter-sterilised) were added and gently mixed. Incubation at room temperature for 5 min changed the appearance of the suspension from turbid to almost translucent. While slowly adding 0.3 ml 3 M potassium acetate, pH 5.5 (autoclaved and stored at +4°C) and gently mixing, a thick white precipitate, of protein and *E. coli* genomic DNA, formed. The sample was placed on ice for 5 to 10 min and centrifuged for 10 min at 14,000 × g. The

supernatant was gently transferred into a fresh tube and centrifuged for 5 min at 14,000 × g. Without transferring any white precipitate, the supernatant of this centrifugation was gently transferred into a tube containing 0.8 ml of isopropanol. Then the tube was inverted a few times to mix and placed on ice for 5 to 10 min. The sample was centrifuged for 15 min, 14,000 × g at room temperature. Without disturbing the pellet, the supernatant was carefully removed. To wash the pellet, 0.5 ml of 70% ethanol were added, the tube was inverted and centrifuged for 5 min at 14,000 × g at room temperature. This step was repeated once. The supernatant of the washing step was removed. To remove as much of the supernatant as possible, the tube was centrifuged again for 10 s and remaining supernatant was removed carefully. The pellet was air dried for no longer than 2 min at room temperature. If the pellet was overdried, the DNA did not dissolve. The bacmid DNA was dissolved in 40 µl of 1 × TE Buffer, pH 8.0. To avoid shearing, the DNA was resuspended by gentle tapping of the bottom of the tube. The DNA was stored at +4 °C, as repeated freezing and thawing may shear the DNA. The DNA was used to analyse the recombinant bacmid DNA by PCR (see Table 2.4) or for the transfection of insect cells (see section 2.3.1.2).

2.3 Protein technologies

2.3.1 Protein production

2.3.1.1 Procaryotic protein production in *E. coli*

Table 2.7: *E. coli* strain used for protein production.

Strain	Genotype	Supplier
E. coli TB1	F- ara Δ(lac-proAB) [ϕ80dlac Δ(lacZ)M15] rpsL(Str^R) thi hsdR	NEB

E. coli TB1 cells (Table 2.7) transformed with pMALHF9 were grown in LB medium (Roth) with 100 µg ampicillin /ml to an OD_{600} of 0.7. Protein expression was induced using IPTG at a final concentration of 0.5 mM and cells were further grown for three hours at 37 °C. Bacteria were harvested by centrifugation (4,500 × g, 10 min, 4 °C) and stored until further processing at

-20 °C.

2.3.1.2 Eucaryotic protein production in insect cells

Spodoptera frugiperda (Sf9) cells (see Table 2.8) were cultivated in Insect XPRESS medium (Lonza) supplemented with 10 µg gentamycine /ml (Invitrogen), in suspension (100 rpm) or monolayer culture at 27 °C. The cells were subcultured to 2.5×10^5 cells /ml every 3 to 4 days.

Sf9 insect cells were transfected with the appropriate plasmid or bacmid using insect gene juice according to the manufacturers instructions (Novagen).

Table 2.8: Insect cells used for production of proteins in eukaryotes.

Host	Cell line	Reference
Sf9	IPLB-Sf-21-AE	Smith *et al.* (1985); Vaughn *et al.* (1977), Invitrogen

Constitutive protein production in insect cells

Wild type Sf9 cells were transfected with pIB/V5 hS39F9 derivates (see section 2.2.3.2). Selection for stable cells was performed for 6 weeks with 80 µg blasticidinS /ml (PAA). Stable cell lines were maintained with 10 µg blasticidinS /ml. Wild type and stable cell lines were disseminated in T25 culture flasks in 4 ml medium at a density of 2.5×10^5 cells /ml. 100 µl of cell culture supernatant were sampled at a daily rate. On day 3 or 4, cell supernatant was harvested by centrifugation (500 × g, 5 min, 4 °C). Cells were dislodged in fresh medium from the culture flask with a cell scraper and separated by gentle pipetting. A new flask was set up with this solution, remaining cells were separated by centrifugation (500 × g, 5 min, 4 °C) and stored at 4 °C.

Baculovirus induced protein production in insect cells

Wild type Sf9 cells were transfected with bacmidTHF9 or bacmidGHF9 DNA (see section 2.2.3.3). Subsequently, the cells produced BacTHF9 or BacGHF9 baculoviruses, respectively. These viruses were amplified in three rounds to a high titre stock which then was used to infect 2×10^6 cells /ml to produce recombinant hS39F9 fusion protein. The optimal MOI for the production

of the recombinant proteins was empirically determined by infection of Sf9 cells in a 24-well plate with serial virus dilutions followed by western blot (see section 2.3.3.1). The determined amount was extrapolated to the culture volume in a sterile plastic or glass Erlenmeyer flask.

2.3.2 Protein isolation

2.3.2.1 Generating protein extracts

Lysis of *E. coli* cells

To prepare *E. coli* cell extract cell pellet from 250 ml culture was resuspended in 25 ml *E. coli* lysis buffer (20 mM Tris pH 8.0, 20 mM Imidazole, 300 mM NaCl, 0.001% Lubrol (ICN), 10 µM PMSF (Roth), 1 µM leupeptin (Roth)). The suspension was sonified on ice with a branson sonifier 250A (output control 3, duty cycle 30, 5 mm microtip) for 10 min and centrifuged (45 min, 14,000 × g, 4 °C). The lysate was used for western blot analysis or activity assays. The pellet was resuspended again in 25 ml lysis buffer, 10 µl of this suspension were used for western blot analysis.

Lysis of insect cells

The cells were harvested 3 days post infection. To extract intracellular hS39F9 from insect cells, cells were resuspended in nickel-nitrilotriacetic acid (Ni-NTA) binding buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM Imidazole) and vortexed for 1 min. The sample was incubated for 10 min on ice and subjected to sonication with a branson sonifier 250A (output control 3, duty cycle 30, 5 mm microtip) for 10 s, the lysate was collected by centrifugation (10 min, 500 × g, 4 °C) and used for activity assays and immobilised metal ion affinity chromatography (IMAC).

Buffer exchange of insect cell culture supernatant

The cell culture supernatant of Sf9 cells post baculovirus infection was concentrated and simultaneously the buffer was exchanged to Ni-NTA binding buffer with a cross flow filtration system (Quixstand Benchtop system, hollow fiber cartridge UFP-10-C-4X2MA [Nominal Molecular Weight Cut-Off 10 kDa], GE Healthcare). For that purpose 200 ml cell culture supernatant was concentrated to 100 ml, next 100 ml Ni-NTA binding buffer was added and concentrated again. This procedure was repeated three times. In a final step

700 ml Ni-NTA binding buffer was added and the mixture was concentrated to 100 ml. This concentrate was used for activity assays and IMAC (see section 2.3.2.2).

2.3.2.2 Chromatography technologies

If not otherwise stated, all chromatographies were carried out in the cold room using the ÄKTA purifier (GE Healthcare). The purity of the resulting protein was determined using Quantity One (Bio-Rad).

Immobilized metal ion affinity chromatography

At a flow rate of 1 ml/min a 1 ml HisTrap column (GE Healthcare) was equilibrated with 7 column volumes (CVs) of Ni-NTA binding buffer. A sample prepared with Ni-NTA binding buffer (see section 2.3.2.1) was loaded on the column. Unspecifically bound proteins were eluted with 10 CVs of the same buffer and a linear imidazol gradient up to 50 mM imidazol. The proteins which specifically bound to the Ni-NTA matrix were eluted with 10 column voulmes Ni-NTA binding buffer containing 500 mM imidazol. For conservation, the column was washed again with 20% ethanol.

GDP affinity chromatography

An empty column was loaded with GDP affinity matrix (10 µmol GDP /ml affinitygel, ASA Spezialenzyme) and equilibrated with 7 CVs GDP binding buffer (25 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 7.0, 100 mM NaCl, 10 mM $MgCl_2$). An elution fraction of IMAC purification (see section 2.3.2.2) was dialysed over night against GDP binding buffer and loaded on the GDP column. The column was washed with 7 CVs GDP binding buffer. Bound protein was eluted with 7 CVs GDP binding buffer containing 3 mM GDP. For conservation, the column was washed again with 20% ethanol.

Anion exchange chromatography (AIEX)

The N-terminal deca-histidine tagged hS39F9 (HF9) containing pool of IMAC purification (see section 2.3.2.2) was dialysed over night against AIEX dialysis buffer (10 mM NaCl, 20 mM Tris-HCl pH 8.5).

- On the one hand, if the following AIEX a Poros HQ/M column (Applied Biosystems) was used, the AIEX dialysis buffer was supplemented with

1% Triton-X-100 to prevent precipitations, the flow rate was adjusted to 4 ml/min and 2 ml fractions were collected.

- On the other hand, if a HiTrapQ column (GE Healthcare) was used the AIEX dialysis buffer was supplemented with β-mercaptoethanol to prevent precipitations, the flow rate was adjusted to 1 ml/min and 1 ml fractions were collected.

The chosen column was equilibrated with with 5 CVs AIEX binding buffer (25 mM 1-methyl piperazine, 25 mM Bis-Tris, 12.5 mM Tris pH 8.5) and the sample was loaded to the column. After washing with 3 CVs AIEX binding buffer, the elution was carried out with binding buffer supplied with NaCl in concentrations increasing linearly to 0.5 M NaCl for 20 CVs and additionally to 1 M NaCl for 10 CVs. For conservation, the column was washed again with 20% ethanol. For storage at -20 °C the protein was mixed with glycerol to a final concentration of 30% glycerol.

2.3.3 Protein determination

2.3.3.1 SDS-PA-gel electrophoresis and detection

Table 2.9: 12% SDS-PA-gel.

Gel	Recipe
Resolving gel	12 ml Rotiphorese Gel 30 (37.5:1) (Roth); 7.5 ml 1.5 M Tris-HCl pH 8.8; 10.4 ml H_2O; 180 µl 10% APS; 48 µl TEMED
Stacking gel	2 ml Rotiphorese Gel 30 (37.5:1); 7.5 ml 0.5 M Tris-HCl pH 6.8; 9.2 ml H_2O; 120 µl 10% APS; 48 µl TEMED

For protein electrophoresis 12% SDS-PA gels were cast in a 5-gel caster for 10 x 8 cm plates (Hoefer) as described in Table 2.9. Electrophoretic separation was performed at 200 V with a limiting setting of 30 mA/ gel in a mighty small II vertical gel electrophoresis unit (Hoefer). Gels were used for western blotting or were coomassie stained (see below). If this did not show all expected proteins an additional silver stain was performed.

The used molecular weight markers were purchased from Fermentas. For western blotting the PageRuler prestained protein ladder, and for coomassie or silver staining PageRuler unstained protein ladder were used . Samples

were prepared using 2× Laemmli buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromphenol blue, 125 mM Tris-HCl pH 6.8 Laemmli (1970)). Prior to loading on a gel the sample-buffer mixture was incubated for 5 minutes at 96 °C and centrifuged (1 min, 16100 × g, 22 °C).

Coomassie staining

Coomassie staining using colloidal coomassie brilliant blue was performed according to Hüseyin (2008).

Silver staining

Used reagents are listed in Table 2.10. The proteins of the SDS-PA gel were fixed for one hour using fixative. The SDS-PA gel was sensitised using thiosulfate reagent for one to twelve hours. Afterwards the gel was washed 3 × with deionised water for 10 min and incubated for 30 min with silver nitrate reagent. Development was performed with developer until desired bands were visible. The reaction was stopped with stop-reagent and the gel was stored therein.

Table 2.10: Silver staining solutions.

Solution	Recipe
Fixative	30% Ethanol, 15% glacial acetic acid, 55% deionised water
Thiosulfate reagent	60.04 g sodium acetate×3 H_2O (or 20.52 g sodium acetate anhydrous), 25% ethanol, fill up to 1000 ml with deionised water; prior to use add 0.05 g $Na_2S_2O_3$ anhydrous (0,08 g $Na_2S_2O_3$×5 H2O) and 125 µl glutardialdehyde (25%) to 25 ml of this solution
Silver nitrate reagent	1 g $AgNO_3$, fill up to 1000 ml with deionised water; prior to use add 7.5 µl formaldehyde (37%) to 25 ml of this solution
Developer	25 g Na_2CO_3, dissolve in 800 ml deionised water, adjust pH with Na_2CO_3/$NaHCO_3$ to 11.5, fill up to 1000 ml with deionised water; prior to use add 7.5 µl formaldehyde (37%) to 25 ml of this solution
Stop-reagent	18.6 g Na_2-EDTA fill up to 1000 ml with deionised water

Western blotting

Accordant samples were separated on a 12% SDS-PA gel. A polyvinylidene fluoride (PVDF) membrane (Roth) was activated with methanol and equilibrated in CAPS-buffer (10 mM CAPS pH 11.0, 10% Methanol). The gel and the membrane were put between equilibrated blotting papers. The proteins

were wet-blotted in CAPS-buffer for 1 hour at 50 V (TE 22 Mini Transfer Tank) to a PVDF membrane and immunostaining was performed with primary and secondary antibodies in TBS buffer with 0.1% bovine serum albumin (BSA) (see Table 2.11). Detection was performed using 10 ‰ NBT, 50 ‰ BCIP in 100 mM Tris-HCl pH 9.5, 4 mM $MgCl_2$.

Table 2.11: Antibodies used for western blot detection.

Source & primary antibody target	Concentration	Secondary antibodies	Concentration
goat anti-FucT-IX (C-17) (Santa Cruz)	1:4,000	anti-goat-alkaline phosphatase (AP) (Sigma-Aldrich)	1:10,000
mouse anti-tetra-His (Quiagen)	1:2,000	anti-mouse-AP (Sigma-Aldrich)	1:10,000
rabbit anti-goat IgG AP (Sigma-Aldrich)	1:10,000	anti-rabbit-AP (Sigma-Aldrich)	1:10,000

AP: alkaline phosphatase conjugated

2.3.3.2 Determination of protein concentration

A serial dilution of BSA in 25 µl buffer was prepared in a 96-well plate (Sarstedt). The buffer used for the dilution was the same buffer as the protein to determine was dissolved in.

Bradford assay

Bradford reagent was prepared by the following steps: 12.5 mg Coomassie Blue G250 were dissolved in 12.5 ml methanol. To this solution 25 ml 85% H_3PO_4 were added. After addition of 125 ml H_2O the solution was filtered to remove precipitates. Finally 87.5 ml H_2O were added and the solution was stored at 4 °C in a brown bottle.

The Bradford assay was performed as duplicate. 200 µl of bradford reagent were added to 25 µl of the sample and a serial BSA dilution (512, 256, 128, 64, 32, 16 and 8 µg/ml).

BCA assay

For BCA assay the serial BSA dilution were 2,000, 1,000, 750, 500, 250, 100 and 20 µg/ml. The assay was performed with duplicate dilution using BCA

assay protein quantitation kit as recommended by the supplier for microplate assays (Uptima).

2.3.3.3 Determination of protein activity

To determine the activity recombinant hS39F9 fusion proteins were measured in a **standard reaction mixture**: the transfer of ^{14}C-fucose from 1.8 µM GDP-^{14}C-fucose (Perkin Elmer) in the presence of 312 µM GDP-β-L-fucose (GDP-Fuc) (Calbiochem) as donor to 4 mM N-acetyllactosamine (LacNAc) (Calbiochem) as acceptor was monitored in a reaction volume of 25 µl. The reaction was performed in different buffers, depending on the application. Usually 10 µl of lysis buffer, cell culture supernatant or elution fraction with or without glycerol were used for the activity assay. Therefore the activity assay contained not only the reaction buffer but also the buffer from the cell lysate, culture supernatant or elution (see Table 2.12).

To separate incorporated from non-incorporated radioactivity the charge of GDP-Fuc was exploited. The substrate GDP-Fuc was negatively charged and the product trisaccharide Lewis x epitope (Le^x) was uncharged. Therefore, the substrate can be separated by anion exchange columns. A 5 ml plastic syringe with centric conus (Luer slip, Becton Dickinson Plastipak; supplied by VWR) was plugged with cotton to keep the matrix in place. For activation 0.5 g anion exchange matrix (AG 1-X8 resin [chloride form] 200-400 dry mesh, 45-106 µm wet bead, BioRad) was resuspended in 2 ml 0.1 M HCl and poured into the prepared plastic syringe. The matrix was further activated with 2 ml

Table 2.12: Final protein buffer concentrations in the activity assay.

Protein buffer	Concentration in the activity assay
E. coli lysis buffer	120 mM NaCl, 8 mM imidazol and 8 mM Tris-HCl pH 8
insect cell lysis buffer	200 mM NaCl, 8 mM imidazol and 8 mM Tris-HCl pH 8
cell culture supernatant	40%
AIEX elution fraction	10 mM 1-methyl piperazine, 10 mM Bis-Tris, 5 mM Tris pH 8.5
AIEX elution fraction with 30% glycerol	7 mM 1-methyl piperazine, 7 mM Bis-Tris, 3.5 mM Tris pH 8.5, 12% glycerol
IMAC elution fraction	200 mM NaCl, 8 mM imidazol and 200 mM Tris-HCl pH 8

1 M NaCl and washed with H_2O, thrice.

Unless otherwise stated the samples were incubated with the desired buffer at 37 °C for one hour. The reaction was stopped by addition of 375 µl ice cold H_2O. The mixture was immediately transferred to the anion exchange column and the product was eluted by addition of 400 µl H_2O, twice. The product was collected in scintillation vials (Sarstedt) and 2.5 ml Ultima Gold scintillation liquid (Perkin Elmer) were added, this mixture was vortexed until it cleared and measured in a Tri-Carb 2500 TR liquid scintillation counter (Perkin Elmer former Packard) to determine counts per minute (cpm). Quantitative conversion of 1.8 µM GDP-^{14}C-fucose resulted in 22,800 cpm. One unit is the amount of enzyme needed to transfer 1 µmol fucose from the donor GDP-Fuc to the acceptor LacNAc at 37 °C within 1 min (NC-IUB, 1979). One site binding (hyperbola) analysis was performed using GraphPad Prism (version 4.03 for Windows, GraphPad Software, San Diego California USA).

All further experiments were performed with the purified AIEX elution fraction in 30% glycerol. This fraction contained 20 ng/µl of the enzyme HF9.

Linearity of the reaction

To assure a linear product formation a time series was carried out. The enzyme was incubated with the MOPS reaction buffer (see Table 2.13) in a standard reaction mixture. These samples were incubated for 15, 30, 45, 60, and 120 min.

Table 2.13: Final reaction buffer concentrations in the activity assay.

Reaction buffer	Concentration in the reaction
Citrate/Phosphate (C/P)	5 mM $MnCl_2$, 15 mM citrate/phosphate pH 5.0
MOPS	10 mM ATP, 4 mM L-Fucose, 0.5% Triton X 100, 10 mM HEPES, 50 mM MOPS/NaOH pH 7.2
acetate-NaOH	250 mM acetate-NaOH pH 3.3, 4.5, or 5.7
MES-NaOH	250 mM MES-NaOH pH 5.7, 6.8, or 7.7
Tris-HCl	250 mM acetate-NaOH pH 7.8, 8.7, or 9.0
carbonate-bicarbonate	250 mM carbonate-bicarbonate pH 9.2, 10.0, or 11.3

pH dependency of the reaction

To determine the optimal pH for the reaction the samples were incubated with 250 mM buffer in a standard reaction mixture. The standard reaction components were mixed with the tested buffer (see Table 2.13) and enzyme was added in a final step. Twelve different buffers were tested: acetate-NaOH pH 3.3, 4.5, 5.7; MES-NaOH pH 5.7, 6.8, 7.7; Tris-HCl pH 7.8, 8.7, 9.0 and carbonate-bicarbonate pH 9.2, 10.0 and 11.3.

Metal ion dependency of the reaction

To test the influence of divalent metal ions the enzyme was incubated in MOPS reaction buffer (see Table 2.13) in a standard reaction mixture. 5 mM EDTA, $CaCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$ or $ZnCl_2$ was added to the standard reaction components.

Temperature dependency of the reaction

To determine the optimal reaction temperature, the standard reaction components and the enzyme were incubated with C/P buffer (see Table 2.13) on ice, at 9.9, 13.9, 19.1, 24.6, 30.6, 35.1, 37.2, 39.2, 44.3, 49.7 and 55.3 °C in a gradient thermocycler (Eppendorf).

Determination of K_m for LacNAc

To determine the K_m for LacNAc 0.05, 0.1, 0.25, 0.5, 1, 2 and 4 mM LacNAc were incubated in the presence of 312 µM GDP-Fuc, C/P reaction buffer (see Table 2.13), 5 mM $MnCl_2$, and the enzyme.

Determination of K_m for GDP-Fuc

To determine the K_m for GDP-Fuc different amounts of unlabelled GDP-Fuc and 0.1 µM labelled GDP-^{14}C-fucose were incubated in the presence of 4 mM LacNAc, C/P reaction buffer (see Table 2.13), 5 mM $MnCl_2$, and the enzyme. The reaction has to be in the linear segment, but with low amounts of GDP-Fuc this linear segment was reached very fast. Therefore, the samples were incubated for a specific time with different concentrations of unlabelled GDP-Fuc (see Table 2.14).

Table 2.14: Incubation times with GDP-Fuc.

Time	Concentration of unlabelled GDP-Fuc in the reaction
30 s	0.2 µM
30 s	0.3 µM
30 s	0.5 µM
40 s	0.7 µM
40 s	1.1 µM
60 s	2.0 µM
60 s	2.9 µM
90 s	5.9 µM
210 s	11.9 µM
360 s	23.9 µM

3 Results

3.1 Recombinant production of fucosyltransferase IX

In order to obtain specific as well as potent inhibitors of fucosyltransferase (FucT) IX it would be valuable to gain insights into the mechanism and structure of the enzyme. The availability of high amounts of pure FucT IX is a prerequisite for such studies. Therefore, the feasibility of producing FucT IX in *Escherichia coli* (*E. coli*) and insect cells using different secretion signals was tested in this thesis.

3.1.1 Production of hS39F9 in *E. coli*

3.1.1.1 Cloning of hS39F9 MBP fusion protein

The sequence encoding the N-terminally truncated human FucT IX starting from serine 39 (hS39F9) in combination with a sequence encoding an N-terminal histidine tag was inserted into vector pMAL-c2X resulting in plasmid pMALHF9. This plasmid encoded the protein MBPHF9 which is a fusion protein of maltose binding protein (MBP), factor X_a protease cleavage site, and a hexa-histidine tag directly followed by hS39F9 (Figure 3.1).

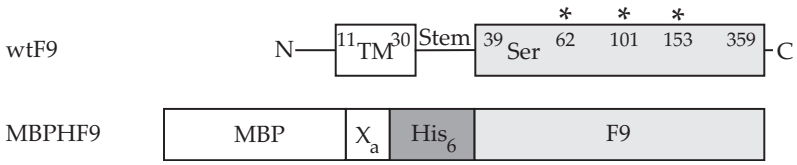

Figure 3.1: Schematic presentation of *E. coli* hS39F9 fusion construct in comparison to wtF9. The schema for full length human FucT IX (**wtF9**) shows the N-terminus (N), a predicted transmembrane domain (TM), important amino acid positions (indicated by numbers), putative N-glycosylation sites ("*") and the C-terminus (C). The MBP-FucT IX fusion construct (**MBPHF9**) contained the maltose binding protein (MBP), a factor X_a protease cleavage site (X_a), and a hexa-histidine-tag (His_6) N-terminal of hS39F9 (F9).

3.1.1.2 Production of MBPHF9 in *E. coli* TB1 cells

After lysis of TB1 cells transformed with pMALHF9, high levels of the fusion protein MBPHF9 was detected by SDS polyacrylamid (SDS-PA)-gel electrophoresis followed by coomassie staining or western blot analysis (Figure 3.2). A band between 70 and 100 kDa was detected, in accordance with the predicted size for MBPHF9 of 78 kDa. The majority of MBPHF9 was insoluble and found in the cell debris. The lysate, however, contained soluble protein.

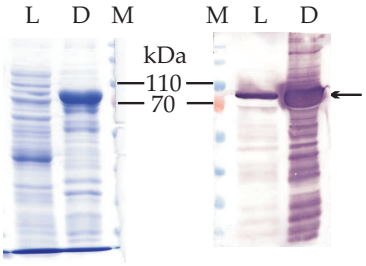

Figure 3.2: Analysis of the production of MBPHF9 in *E. coli*. After lysis of TB1 pMALHF9 cells a 12% SDS-PA-gel was loaded with cell lysate (L) and according amounts of cell debris (D). Left: coomassie staining; right: western blot analysis using goat anti-FucT IX and rabbit anti-goat-AP antibodies. M: PageRuler prestained protein ladder. The arrow indicates the fusion construct MBPHF9, which is outlined in Figure 3.1.

3.1.1.3 Determination of fucosyltransferase activity

In order to determine the activity of MBPHF9, the transfer of ^{14}C-fucose from GDP-^{14}C-fucose to N-acetyllactosamine (LacNAc) was monitored. The fucosyltransferase activity of the TB1 pMALHF9 *E. coli* lysate and TB1 pMAL-c2X *E. coli* lysate was assayed (Table 3.1). The activity of the TB1 pMALHF9 *E. coli* lysate was not significantly higher than that of the control. The produced MBPHF9 was inactive, therefore another production method had to be developed.

Table 3.1: FucT activity in *E. coli* TB1 lysates.

Construct	Determined activity ± standard deviation
c2X	31 ± 13 mU/l
MBPHF9	38 ± 32 mU/l

Transfer of fucose from GDP-β-L-fucose (GDP-Fuc) to LacNAc in the presence of citrate/phosphate (C/P) reaction buffer and *E. coli* lysis buffer was monitored in lysates in three independent experiments. **c2X**: lysate of *E. coli* TB1 transformed with pMAL-c2X (control plasmid), **MBPHF9**: lysate of *E. coli* TB1 transformed with pMALHF9.

3.1.2 Production of hS39F9 fusion proteins in insect cells

3.1.2.1 Constitutive production of hS39F9 fusion proteins in insect cells

Production with pIB/V5 based expression vectors

Various FucTs have already been produced constitutively in insect cells (see section 1.4.2.1). Therefore, it was tested whether constitutive production is a feasible method to produce FucT IX. To this end different hS39F9 fusion constructs were cloned. The accordant fusion proteins were produced constitutively in *Spodoptera frugiperda* (Sf9) insect cells and tested for the highest secretion rates in order to obtain optimal production conditions for the protein (Figure 3.3).

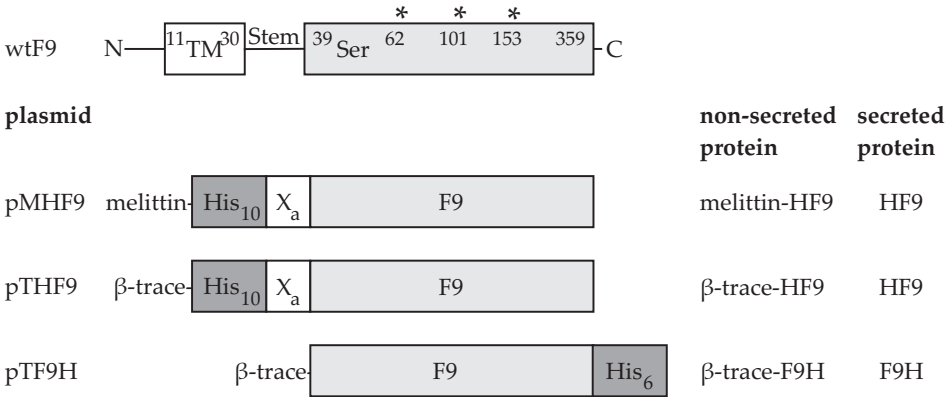

Figure 3.3: Schematic presentation of hS39F9 fusion constructs used for constitutive production in comparison to wtF9. The schema for full length human FucT IX (**wtF9**) shows the N-terminus (N), a predicted transmembrane domain (TM), important amino acid positions (indicated by numbers), putative N-glycosylation sites ("*") and the C-terminus (C). The **plasmid** pMHF9 encodes the protein melittin-HF9, pTHF9 encodes β-trace-HF9, and pTF9H encodes β-trace-F9H, respectively. The produced **secreted protein**s contained a hexa- or deca-histidine-tag (His_6 or His_{10}, respectively) and a factor X_a protease cleavage site (X_a) N-terminal of hS39F9 and were named HF9 and F9H. If unintentionally **non-secreted protein**s were produced, HF9 and F9H additionally contained the signal peptides melittin or β-trace at the N-terminus and was named melittin-HF9, β-trace-HF9, and β-trace-F9H, respectively.

The melittin and β-trace signal sequences are known to be strong export signals for secreted proteins in insect cells (Grunwald *et al.*, 2006; Münster *et al.*, 2006). Both were tested for their secretion rate. In addition it was analysed if the protein activity was influenced by fusion of a histidine tag to the N- or C-terminus.

The protein encoded by the plasmids pMHF9 and pTHF9 was named HF9 in case of secretion to the cell culture medium. It contained the restriction site derived amino acids, an N-terminal deca-histidine tag followed by factor X_a protease cleavage site and hS39F9. If the protein was secreted the signal peptides melittin or β-trace wes removed. After transfection of Sf9 insect cells with plasmid pTF9H the protein was secreted to the culture supernatant, it is then referred to as F9H. It contained hS39F9, some restriction site derived amino acids and a C-terminal hexa-histidine tag. If any of the proteins were not secreted the proteins additionally contained the according signal peptide and were named melittin-HF9, β-trace-HF9 or β-trace-F9H, respectively (Figure 3.3).

To evaluate the production rate of hS39F9 fusion proteins, samples from the supernatant of monolayer cultures were assayed four days after dissemination. The amounts of soluble protein in the culture supernatant and cell pellet were estimated by western blot analysis (Figure 3.4). As expected, non-transfected Sf9 cells did not secrete hS39F9 protein into the supernatant, while all of the different cell lines stably producing hS39F9 fusion constructs secreted the protein into the supernatant. Western blot analysis and quantitation displayed approximately 2-fold more hS39F9 fusion protein from F9H than HF9 (arrow in Figure 3.4). Only a weak fraction of non-secreted protein was detected in the cell pellet.

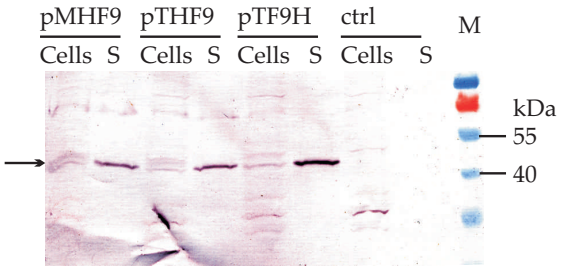

Figure 3.4: Constitutive protein production of hS39F9 fusion constructs in Sf9 insect cells. Samples from Sf9 cells constitutively producing hS39F9 fusion proteins were taken four days after dissemination. Cell lysates (Cells) and cell culture supernatants (S) were loaded on a 12% SDS-PA-gel. Western blot detection was performed using goat anti-FucT IX and rabbit anti-goat-AP antibodies. M: PageRuler prestained protein ladder; the hS39F9 fusion proteins are indicated by an arrow and their constructs are outlined in Figure 3.3.

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Activity assays of culture supernatants

The activity in the cell culture supernatant was assayed one to four days after dissemination (Figure 3.5). Maximal secretion of enzymatic activity was detected by transfection with the plasmids pMHF9 and pTHF9 expressing N-terminal histidine tagged protein HF9 with approximately 240 mU/l and 130 mU/l on day 3 after dissemination, respectively. In contrast, the activity of C-terminally histidine tagged hS39F9 fusion protein F9H did not significantly differ from the activity of non-transfected cells. From these results it could be concluded, that the C-terminal histidine tag led to inactivity of F9H.

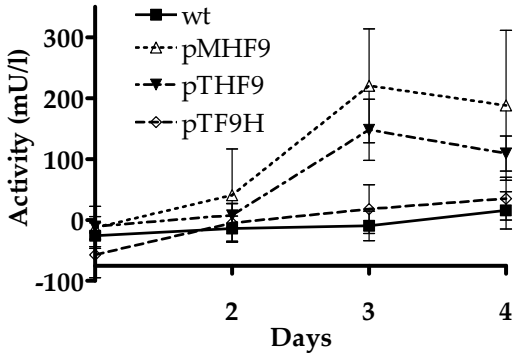


Figure 3.5: Time course of FucT activity in cell culture supernatants after stable transfection of Sf9 cells with hS39F9 plasmid constructs. Samples from cell culture supernatants were taken one to four days after dissemination of Sf9 cells (■ wt) and stable Sf9 cells transfected with plasmids pMHF9 (△), pTHF9 (▼) and pTF9H (◇). In three independent experiments FucT activity was assayed with MOPS reaction buffer, mean values and standard deviations are displayed. The tested hS39F9 fusion proteins are outlined in Figure 3.3.

3.1.2.2 Production of hS39F9 fusion proteins using a baculovirus production system

The constitutive protein production system produced only low amounts of hS39F9 fusion protein. Thus, an alternative production system, the baculovirus production system, was used to test its capability to yield higher amounts of active protein. This system was already applied for the production of several other FucTs (see section 1.4.2.2).

Generation of bacmids

For baculoviral overproduction of hS39F9 fusion proteins in Sf9 cells an approach described by Ihara *et al.* (2006) for FucT VIII was chosen. Constructs

containing the baculoviral secretion signal gp67 or β-trace, a deca-histidine tag and a factor X_a protease cleavage site located N-terminally of hS39F9 were designed (Figure 3.6). These constructs were cloned into a pFastBac1 plasmid and the resulting plasmids were named pFastGHF9 and pFastTHF9, respectively. The plasmids were used to transform *E. coli* DH10Bac cells. The bacmids bacmidGHF9 and bacmidTHF9 were isolated and used for transfection of Sf9 cells.

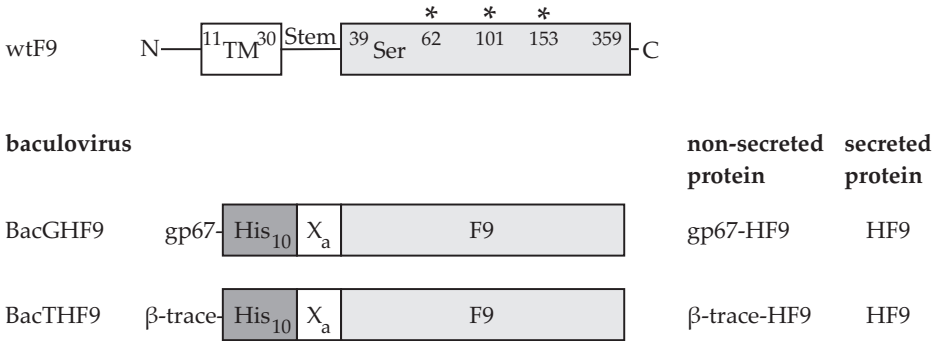


Figure 3.6: Schematic presentation of hS39F9 constructs used for baculoviral protein production in comparison to wtF9. The schema for full length human FucT IX (**wtF9**) shows the N-terminus (N), a predicted transmembrane domain (TM), important amino acid positions (indicated by numbers), putative N-glycosylation sites ("*") and the C-terminus (C). The **baculovirus** BacGHF9 encodes gp67-HF9 and the virus BacTHF9 encodes β-trace-HF9. The produced **secreted protein** contained a deca-histidine-tag (His_{10}) and a factor X_a protease cleavage site (X_a) N-terminal of hS39F9 and was named HF9. If unintentionally **non-secreted protein**s were produced, HF9 additionally contained the signal peptides gp67 or β-trace at the N-terminus and was named gp67-HF9 and β-trace-HF9, respectively.

Generation of recombinant baculovirus and protein production

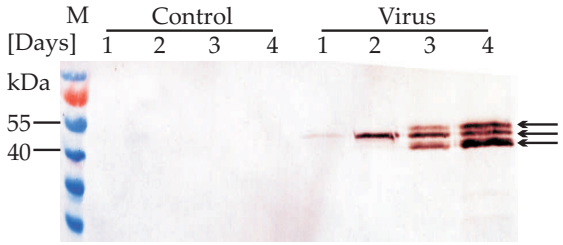
Transfection of Sf9 insect cells with recombinant bacmids bacmidGHF9 and bacmidTHF9 resulted in the production of the viruses BacGHF9 and BacTHF9, respectively. The viruses were used to infect Sf9 insect cells for the production of recombinant hS39F9 fusion constructs. Similar to the constitutive production, the secreted protein HF9 contained the restriction site derived amino acids and an N-terminal deca-histidine tag followed by factor X_a protease cleavage site but no signal peptides. Unintentionally, non-secreted protein was also produced. In this case, the intracellular protein still contained the gp67 or β-trace signal peptide, respectively. The resulting proteins were named gp67-HF9 and β-trace-HF9 (Figure 3.6).

Insect cells, as well as cell culture supernatant, were sampled one to four

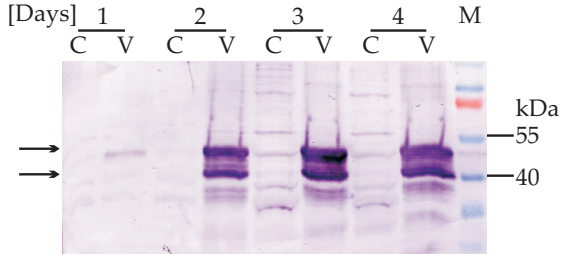
days after infection with BacTHF9. Western blot analysis revealed efficient production of hS39F9, yielding both secreted and intracellular protein. Infected cells secreted only a small amount of the produced hS39F9 fusion construct, the majority remained intracellular (Figure 3.7).

In contrast to uninfected cells, a 40 kDa band corresponding to HF9 was visible for cells infected with the BacTHF9 baculovirus. Three days after infection, two other unidentified bands in the range of 40 to 55 kDa appeared (Figure 3.7a, arrows).

The intracellular proteins displayed two prominent bands: one with an approximate molecular weight of 40 kDa and the other at about 50 kDa (Figure 3.7b, arrows). Similar results for secreted and non-secreted hS39F9 fusion proteins were obtained with the gp67 secretion signal (data not shown).



(a) Cell culture supernatant.



(b) Cells.

Figure 3.7: Baculovirus induced production of hS39F9 fusion proteins in insect cells. Western blot analysis was performed using goat anti-FucT IX and rabbit anti-goat-AP antibodies. M: PageRuler prestained protein ladder; numbers indicate the day after infection; The arrows indicate out prominent FucT IX positive bands. (a) Cell culture supernatants of cells infected with BacTHF9 (Virus) or uninfected cells (Control) were collected one to four days after infection. Samples were loaded on a 12% SDS-PA-gel. (b) Cells infected with BacTHF9 (V) or uninfected cells (C) were collected one to four days after infection. Cells were lysed with SDS-PA loading buffer and samples were separated on a 12% SDS-PA-gel.

Activity assays

The enzymatic activities of hS39F9 fusion proteins were determined using cell culture supernatants from Sf9 cells infected with either BacGHF9 or BacTHF9 baculovirus (Figure 3.8). Two days after infection, a maximum activity of 500 mU/l for the cell culture supernatant was achieved. A comparison of the supernatant activities revealed the maximal activity one day earlier in baculovirally infected Sf9 insect cells than in Sf9 cells constitutively producing HF9 fusion proteins. Additionally a 2-fold increase in active protein was detected (compare Figure 3.5 and 3.8).

To analyse the activity of non secreted hS39F9 three days after infection with BacTHF9, insect cells were lysed. The enzymatic activity of the lysate was 950 mU/l culture (data not shown). This was 2-fold higher than the activity detected in the baculovirally produced supernatant and 4-fold higher than the yield from the supernatant of cells constitutively producing the protein.

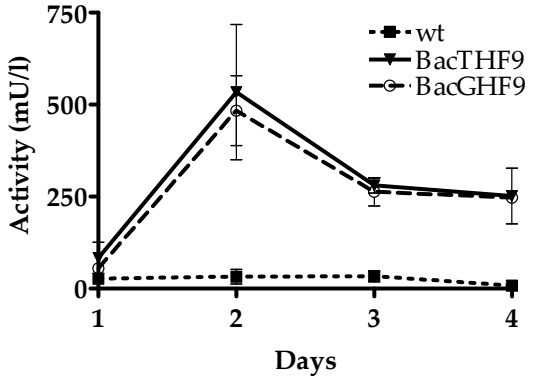


Figure 3.8: Time course of FucT activity in cell culture supernatant of Sf9 cells infected with recombinant hS39F9 baculovirus. Sf9 (■ wt) monolayer cultures were infected with baculoviruses BacGHF9 (○) or BacTHF9 (▽), respectively. Samples of the cell culture supernatant were taken one to four days after dissemination. Activity in the cell culture supernatant was assayed in three independent experiments using C/P reaction buffer, mean values and standard deviations are displayed..

3.2 Purification of hS39F9 fusion proteins produced with the baculovirus production system

3.2.1 Immobilised metal ion affinity chromatography

3.2.1.1 Purification of non-secreted β-trace-HF9 protein from lysed Sf9 insect cells

Three days after BacTHF9 infection, Sf9 cells were lysed. Most of the β-trace-HF9 protein was insoluble (compare lanes C to D in Figure 3.9). The soluble β-trace-HF9 was used for immobilised metal ion affinity chromatography (IMAC), bound proteins were eluted with imidazol. Fractions were analysed on SDS-PA-gels by coomassie staining and western blot (Figure 3.9).

After purification, elution fractions (see lanes E2 and E3 in Figure 3.9) still contained other proteins, β-trace-HF9 fragments or isoforms, but an enrichment of β-trace-HF9 in comparison to the lysate was achieved (arrows,

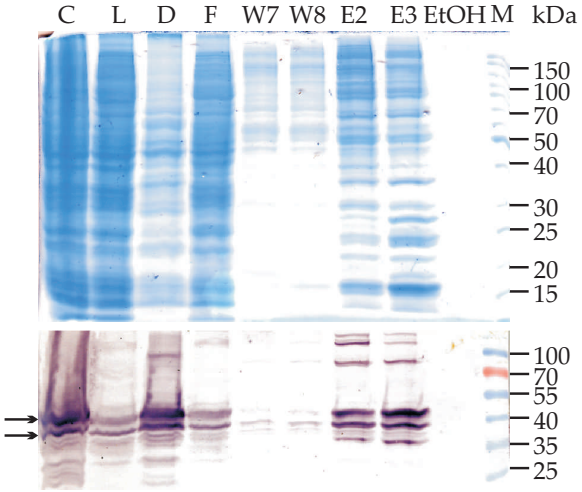


Figure 3.9: Purification of non-secreted β-trace-HF9 protein from lysed Sf9 cells. BacTHF9 infected Sf9 cells (C) were lysed in Ni-NTA column binding buffer, by vortexing. This suspension was sonified to shear DNA and centrifuged to separate cell debris (D) from lysate (L). Lysate was applied to a Ni-NTA column and flow through (F) was collected. The column was washed (W7, W8) with Ni-NTA column binding buffer and bound proteins were eluted (E2, E3) with the same buffer containing 500 mM imidazol. To remove residual proteins the column was washed again with 20% ethanol (EtOH). For analysis of the purification, cells, lysate, debris, flow through and fractions were loaded on a 12% SDS-PA-gel. Top: coomassie staining; bottom: western blot detection using goat anti-FucT IX and rabbit anti-goat-AP antibodies; M: top: PageRuler unstained protein ladder, bottom: PageRuler prestained protein ladder; The arrows indicate the main β-trace-HF9 bands.

Figure 3.9). After washing with 20% ethanol no proteins could be detected (see lane EtOHin in Figure 3.9), implying that all bound proteins were eluted with imidazol. Due to difficulties obtaining pure HF9 from insect cell lysate, a new purification procedure, starting from the cell culture supernatant, was tested.

3.2.1.2 Purification of secreted HF9 protein from Sf9 cell culture supernatant

The cell culture supernatant of Sf9 cells, collected three days after BacTHF9 infection, was concentrated and simultaneously the buffer was replaced by Ni-NTA binding buffer with a cross flow filtration. The resulting concentrate was applied to a IMAC column. The column was washed and bound proteins were eluted with imidazol. Appropriate fractions were analysed on SDS-PA-gels by coomassie staining and western blot (Figure 3.10).

The supernatant (lane S in Figure 3.10) displayed a band corresponding to HF9 only in the western blot, indicating that the coomassie staining was not sensitive enough to detect the band. No HF9 could be detected in the flow through (lane F in Figure 3.10) of the Ni-NTA column, implying all HF9 was bound to the Ni-NTA column. In the elution fractions (lanes E2 and E3 in Figure 3.10) a band at about 40 kDa corresponding to HF9 was detected with coomassie staining and western blot. Bands other than HF9 were detectable in the coomassie gel. No proteins were detected after washing with H_2O and EtOH, implying that all bound proteins were eluted.

Protein containing fractions were assayed for fucosyltransferase activity and protein content (Table 3.2). The supernatant showed a specific activity of 0.06 U/g. When small proteins and amino acids were removed by cross flow filtration the specific activity already increased to 0.28 U/g. After elution from the Ni-NTA column specific activity increased further to 2.1 U/g and 7.3 U/g in fraction IMAC E2 and E3, respectively. After this purification procedure quantitation of the protein containing lanes showed that the protein in fraction E2 and E3 were $\sim$ 55% and $\sim$ 58% pure, respectively.

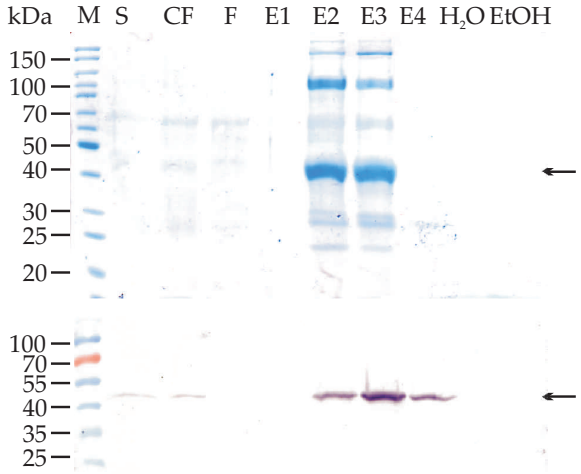


Figure 3.10: Purification of secreted HF9 protein from Sf9 cell culture supernatant. Three days after BacTHF9 infection, cell culture supernatant (S) of Sf9 cells was concentrated by cross-flow filtration and buffer was replaced by Ni-NTA binding buffer. The concentrate (CF) was applied to a Ni-NTA column and flow through (F) was collected. The column was washed with Ni-NTA binding buffer and bound proteins were eluted (E1-4) with the same buffer containing 500 mM imidazol. To remove residual proteins the column was washed with H_2O (H_2O) followed by 20% ethanol (EtOH). Purification was analysed by coomassie staining (top) and western blot (bottom) using a 12% SDS-PA-gel. Western blot detection was performed using goat anti-FucT IX and rabbit anti-goat-AP antibodies. M: top: PageRuler unstained protein ladder, bottom: PageRuler prestained protein ladder; The arrows indicate HF9 containing bands.

Table 3.2: IMAC purification of secreted HF9 protein from Sf9 cell culture supernatant.

Step	Protein (mg)	Total activity (mU)	Specific activity (U/g)	Yield (%)
S	228	14.2	0.06	100.0
CF	35	9.7	0.3	68.6
IMAC E2	0.57	1.2	2.1	8.4
IMAC E3	0.64	4.7	7.3	33.0

To determine extent of purification of HF9 the supernatant (S), cross flow concentrate (CF) and IMAC elution fractions 2 and 3 (IMAC E2, E3) were assayed for protein content and fucosyltransferase activity. The protein content was determined by BCA assay using anion exchange chromatography (AIEX) binding buffer containing 200 mM NaCl. The activity assay was carried out in the according protein buffer with MOPS reaction buffer. The values were obtained in a single experiment.

3.2.2 GDP affinity chromatography of pre-purified HF9

GDP affinity matrices can be used for the purification of glycosyltransferases (Beyer *et al.*, 1980). Fucosyltransferase IX belongs to the glycosyltransferase family 10. Therefore it might be possible to purify HF9 with GDP-affinity matrices. Hence the feasibility of this method was tested to further purify the HF9 containing Ni-NTA fractions (see section 3.2.1.2).

The HF9 containing fractions of the Ni-NTA purification of Sf9 cell culture supernatant were dialysed. After dialysis the protein was applied to a GDP-affinity matrix. The matrix was washed and protein was eluted with 3 mM GDP. Fractions were analysed on SDS-PA-gels by coomassie staining and western blot (Figure 3.11).

HF9 was found predominantly in the wash fraction (lane W1 in Figure 3.11). Only small amounts of HF9 were detected in the elution fraction by

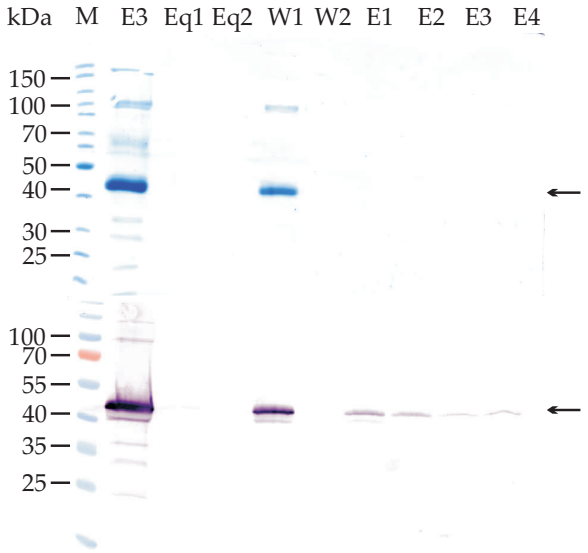


Figure 3.11: Purification of HF9 via GDP affinity chromatography. A column was loaded with GDP affinity matrix and equilibrated with GDP binding buffer (Eq1 and Eq2). Elution fraction IMAC E3 (see Figure 3.10) was dialysed against GDP binding buffer and loaded on the GDP column. The column was washed with GDP binding buffer (W1 and W2). Bound HF9 was eluted with GDP binding buffer containing 3 mM GDP (E1-4). Purification was analysed with 12% SDS-PA-gels which were coomassie stained (top) and western blotted (bottom). Western blot detection was performed using goat anti-FucT IX and rabbit anti-goat-AP antibodies. M: top: PageRuler unstained protein ladder , bottom: PageRuler prestained protein ladder; The arrows indicate the HF9 the containing bands.

western blotting (see lane E1-4, bottom in Figure 3.11) but not after coomassie staining (E1-4, top in Figure 3.11) due to lower sensitivity. That showed that the binding capacity of the matrix was too low to bind HF9. Therefore a method promising better yields was tested.

3.2.3 Ion exchange chromatography of pre-purified HF9

For further purification of the HF9 containing Ni-NTA fractions (E2 and E3), ion exchange chromatography was chosen. Isoelectric point analysis according to Gasteiger *et al.* (2003) of FUT9_HUMAN (Q9Y231) from amino acid 39 to 359 with N-terminal addition of $GSD(H)_{10}W$ LEIEG RVDSR (1-letter code for amino acids) gave a theoretical isoelectric point of 6.66. Therefore anion exchange chromatography at pH 8.5 was chosen. At this pH the theoretical net charge of HF9 should be negative and the protein should bind to the anion exchange column. Preliminary dialysis against AIEX dialysis buffer was necessary to lower the high sodium chloride concentration which was present after Ni-NTA purification as well as to adjust the pH.

Constitutively produced HF9, with hexa-histidine tag but without factor X_a protease cleavage site, precipitated under low sodium chloride concentrations (Figure 3.12). To avoid precipitation of baculovirally produced, pre-purified HF9, dialysis was performed in presence of either 1% Triton X-100 or 5 mM β-mercaptoethanol. Two different columns were tested for purification of HF9.

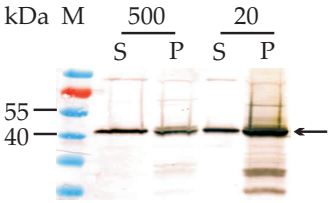


Figure 3.12: Dialysis of constitutively produced cell culture supernatant. Cell culture supernatant of Sf9 insect cells constitutively producing HF9 (with hexa-histidine tag but without factor X_a cleavage site) was dialysed over night against buffer containing 50 mM Na_2HPO_4 pH 8.0 and 500 mM (500) or 20 mM (20) NaCl, respectively. Supernatant (S) and precipitate (P) were separated by centrifugation (5 min, 5000 × g, 4 °C). One fifth of the precipitate and $\frac{1}{1500}$ of the supernatant were separated on a 12% SDS-PA-gel. Western blot detection was performed using goat anti-FucT IX and rabbit anti-goat-AP antibodies. M: PageRuler prestained protein ladder. The arrow indicates the HF9 containing band.

3.2.3.1 Poros HQ/M anion exchange chromatography after dialysis of HF9 with Triton X-100

To prepare IMAC elution fraction E2 for anion exchange chromatography dialysis in the presence of 1% Triton X-100 was performed. The Ni-NTA fraction E2 was applied to a Poros HQ/M column, the column was washed and bound proteins were eluted using a linear increasing NaCl gradient (Figure 3.13a). The dialysed fractions, the pellet after dialysis and the fractions after anion exchange chromatography were applied to a SDS-PA-gel and silver stained (Figure 3.13b).

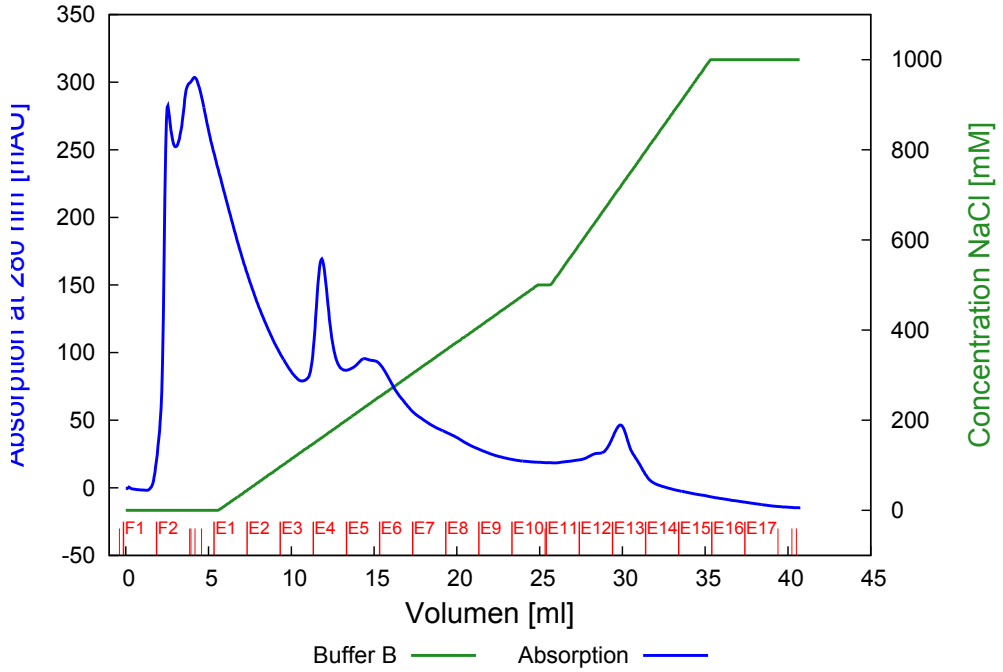
The addition of 1% Triton X-100 eliminated precipitates of HF9 (see Figure 3.13a, lane Pt Dia). Fractions E4-E6 contained HF9 (Figure 3.13b, arrow). HF9 eluted at NaCl concentrations between 200 mM and 300 mM. The other signals visible at about 50 to 68 kDa resulted from skin keratins which contaminated the protein samples or the buffers of the SDS-PA-gel electrophoresis (Ochs, 1983). Quantitation of the protein containing lanes revealed that the protein obtained with this purification procedure was ∼70% pure.

The specific activity of the eluted fractions was determined (Table 3.3). The activity in the E2 Ni-NTA fraction used for anion exchange chromatography was 2.1 U/g. After elution from the Poros HQ/M column the specific activity increased to 4.0 U/g and 2.2 U/g for fraction E5 and E6, respectively.

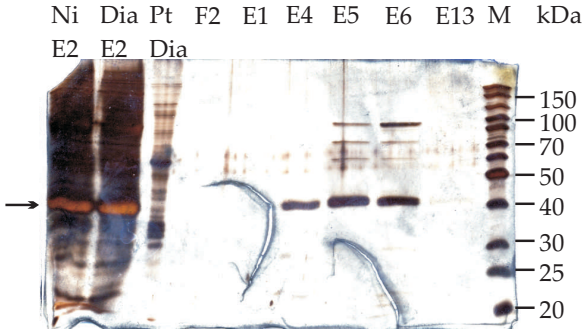
Table 3.3: HF9 Poros HQ/M purification.

Step	Protein (mg)	Total activity (mU)	Specific activity (U/g)	Yield (%)
IMAC E2	0.57	1.2	2.1	8.4
Poros HQ/M E5	0.09	0.4	4.0	2.6
Poros HQ/M E6	0.09	0.2	2.2	1.5

The IMAC elution fraction (IMAC E2) and the Poros HQ/M AIEX elution fractions (Poros HQ/M E5, E6) were obtained from purification of HF9. To determine their yields and the specific activities the fractions were assayed for protein content and fucosyltransferase activity. The protein content was determined by BCA assay using AIEX binding buffer containing 200 mM NaCl. The activity assay was carried out in the according protein buffer and MOPS reaction buffer. The values were obtained in a single experiment.



(a) Chromatogram.



(b) Silver stained 12% SDS-PA-gel.

Figure 3.13: Purification of HF9 using a Poros HQ/M anion exchange column. Elution fraction E2 of Ni-NTA purified HF9 (E2, see Figure 3.10) was dialysed against AIEX dialysis buffer pH 8.5 containing 1% Triton X-100. The dialysed solution was centrifuged to remove any potential precipitate (Pt Dia). Supernatant (Dia E2) was applied to a Poros HQ/M anion exchange column equilibrated with AIEX binding buffer pH 8.5 and flow through (F1-4) was collected. Elution was performed using AIEX binding buffer with a linearly increasing NaCl gradient (E1-17). (a) Chromatogram of purification. Green: NaCl concentration; Blue: Absorption at 280 nm; Red: Fraction number. (b) The purification was analysed by silver staining of a 12% SDS-PA-gel. M: PageRuler unstained protein ladder; F2: flow through fraction 2; E4, E5, E6, E13: elution fractions 4, 5, 6 and 13; The arrow indicates the HF9 containing band.

3.2.3.2 HiTrapQ anion exchange chromatography after dialysis of HF9 with β-mercaptoethanol

After the purification described in the previous section, contaminating proteins were still present, making a further optimisation of the method necessary. Therefore another column was used and dialysis with 5 mM β-mercaptoethanol instead of 1% Triton X-100 were tested. Sample preparation and anion exchange chromatography procedure were performed as described for PorosHQ/M purification, except that dialysis was performed with β-mercaptoethanol instead of Triton X-100. Furthermore, as the Poros HQ/M matrix was susceptible to reducing agents, a HiTrapQ column was chosen instead (Figure 3.14).

To analyse the result of this purification method, IMAC fraction E3 after dialysis, pellet after dialysis and fractions after anion exchange chromatography were applied to a SDS-PA-gel which was silver stained (Figure 3.14b). Similarly to the addition of 1% Triton X-100, addition of 5 mM β-mercaptoethanol eliminated precipitates of HF9 (lane Pt Dia). Fractions E10 and E11 contained HF9 (lane E10 and E11). As before, HF9 was eluted at NaCl concentrations between 200 mM and 300 mM. Again, other signals were visible at about 50 to 68 kDa, resulting from contamination (see section 3.2.3.1). The protein obtained with this purification procedure was ∼100% pure.

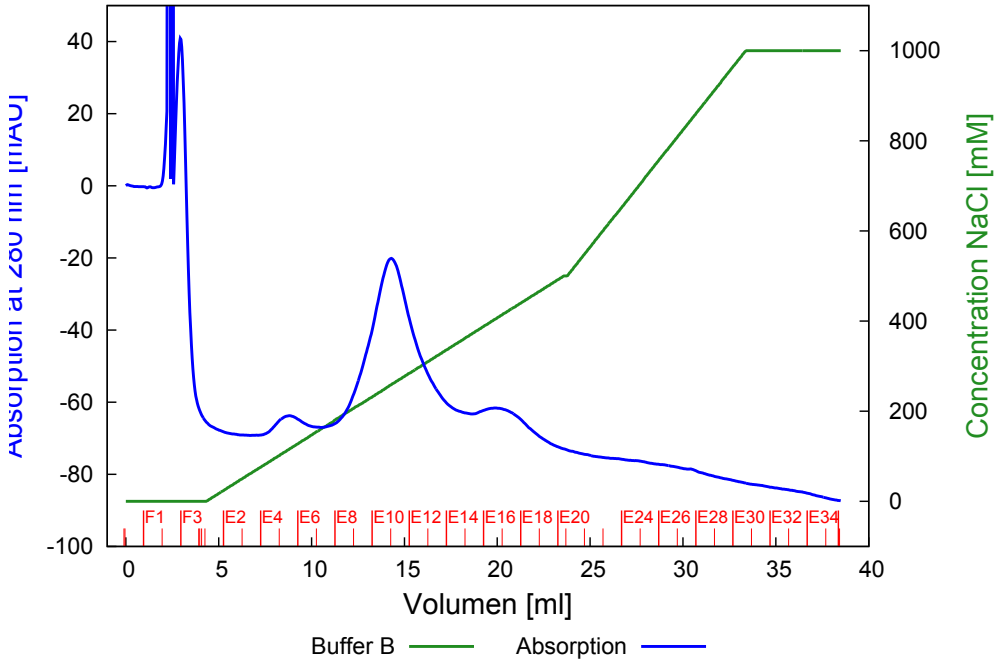
Table 3.4: HF9 HiTrapQ purification.

Step	Protein (mg)	Total activity (mU)	Specific activity (U/g)	Yield (%)
IMAC E3	0.64	4.7	7.3	33.0
HiTrapQ E10	0.03	0.2	9.3	1.7
HiTrapQ E11	0.04	0.2	4.7	1.5

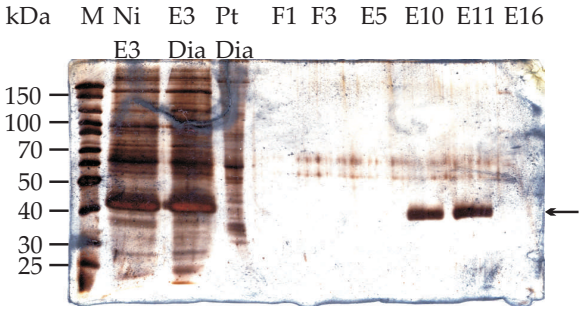
The IMAC elution fraction (IMAC E3) and the HiTrapQ AIEX elution fractions (Poros HQ/M E10, E11) were obtained from purification of HF9. To determine their yields and the specific activities the fractions were assayed for protein content and fucosyltransferase activity. The protein content was determined by BCA assay using AIEX binding buffer containing 200 mM NaCl. The activity assay was carried out in the according protein buffer and MOPS reaction buffer. The values were obtained in a single experiment.

The specific activity of the IMAC fraction E3 used for anion exchange

chromatography was 7.3 U/g. After elution from the Poros HQ/M column the specific activity of fraction E10 increased to 9.3 U/g and of fraction E11 decreased to 4.7 U/g (see Table 3.4). In addition the silver stained gel displayed a purification to homogeneity of HF9 protein (Figure 3.14). These fractions were suitable for enzymological studies.



(a) Chromatogram.



(b) Silver stained 12% SDS-PA-gel.

Figure 3.14: Purification of HF9 with HiTrapQ anion exchange column. Fraction E3 of Ni-NTA purification of supernatant (E3) was dialysed against AIEX dialysis buffer pH 8.5 containing 1% Triton X-100. The dialysis was centrifuged to remove any potential precipitate (Pt Dia). Supernatant (E3 Dia) was applied to a HiTrapQ anion exchanger column equilibrated with AIEX binding buffer pH 8.5 and flow through (F1-3) was collected. Elution was performed using AIEX binding buffer with a linearly increasing NaCl gradient (E1-34). (a) Chromatogram of purification. Green: NaCl concentration; Blue: Absorption at 280 nm; Red: Fraction number. (b) The purification was analysed by silver staining of a 12% SDS-PA-gel. M: PageRuler unstained protein ladder; F1, F3: flow through fraction 2; E5, E10, E1, E16: elution fraction 5, 10, 11 and 16; The arrow indicates the HF9 containing band.

3.3 Characterisation of HF9

The HF9 produced with the baculovirus expression system and purified by IMAC and HiTrapQ anion exchange chromatography was used for enzymological characterisation. The activity was monitored by measuring the transfer of ^{14}C-fucose from GDP-^{14}C-fucose to LacNAc. To assure a linear product formation a time series was carried out. This series showed that under standard reaction conditions the reaction remained linear within one hour and allowed about 20% conversion of the GDP-Fuc substrate. Therefore, all experiments were carried out either for one hour or until 20% conversion. To determine optimal reaction conditions, temperature preferences, pH dependency and metal ion preferences were analysed.

3.3.1 Temperature sensitivity of HF9

To determine the optimal reaction temperature for HF9, the thermal stability of HF9 was measured. Samples were incubated for one hour in C/P reaction buffer at temperatures ranging from 4 to 55 °C (Figure 3.15). The optimal reaction temperature was 37 °C. Therefore all further experiments were performed at this temperature.

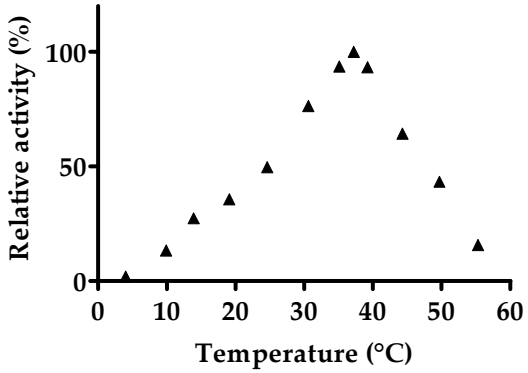


Figure 3.15: Thermal stability of HF9. The activity assay was performed in C/P reaction buffer pH 5.0 at temperatures ranging from 4 to 55 °C. The relative activities at different temperatures were compared to 37 °C, which was set as 100%. Three replicates were used per data point.

3.3.2 pH dependency of HF9

To determine the pH dependency of HF9 enzyme activity, four different buffer systems were used: acetate-NaOH (3.5-5.7), 2-(N-morpholino)ethanesulfonic acid (MES)-NaOH (5.7-7.7), Tris-HCl (7.8-9.0) and carbonate-bicarbonate (9.2-11.3) as used earlier for fucosyltransferase VIII by Yanagidani *et al.* (1997). HF9 and its substrates were incubated in reaction buffer with different pH values (Figure 3.16). The optimum pH was from 5.7 (acetate-NaOH) to 7.8 (Tris-HCl). At pH values higher than 9.0 or lower than 5.7 the activity droped significantly. All following experiments were performed with a combined buffer containing C/P-buffer pH 5.0 and the AIEX elution fraction with 30% glycerol buffer pH 8.5, resulting in an optimal pH for the reaction.

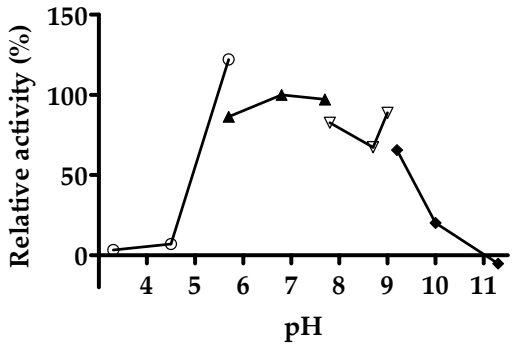


Figure 3.16: Effect of pH on HF9 activity. The activities for different pH values were obtained in (○) acetate-NaOH (3.5-5.7), (▲) MES-NaOH (5.7-7.7), (▽) Tris-HCl (7.8-9.0) and (◆) carbonate-bicarbonate (9.2-11.3). The relative activities at different pH values compared to pH 6.8 (100%) are displayed. Three replicates were used per data point.

3.3.3 Metal ion preferences of HF9

The activities of several fucosyltransferases depend on or are inhibited by metal ions. Therefore the effect of divalent cations on HF9 enzyme activity was determined at a concentration of 5 mM metal chloride. 5 mM EDTA and H_2O were used (Figure 3.17) as controls. Under influence of Mn^{2+} HF9 enzyme activity showed a 1.3-fold increase of activity compared to H_2O. EDTA, Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Ni^{2+}, and Zn^{2+} also enhanced the activity of HF9 compared to H_2O, respectively.

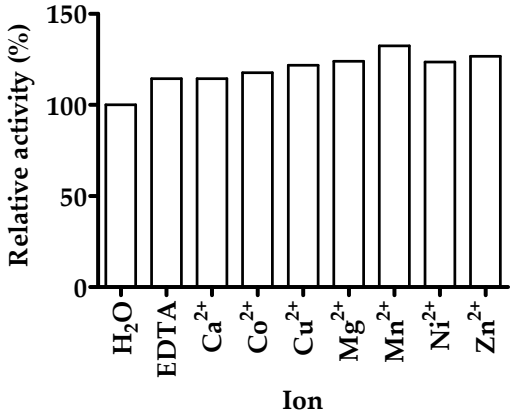


Figure 3.17: Effect of metal ions on HF9 activity. Activity assay was perfomed in C/P reaction buffer supplied with 5 mM of the according metal chloride. The relative HF9 activities in presence of different metal ions were compared to H_2O (100%). Three replicates were used per data point.

3.3.4 Determination of kinetic parameters for the enzymatic reaction of HF9

Different kinetic parameters characterise an enzyme and give hints to the suitability for different chemoenzymatic purposes. The parameters for the minimal substrate LacNAc and the donor GDP-Fuc were determined.

The data for GDP-Fuc were analysed by a double reciprocal Lineweaver-Burk plot (data not shown). Linearity in this plot suggested Michaelis-Menten kinetics. Therefore the data were evaluated by a Michaelis-Menten plot using one site binding analysis (Figure 3.18). The obtained K_m value of GDP-Fuc for the HF9 catalysed reaction was 1.58 µM (Table 3.5).

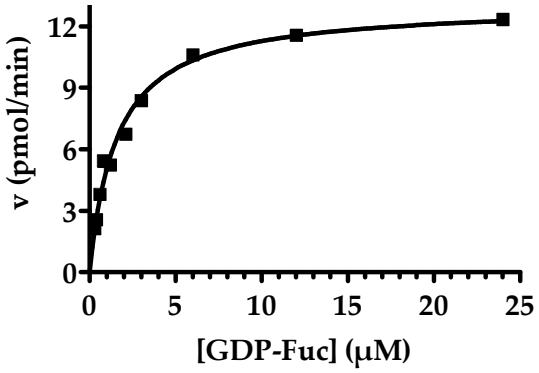


Figure 3.18: Michaelis-Menten plot for determination of the K_m value for GDP-Fuc of HF9. To determine the K_m value for GDP-Fuc, 200 ng HF9 were incubated with 0.1 µM GDP-[Fucose-^{14}C] and 0.2-23.9 µM GDP-Fuc at constant concentration of 4 mM LacNAc. The samples were incubated at 37 °C in C/P buffer for 0.5-6 min until approximately 20% GDP-Fuc was converted. Three replicates were used per data point. The non-linear curve was fitted using one site binding (hyperbola) analysis with GraphPad Prism.

Data analysis for LacNAc was performed similar to the analysis of GDP-Fuc (Figure 3.19). The obtained LacNAc K_m value for the HF9 catalysed reaction was 0.70 mM (Table 3.5). The V_{max} value was 880 nM/min. As 200 ng HF9 were used for this analysis the V_{max} corresponds to a k_{cat} of 4.6 min^{-1}.

The specificity constant k_{cat}/K_m for GDP-Fuc was 490×10^2 M^{-1}s^{-1} and 1×10^2 M^{-1}s^{-1} for LacNAc.

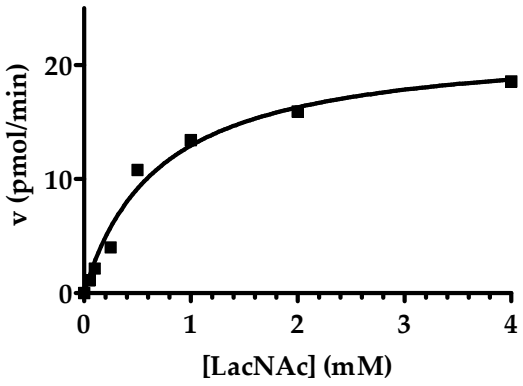


Figure 3.19: Michaelis-Menten plot for the determination of the K_m value for LacNAc of HF9. To determine the K_m value for LacNAc, 200 ng HF9 were incubated with 0.05-4 mM LacNAc at constant concentration of 1.8 µM GDP-[Fucose-^{14}C] and 312 µM GDP-Fuc. The samples were incubated at 37 °C in C/P buffer for one hour. Three replicates were used per data point. The nonlinear curve was fitted using one site binding (hyperbola) analysis with GraphPad Prism.

Table 3.5: Kinetic parameters for recombinant HF9.

Kinetic parameter	Value
K_m GDP-Fuc	1.6 µM
K_m LacNAc	0.7 mM
k_{cat}	4.6 min^{-1}
k_{cat}/K_m GDP-Fuc	490×10^2 $M^{-1}s^{-1}$
k_{cat}/K_m LacNAc	1×10^2 $M^{-1}s^{-1}$

4 Discussion

4.1 Recombinant production of fucosyltransferase IX

4.1.1 Production of hS39F9 fusion protein MBPHF9 in *E. coli*

Recombinant production of proteins in *Escherichia coli* (*E. coli*) is unmatched regarding yield and feasibility. Therefore the possibility to recombinantly produce soluble and active human fucosyltransferase (FucT) IX in *E. coli* was tested. In order to increase the chances to obtain soluble protein, the intracellular and the transmembrane domains of FucT IX were deleted and at the N-terminus a maltose binding protein (MBP) (Kapust and Waugh, 1999) was introduced. The fusion protein MBPHF9, consisting of MBP and N-terminally truncated human FucT IX starting from serine 39 (hS39F9), was cloned, produced in *E. coli*, and analysed for activity. MBPHF9 was investigated by SDS polyacrylamid (SDS-PA)-gel electrophoresis followed by coomassie staining or western blot. This investigation revealed that only small a amount of the protein was soluble. Furthermore, activity assays demonstrated that the protein was inactive.

Until today no active human α-1,3 fucosyltransferase has been produced in *E. coli*. However, in principle it is possible to produced active human glycosyltransferases in *E. coli*. Hidari *et al.* (2005) produce soluble and active ST6 β-galactosamide α-2,6 sialyltransferase-1 with a MBP-tag. Hence it was unlikely that the MBP-tag used in this work was responsible for the inactivity of MBPHF9. The inactivity of the protein was more likely caused by the lack of glycosylation, which is absent in *E. coli*. This is supported by previous findings from Morais *et al.* (2003) who showed that glycosylation might be crucial for folding and FucT III activity.

4.1.2 Production of hS39F9 fusion protein in insect cells

4.1.2.1 Constitutive system

Constitutive production of hS39F9 fusion protein in Sf9 insect cells

FucT IX (Brito *et al.*, 2007b) as well as FucT V (Münster *et al.*, 2006) were already produced using constitutive production systems. Brito *et al.* (2007b) used a IL-2 secretion signal to promote secretion of N-terminally truncated human fucosyltransferase IX starting from proline 40 (hP40F9). By this method they could obtain soluble, active truncated human FucT IX. The amounts they could produce were not enough for purification of the enzyme and structural studies. Therefore, this system was optimised to produce active, truncated, recombinant FucT IX in higher quantities.

The N-terminal secretion signals melittin and β-trace should enable insect cells to secrete hS39F9 fusion proteins into the cell culture supernatant. These fusion proteins were produced constitutively in insect cells with N- or C-terminal purification tags. The produced hS39F9 fusion proteins were analysed by western blot (Figure 3.4). The blot showed the strong capability of both melittin and the β-trace signal sequence to promote secretion of recombinant hS39F9 fusion proteins into the cell culture supernatant.

Activity assay of hS39F9 fusion proteins constitutively produced in Sf9 insect cells

The activity assay of cell culture supernatants from different secreted fusion proteins showed that a maximal activity of 240 mU/l could be detected on day 3 after dissemination with the N-terminal melittin signal sequence and deca-histidine-tag. Hence, the melittin signal sequence enables efficient secretion of N-terminal deca-histidine tagged hS39F9 (HF9). In a similar way slightly lower amounts of active protein (130 mU/l) were produced using the N-terminal β-trace signal.

In Experiments by Brito *et al.* (2007b), who used an N-terminal IL-2 secretion signal without additional tag (soluble hP40F9), showed a maximum activity of about 15 mU/l on day 2 after dissemination. In the present work the maximal observed activities were 16 (melittin) and 8.7-fold (β-trace) higher than activites measured by Brito *et al.* (2007b). The comparison of these results showed that the melittin or the β-trace signal sequence were more applicable

than the IL-2 signal sequence, for the secretion of active, recombinant HF9 from insect cells.

In contrast, a hS39F9 fusion protein with an N-terminal β-trace signal sequence in combination with a C-terminal hexa-histidine-tag (F9H) had a strongly diminished activity. The position of the tag therefore seems crucial for enzymatic activity. This is in accordance with findings from Xu *et al.* (1996), who proved that deletion of only a few amino acids from the C-terminus of the α-1,3 FucT III or V resulted in complete loss of enzymatic activity. The findings presented here support the assumption that alterations at the C-terminus of α-1,3 FucTs reduced enzymatic activity.

The constitutive production already yielded 16-fold more active hS39F9 fusion protein than the method by Brito *et al.* (2007b) produced. However to investigate if it is possible to increase the amount of active protein, the baculoviral expression system was tested in parallel.

4.1.2.2 Baculoviral system

Production of fucosyltransferase IX using a baculovirus induced protein production system

α-1,6 FucT VIII (Ihara *et al.*, 2006), α-1,3/4 FucT III (Morais *et al.*, 2003) and α-1,3 FucT VII (Shinkai *et al.*, 1997) were already produced in high yields by a baculovirus production system. Therefore, the feasibility of this system to produce FucT IX was tested and its yield was compared to the constitutive production system.

For secretion of HF9 into the cell culture supernatant, a β-trace and a gp67 signal sequence were used alternatively. Secreted (HF9, Figure 3.7a) and non-secreted (β-trace-HF9, Figure 3.7b) protein could be detected by western blots. From day 3 onwards, two protein bands were visible within the cells. In the cell cutlure supernatant three different proteins were detectable from day 3 onwards. This result was found with both the gp67 and β-trace secretion signals, displaying that there was no difference in the used signal sequence. Furthermore, these multiple bands might be present due to different glycosylation states of the proteins. The intracellular localisation of the non-secreted protein indicates that the baculovirus-driven overproduction saturates the insect cell secretion pathway as previously demonstrated by Rodriguez

et al. (1998). The saturation of the secretion pathway might have correlated with a saturation of the glycosylation apparatus. Because non glycosylated proteins might not fold correctly and therefore are not secreted (Morais *et al.*, 2003).

Activity assay of baculovirally produced HF9

Activity assays demonstrated active protein in the cell culture supernatant. The highest activity was detected two days after infection (Figure 3.8). It was 2-fold higher than the yield obtained in the cell culture supernatant after constitutive production and 35-fold higher than the yield obtained by Brito *et al.* (2007b). Furthermore, when cells were lysed, activity assays of lysate demonstrated an even higher activity. The measured activity per litre of cell culture was about 2-fold higher than that in the culture supernatant. Additionally, the enzyme production time was one day shorter using the baculovirus production system (BEVS) compared to the constitutive system. This data indicated the suitability of the baculovirus production system to recombinantly produce HF9 in amounts suitable for kinetic analyses and enzyme characterisations. Consequentially, all following experiments were conducted using the baculovirally produced protein.

4.2 Purification of human fucosyltransferase IX produced by the baculovirus production system

4.2.1 Immobilised metal ion affinity chromatography

A purification procedure for recombinantly produced HF9 was developed. According to Ihara *et al.* (2006), a histidine-tag was introduced to facilitate this process.

Because baculoviral cell lysate contained the highest amount of active protein (β-trace-HF9), it was used instead of cell culture supernatant for immobilised metal ion affinity chromatography (IMAC) (Figure 3.9). SDS-PA-gel electrophoresis of the purified fractions, followed by coomassie staining as well as western blot, detected multiple bands different from FucT IX. This showed that the purification of β-trace-HF9 from the lysate was not suitable to purify the enzyme. Western blot detection showed that these protein bands

contained not only other histidine-rich proteins which copurified from insect cell lysate, but also isoforms or different glycosylation states of β-trace-HF9, and the β-trace signal peptide was not removed. Therefore, despite the higher amount of overall active protein, this method was not further improved and purification optimisation was focused on cell culture supernatant.

After cross flow filtration concentration and buffer exchange, IMAC was used to purify HF9 from cell culture supernatant of infected *Spodoptera frugiperda* (Sf9) cells. This new purification method was superior to the purification from the cell lysate. Compared to the cell culture supernatant, the specific activity increases 33.6- and 117.6-fold for elution fractions E2 and E3, respectively (Table 3.2). Western blot analysis confirmed that the 40 kDa band detected in the coomassie stained gel corresponded to HF9. The overall yield of this purification step was 41.4 % of the total active protein in the cell culture supernatant. This yield was 1.5-fold higher than the 28 % yield from IMAC purification which was obtained by Ihara *et al.* (2006) for α-1,6 FucT VIII. It turned out that the new purification procedure used in this study was more suitable to purify fucosyltransferases from insect cell culture supernatant than the ammoniumsulfate precipitation followed by a dialysis performed by Ihara *et al.* (2006). The protein obtained by this purification procedure was already 55 – 60 % pure.

4.2.2 GDP affinity chromatography

The purification using a GDP affinity matrix should result in pure recombinant FucT IX (Beyer *et al.*, 1980). Coomassie stained SDS-PA-gel and western blot showed very low recovery rates of HF9 after GDP matrix affinity purification (see section 3.2.2). Due to this low recovery rate no protein could be detected by a SDS-PA-gel in the elution fractions. Therefore no information about the purity of the enzyme after this purification is known. A possible reason for this low recovery could be the binding capacity of the matrix.

On the used matrix more than 10 µmol GDP were bound per ml affinitygel (see section 2.3.2.2). Correspondingly, if every GDP on the matrix can bind a glycosyltransferase enzyme 10 µmol glycosyltransferase should be able to bind to the matrix. The matrix should therefore be able to bind 400 mg of

a 40 kDa glycosyltransferase. Under the applied conditions the GDP on the matrix was probably not accessible for recombinant HF9.

4.2.3 Ion exchange chromatography

The purification by GDP affinity matrix was not as successful as expected. Therefore, ion exchange chromatography was used to further purify HF9. To prevent HF9 to form precipitates during overnight dialysis, the buffer was supplemented with Triton X 100 or β-Mercaptoethanol. With these supplements no formation of HF9 precipitates was observed. The silver stained SDS-PA-gels ran after anion exchange chromatography with columns Poros HQ/M or HiTrapQ, showed nearly homogeneous HF9. However, in the SDS-PA-gel after HiTrapQ purification no other protein bands were detected which corresponded to a purity of approximately 100 % (Figure 3.14b), whereas Poros HQ/M purification showed 3 bands of other proteins corresponding to a purity of 70 % (Figure 3.13b).

The purification process of HF9 from Sf9 insect cell culture supernatant by cross flow filtration, IMAC and anion exchange on HiTrapQ developed in this work is the first report for the purification of human FucT IX. Several other human fucosyltransferases have also been purifed to homogeneity by various approaches, some examples are: Full length FucT III was purifed from BHK cells by cation exchange on SP-Sepharose, GDP-fractogel and filtration on Superdex 200 by Sousa *et al.* (2001). Truncated FucT V was purified from supernatants of Tn insect cells by GDP-affigel column and cation exchange on a MonoS HR5/5 column (Münster *et al.*, 2006). The purification of truncated FucT VIII from the culture medium of insect cells was perfomed by ammonium sulfate precipitation followed by IMAC (Ihara *et al.*, 2006).

Not all HF9 containing fractions were pooled and analysed, but only the ones with the highest UV absorption at 280 nm. Therefore, a comparison of yields is difficult. Combined elution fractions E5 and E6 from Poros HQ/M purification contained 4.1 % of the total produced protein. Thus, approximately 50 % of the protein could be recovered from the column, because the elution fraction IMAC E2 which was loaded to the Poros HQ/M column contained 8.4 % of the total produced protein.

Only a recovery rate of 10 % was found for the HiTrapQ column purification. Because HiTrapQ elution fractions E10 and E11 together contained 3.2 % of the total produced protein and the IMAC elution fraction E3 which was used to load the HiTrapQ column contained 33.0 % of the total produced protein.

As mentioned before, the purity of the protein was higher after HiTrapQ compared to Poros HQ/M purification. Therefore, despite lower recovery, the fractions obtained by the HiTrapQ column were used for further analysis of protein activity. For applications which need higher protein amounts the contaminations present after Poros HQ/M purification might be acceptable because the overall yield was much higher.

4.3 Characterisation of HF9

4.3.1 Temperature sensitivity of HF9

The optimal temperature for enzymatic acitvity of HF9 was 37 °C. This correlated with findings for other fucosyltransferases: during characterisation of FucT VII, Shinoda *et al.* (1997) found that 37 °C was the optimal reaction temperature for this enzyme. Likewise, human α-1,6 FucT VIII produced in MKN45 cells displayed an optimal reaction temperature of 30-37 °C (Yanagidani *et al.*, 1997). Optimal reaction temperature for *Caenorhabditis elegans* FucT was at approximately 23 °C, the natural temperature of the organism (Nguyen *et al.*, 2007). As expected, glycosyltransferases had their optimal reaction temperature at optimal temperatures for each organism.

At temperatures higher than 37 °C heat inactivation of HF9 started. However, at temperatures of 50 °C at least half of the maximal activity of HF9 was obtained after one hour of incubation. These values also correspond to the heat stability reported by Cailleau-Thomas *et al.* (2000) for enzyme activities from extracts of COS7 cells transfected by FUT9 encoding full length FucT IX. The mentioned authors also report that FucT IV had the same temperature stability as FucT IX, whereas FucT VI was almost completely inactivated at 50 °C after 30 min.

4.3.2 pH dependency of HF9

The pH tolerance of FucTs is defined to be narrow, if the minimal and maximal tolerated pH differed by less than one pH value. A broad pH tolerance was present if the minimal and maximal tolerated pH differed by more than one pH value.

It was shown that FucT IX had an optimal activity at pH 5.5 – 9.0, thus belonging to the broad pH optimum group. Other human FucTs being part of the same group are summarised in Table 4.1. In contrast, a narrow pH optimum was found for FucT IV, FucT VI, full length FucT VII, and FucT VIII (Table 4.1).

FucT VII was found in both groups. It is imaginable that the truncation introduced by de Vries *et al.* (2001a) to obtain optimal protein yield of recombinant enzyme was responsible for this bias. Therefore it might be possible that the pH optimum measured for HF9 was not the same as for full length FucT IX.

Table 4.1: pH optima of FucTs.

optimum	FucT	pH range	References
narrow	IV	8 – 8.7	Mollicone *et al.* (1990)
	VI	7 – 8	Johnson *et al.* (1995)
	VII (full length)	7 – 7.7	Shinoda *et al.* (1997)
	VIII (chicken heart)	5.5	Struppe and Staudacher (2000)
broad	III	6 – 9 with Mn^{2+}	Palma *et al.* (2004)
	V	5 – 7	Murray *et al.* (1996)
	VII (truncated)	6 – 10.5	de Vries *et al.* (2001a)
	VIII (rabbit brain)	5.8 – 8	Struppe and Staudacher (2000)
	VIII (porc brain)	5 – 8	Uozumi *et al.* (1996)
	IX	5.5 – 9	this work

4.3.3 Effect of metal cofactor substitution on activity of HF9

A small effect on HF9 activity was found for all tested divalent ions (5 mM Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, respectively). The enzyme was fully active even in the presence of 5 mM EDTA.

4.3.3.1 Effect of manganese ions

Brito *et al.* (2008) demonstrated that dependant on different Mn^{2+} concentrations the enzymatic activity of full length human FucT IX was improved or inhibited. They found that 2.5 to 10 mM Mn^{2+} enhanced the activity 1.9-fold, whereas 40 mM Mn^{2+} decreased the activity compared to assays without addition of Mn^{2+}. The maximal effect of Mn^{2+} ions on HF9 measured here was 1.3-fold, which was smaller than the values obtained by Brito *et al.* (2008).

This 1.3-fold activation by Mn^{2+} was untypically low for α-1,3 FucTs. Activation by Mn^{2+} ions was 2.7-fold for FucT III (Palma *et al.*, 2004), 3.0-fold for FucT IV (Mollicone *et al.*, 1990), 11-fold for FucT V (Murray *et al.*, 1996), 8-fold for FucT VI (Johnson *et al.*, 1995) and 12-fold for FucT VII (de Vries *et al.*, 2001a, see Table 4.2). Thus, even the lowest activation was still 2.1-fold higher than the activation of FucT IX measured in this work.

The DxD sequence in motif IV (see Figure 1.5), which is present in highly divergent cation-dependent glycoprotein glycosyltransferases (Wiggins and Munro, 1998; Zhang *et al.*, 2001) was postulated by Palma *et al.* (2004) to interact with Mn^{2+} to complex the phosphates of GDP-β-L-fucose (GDP-Fuc). Substitution of either one of the aspartates in this motif by asparagine abolished activity of α-1,3-galactosyltransferase (Zhang *et al.*, 2001). In contrast to findings for α-1,3 FucT, activity of α-1,3 FucT IX is not strongly dependent on Mn^{2+}, although it contains a DxD sequence in motif IV. It is likely that additional factors different from the two aspartates and independent of Mn^{2+} ions were involved in the complexation of GDP-Fuc phosphates.

The measured effect could also originate from the purification procedure. One could imagine that cofactors required for a stronger effect of Mn^{2+} were removed during purification. Another possibility is that Mn^{2+} ions did not have any effect, as all possible binding sites were already occupied and bound Mn^{2+} remains bound to the enzyme during the whole purification procedure. It might be possible that addition of EDTA did not remove them. Furthermore the truncation of the protein or the addition of the purification tag might have influenced the enhancement of FucT IX activity by Mn^{2+} ions.

In summary, two different scenarios are possible: either purified FucT IX is

independent from Mn^{2+} ions or strongly binds them. A crystallisation of the enzyme could reveal details of metal ion involvement.

Table 4.2: Influence of Mn^{2+} ions on activity of human FucTs.

FucT	Influence of Mn^{2+} ions on FucTs activity	References
III	2.7-fold	Palma *et al.* (2004)
IV	3.0-fold	Mollicone *et al.* (1990)
V	11-fold	Murray *et al.* (1996)
VI	8-fold	Johnson *et al.* (1995)
VII	12-fold	de Vries *et al.* (2001a)
VIII	0.8-fold	Uozumi *et al.* (1996)
IX	1.3 / 1.9-fold	this work / Brito *et al.* (2008)

4.3.3.2 Effect of other metal ions

Other divalent metal ions than Mn^{2+} also had an influence on the activity of different human α-1,3 fucosyltransferases. FucT IX was not influenced strongly by any of the tested ions, whereas the addition of Co^{2+} ions to FucT III enhanced the activity about 2.8-fold (Palma *et al.*, 2004). Also FucT VII was stimulated by divalent cations such as Mg^{2+}, Ca^{2+} and Co^{2+} (Shinoda *et al.*, 1997). In contrast, the respective presence of Co^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+} suppressed the activity of human α-1,6 FucT VIII produced in MKN45 cells (Yanagidani *et al.*, 1997).

FucTs of adult and 7-week old human embryonic brain samples in studies by Cailleau-Thomas *et al.* (2000) were not inhibited by Co^{2+} ions. On the contrary, 5- and 6-week old human embryonic brain samples and cloned FucT IV and FucT IX enzymes were inhibited by Co^{2+} ions. Cailleau-Thomas *et al.* (2000) suspected that fucosyltransferases other than FucT IV and FucT IX are the fucosyltransferases in the brain which are not inhibited by Co^{2+} ions.

The results presented in this thesis suggest that the FucT IX activity in the human brain could be both, inhibited and not inhibited by Co^{2+}. Full length enzyme FucT IX inhibited by Co^{2+} ions was present in COS7 cells transfected with FUT9 (Cailleau-Thomas *et al.*, 2000) and might also be present in 5- and 6-week old human embryonic brain. In this thesis, truncated FucT IX was not

inhibited by Co^{2+} ions. Truncated FucT IX might also be present in adult and 7-week old human embryonic brain samples and truncated FucT IX could thus be the FucT responsible for the observations of Co^{2+} independency in these samples. Probably proteolysis of the enzyme is regulated from 7^{th}-week onwards and upon proteolysis the amino acids, relevant for inhibition by Co^{2+} ions, are cut by a still unknown process.

4.3.4 Determination of kinetic parameters of HF9

4.3.4.1 Michaelis-Menten constant of GDP-Fuc for HF9

Different kinetic parameters characterise an enzyme and give hints on the suitability for different chemoenzymatic purposes or design of inhibitors. The parameters for the minimal substrate N-acetyllactosamine (LacNAc) and the donor GDP-Fuc were determined in this thesis. Kinetic constants were determined by non-linear fit of the data. The observed K_m value of 1.6 µM for GDP-Fuc was in the range of other human FucTs which show values for the K_m value of GDP-Fuc from 0.1 to 60 µM (Table 4.3).

Table 4.3: K_m values of different human FucT for GDP-Fuc.

FucT	K_m (µM) value for GDP-Fuc	References
III	12	Cailleau-Thomas *et al.* (2000)
IV	40	Cailleau-Thomas *et al.* (2000)
V	60	Murray *et al.* (1996)
VI	6	Cailleau-Thomas *et al.* (2000)
	21	Miyashiro *et al.* (2004)
	5.5	Maeda and Nishimura (2008)
VII	6.2	Cailleau-Thomas *et al.* (2000)
	21	Miyashiro *et al.* (2004)
	0.1	von Ahsen *et al.* (2008)
VIII	19.3	Ihara *et al.* (2006)
IX	21	Cailleau-Thomas *et al.* (2000)
	1.6	this work

In this thesis, the K_m value for GDP-Fuc using LacNAc as the acceptor sugar was 13-fold lower compared to the literature value of 21 µM using LacNAc-

$(CH_2)_8COOCH_3$ as acceptor (Cailleau-Thomas *et al.*, 2000). The difference in K_m values may be due to the utilisation of different acceptor sugars. Another reason for the different K_m values could be the fact that Cailleau-Thomas *et al.* (2000) used non-purified cell lysate to determine the K_m value for GDP-Fuc.

4.3.4.2 Michaelis-Menten constant of LacNAc for HF9

The observed K_m value of 700 µM for LacNAc was in the range reported for other human FucTs. The published K_m value for LacNAc derivatives for human FucTs ranges from 12.9 to 8800 µM (Table 4.4).

Table 4.4: K_m values of different human FucT for LacNAc derivatives.

FucT	K_m (µM) value	LacNAc derivative	References
III	1330	Fucα-1,2 LacNAcβ-R1	Cailleau-Thomas *et al.* (2000)
IV	360, 170	LacNAcβ-R1, Fucα-1,2 LacNAcβ-R1	de Vries *et al.* (1995)
	130	Fucα-1,2 LacNAcβ-R1	Cailleau-Thomas *et al.* (2000)
V	8800, 170	LacNAc, LacNAcβ-R2	Murray *et al.* (1996)
VI	4600, 64	LacNAc, LacNAcβ-R3	Lee *et al.* (2003)
	300	LacNAc	Miyashiro *et al.* (2004)
VII	7800	sia-LacNAc	Miyashiro *et al.* (2004)
	50	6-O-sulfo-3'-sia-LacNAc	von Ahsen *et al.* (2008)
VIII	12.9	fluorescence labelled Asn-linked agalacto-biantennary sugar chain	Ihara *et al.* (2006)
IX	68	Fucα-1,2 LacNAcβ-R1	Cailleau-Thomas *et al.* (2000)
	700	LacNAc	this work

The residues are: R1=-O-$(CH_2)_8COOCH_3$, R2 = -O-$(CH_2)_5COOCH_3$, R3 = -O-(2-naphtyl)oxyethylene, sia = Neu5Acα-2,3.

In this work a 10.3-fold higher K_m value of LacNAc was observed, compared to the published value of 68 µM of Fucα-1,2 LacNAcβ-O-$(CH_2)_8COOCH_3$ (Cailleau-Thomas *et al.*, 2000). This difference may be explained by the use of different acceptor sugars or that the initial values by Cailleau-Thomas *et al.* (2000) were apparent values from non-purified cell lysate.

For other human FucTs huge differences for the minimal acceptor LacNAc

and elongated acceptors were reported. FucT V for example, shows a K_m value of 8800 µM with LacNAc and 170 µM for LacNAcβ-O-$(CH_2)_5COOCH_3$ (Murray *et al.*, 1996) or FucT VI with 4600 µM for LacNAc and 64 µM for LacNAcβ-O-(2-naphtyl)oxyethylene (Lee *et al.*, 2003). Those longer substrates seem to be better acceptors because they are more alike sugar chains on glycoproteins, the natural substrates of human fucosyltransferases than the minimal acceptor LacNAc. Results from Brito *et al.* (2007b) support this hypothesis because they showed that on the glycoproteins asialo-fetuin, erythropoietin and asialo-erythropoietin FucT IX had a 11 to 12-fold higher relative activity than on LacNAc-O-$(CH_2)_3NHCO(CH_2)_5$-NH-biotin.

Acceptor specificity of FucT IX has been investigated in detail by Toivonen *et al.* (2002). For Fucα-1,2 LacNAc they show a 2-fold lower relative activity than for LacNAc. In contrast, Cailleau-Thomas *et al.* (2000) showed a 1.15-fold better relative activity for Fucα-1,2 LacNAcβ-O-$(CH_2)_8COOCH_3$ than for LacNAcβ-O-$(CH_2)_8COOCH_3$. Brito *et al.* (2007b) also showed a 1.6-fold better relative activity for Fucα-1,2 LacNAc-O-$(CH_2)_3NHCO(CH_2)_5$-NH-biotin than for LacNAc-O-$(CH_2)_3NHCO(CH_2)_5$-NH-biotin. These results suggest that the addition of extra linkers to the acceptor substrates alters the reactivity of FucT IX. This has to be kept in mind when measuring the reactivity of FucT IX by adding detection markers like fluorescent labels.

4.3.4.3 Turnover number of HF9

The observed k_{cat} for FucT IX of 4.6 min^{-1} was in the range of other glycosyltransferases. Studies listed in Table 4.5 mentioned k_{cat} values from 0.8 to 1570 min^{-1}, depending on the enzyme and the used acceptors.

The turnover number measured in this thesis was on the lower end of reported values. Because glycosyltransferases show different turnover numbers for different acceptors the found value might increase with another acceptor, for example a glycoprotein.

Table 4.5: Values for k_{cat} of different glycosyltransferases.

Enzyme	k_{cat} values (min^{-1})	Acceptor	References
human FucT V	40, 10	LacNAc, LacNAcβ-R2	Murray *et al.* (1996)
Helicobacter pylori (*H. pylori*) α-1,4 FucT	1570, 4	Galβ-1,3GlcNAcβ-R1, LacNAcβ-R1	Rabbani *et al.* (2005)
Human blood group glycosyltransferase A	294, 1.2	UDP-GalNAc, UDP-Gal on Fucα(1-2)Galβ-R4	Seto *et al.* (1997)
Rat FLAG-tagged ST6GalI	68, 5	LacNAcβ-R3, Lactoseβ-R3	Ogata *et al.* (2009)
Staphylococcus aureus monofunctional Glycosyltransferase	0.8	meso-[^{14}C]A2pm-labeled C55 lipid II	Terrak and Nguyen-Distèche (2006)
human α-1,3 Galactosyltransferase	384	Lactose	Zhang *et al.* (2004)
human FucT VIII	24.6	fluorescence labelled Asn-linked agalacto-biantennary sugar chain	Ihara *et al.* (2006)
human FucT IX	4.6	LacNAc	this work

R1=-O-$(CH_2)_8COOCH_3$, R2=-O-$(CH_2)_5COOCH_3$, R3= 5-(5-Dimetylaminonaphthalene-1-sulfonyl-2-(2-aminoethoxy))ethyl, R4=-O-$(CH_2)_7CH_3$.

4.3.4.4 Specificity constant of HF9

The calculated specificity constant k_{cat}/K_m was 490×10^2 $M^{-1}s^{-1}$ for GDP-Fuc and 1×10^2 $M^{-1}s^{-1}$ for LacNAc. Both values were in the range of values reported

for other FucTs (Table 4.6). These values depend on the enzyme and the used substrates.

Table 4.6: Values for k_{cat}/K_m of different FucTs.

Enzyme	k_{cat}/K_m values ($\times 10^2$ $M^{-1}s^{-1}$)	Substrate	References
human FucT V	110	GDP-Fuc	Murray *et al.* (1996)
	1	LacNAc	
human FucT VIII	210	GDP-Fuc	Ihara *et al.* (2006)
	320	fluorescence labelled Asn-linked agalacto-biantennary sugar chain	
human FucT IX	490	GDP-Fuc	this work
	1	LacNAc	

The measurement of enzyme properties for the purified FucT IX laid the basis to further characterise the enzyme's kinetic mechanism or structure. Thus, it will be possible to understand the functionality of the enzyme and develop specific inhibitors to treat pathological processes associated with excess fucosylation.

5 Outlook

The production, purification and basic characterisation of fucosyltransferase (FucT) IX conducted in this work, enable its detailed characterisation. Thus, the basis is laid to reveal the reaction mechanism of recombinant FucT IX by measuring a secondary isotope effect or conducting inhibitor studies. Similar studies were already successfully realised by Murray *et al.* (1997) and Ihara *et al.* (2006) to reveal the reaction mechanism of FucT V and FucT VIII, respectively.

Maeda and Nishimura (2008) used a Förster resonance energy transfer (FRET)-based test to monitor the generation of sialyl-Lewis x epitope (Le^x) by α-1,3 FucT VI *in vitro*. They used a naphtyl fluorescence donor on GDP-β-L-fucose (GDP-Fuc), which was exited at 290 nm, and monitored the fluorescence emitted by the dansyl fluorescence acceptor at 540 nm on the sialyl-Le^x tetrasaccharide product. This method provides a highly sensitive, direct, and continuous *in vitro* monitoring of the catalysed reaction. It might be possible to use this FRET-based assay, to measure the enzyme activity in living cells. However, excitation at 290 nm induces mutagenic pyrimidine dimers in the DNA. Therefore another fluorescence donor-acceptor pair should be used for the cellular assay. The Atto dyes 550 and 647N, for example, are a FRET pair, which is exited at 554 nm and emits at 669 nm.

Furthermore, it was hypothesised in this thesis that the full length and truncated FucT IX possess a different Co^{2+} dependency. This hypothesis should be verified in further detail. To this end, the full length FucT IX should be produced. This could be accomplished using a pCR3.1 expression vector to produce the full length FucT IX in BHK cells. Morais *et al.* (2003) used this method earlier to produce full length FucT III and measured its activity in the cell lysate.

Christensen *et al.* (2000) analysed the functional role of N-glycosylation sites

in FucT III, -V, and -VI. They found that the replacement of the C-terminal N-glycosylation site, decreased the activity to one fourth of the wild type activity, whereas the K_{m} value for substrate and acceptor was not affected. This C-terminal N-glycosylation site is not present in FucT IX. It is possible that reintroduction of this site enhances the overall activity of FucT IX. Thus, it would be interesting to analyse the glycosylation state of the putative glycosylation sites in FucT IX.

The analysis of cysteine residues and their involvement in disulfide bonds could give hints about the protein structure. Free cysteines have to be modified and cysteines involved in disulfide bonds should be reduced prior to protease digestion of FucT IX. Finally, the protein fragments could by analysed by mass spectrometry (Holmes *et al.*, 2000; de Vries *et al.*, 2001b).

Alternatively most of these informations could be gained by crystallisation of the enzyme. A crystallisation is possible, as the production and purification method for recombinant FucT IX was developed in this work. These structural studies could also help to reveal the catalysis mechanism. Furthermore they could confirm or exclude the involvement of metal ions in the catalysis mechanism.

Finally, the purified enzyme enables a screening for potent inhibitors. It would be possible to use the library designed by Lee *et al.* (2003). In order to design this library of organic compounds the authors retained the GDP core of GDP-Fuc, while attaching hydrophobic groups and varying the linker length. If an inhibitory organic compound would be found by this screening it could be optimised systematically with the informations gained by the structural analysis of FucT IX. This optimised inhibitor could later on be used for medical applications *i.e.* to prevent tumour metastasis or viral infections.

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Declaration

With this statement I declare that there were no earlier attempts for granting of a doctorate. Furthermore, this thesis was not previously submitted to another academic institution. I have independently completed the above thesis. The thoughts taken directly or indirectly from external sources are properly marked as such.

Hamburg, October 21, 2010 Christina Stacke