universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Expression of Recombinant Flavivirus E Proteins

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

Verfasserin:	Beate Geller
Matrikel-Nummer:	9809785
Studienrichtung /Studienzweig (lt. Studienblatt):	A490 Molekulare Biologie
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Wien, am 31. August 2009

Danksagung

Diese Arbeit wurde an der Medizinischen Universtität Wien in der Forschungsgruppe von O. Univ. Prof. Dr. Franz X. Heinz am Klinischen Institut für Virologie durchgeführt.

Ich möchte mich bei Univ. Prof. Dr. Heinz für die Möglichkeit in seiner Gruppe zu arbeiten herzlich bedanken. Während dieser Zeit konnte ich viele wertvolle Erfahrungen für meine Zukunft sammeln. Bei Gabriel O´Riordain bedanke ich mich für das Korrekturlesen meiner Arbeit.

Ganz speziell danke ich meinen Eltern und meiner Familie die mir meine Ausbildung ermöglicht haben und mir Kraft, Liebe und Unterstützung während meiner gesamten Studienzeit gegeben haben. Ein großes Dankeschön auch an all meine Freunde und ArbeitskollegenInnen die immer ein offenes Ohr für mich hatten und mir mit Rat und Tat zur Seite gestanden sind.

Vielen Dank

Zusammenfassung

Frühsommer-Meningoenzephalitis Virus (FSME-Virus) ist ein human-pathogenes Virus aus der Familie *Flaviviridae*. Flaviviren sind einzelstrang-RNA-Viren, die von einer Hüllmembran umgeben sind. Das Glycoprotein E an der Virusoberfläche ist essentiell für das Eindringen des Virus in die Wirtszelle. Nachdem das Virus über Endocytose von der Zelle aufgenommen wurde, bewirkt die Ansäuerung des Endosominneren die Fusion der viralen mit der endosomalen Membran. Dieser Fusion liegt eine Konformationsänderung des E Proteins zu Grunde, welches im nativen Zustand ein Dimer darstellt und sich bei saurem pH irreversibel in ein Trimer umlagert. Obwohl die atomaren Strukturen des C-terminal-verkürzten E Proteins bekannt sind, ist der Fusionsmechanismus im Detail noch nicht aufgeklärt. Biochemische Daten haben gezeigt, dass die „Stamm-Anker-Region", welche in den gelösten Kristallstrukturen fehlt, vermutlich eine wichtige Rolle im Fusionsprozess spielt. Um die Funktion der „Stamm-Region", welche das sE mit dem Transmembrananker verbindet genauer zu untersuchen wurden im Rahmen dieser Diplomarbeit hoch aufgereinigte FSME-Virus E Proteine, welche die „Stamm-Region" beinhalten, rekombinant hergestellt. Die produzierten Proteine sollen später in ihrer trimeren, postfusionären Form kristallisiert werden, um die Rolle des „Stammes" während des Fusionsprozess aufklären zu können. Für diese Zwecke wurde das Protein TBEsE H1/H2_dstrep, das den gesamten Stamm und TBEsE H1_dstrep, das nur Teile des Stammes beinhaltet, in stabil transfektierten *Drosophila Z*ellen exprimiert. TBEsE H1_dstrep wurde in seiner dimeren Form exprimiert und wies eine Reinheit von 70-95% nach der Aufreinigung auf. Das aufgereinigte rekombinante Protein wurde mit saurem pH behandelt, was zur Umlagerung in den trimeren Zustand führte. Dieses Ergebnis ermöglicht eine weitere Verwendung des Proteins für Kristallisierungsstudien.

Abstract

Tick-borne encephalitis virus (TBEV) is an enveloped virus and an important human pathogen, which belongs to the family *Flaviviridae*. Flaviviruses, like other enveloped viruses, enter cells via a membrane fusion reaction driven by conformational transitions in the major envelope glycoprotein E. This fusion mechanism is triggered by the acidic pH in endosomes after virus uptake by receptor-mediated endocytosis. Although the dimeric pre- and trimeric postfusion forms of a C-terminally truncated form of E (sE) have been determined by atomic resolution, some steps of the fusion mechanism are still unexplained. The crystallized, truncated E proteins lack the so-called stem-anchor region, which has been hypothesized to play an important role in the fusion process by bringing the host and viral membrane into close proximity through a so-called zippering action. This work focused on the preparation of highly purified TBEV E proteins that include the stem region for the structural determination of the trimeric post-fusion form. Knowledge of this structure should elucidate the role of the stem during membrane fusion. This work reports the expression of the proteins: TBEsE H1/H2_dstrep – containing the whole stem region - and TBEsE H1_dstrep – containing only parts of the stem, in stable transfected *Drosophila* cell lines. TBEsE H1_dstrep was purified using Strep tag technology. The oligomeric form, TBEsE H1_dstrep, was dimeric and we were able to obtain it at a high purity of 70-95%. The exposure to low pH allowed the transition into the trimeric post-fusion form which will be used in subsequent crystallization studies.

List of Abbreviations

FSME – Frühsommer-Meningoenzephalitis; TBEV – tick-borne encephalitis virus; sE – soluble E protein; YFV - yellow fever virus; JEV – japanese encephalitis virus; WNV – west nile virus; DenV – dengue virus; ORF - open reading frame; C – capsid protein; prM – precursor of the membrane protein; M – membrane protein; E – envelope protein; ER – endoplasmatic reticulum; RNA – ribosonucleic acid; TGN – *trans*-Golgi network; DI/II/III – domain I/II/III; CHO – carbohydrate side chain; cd loop / fp loop – internal fusion peptide loop; cryo-EM – cryo-electron microscopy; SLEV – St. Lous encephalitis virus; MVEV – Murray Valley encephalitis virus; H1 - helix 1 of the stem; H2- helix 2 of the stem; TM1 – transmembrane helix 1 of the membrane anchor; TM2 – transmembrane helix 2 of the membrane anchor; His323 – histidin residue 323; *E.coli* – *Escherichia coli*; *Pichia pastoris* – *P.pastoris*; Sf9 - *Spodoptera frugiperda* 9; S2 – Schneider 2; DES – *Drosophila* expression system; pMT – metallothionin promoter; TBE buffer - Tris-(hydroxymethyl)-aminomethan Borat Ethylenediaminetetraacetic acid; PCR – polymerase chain reaction; DNA - Deoxyribonucleic acid; dATP - 2'-deoxyadenosine 5'-triphosphate; dCTP - 2'-deoxycytitine 5'-triphosphate; dGTP - 2'-deoxyguanosine 5'-triphosphate; dTTP - 2'-deoxytyrosine 5'-triphosphate; ddH_2O – double destilled water; LB medium – Luria Bertani medium; SOC - Super Optimal broth with Catabolite repression; HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LSB – Laemmli sample buffer; TAN – Triethanolamine NaCl; MES – 1-Morpholino Etahnesulfonic acid; FPLC- fast protein liquid chromatography; ELISA –enzyme linked immunosorbent assay; SDS - sodium dodecylsulfate; PBS - phosphate buffered saline; Ig- Immunflobulin; DOC – deoxycholic acid; TCA – tricholoroacetic acid; APS – ammonium persulfate; pT350 – expression plasmid; pCoBlast – selection vector; PSA-penicillin streptomycin antimycoticum; DMSO – Dimethylsulfoxid; cDNA – complementary DNA; BipSS – Drosophila Bip secretion signal; Bip - Drosophila homologue of the immunoglobulin binding chaperone protein; EK – enterokinase; FBS – fetal bovine serum; kDa – kilodalton; TBEsE H1_dstrep CC-SN – TBEsE H1_dstrep from the S2 cell culture supernatant; TBEsE H1_dstrep purif. – TBEsE H1_dstrep after purification; TBEsE H1_dstrep acid. – TBEsE H1_dstrep after purification and low pH treatment; D – dimers; T – trimers; FACS – fluorescence-activated cell sorting.

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1. Introduction

1.1. Flaviviruses

The genus *Flavivirus* belongs to the virus family *Flaviviridae* and contains more than 70 different viruses. Most of them are transmitted to their vertebrate hosts by infected ticks or mosquitoes and they can cause serious, sometimes fatal infections in humans [1]. The most important human pathogenic flaviviruses are yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), dengue virus (DenV) and tick-borne encephalitis virus (TBEV) [2].

1.1.1. Flavivirus genome

The genome, a positive stranded RNA of about 10,800 nucleotides, consists of a single open reading frame (ORF) and is flanked by non-coding regions at its 5′ and 3′ ends (Figure 1). It codes for three structural proteins, the capsid protein (C), the precursor of the membrane protein (prM/M) and the envelope protein (E). The genomic RNA is not polyadenylated, but contains a cap structure at its 5′end (m^7G5′ppp5′A) [3].

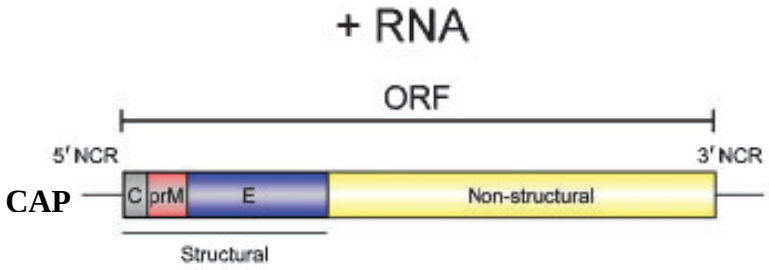

Figure 1. Schematic representation of the flavivirus genome organization.
The positive-stranded RNA genome is unsegmented. The 10.8-kDa genome has one open reading frame (ORF) encoding the structural proteins C, prM/M and E and a set of seven non-structural proteins. The ORF is flanked by non-coding regions (NCRs) at both ends. The genomic RNA is not polyadenylated but has a CAP structure at its 5′end (modified from [4]).

1.1.2. Flavivirus lifecycle

The assembly of flavivirus particles takes place at the endoplasmatic reticulum (ER) (Figure 2A), where the nucleocapsid, formed by the genome in complex with the capsid proteins, is packed into non-infectious immature virions (Figure 2B). These particles show a spiky surface that is formed by a heterodimeric complex of prME preproteins [5]. During the maturation process, which takes place during transportation through the *trans*-Golgi network, the particle surface undergoes a conformational change. This change is triggered by low pH, which enables the maturation cleavage of the prM protein by a host-encoded furin-like protease. This cleavage causes major rearrangements of the E proteins leading to mature, infectious particles with a smooth particle surface morphology (Figure 2B) [6, 7]. Once cleaved, the pr peptide remains associated with the particle until its release into the neutral pH of the extracellular environment [8]. The E proteins, on the surface of mature virions, are in a dimeric metastable conformation, and are primed to mediate low pH triggered membrane fusion in the endosomes after uptake by receptor-mediated endocytosis (Figure 2A).

After the virus uptake, the acidic pH in the endosome induces strucutral alterations which lead to membrane fusion and the release of the nucleocapsid into the cytoplasm. There, the positive-stranded RNA genome is translated to initiate virus replication.

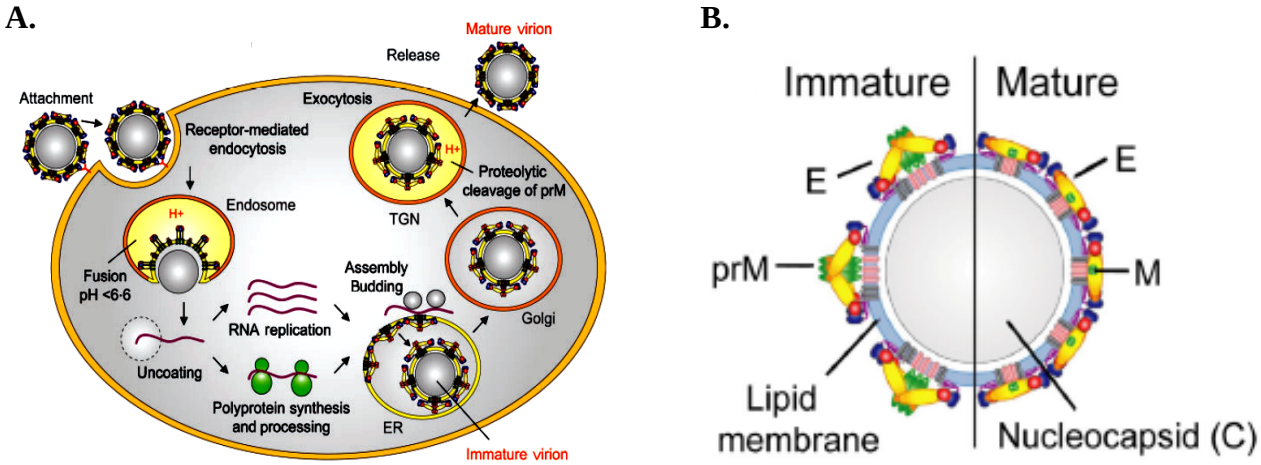

Figure 2. Schematic of the flavivirus lifecycle (A) and organisation of the flavivirus particle (B). (A) Virus entry occurs by receptor-mediated endocytosis, acidic pH in the endosome induces structural rearrangement in the E protein, leading to membrane fusion and the release of the genome into the cytoplasm. Synthesized RNA and structural proteins are assembled in the endoplasmic reticulum (ER) and lead to the formation of immature (prM-containing) particles, which are transported through the exocytic pathway. Shortly before release through exocytosis, the particle is cleaved by the host protease furin which leads to the formation of mature, infectious particles (modified from [4]). (B) Schematic representation of a flavivirus particle in its immature (prM-containing) and mature form after proteolytic cleavage of prM [9].

1.2. Structural perspectives of the flavivirus fusion machinery

1.2.1. Structure of the E protein pre-fusion form

The E protein of flaviviruses consists of about 500 amino acid residues and is glycosylated in some flaviviruses. The atomic structures of the E proteins from TBEV, WNV, DenV2, and DenV3 have been determined by X-ray crystallography using E proteins purified from infectious virus (TBEV) or expressed as recombinant proteins in insect cell expression systems (DenV2, DenV3, and WNV) [10-13]. In all cases, a soluble form of E (sE) was used, lacking the 100 C-terminal amino acids, including the so-called stem and the transmembrane helices. The basic structural feature – an antiparallel orientated homodimer – is common in most flavivirus E proteins crystallized so far, although they have less then 40% amino acid identity. Each monomer of the E protein is composed of three distinct domains – domain I (DI), domain II (DII) and domain III (DIII). DI (Figure 3, indicated in red) is the central β-barrel domain, which lies in parallel to the viral membrane and contains the amino terminus. In many of the flaviviruses, it carries the N-linked carbohydrate side chain (CHO). Two long disulfide stabilized loops emanate out of this central domain and interact to form DII (Figure 3, indicated in yellow). This elongated dimerization region provides inter-subunit contacts ("dimerization domain"). DIII (Figure 3, indicated in blue) flanks DI on the opposite side and has an Ig-like β-barrel fold. DIII constitutes the carboxy terminus of the crystallized fragment [10]. The three domains are associated by flexible junctions. They are capable of hinge-like motions, which are important in the conversion of immature to mature virions [13] and during the fusion process [14, 15].

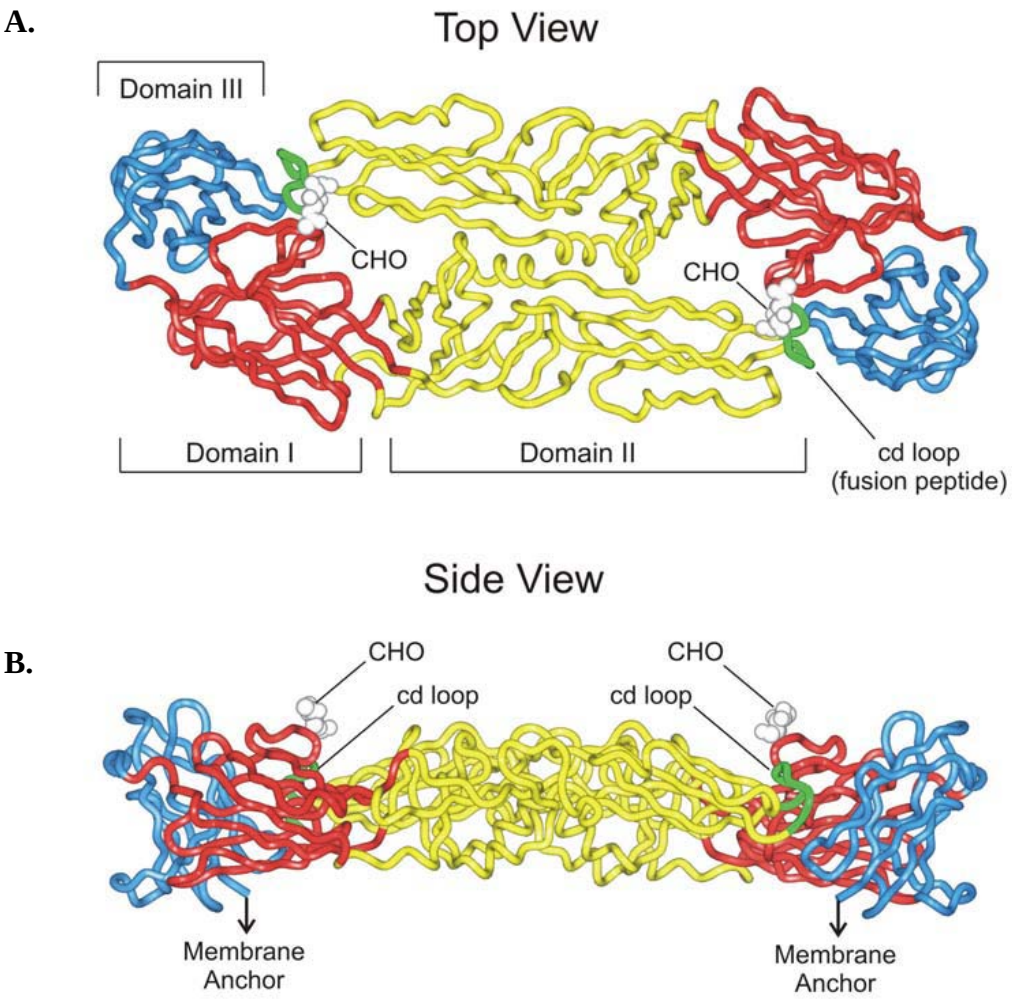

Figure 3. Ribbon diagram of the TBE virus E protein dimer in top view (A) and side view (B). Each of the monomers is composed of three distinct domains, domain I (red), domain II (yellow), domain III (blue). The cd loop at the tip of domain II is highlighted in green. The positions of the carboxy terminal stem-anchor regions are indicated, and the first residue of the carbyhydrate (CHO) attached to Asn^{154} is shown [10].

The cd loop structure at the tip of DII (Figure 3, indicated in green) plays a significant role in the fusion process. It is almost fully conserved among all flaviviruses. The cd loop functions as an internal fusion peptide during membrane fusion and is therefore also called fusion peptide loop (FP loop) [16]. At neutral pH, the fusion loop is buried in a hydrophobic pocket, which is formed by DI and DIII of the opposite subunit and an oligosaccharide attached

to DI. This shields the fusion loop from interactions with membranes in native configurations [10, 11, 13, 17]. The E protein also mediates receptor binding, in additionl to its function during the fusion process, which leads to the uptake of the virus into the host cell. [18-25]. To date, the crystal structure of the full length E protein is not known.

1.2.2. Structural organization of flavivirus particles

Cryo-electron microscopy (cryo-EM) and image reconstructions of mature DenV, YFV and WNV revealed an icosahedral, so-called herringbone organization of 180 E proteins, consisting of 30 rafts of three parallel E protein dimers (Figure 4).

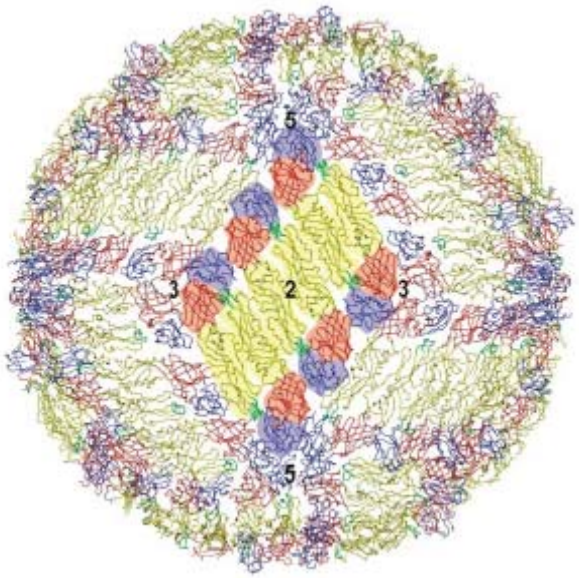

Figure 4. The mature flavivirus particle. Packing of the E proteins in the mature virus, showing the herringbone pattern. One of the 30 rafts, each containing three parallel dimers, is highlighted. Domains I, II, and III are colored red, yellow, and blue, respectively, with the fusion peptide in green. Symmetry axes are labeled 5 (fivefold), 3 (threefold) and 2 (twofold) respectively modified from [13].

This arrangement makes the viral membrane largely inaccessible from the viral exterior and DIII protrudes from the smooth viral surface.

Structural data from cryo-EM reconstructions revealed also that the flavivirus E protein contains a double transmembrane anchor (Figure 5), exposing the

carboxy termini at the external surface of the membrane. The two amphipathic α-helices of the stem are oriented parallel to the viral membrane. Through their hydrophobic sides, they are half buried in the outer leaflet of the lipid bilayer (Figure 5) [26].

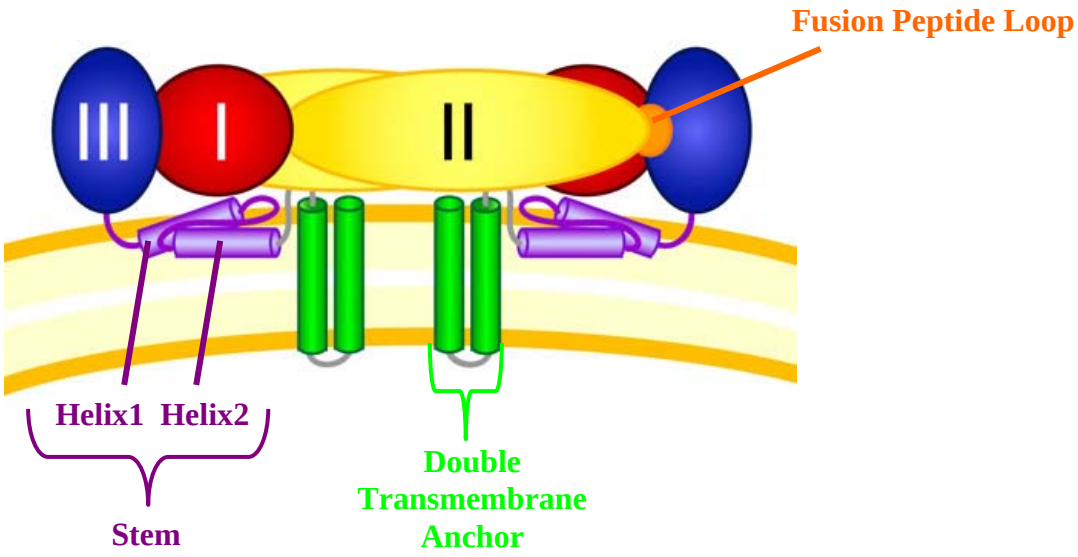

Figure 5. Schematic diagram of the TBEV E protein homodimer containing stem and anchor regions. Illustration of the E protein homodimer of the mature virus particle, lying flat on the virus surface. The domains of the E protein homodimer are indicated in the colors red (Domain I), yellow (Domain II) and blue (Domain III). The double transmembrane anchor is indicated in green and the stem region, containing the two α-helices (Helix1 and Helix2), indicated in purple, are oriented parallel to the viral membrane and are half buried in the outer leaflet of the lipid bilayer (illustration modified, obtained from Stiasny, K.).

1.2.3. Structure of the E protein post-fusion form

Studies on flavivirus membrane fusion have shown that this process is triggered by a slightly acidic pH with a threshold around 6.6. This fact suggests that fusion of the viral with the host membrane can already occur in early endosomes. Such studies were done using cell-cell fusion or virus-liposome fusion assays with DenV, JEV, St. Louis encephalitis virus (SLEV), WNV, Murray Valley encephalitis virus (MVEV) and TBEV [27-37]. So far, detailed kinetic data have only been published for TBEV [37]. Experimental data demonstrated that, during the fusion process, exposure to acidic pH leads to a complete oligomeric rearrangement of the E proteins in the viral membrane. The dimeric pre-fusion form of the E protein converts to a trimeric post-fusion form [37]. This post-fusion form was shown to be significantly more thermostable than the E dimers (Figure 6A) [38]. Up to now, no structural data exist for the full-length E trimer but X-ray crystal structures are available for sE trimers (Figure 6B) [14], which were produced by trimerization at low pH values in the presence of liposomes [39].

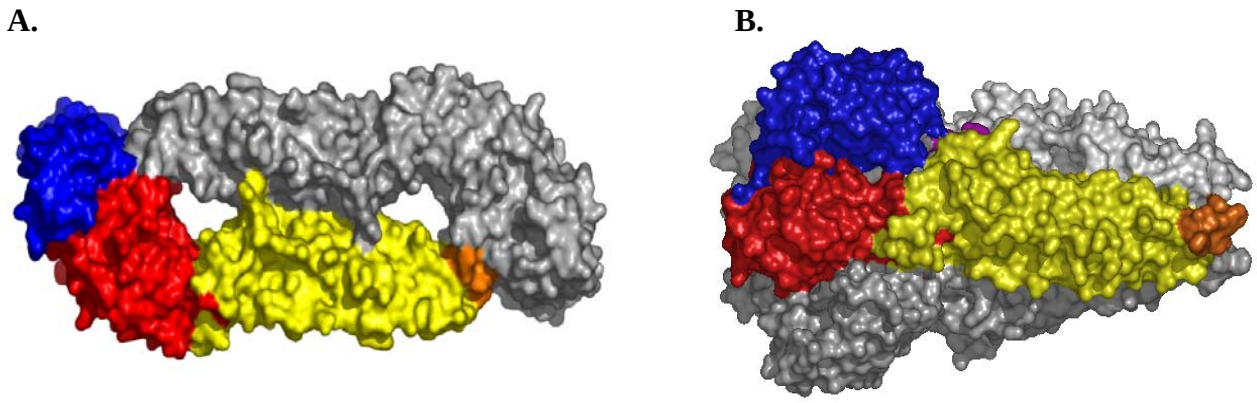

Figure 6. Surface representation of the crystal structure of the TBEV E protein dimer (A) and the E protein trimer (B). The domains of one monomer are highlighted, indicating domain I (red), domain II (yellow) and domain III (blue). The fusion peptide loop is colored in orange and can be seen to be covered in the pre-fusion, dimeric structure and to be exposed in the post-fusion trimeric structure. The crystallized glycoproteins lack the stem and anchor region [6, 10].

The structural analyses revealed that the domains change their arrangement relative to each other but are not altered in their structural organization. This can be seen by comparison of a single monomer of the TBEV E protein in its native pre-fusion (Figure 7A) and post-fusion form (Figure 7B) [14].

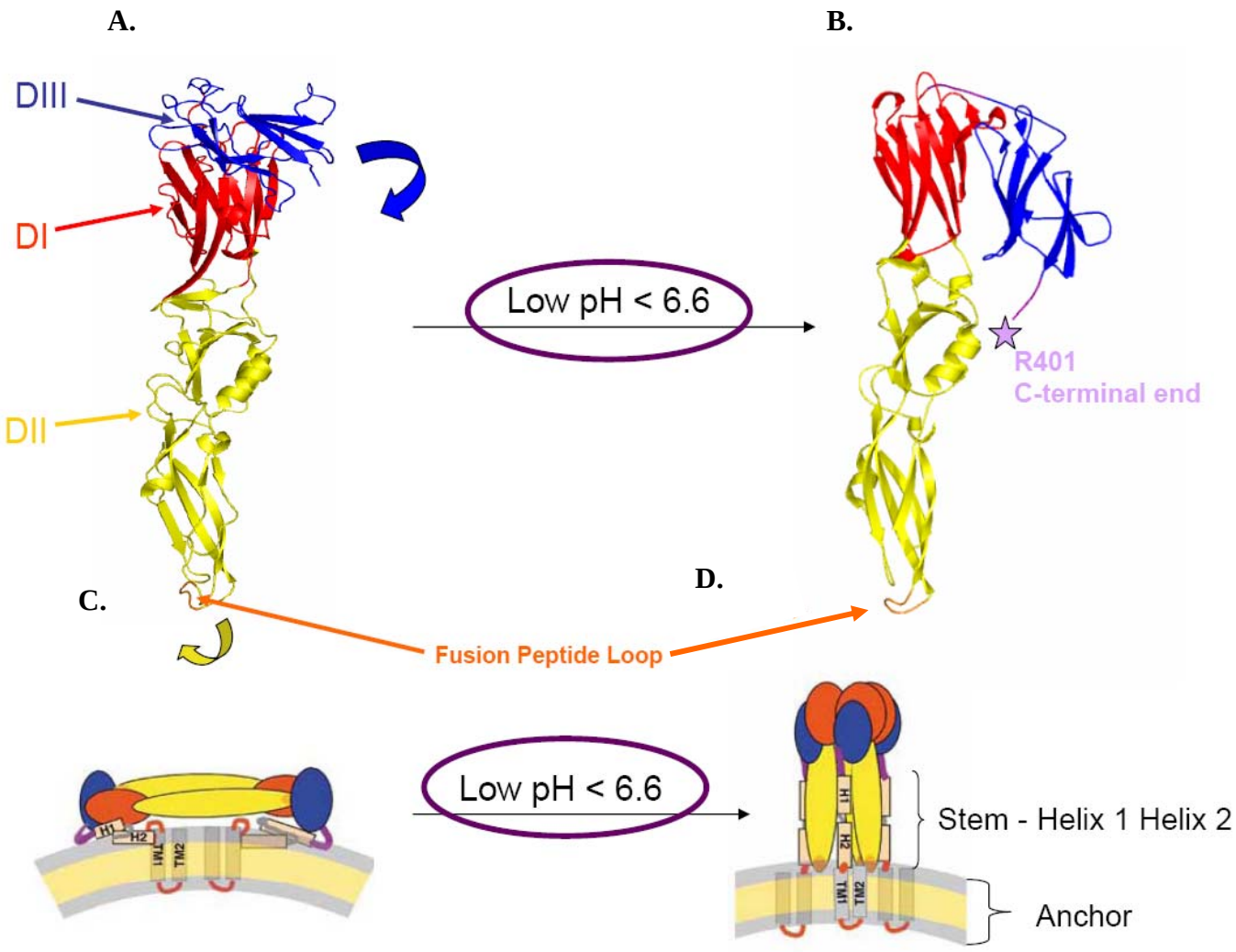

Figure 7. Ribbon diagram (A-B) and schematic (C-D) of TBEV E proteins in their pre- and post-fusion conformations.
(A-B) Curved arrows indicate the movements of DII (yellow arrow) and DIII (blue arrow) to reach their positions in the post-fusion conformation. The open star indicates the position of the last residue of the crystallized sE fragment [14].
(C) Side view of the full-length E homodimer in its pre-fusion conformation.
(D) Side view of the full-length E trimer in its post-fusion conformation.
Color codes: DI, red; DII, yellow; DIII, blue; fusion peptide loop, orange; helices 1 and 2 in the stem indicated as H1 and H2; C-terminal transmembrane helices of the membrane anchor, indicated as TM1 and TM2 [4]

The ribbon diagram (Figure 7) depicts the conformational switch, including a reorganization of the molecule. In the course of this reorganization the anti-parallel dimer, oriented horizontally with respect to the viral membrane (Figure 7C), switches into a perpendicularly oriented trimer (Figure 7D). In the trimeric, post-fusion conformation, the monomeric subunits are arranged in parallel. It is remarkable that the polypeptide chain and the structural integrity of the three domains remain largely unchanged. These transitions are only possible because of the hinge-like structures and the flexibility at the junctions between DI and DII and between DI and DIII. This flexibility is important to facilitate the reorientation of the domains relative to each other, which is the major change during the dimer-trimer conversion (Figure 7A-B). The position of DII relative to DI shows minimal differences in both forms. In contrast, DIII adopts a completely new position. It relocates from the end of the native E protein rod to the side of DI (Figure 7A-B). The interface of DI-DIII of the native E monomer possesses tight interactions, involving a number of van der Waals contacts, salt bridges and hydrogen bonds. These tight junctions must be broken to allow domain movement. It has been shown that the protonation of one of the conserved histidines (His323) at this interface is an essential driving force for this dissociation [9]. After the folding back of DIII, the E protein structure acquires a hairpin-like orientation. The position of the carboxy terminus of DIII indicates that the stem follows the grooves in the full-length post-fusion structure. The grooves are generated at the interface between the DIIs, leading to the juxtaposition of the membrane anchor and the membrane inserted FP loops, as schematically shown in figure 7D.

In modeling studies, helix 1 of the stem was fitted into the existing trimeric, post-fusion structure (Figure 8). It was shown that the best match for the "stem" region is obtained when conserved residues of helix 1 are directed toward the groove formed by the two DIIs [14] .

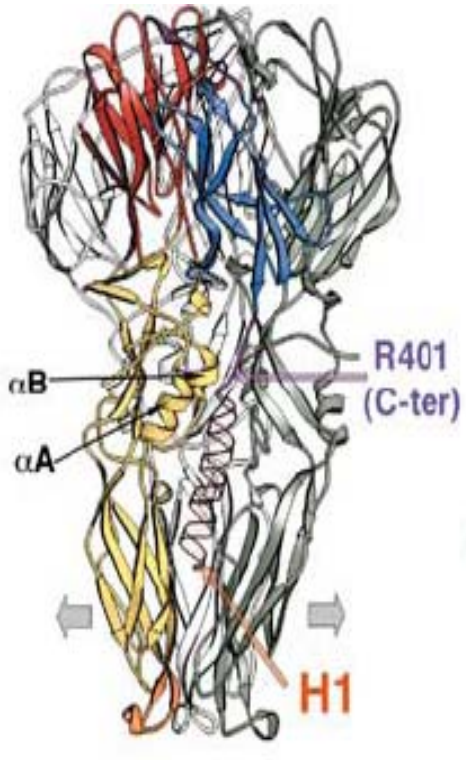

Figure 8. Ribbon representation of the side view of the sE trimer with the modelled helix 1 (H1) of the stem. The αA and αB helices of domain II are labeled. The modeled H1 helix is indicated in a semi-transparent purple ribbon, showing its path in between domain II. In this modeling study, it was postulated that the presence of H1 will force the tips of domain II to move apart (indicated by the gray arrows at either side of the sE trimer) [14]. Color codes: Domain I (red); Domain II (yellow); Domain III (blue).

1.3. The mechanism of flavivirus fusion

The proposed membrane fusion mechanism of flavivirus is a multistep process. Most of the intermediate stages and structures are still only hypothetical and need further experimental confirmation (Figure 9A-D).

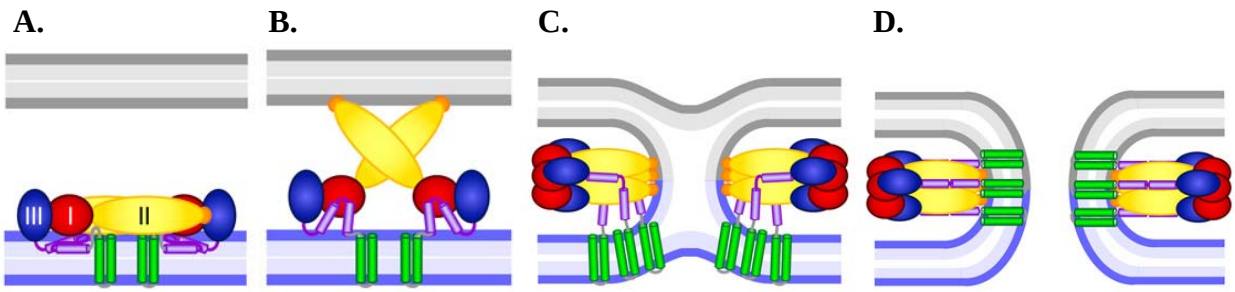

Figure 9. Model of the flavivirus membrane fusion mechanism. (A) Metastable E dimer in mature virions. (B) Dissociation of the E dimers at acidic pH, outward projection of E monomers, and interaction of the FP with the target membrane. (C) Trimerization, DIII relocation, and "zippering action" of the stem. (D) Formation of the post-fusion trimer and opening of the fusion pore. Red, DI; yellow, DII; blue, DIII; orange, FP; purple, stem (linker between DIII and the transmembrane anchors); green, transmembrane anchors

It was shown that the flavivirus membrane fusion initiates with the dissociation of the E dimers into monomers (Figure 9B) [36]. The E monomers are brought in a position which makes an interaction between target membrane and the fusion loops possible (Figure 9B). Trimerization of the E protein occurs by the relocation of DIII and it is believed that a "zippering action" of the stem brings the host cell and viral membranes in close proximity, which leads then to the formation of a lipid stalk (hemifusion intermediate) (Figure 9C). In the last step the post-fusion trimer is formed leading to the opening of the fusion pore (Figure 9D) [4].

1.4. Expression of recombinant flavivirus E proteins

Different methods have been used for the expression of flavivirus proteins previously.

1.4.1. Bacterial expression systems

DenV envelope protein was expressed, using *Escherichia coli* (*E.coli*) as host cells [40]. With this expression system, it was possible to yield up to 2 mg of purified recombinant E protein from 1 liter bacterial culture. The achieved recombinant protein had a correct size of about 55kDa, but was non-glycosylated.

1.4.2. Mammalian expression systems

Hela and Cos cell lines are mammalian cells, which were used for the expression of flavivirus particles and subviral particles [41-47].

1.4.3. Yeast expression systems

Expression of recombinant flavivirus proteins in *Pichia pastoris* (*P.pastoris*) showed extensive proteolytic degradation of the recombinant E protein [40]. Expression of the WNV E protein was performed using the yeast two-hybrid system [48].

1.4.4. Insect cell expression systems

Recombinant JEV prM and E proteins were produced using Sf9 insect cells. The intracellular E protein was shown to be protective in mice against lethal JEV challenges [49, 50].

Bac-to-Bac® TOPO® Expression System (Invitrogen Corporation, Carlsbad, CA) was used to generate a recombinant baculovirus, providing some advantages in the expression of recombinant proteins. This method reduces the need for multiple rounds of plaque purification as well as requiring less than two weeks to identify a recombinant baculovirus. The crystallization of the WNV sE protein was possible because of its successful expression in this system [51].

Drosophila melanogaster Schneider (S2) cells have become increasingly utilized over the past few years for the expression of heterologous proteins.

Drosophila expression system (DES) was used to produce, for example, DenV E protein [11]. In this system, a simple plasmid vector for the expression of heterologous proteins, carrying a metallothionin (pMT) promoter for heavy metals inducible expression [52, 53], is the basic tool of this expression system. High levels of protein expression can be achieved by the co-transfection of the expression plasmid with a plasmid encoding resistance to blasticidin for selection. After three weeks of selection, a stable cell line is obtained that expresses the protein of interest. Proteins of prokaryotic, eukaryotic and viral origin expressed in this system were properly processed and biologically active [54-56]. Based on this evidence, DES has become a very effective gene expression system and it was selected in this work to express different truncated recombinant TBEV E proteins, including the stem region.

2. Objectives

2.1. Definition and scientific question of the project

The objective of this study was the expression, purification, and conversion into the trimeric postfusion form of two different truncated TBEV E proteins, (i) E protein missing helix 2 of the stem and the whole transmembrane anchor (TBEsE H1_dstrep) and (ii) E protein missing only the transmembrane anchor (TBEsE H1H2_dstrep).

The trimeric post-fusion form of these proteins will then be used in additional studies to determine their X-ray crystal structure.

Knowledge about the X-ray structure of these post-fusion forms of the flavivirus E protein will help with explaining the impact of the stem region during the membrane fusion process, thus contributing significantly to our understanding of the fusion mechanism.

3. Materials and Methods

The chemicals and media which were used in this work were purchased from Sigma, Fluka, Qiagen, Promega, Invitrogen, Schneider and Lonza. Restriction enzymes were obtained from Fermentas.

3.1. Manipulation of nucleic acids

3.1.1. Buffers and oligonucleotides

10x TBE buffer	108g Tris/HCl
	55g Boric acid
	8.3g EDTA (Tritiplex III)
	Made up to 1 liter with ddH_2O
	adjusted to pH 8.0
DNA loading buffer	30% (v/v) glycerol
	0.25% (w/v) bromphenol blue
	0.25% (w/v) xylene cyanol

Table 1. List of primers. Primers were purchased from VBC Biotech (Austria). The respective primer for each construct contained the cleavage sites for *Bsp120I* (red) and *BglII* (blue).

OLIGONUCLEOTIDE	PRIMER SEQUENCE
Forward [3´- 5´]	TTTAGATCTGCAACGGTGAGGAAAGAAAG
Reverse [5´- 3´] TBEsE H1	AAGGGCCCGTGCTCTCCTATCACTGTCA
Reverse [5´- 3´] TBEsE H1H2	AAGGGCCCGATGCTGTTGAAAGCGCCAC

3.1.2. Polymerase chain reaction (PCR)

The desired constructs were amplified using the method of Saiki [57]. For the reaction mixture, 10-50ng DNA (template) in 10µl water, 1µl each of a forward and a reverse specific primer (100µM), 16µl nucleotide mix (1.25mM dATP, dCTP, dGTP, dTTP), 10µl high fidelity buffer [10x] and 1µl Advantage high fidelity-2-polymerase (Clontech-Takara Europe, France) were mixed and filled up to a final volume of 100µl with water. The reactions were performed in a thermal cycler (PerkinElmer).

3.1.3. Digestion and restriction enzymes

Digestion of DNA was performed using enzymes purchased from Fermentas (Canada) according to the manufacturer´s protocols. For efficient digestion, 2-10µg DNA as well as the appropriate volume of enzyme and buffer was incubated for 60 minutes at 37°C.

3.1.4. Agarose gel electrophoresis

Agarose gel electrophoresis was performed in a BioRad electrophoresis cell (BioRad Laboratories, Hercules, CA). A 1% (w/v) solution of agarose gel was prepared by dissolving the agarose (Serva GmbH, Heidelberg, Germany) in 1xTBE buffer with Ethidium bromide to a final concentration of 0.0025mg/ml. The gel solution was poured into a casting form provided with a gel comb for polymerisation. 1xTBE buffer was used as an electrophoresis buffer. The DNA samples were mixed with 20% DNA loading buffer and pipetted into the wells formed by the comb in the gel. Electrophoresis was carried out for 20min at 110V. The detection of the DNA fragments was done using a trans-illuminator (wavelength 302nm) combined with a camera for gel imaging.

3.1.5. DNA gel extraction and PCR-fragment purification

The extraction of the DNA from the gel and PCR purification protocols were performed using the QIAquick gel extraction kit and QIAquick PCR purification kit (Qiagen, Valencia, CA), according to the manufacturer´s instructions.

3.1.6. DNA ligation

DNA fragments were ligated using T4 DNA ligase (30 Weiss U/µl, Fermentas, Canada). An insert:vector ratio of 19:1 was used to ensure high ligation efficiency. The ligation reaction was performed overnight at room temperature, according to the manufacturer´s protocol. After ligation, the proteins were removed using the PCR purification kit (Qiagen, Valencia, CA).

3.1.7. DNA sequencing

Sequencing was performed using fluorescence-labeled dideoxynucleotides as first described by Sanger [58]. The reaction was carried out with ABI Prism Big Dye Terminator Cycle Sequencing Kit. 0.5µg DNA was resuspended in 10µl water and mixed with 6µl specific primer (0.1M) and 4µl Big Dye Ready mix (containing nucleotides and polymerase). The reaction was performed in a thermal cycler (PerkinElmer) with the following settings: 20s, 96°C →[25x 90s, 96°C → 15s, 50°C → 4min, 60°C → 4°C ∞]

3.2. Bacterial cultures

3.2.1. Media and buffers

LB medium
10g Bactotryptone
5g Yeast extract
10g NaCl,
adjusted to pH 7.0
made up to 1L with ddH_2O
autoclaved

LB agar medium:
LB medium
20g/l Agar
made up 1L with ddH_2O
autoclaved

SOC Medium
20g Bactotryptone
5g Yeast extract
0.5g NaCl
made up 1L with ddH_2O
autoclaved,
10ml of 1M $MgCl_2$
10ml of 1M $MgSO_4$
20ml of 1M Glucose
} added prior to use
sterile filtered

Antibiotic stock soloution:
Ampicillin
100mg/ml in ddH_2O stock solution,
sterile filtered
final concentration: [100µg/ml]
stored at -20°C

3.2.2. Bacterial strains:

The following bacterial strain purchased from Invitrogen (Invitrogen Corporation, Carlsbad, CA) was used:

Table 2. Bacterial strain for cloning.

HOST STRAIN	GENOTYPE
Escherichia coli DH5α chemically competent cells	*F*-, φ 80Δ lacZΔM15Δ(lacZYA-argF)U 169 deoR recA1 endA1 *hsdR17(rk-mk+)* phoA supE44 thi-1 gyrA96 relA1

3.2.3. Transformation of chemically competent cells

Chemically competent cells were thawed on ice, 20µl of ligation product was mixed with 50µl of the cells followed by incubation on ice for 20 minutes before heat shock at 42°C for 30 seconds and then placed on ice again for 2 minutes. 1ml SOC medium was added and the cells were incubated for one hour at 37°C shaking at 225 rpm in an incubator. 100µl from the transformation mix were spread on LB agar plates, containing the appropriate antibiotic, and incubated at 37°C overnight.

3.2.4. Overnight culture

A single colony, picked from a LB-agar plate was grown overnight in a shaking incubator at 37°C and 225 rpm in 5ml LB medium containing the appropriate antibiotic.

3.2.5. Isolation of plasmid DNA from transformed *E.coli*

The Plasmid Mini Kit (Qiagen, Valencia, CA) was used for DNA isolation to identify colonies containing the correct plasmid. The cells were harvested by centrifugation and purified by the alkaline lysis method following the manufacturer´s instructions.

3.3. Protein chemistry

3.3.1. Materials and buffers

2 x HEPES Buffered Saline	0.05M HEPES 0.0015M Na_2HPO_4 0.280M NaCl made up with ddH_2O, adjusted to pH 7.1
$CaCl_2$	2M $CaCl_2$ made up with ddH_2O, stored at -20°C
Wash Buffer W	0.10M Tris/HCl 0.15M NaCl 0.001M EDTA made up with ddH_2O, adjusted to pH to 8.0
Elution Buffer E	0.10M Tris/HCl 0.15M NaCl 0.001M EDTA 0.0025M Biotin made up with ddH_2O adjusted pH to 8.0

Phosphate Buffered Saline	16.0g NaCl 0.4g KCl 0.4g KH_2PO_4 5.8g Na_2HPO_4 x 12 H_2O, made up with 2L ddH_2O adjusted to pH to 7.4
Carbonate Buffer	1.59g Na_2CO_3 2.59g $NaHCO_3$ made up with 0.5L ddH_2O adjusted to pH to 9.6
Laemmli sample buffer (LSB)	0.125M Tris pH 6.8 2% SDS 10% Glycerine, 0.0025% Bromphenol blue 5% β-Mercaptoethanol
TAN-Buffer	0.05M Triethanolamine 0.10M NaCl made up with ddH_2O adjusted pH to 8.0
MES-Buffer	0.05M 1-Morpholino Ethanesulfonic acid 0.10M NaCl made up with ddH_2O adjusted pH to 5.5

3.3.2. Protein purification

Purification was performed with the Strep-tag technology using the chromatographic ÄKTA fast protein liquid chromatography (FPLC) system (GE Healthcare, UK Limited). The cell culture supernatant was clarified by centrifugation (10,000 g, 45 minutes, 4°C) and frozen at -20°C. For purification, 1.5 liters were thawed, centrifuged (10,000 g, 30 minutes, 4°C) and filtrated through a 0.22µm filter. After this procedure, the solution was concentrated by ultra filtration (Sartorius, Germany) according to the manufacturer´s protocol. The Strep-tag column was pre-washed and equilibrated by washing with buffer W, with twice the column volume. The supernatant was loaded onto the column (2ml/min). The column was washed with 25ml buffer W until a constant baseline was reached. The elution of the protein was performed with buffer E.

3.3.3. Denatured enzyme linked immunosorbent assay (ELISA)

Nunc microtiterplates were coated at room temperature in a humid chamber with polyclonal guinea pig anti-TBEV-Immunglobulin (2.5µg/ml in carbonate buffer pH 9.6). The samples were denatured for 30 minutes with 0.4% SDS at 65°C. Further dilutions were carried out in PBS pH 7.4 + 2%Tween-20 + 2% filtered sheep serum filtered. Aliquots of 50µl of the diluted antigen were applied to the microtiterplates. After incubation for 2 hours at 37°C in a humid chamber, the plates were washed three times with PBS pH 7.4 + 0.05% Tween-20. 50µl of a TBEV-specific polyclonal rabbit antibody was added and after incubation in a humid chamber at 37°C, the plates were washed again three times with PBS pH 7.4 + 0.05% Tween-20. For detection of the bound antibody, 50µl of a peroxidase conjugated anti-rabbit Ig (horseradish peroxidase linked whole antibody from donkey, GE Healthcare, UK Limited) was used. After an additional hour of incubation at 37°C in a humid chamber, the plates were washed with PBS pH 7.4 + 0.05%Tween-20. 50µl of ortho-Phenylendiamine (Sigma-Aldrich, St. Louis, USA) in phosphate-citrate buffer pH 5.0 (1mg/ml) + 1µl/ml 30% H_2O_2 was used as a substrate. The developed colour was measured at 490nm extinction and the antigen concentration was determined using purified TBEsE H1 protein with a known protein concentration as standard.

3.3.4. Protein precipitation with deoxycholic acid (DOC) –Tricholoroacetic acid (TCA) for SDS-PAGE

Protein solutions were incubated with 0.015% DOC at room temperature for 10 minutes and afterwards precipitated with 5% TCA for 30 minutes at 4°C, followed by centrifugation (4,000 g, 20 minutes, 4°C, Multifuge 4KR, Heraeus). The pellet was washed three times with ice cold acetone (4,000 g, 10 minutes, 4°C) and dissolved in 20µl 1x sample buffer (LSB + β-mercaptoethanol).

3.3.5. SDS – Polyacrylamide gel electrophoresis with Coomassie staining [59]

The electrophoresis was performed at 200V using 0.75mm thick gels with a 15% separating gel and a 5% stacking gel.

Stacking Gel (5%)	40% Acrylamide-Bisacrylamide (37.5:1)	0.630ml
	1M Tris pH 6.8	0.630ml
	ddH_2O	3.660ml
	10% SDS	0.050ml
	TEMED	0.005ml
	10% Ammonium persulfate (APS)	0.025ml

Separating Gel (15%)	40% Acrylamide-Bisacrylamide (37.5:1)	3.75ml
	1M Tris pH 6.8	2.50ml
	ddH_2O	3.59ml
	10% SDS	0.10ml
	TEMED	0.01ml
	10% Ammonium persulfate (APS)	0.05ml

Buffer for Electrodes 288g Glycine
60g Tris
made up with 2L ddH_2O (= 5 x buffer)
shortly before use 0.1% SDS was added

Fixating Solution : 50% (v/v) ethanol in water with 10%(v/v) acetic acid

Ethanol 95% (v/v)	500ml
Acetic Acid	100ml

made up with water to a final volume of 1000ml

Coomassie staining solution:

0.1% (w/v) Coomassie blue R350
20% (v/v) Methanol
10% (v/v) Acetic Acid

Destaining solution:

50% (v/v) Methanol
10% (v/v) Acetic Acid

For Coomassie staining, the gel was first shaken in a fixing solution for 30 minutes at room temperature. Staining with the Coomassie solution was performed by shaking at room temperature for a further 30 minutes. The destaining was performed with the destaining solution, by shaking the gel at room temperature and changing the solution three times every 10 minutes. Finally the gel was destained overnight at room temperature by shaking in destaining solution. After destaining, the gel was dried for 2 hours, using a gel dryer (Model 543, BioRad) according to the manufacturer´s manual.

3.3.6. Liposomes

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids, Inc., Birmingham,Ala.) and 1-cholesterol (Sigma) were mixed in a molar ratio of 1:1:1 from stock solutions in chloroform. The mixture was dried to a thin film with a rotary evaporator and then dried further under high vacuum for at least 2 h. The lipid film was hydrated in 10 mM triethanolamine (pH 8.0)–140 mM NaCl and subjected to 5 cycles of freeze-thawing, followed by 21 cycles of extrusion through two 200-nm pore size polycarbonate membranes with a Liposofast syringe-type extruder (Avestin, Ottawa, Canada).

3.3.7. Low pH treatment of E proteins

10µg of the purified proteins at a concentration of 100 µg/ml in buffer E (100mM Tris/HCl, 150mM NaCl, 1mM EDTA, 5mM Desthiobiotin, pH 8.0) were mixed with liposomes in a ratio of 1 µg of E protein to 15 nmol of lipid and incubated for 5 min at 37°C. The samples were acidified using a buffer consisting of 0.35 M morpholineethanesulfonic acid (MES), which had been pre-titrated to yield the desired final pH of 5.4 when the sample was added. The mixture was incubated for 30 min at 37°C.

As a control, TBEV [10µg E protein] at a concentration of 100µg E/ml were incubated with 40µl 350mM MES for 10 min at 37°C.

3.3.8. Sedimentation analysis

Sucrose gradient 7% 7 g Sucrose (w/w)
1ml 10% TX-100
made up to 0.1L with 100 ml TAN pH 8.0 (w/w)

Sucrose gradient 20% 20 g Sucrose (w/)
1ml10% TX-100
made up to 0.1L with 100 ml TAN pH 8.0 (w/w)

To analyze the oligomeric structure of the expressed proteins, the samples were adjusted to a Triton X-100 final concentration of 1%, incubated for 1 h at 37°C and then applied to 7 to 20% (wt/wt) continuous sucrose gradient in TAN buffer (pH 8.0) containing 0.1% Triton X-100. As controls, TBEVpH 8.0 and low pH treated TBE virus were used. The centrifugation was carried out for 20 h at 38,000 rpm at 15°C in a Beckman SW40 rotor and fractions of 600µl were collected by upward displacement with an ISCO model 640 fraction collector. The amount of E protein in each fraction was determined by a quantitative four-layer ELISA after denaturation of the samples with 0.4% SDS as described above.

3.4. Expression of TBEsE H1_dstrep and TBEsE H1/H2_dstrep in *Drosophila* cells

3.4.1. Vectors and plasmids

For the recombinant expression of TBEsE H1_dstrep and TBEsE H1/H2_dstrep, the expression plasmid pT350 and the selection vector pCoBlast, both described in table 3, were used.

Table 3. Vector systems for protein expression.

VECTOR	PROPERTIES
plasmid pT350 provided by the Institut Pasteur (France)	Modified plasmid for inducible expression of recombinant protein from the metallothionein (pMT) promotor [52, 53]. Cloning Sites: BglII 3´- *AGATCT* – 5´ Bsp120I 3´- *CCCGGG* – 5´ Bip-Export Sequence Enterokinase Cleavage Site Double Strep Tag Stop 3´ - *AGT* – 5´
pCoBlast Vector purchased from Invitrogen	Selection vector, lyophilised in TE pH8.0 For selection with blasticidin

3.4.2. Initiation of a cell culture from a frozen stock

S2 cells ($1x10^7$cells) from a frozen stock were quickly thawed at 30°C. The outside of the vial was decontaminated with 70% ethanol and the cells were transferred into a $25cm^2$ cell culture flask with 5ml of room temperature Schneiders complete *Drosophila* medium (Invitrogen Corporation, Carlsbad, CA) containing 10% foetal bovine serum (Invitrogen Corporation, Carlsbad, CA) (FBS, thawed and inactivated before use at 65°C for 30 minutes) and 1% Penicillin Streptomycin Antimycoticum (PSA, Invitrogen Corporation, Carlsbad, CA). The cells in the flask were incubated at 28°C for 30 minutes followed by resuspension the cells and centrifugation at 1,000 g for 7 minutes at room temperature. The medium was decanted and the cells were plated in 5ml fresh Schneiders complete *Drosophila* medium. The cells were incubated again at 28°C until they reached a density of 6 to 20 x 10^6 cells/ml.

3.4.3. Measuring the cell density

Cell densities were determined using a Casey cell counter (1TT, Schärfe System) according to the manufacturer´s protocol.

3.4.4. Transfection of S2 cells

S2 cells were prepared for transfection by seeding 1x 10^6cells/ml in a 35 mm petri-disk in 3 ml Schneiders complete *Drosophila* medium. The cells were grown for 16 hours at 28 °C until they reached a density of 2 to 4x10^6cells/ml. The transfection mix (for each 35 mm petri-disk), (solution A) was prepared in microcentrifuge tubes with the following components: 36µl 2M $CaCl_2$, 19µg recombinant DNA, 1µg selection vector. Sterile water was used to fill up the mixture to a final volume of 300µl. In a second microcentrifuge tube, 300µl 2x HEPES-buffered saline (solution B) was added. Solution A was then added drop wise to solution B under continuous vortexing. The resulting solution was incubated at room temperature for 40 minutes. During the incubation time, a fine precipitate formed and the solution was then added drop-wise to the cells under continous shaking of the cultures. The transfected cultures were incubated for 24 hours at 28°C, then the Calcium phosphate solution was removed and the cells were washed twice with complete medium. Fresh Schneiders complete *Drosophila* medium was added and the cells were replated in a new well. After incubation for 24 hours at 28°C, the cell medium was removed and the cells were washed. Medium for selection was prepared by adding blasticidin in a final concentration of 25µg/ml to complete Schneiders complete *Drosophila* medium. The washed cells were resuspended in the new medium containing blasticidin in a final concentration of 25µg/ml. This selection medium was replaced every 3 days until resistant cells started growing.

3.4.5. Amplification of stable transfected cells

After 3 weeks of selection, stable transfected cells were established and the amplification of the cell culture was started. For this purpose the stable transfected cells were centrifuged at 1,000 g for 7 minutes, followed by decanting of the supernatant and resuspension of the cells in Schneiders complete *Drosophila* medium containing blasticidin. The cells were passaged at a 1:2 to 1:5 dilution after reaching a density of 6 to 20 x 10^7 cells/ml.

3.4.6. Test of expression

The stably transfected cells with a density of 1x10^6cells/ml were passaged in 5ml Schneiders complete *Drosophila* medium. Protein expression was induced by adding copper sulfate to a final concentration of 0.5M. The cell cultures were incubated at 28°C. After 5 days of incubation, cells were centrifuged at 1000 g for 7 minutes and the supernatant was then used to test the expression of the recombinant protein using ELISA.

3.4.7. Preparation of frozen stocks

Cryovials were labeled and placed on ice. Freezing medium was prepared, containing 75% Schneiders complete *Drosophila* medium, 10% heat-inactivated FBS, 5% PSA and 10% DMSO.The cells were pelleted by centrifugation at 1000 x g for 7 minutes and resuspended in freezing medium at a density of 1 x 10^7 cells/ml. The cell suspension was divided into 1ml aliquots per vial and put on ice at 4°C for 30 minutes. Then the vials were placed into a special storage unit at -80°C, which provided a slow freezing process of the cells. After 24 hours, they were transferred into liquid nitrogen for long term storage.

4. Results

4.1. Generation of recombinant plasmids for the expression of the recombinant TBEsE H1_dstrep and TBE sE H1/H2_dstrep proteins

Two different expression plasmids – pT350-TBEsE H1 and pT350-TBEsE H1/H2 - for the recombinant expression in S2 cells of the corresponding proteins were generated. For this purpose, the cDNAs, designated TBEsE H1, and TBEsE H1/H2 were amplified by polymerase chain reaction (PCR) from the wild-type plasmid *SV-Pwt* [60]. This plasmid codes for the cDNA of the prM and E genes of TBE virus strain, Neudoerfl (GenBank accession no. U27495). PCR was performed using specific primers (Table 1, Materials & Methods). The cDNA named TBEsE H1 codes for the TBEV prM protein and the E protein including helix 1 of the stem (amino acids 1-428) (Figure 10A). The cDNA TBEsE H1/H2 codes for the TBEV prM protein and the E protein including the whole stem (helix 1 and helix 2) (amino acids 1-450), (schematically shown in Figure 10B).

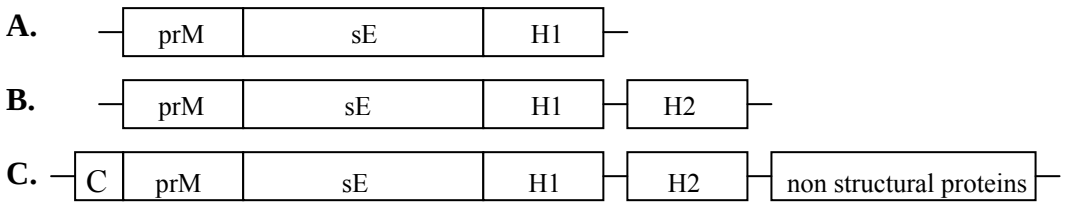

Figure 10. Schematic representation of the TBEV cDNA constructs TBEsE H1 (A), TBEsE H1/H2 (B) and TBE full length E (C). (A) TBEV cDNA coding for the prM protein, sE protein, and helix 1 of the E protein stem named TBEsE H1.(B) TBEV cDNA coding for the prM protein, sE protein and helix 1 and helix 2 of the E protein stem, named TBEsE H1/H2. (C) TBE full length E

After amplification of the cDNAs (TBEsE H1 and TBEsE H1/H2), they were separated by preparative agarose gel electrophoresis. The amplified cDNA molecules showed the expected size of about 2kbp (Figure 11).

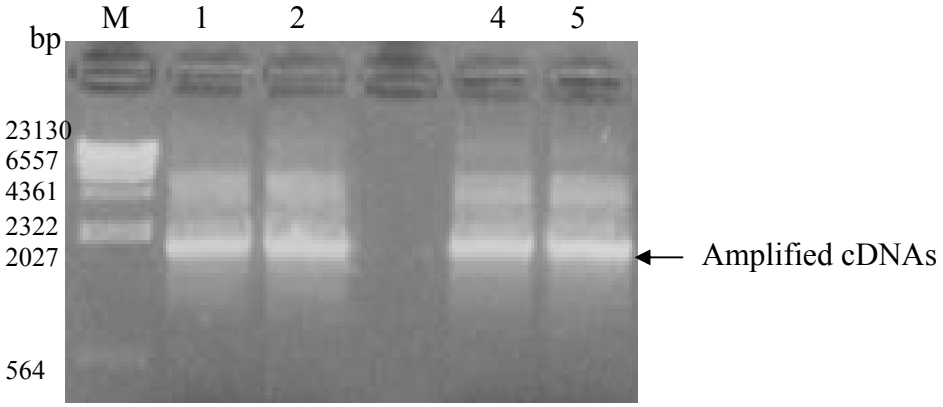

Figure 11. Agarose gel electrophoresis of PCR amplified cDNAs TBEsE H1 and TBEsE H1/H2. Amplified double stranded cDNAs, coding for TBEsE H1 and TBEsE H1/H2 were separated according to their molecular weight by a 1% agarose gel. Lanes 1 and 2 show the PCR fragments of TBEsE H1 and lanes 4 and 5 the PCR fragments of TBEsE H1/H2. Both fragments showed the expected size of around 2kbp (indicated by the black arrow). Size marker (M): Lambda DNA-HindIII Digest (Fermentas, Canada).

The PCR fragments were excised out of the agarose gel, and cloned into the expression vector pT350 (Figure 12), kindly provided by F. Rey, Institut Pasteur. This vector allows the expression of the protein of interest in *Drosophila melanogaster* Schneider 2 cells (Invitrogen Corporation, Carlsbad, CA, Cat.No:R690-07) under the control of a metallothionein promoter. In this system, the protein is expressed as a fusion protein, with an N-terminal Bip-Export Sequence (BipSS) and a C–terminal double Strep-tag (Figure 12). BipSS is essential for the export of the recombinant protein out of the cell into the cell culture supernatant and is cleaved by cellular proteases during export. The double Strep-tag can be used for purification and due to the presence of an enterokinase cleavage site (Figure 12), it can be cleaved off *in vitro* with enterokinase (EK).

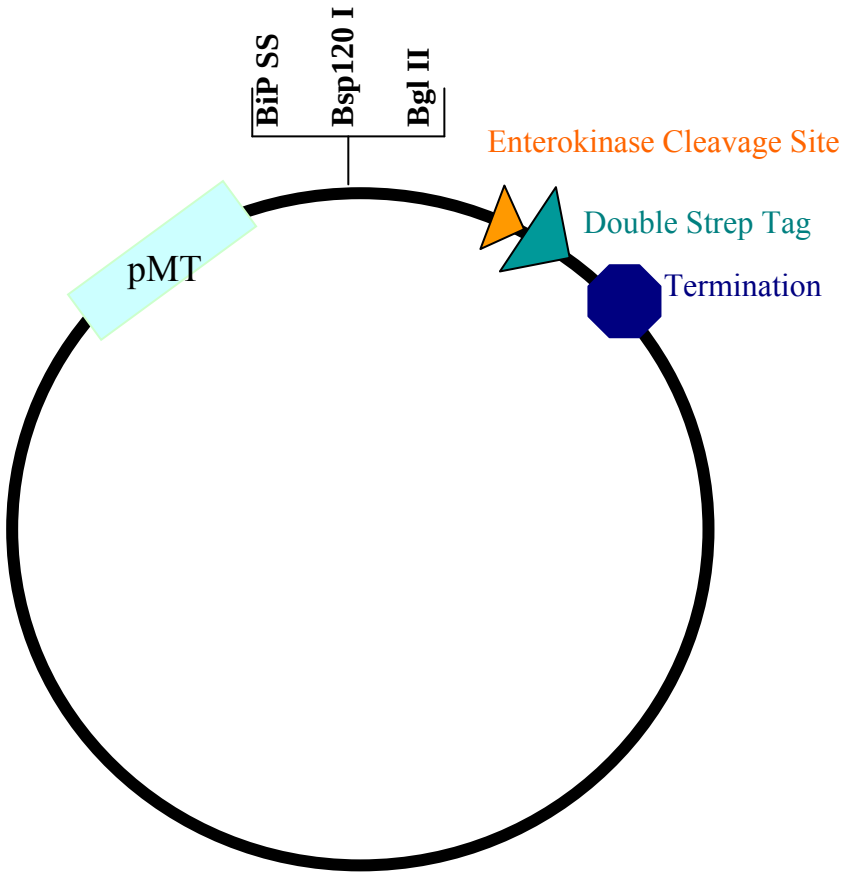

Figure 12. Schematic representation of the plasmid pT350, used for the expression of TBEsE H1_dstrep and TBEsE H1/H2_dstrep. The recombinant plasmids pT350-TBEsE H1 and pT350-TBEsE H1/H2 were generated by cloning the cDNA sequence of TBEsE H1 and TBEsE H1/H2 into the multiple cloning site of the vector pT350 after enzymatic digestion. The expression of the recombinant proteins is under the control of a metallothionin promoter (pMT). The vector contains a Bip-signal sequence (Bip SS), which mediates the export of the recombinant protein out of the cell, a double Strep-tag (green) for protein purification, an enterokinase cleavage site (orange) - to cleave the tag after purification of the protein - and a stop sequence (termination).

The PCR fragments of TBEsE H1 and TBEsE H1/H2 and the expression vector pT350 were digested by a double digestion reaction with the restriction enzymes, *Bgl*II and *Bsp*I120. To avoid religation of the restricted plasmid, a dephosphorylation reaction was performed (see Materials&Methods). The cleaved inserts and the linearized vector were separated by agarose gel electrophoresis. The fragments of TBEsE H1 and TBEsE H1/H2 showed the expected size of about 2kbp. The digested fragments and plasmid were purified by Qiaex II Gel Extraction Kit (Qiagen, Valencia, CA). The amount of cDNA was estimated by analytical agarose gels (Figure 13).

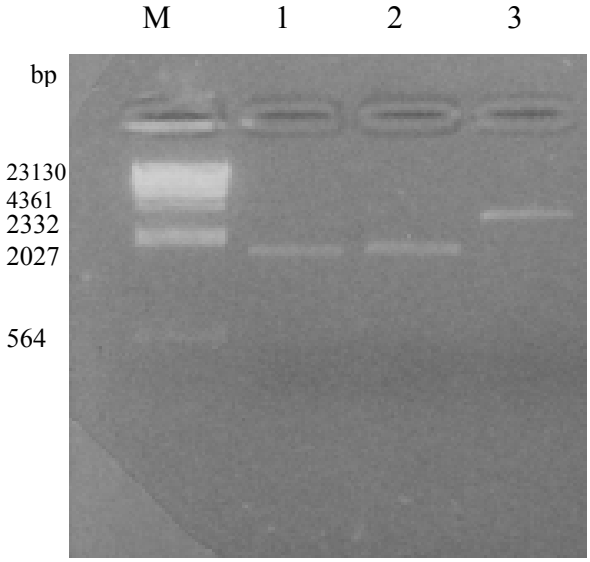

Figure 13. Analytical agarose gel electrophoresis of the inserts TBEsE H1 (lane 1) and TBEsE H1/H2 (lane 2) and the linearized expression vector pT 350 (lane 3). Size marker (M): Lambda DNA-HindIII Digest (Fermentas, Canada).

Recombinant plasmids pT350-TBEsE H1 and pT350-TBEsE H1/H2 were generated by performing a ligation reaction (see Material&Methods) using 1:10 molar ratios of the respective fragment and the linearized vector. The ligation mixtures were used to transform the chemically competent *Escherichia coli* (*E.coli*) strain DH5α (Invitrogen Corporation, Carlsbad, CA). After transformation, plasmid DNA was isolated from small scale plasmid preparations of a 4ml overnight culture from colonies. The correct DNA sequence was verified by sequence analysis (see Materials&Methods). A retransformation of *E.coli* DH5α (Invitrogen Corporation, Carlsbad, CA) with the correct, isolated, recombinant plasmid DNA was performed, followed by an additional large scale plasmid preparation. The correct sequences of both recombinant plasmids were again verified by sequence analysis (see Materials&Methods).

4.2. Expression of the recombinant proteins TBEsE Helix1_dstrep and TBEsE H1/H2_dstrep in *Drosophila melanogaster* - (S2) – cells

4.2.1. Transfection

Drosophila melanogaster (Schneider, S2) were transfected with the recombinant plasmids pT350-TBEsE H1 and pT350-TBEsE H1/H2 together with a blasticidin resistance selection vector (pCoBlast) (see Materials&Methods). Briefly, for transfection, S2 cells [$3x10^6$cells/ml] were seeded into 6 well plates and grown for 20 hours at 28°C. After transfection, the cell cultures were first incubated at 28°C for 24 hours in complete medium and then further grown in the presence of blasticidin for the selection of resistant cells, as described in Materials&Methods. Within the three week selection period, the cells were further propagated at 28°C, by continuously changing the cell culture medium containing blasticidin at the appropriate concentration of 25µg/ml. Protein expression was induced by the addition of copper sulphate to 5 ml cultures of stable transfected cells. The induced cell cultures were then incubated for 5 days at 28°C. After this period, the cells expressed the recombinant proteins and secreted them into the cell culture supernatant which was tested by measuring the E protein concentration. Aliquots of the supernatants were taken and a quantitative ELISA was performed (see Materials&Methods). The results of quantitative ELISA obtained with the cell culture supernatants from the transfections are displayed in tables 4 and 5. The expression levels ranged between 0.0 – 1.86µg/ml for TBEsE H1_dstrep and 0.0 – 0.82 µg/ml for TBEsE H1/H2_dstrep.

Table 4. Concentration of the TBE E protein in the supernatants of S2 cells transfected with pT350-TBEsE H1_dstrep.

Sample Number	E Protein Concentration [µg/ml]
1	0.027
2	0.000
3	0.171
4	0.200
5	0.950
6	0.437
7	1.860

Table 5. Concentration of the TBE E protein in the supernatants of S2 cells transfected with pT350-TBEsE H1/H2_dstrep.

Sample Number	E Protein Concentration [µg/ml]
8	0.000
9	0.543
10	0.018
11	0.823
12	0.000
13	0.321
14	0.179

The cultures that expressed the highest amount of recombinant TBEsE H1_dstrep (Table 4, No.7) and TBEsE H1/H2_dstrep (Table 5, No.11) were later used for large scale cultures (up to five liters). During this amplification process, regular aliquots were taken and frozen in liquid nitrogen for long term storage (see Materials&Methods).

4.2.3. Upscaling of protein expression

For the production of larger quantities of recombinant proteins, an upscaling of cell culture conditions was requested. Transfected *Drosophila* cells can grow to high cell densities in cell cultures under shaking conditions. With the complete cell culture medium, containing fetal bovine serum (FBS), cells are more likely to clot under shaking conditions which would decrease cell viability. Therefore, the stable transfected cells were adapted to serum-free medium, according to the manufacturer´s instructions (see Materials&Methods). After adaption to serum-free medium, containing blasticidin [12.5µg/ml], the cells were transferred to shaking conditions at 28°C and 120rpm (see Materials&Methods). Further upscaling was achieved by the using a bioreactor (WAVE, GE Healthcare, UK Limited). This system allows cells to be cultured up to a volume of five liters. The bioreactor was filled with cells diluted to a final density of $1.5x10^6$ cells/ml in five liters of medium. When the cells were growing exponentially, protein expression was induced with 0.5M copper sulfate. Samples were taken at different intervals and E protein concentrations were determined by a quantitative SDS-ELISA (see Materials&Methods). The resultant E protein concentrations for TBEsE H1_dstrep are displayed in table 6 and for TBEsE H1/H2_dstrep are displayed in table 7.

Table 6. Concentration of the TBE E protein in the supernatants of S2 cells transfected with pT350-TBEsE H1_dstrep in the bioreactor.

Days post-induction	TBEsE H1_dstrep [µg/ml]
0	0
2	0,312
4	1,021
7	1,521
8	1,576

Table 7. Concentration of the TBE E protein in the supernatants of S2 cells transfected with pT350-TBEsE H1/H2_dstrep in the bioreactor.

Days post-induction	TBEsE H1/H2_dstrep [µg/ml]
0	0
2	0,350
6	1,750
7	2,973

4.2.4. Purification of the recombinant proteins from cell culture supernatants

The cell culture supernatant was concentrated by ultra filtration using the concentrator Viva-flow (Sartorious AG, Germany). Typically, 1500ml were concentrated to a final volume of 100ml, which corresponds to a 15-fold concentration. After this step, the recombinant protein was loaded onto a 5 ml Strep-tactin Superflow cartridge H-PR (IBA, Germany) using the chromatographic ÄKTA FPLC system (GE Healthcare, UK Limited) (see Materials&Methods). After a washing step, the sample was eluted with 0.025M desthiobiotin (Figure 14).

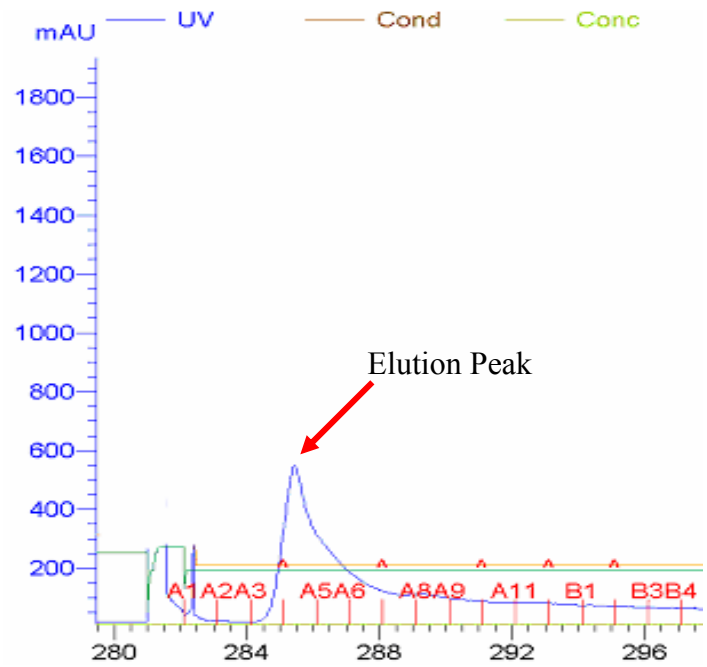

Figure 14. Chromatogram of purification of TBEsE H1_dstrep. The ultra filtrated supernatant containing TBEsE H1_dstrep was purified using a Strep-tactin Superflow cartridge H-PR in combination with the chromatographic ÄKTA FPLC system. The sample was eluted from the column with 0.025M desthiobiotin, as specified by the elution peak (red arrow) in the fractions A3 to A7.

The E protein concentrations during the production process of TBEsE H1_dstrep and TBEsE H1/H2_dstrep, including concentration and purification, were determined by SDS-ELISA (see Materials&Methods). The E protein concentrations were calculated as a percentage of the total initial protein

amount. The results obtained with TBEsE H1_dstrep are displayed in the diagram figure 15. A loss of about 60% E protein was detected after the ultra filtration step. During further purification steps, almost no additional loss of recombinant TBEsE H1_dstrep occurred (Figure 15, lane 10).

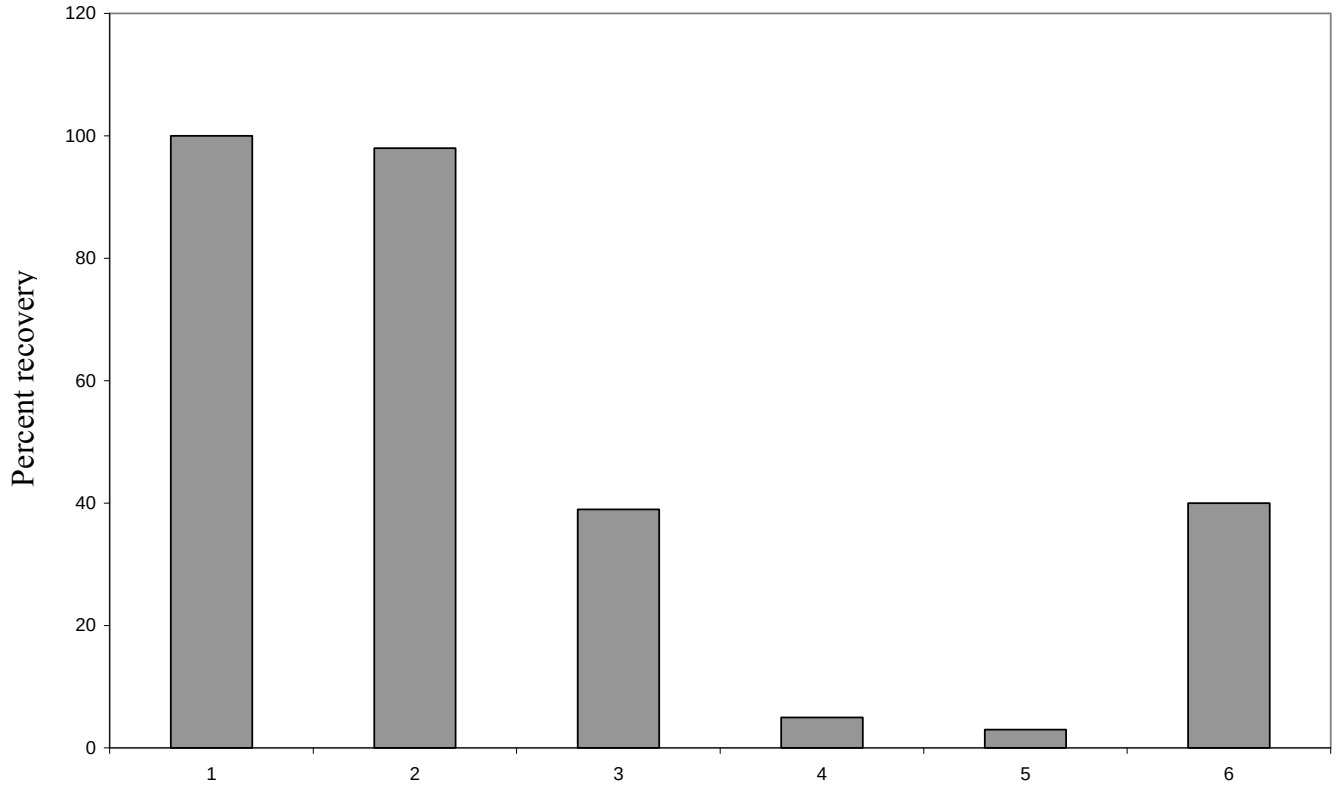

Figure 15. Recovery of E protein during the main steps of purification of TBEsE H1_dstrep. 1. Supernatant before clarification, 2. clarified supernatant, 3. supernatant after ultra filtration, 4. flow through of the purification column, 5. wash of the purification column, 6. pooled peak fractions (A3 to A7, Figure 14).

The results obtained with TBEsE H1/H2_dstrep are displayed in figure 16, in this case no detectable loss of protein was observed during the ultra filtration step. However, due to suboptimal binding of the protein to the affinity strep-tactin column, the recovery of purified product was maximal only about 20%.

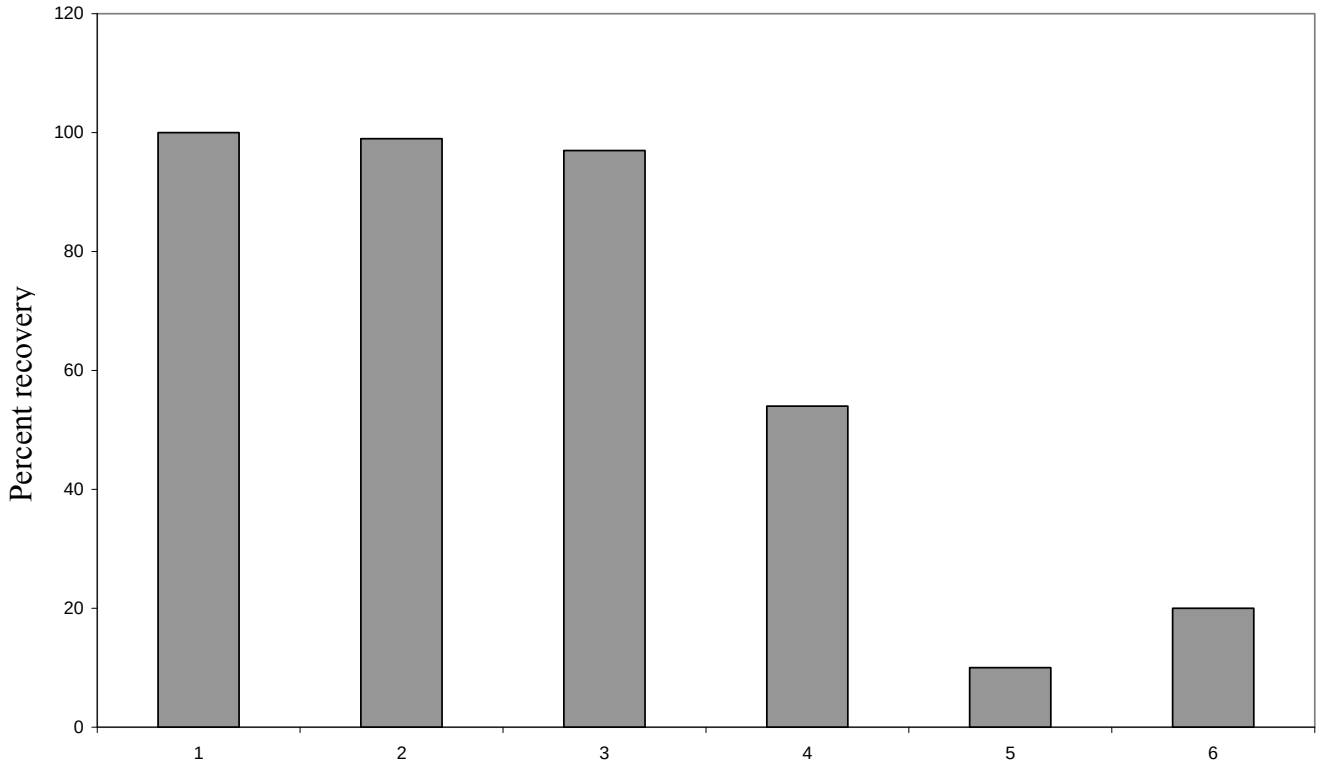

Figure 16. Recovery of E protein during the main steps of purification of TBEsE H1/H2_dstrep. 1. Supernatant before clarification, 2. clarified supernatant, 3. supernatant after ultra filtration, 4. flow through of the purification column, 5. wash of the purification column, 6.pooled peak fractions.

4.2.5. Determination of the purity of the recombinant TBEsE H1_strep

The purity of the purified recombinant TBEsE H1_dstrep protein was densitometrically determined after separation of the sample by SDS-PAGE. For this process, an aliquot of the purified recombinant protein was precipitated by DOC-TCA, subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (see Materials&Methods). The protein of approximately 52kDa was typically calculated to be between 70-95% pure (Figure 17).

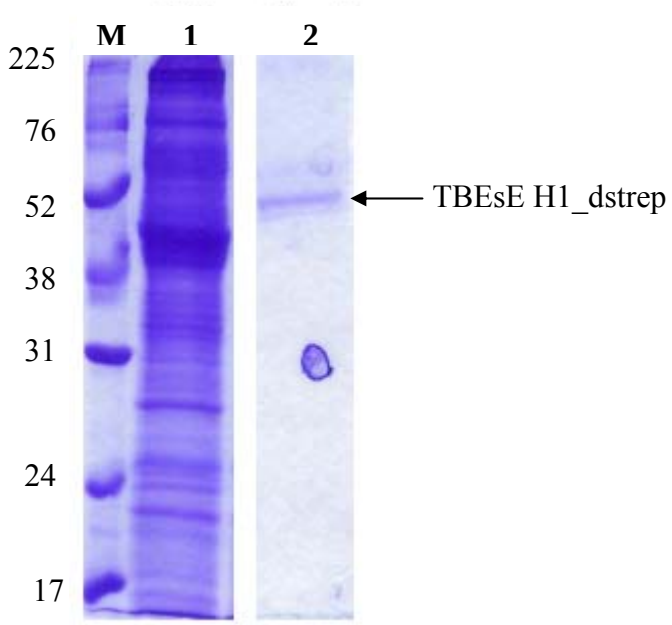

Figure 17. SDS-PAGE of the recombinant TBEsE H1_dstrep. M: Molecular weight marker in kDa, lane 1: clarified cell culture supernatant, lane 2: purified TBEsE H1_dstrep

4.2.6. Determination of the oligomeric state of TBEsE H1_dstrep

In order to determine the oligomeric state of the expressed TBEsE H1_dstrep proteins at different stages of the purification process, aliquots of TBEsE H1_dstrep protein from (i) the S2 cell culture supernatant, (ii) after elution from the Strep-tactin Superflow cartridge H-PR, and (iii) after low pH treatment in the presence of liposomes with 1% Trition X-100 were used for sedimentation analysis (see Materials & Methods). As can be seen in figure 18, the vast majority of the TBEsE H1_dstrep proteins were in a dimeric arrangement directly after expression. After purification with a Strep-tactin column, the predominate form of the TBEsE H1_dstrep proteins was still a dimer. As expected, after low pH treatment in the presence of liposomes, the oligomeric state changed to a trimer, although only with a transition efficiency of about 55%. 45% of the TBEsE H1_dstrep proteins remained in the dimeric conformation.

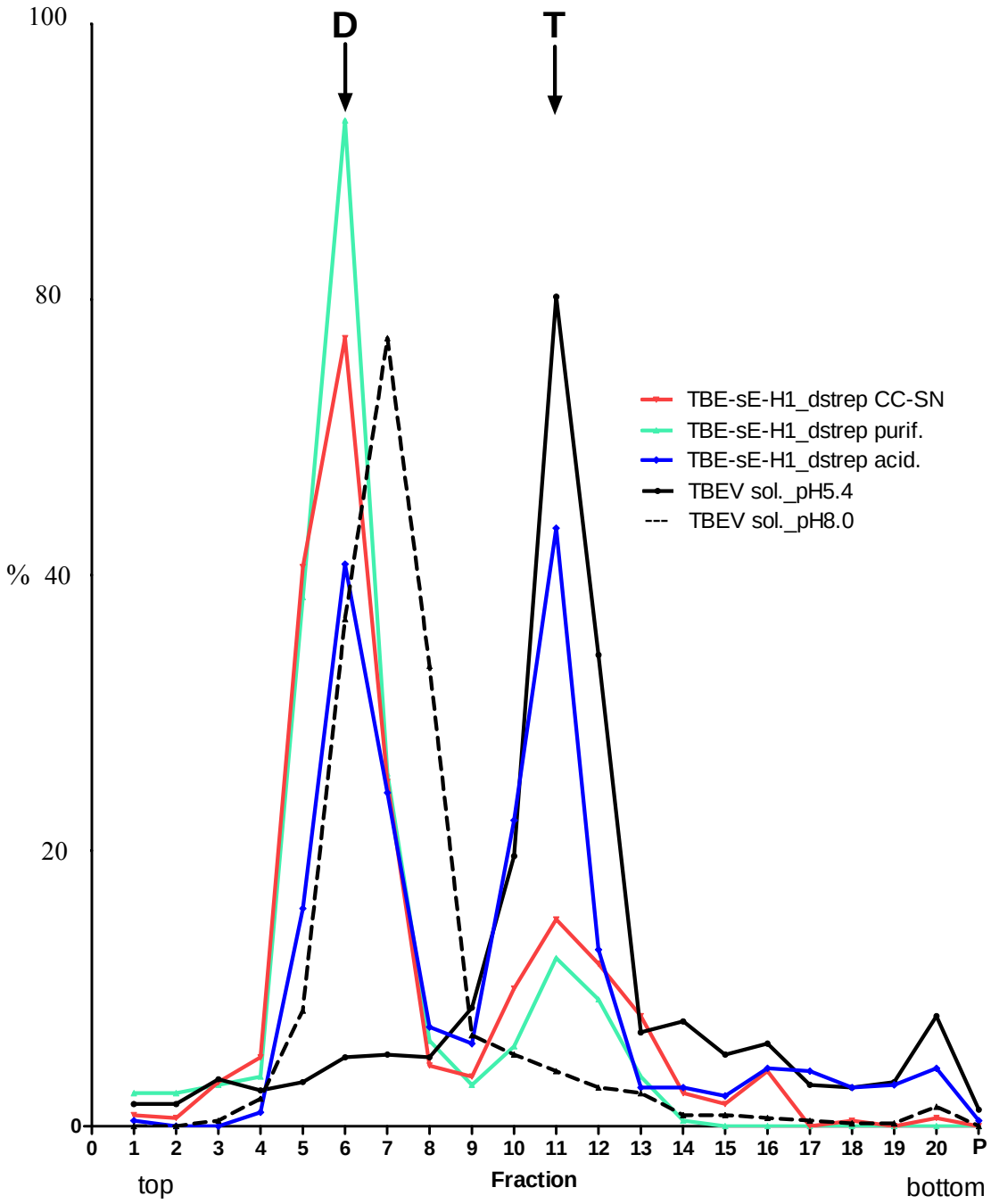

Figure 18. Sedimentation analysis of TBEsE H1_dstrep protein in the presence of deteregent. TBEsE H1_dstrep from the S2 cell culture supernatant (TBEsE H1_dstrep CC-SN, red), TBEsE H1_dstrep after the Strep-tactin purification (TBEsE H1_dstrep purif., turquoise) and TBEsE H1_dstrep after Strep-tactin purification and low pH treatment (TBEsE H1_dstrep acid., blue), were solubilised with 1% Triton X-100, and centrifuged into 7 to 20% sucrose gradients (containing 0.1% Triton X-100) at pH 8.0. As controls, TBEV was incubated at pH 5.4 for 10 min (TBEV sol._ pH 5.4, black), or at pH 8.0 (TBEV sol._pH 8.0, black dotted line), solubilized, and analyzed as described above. The sedimentation direction is from left to right, and the positions of dimers (D) and trimers (T) are indicated.

5. Discussion

In recent years, structural determinations of the fusion protein E in its pre- and post-fusion conformations have rapidly advanced our understanding of the flavivirus fusion mechanism [4, 10-15, 36]. Although only truncated forms of E, lacking the so-called stem-anchor region, were used for these studies, the knowledge of these structures led to a mechanical type model of the flavivirus fusion process (Figure 9) [4]. This suggested process involves a conformational switch from the pre-fusion dimer to the post-fusion trimer and thus a transition of the E molecule from an antiparallel dimer that is oriented horizontally with respect to the viral membrane into a parallel trimer, oriented perpendicular to the membrane. During this process, it is believed that the viral and cellular membranes are brought into close proximity, which is a prerequisite for the membrane fusion, by a so-called “zippering action”. For this mechanism, it was assumed that the stem follows the grooves, generated at the interfaces between the DIIs in the post-fusion trimer, which leads to the juxtaposition of the E membrane anchors and the insertion of the FP loops into the cellular membrane [4]. This mechanism had been confirmed by modelling studies which have shown that the best fit was obtained when conserved residues of the helix 1 of the stem were directed towards the DII of the same E monomer in the post-fusion trimer [14]. The model of the flavivirus fusion process still needs further experimental conformation as the roles of the flavivirus stem region and most of the intermediate stages and structures are hypothetical. For these reasons, we conducted a study to generate a system for the recombinant expression of the TBEV E protein, including parts of or the whole stem region without the membrane anchor region, which could be used for further structural determination studies. Important for subsequent crystallization studies with the recombinant E protein are: the folding of the protein (including the formation of the disulfide bridges), achievable expression levels and adequate culture sizes.

Previous studies have shown that protein expression with *Drosophila melanogaster* cell lines, derived from primary culture of late stage embryos [61], was successfully used for the expression and crystallization of pre- and post-fusion forms of several flavivirus E proteins [11-13, 15].

The *Drosophila* expression system (Invitrogen Corporation, Carlsbad, CA) used in this study is a two-vector based expression system. One vector codes for blasticidin resistance, which is the selection marker, while the other codes for the protein of interest. Generation of stable, transfected cells expressing the protein of interest requires the simultaneous transfection of the cells with both plasmids. A major disadvantage of this system and its selection strategy is that cells, which were transfected only with the selection plasmid, are resistant to the selection but do not provide any expression of the protein of interest. Such cells can cause problems especially during long-term expression, for example by overgrowing expression-positive cells. Another drawback of such a two-vector based system is that if different cell cultures are transfected with the same construct in parallel, the transfection rate and therefore the resulting expression levels can vary massively - ranging in our case from no expression up to 1.86µg/ml for TBEsE H1_dstrep (Table 4) and from no expression up to 0.82µg/ml for TBEsE H1/H2_dstrep (Table 5). Monitoring the transfection efficiency of the cells with the expression plasmid is possible with immunofluorescence or a fluorescence- activated cell sorting (FACS) assay, however during the time period of this work, no established assay was available. A possible way to overcome the suboptimal conditions of the expression system would be the generation of a one-vector based expression system in which the selection marker, as well as the protein of interest, are encoded on the same plasmid. This would guarantee that the selected cells are also expressing the protein of interest, but it is not clear if such a selection-expression plasmid can be stable inserted into the *Drosophila* genome. The recombinant E protein wasexpressed as a TBEV prME pre-protein to ensure proper folding, as

previously described [12, 62]. This pre-protein was then processed by cellular proteases during the secretion pathway and expressed with an N-terminal secretion signal. This signal is an 18 amino acids long secretion signal of the *Drosophila* BiP protein (BiPSS, Figure 12), which is an immunoglobulin binding chaperone protein [63]. Both the prM protein as well as the BipSS are cleaved off by cellular proteases in the exocytotic pathway, leading to secretion of E protein into the cell culture medium. This allows one-step affinity purification via the C-terminal double Strep-tag directly from the concentrated cell culture supernatant. It transpired that the double Strep-tag is an optimal tag for the purification of proteins expressed in S2 cells, because (i) of its insensibility to Cu^{2+} ions, which were used for the induction of the expression, and (ii) of the particular suitability of the double tag for the binding of proteins expressed as oligomers. An enterokinase cleavage site for a possible elimination of the tag after purification was inserted, to prevent influences of the tag on the formation of the E trimer and especially of the incorporation of the stem region into the groove formed by the DIIs of the E protein. Sedimentation analyses have shown that only about 55% of the TBEsE H1_dstrep proteins, which were expressed as dimers, could be converted by low pH in the presence of liposomes into trimers, which represents a very low transition rate, compared to TBEsE, where 82% of the dimer proteins could be rearranged into trimers [37]. This result was quite surprising as the addition of the helix 1 of the stem was expected to increase the stability of the resulting trimer, compared to the sE trimers. Steric clashes during the transition process from dimer to trimer, caused by the double Strep-tags, are possible explanations for this result. During this work, no trimerization experiments in the absence of C-terminal tags were performed to verify this speculation. In the case of TBEsE H1/H2_dstrep, it was not possible to bind the protein onto the Strep-tactin column (Figure 16). This result could be explained by steric hindrances which prevent binding of the tag onto the column.

Another explanation can be that the tag was probably cleaved off during the expression, even without the addition of enterokinases. Unfortunately it was not possible to verify this phenomenon. Possible alternative purification strategies for the TBEsE H1/H2_dstrep proteins could be ion-exchange chromatography or affinity purification via monoclonal antibodies.

In conclusion, it was possible to express correctly folded TBEsE H1_dstrep and TBEsE H1/H2_dstrep proteins in *Drosophila* S2 cells at high expression rates. We were able to establish a purification strategy for TBEsE H1_dstrep trimers which were produced in amounts sufficient for structural determination studies.

6. References

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