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Production, Purification and Evaluation of Insect Cell-expressed Proteins with Diagnostic Potential

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ABSTRACT

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Yhteenveto: Diagnostisesti tärkeiden proteiinien tuotto hyönteissolussa sekä niiden puhdistus ja karakterisointi
Diss.

Living organisms are widely used for the production of recombinant proteins. The baculovirus expression vector system (BEVS) has become a popular choice for diverse recombinant protein production. Moreover, the insect cells used in the BEVS are able to perform most post-translational modifications of proteins with eukaryotic or viral origin. Recombinant proteins can be used in many fields such as in enzyme technology or in disease diagnostics. In general, the proteins must be extracted from the insect cells before their final use. For that, there are various schemes available. The conventional methods are often time consuming and laborious. In addition, they usually allow only limited working volumes to be processed. The addition of purification tags such as a polyhistidine tag to the N- or C terminus of the protein enables the use of affinity chromatography without affecting correct protein folding. Immobilized metal-ion affinity chromatography (IMAC) is the technique used for polyhistidine-tagged proteins. IMAC can be coupled with expanded-bed adsorption chromatography (EBA) for large-scale applications. Here, the coleopteran firefly luciferase of *Photinus pyralis*, the Epstein-Barr virus (EBV) protein, the Epstein-Barr nuclear antigen-1 (EBNA-1) and finally the viral capsid proteins VP1 and VP2 of human parvovirus B19 were produced using the BEVS. The proteins were genetically modified to include a polyhistidine tag and purified by using IMAC. In addition, the scalability of the system was assessed by purifying the firefly luciferase with the EBA. The abilities of the recombinant protein products were shown to have similar properties compared to the authentic counterparts or to previously characterized recombinant proteins. The tagged firefly luciferase was an excellent reagent in ATP monitoring. Similarly, the recombinant antigens for diagnosis of oncogenic virus EBV and human pathogenic the B19 infections appeared to work very well in ELISA:s. In addition, complex formation between VP2 virus-like particles of B19 and antibodies present both in acute and past-immunity sera was shown by fluorescence correlation spectroscopy. Together, the results presented in this thesis demonstrate that the addition of a polyhistidine tag to the recombinant proteins produced in insect cells can facilitate the purification process without affecting the final use. The set-up is therefore attractive when large-scale production and purification of proteins with diagnostic potential is under study.

Key words: Baculovirus; Epstein-Barr nuclear antigen 1; firefly luciferase; human parvovirus B19; polyhistidine tag; virus-like particle.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following scientific articles, which will be referred to in the text by their Roman numerals (I-IV).

I Michel, P., Torkkeli, T., Karp, M. & Oker-Blom, C., 2001. Expression and purification of polyhistidine-tagged firefly luciferase in insect cells – a potential alternative for process scale-up. Journal of Biotechnology, Jan. 23; 85 (1), 49-56.

II Toivola, J., Michel, P.O., Gilbert, L., Lahtinen, T., Marjomäki, V., Hedman, K., Vuento, M. & Oker-Blom, C., 2004. Monitoring human parvovirus B19 virus-like particles and antibody complexes in solution by fluorescence correlation spectroscopy. Biological Chemistry, Jan. 385 (1), 87-93.

III Schenk, B.I., Michel, P.O., Enders, G., Thilo, N., Radtke, M., Oker-Blom, C. & Franke, D., 2007. Evaluation of a new ELISA for the detection of specific IgG to the Epstein-Barr nuclear antigen 1 (EBNA-1). Clinical Laboratory, 53 (3-4), 151-155.

IV Michel, P.O., Mäkelä, A.R., Korhonen, E., Toivola, J., Hedman, L., Söderlund-Venermo, M., Hedman, K. & Oker-Blom, C., 2008. Purification and analysis of polyhistidine-tagged human parvovirus B19 VP1 and VP2 expressed in insect cells. Journal of Virological Methods, Sep. 152 (1-2), 1-5.

In addition, some unpublished data are presented.

RESPONSIBILITIES OF PATRIK MICHEL IN THE ARTICLES OF THE THESIS

Article I: I am responsible for the study and I also wrote the major parts of the article. Tuula Torkkeli performed the construction of the recombinant baculovirus. Matti Karp participated in designing the luciferase gene and writing of the manuscript.

Article II: I am responsible for the production and purification of the B19 VP2 VLPs as well as parts of the characterization process. Jouni Toivola was responsible of the FCS part. We wrote the major parts of the manuscript together. Leona Gilbert performed the CPV baculovirus construct. Tomi Lahtinen participated in the characterization of the protein. Varpu Marjomäki carried out the electron microscopy studies and Klaus Hedman the ELISA testing. Matti Vuento supervised the FCS part of the study.

Article III: I am responsible for the production and purification of the EBNA-1 recombinant protein and I participated in the writing process of the manuscript. Brigit Schenk was responsible for the ELISA and wrote the major parts of the article. Gisela Enders, Niklas Thilo and Martina Radtke performed and evaluated the ELISA. Dieter Franke supervised the ELISA and participated in writing the manuscript.

Article IV: I am responsible for the study and I also wrote the article. Anna Mäkelä participated in the construction of the recombinant baculoviruses. Eila Korhonen and Jouni Toivola participated in the laboratory analysis. Lea Hedman, Maria Söderlund-Venermo and Klaus Hedman were involved in the diagnostic part of the study as well as in the revision of the manuscript.

All these studies were carried out under the supervision of Professor Christian Oker-Blom.

ABBREVIATIONS

AAV	adeno-associated virus
*Ac*MNPV	*Autographa californica* multiple nucleopolyhedrovirus
ADP	adenosine diphosopahte
AdV	adenovirus
AMDV	Aleutian mink disease parvovirus
AMP	adenosine monophosopahte
AP	alkaline phosphatase
ATP	adenosine triphosphate
B19	human parvovirus B19
BCCP	biotin carboxyl carrier protein
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BDVS	baculovirus display vector system
BEVS	baculovirus expression vector system
bp	base pair
BV	budded virus
CMV	cytomegalovirus
CPV	canine parvovirus
DAB	3, 3′-diaminobenzidine tetrahydrochloride
dsDNA	double stranded deoxyribonucleic acid
DTT	dithiothreitol
E.coli	*Escherichia coli*
EA	early antigen
EBA	expanded bed adsorption
EBNA-1	Epstein-Barr nuclear antigen -1
EBV	Epstein-Barr virus
EDTA	ethylenediamine tetraacetic acid
EIA	enzyme immuno assay
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
FCS	fluorescence correlation spectroscopy
GFP	green fluorescent protein
gp64	major envelope glycoprotein 64
GV	granulosis virus
HAV	hepatitis A virus
HBV	hepatitis B virus
HCMV	human herpesvirus type 5 or human cytomegalovirus
Hepes	N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid)
HHV-4	human herpes virus 4
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
IDA	iminodiacetic acid
IFA	immunofluorescence assay

IgG	immunoglobulin G
IgM	immunoglobulin M
IM	infectious mononucleosis
IMAC	immobilized metal-ion affinity chromatography
kb	kilobase
kDa	kilodalton
LMP	latent membrane protein
Luc	firefly luciferase of *Photinus pyralis*
MA	membrane antigen
MEV	mink enteritis virus
MNPV	multiple nucleocapsid virus or multiple nucleopolyhedrovirus
MOI	multiplicity of infection (plaque-forming units/cell number)
MVM	minute mice of virus
MW	molecular weight
NBT	nitro blue tetrazolium
NPV	nucleopolyhedrovirus or nuclear polyhedrosis virus
NSP	non-structural protein
OV	occluded virus
p.i.	post infection
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFU	Plaque-forming unit = infectious virus
PPV	porcine parvovirus
RNA	ribonucleic acid
RV	rubella virus
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sf	*Spodoptera frugiperda*
ssDNA	single stranded deoxyribonucleic acid
SV40	simian virus 40
Tn-368	*Trichoplusia ni* or High-Five™
Tris	Tris(hydroxymethyl)aminomethane
Triton	polyethylene glycol tert-octylphenyl ether
UV	ultra violet
VCA	viral capsid antigen
VLP	virus-like particle
VP	viral protein

CONTENTS

1 INTRODUCTION

The main focus of biotechnology is to exploit the cell machinery of living organisms. The exploitation of living organisms gives efficient alternatives to traditional chemical synthesis procedures. This includes processes like the use of yeast to bake bread or the use of cultured cells to produce new recombinant vaccines, other proteins, or gene therapy vectors (Smith 2001). Biotechnological processes are widely used, but there are still plenty of areas that need to be studied. For example, recombinant proteins have an inherent position in diagnostic technology (Kresse 2001). They are valuable alternatives to native proteins, which are also rather difficult to extract from their natural host, the natural host availability is often lacking (Hatti-Kaul & Mattiasson 2001). On the other hand, efficient biotechnological tools such as insect cells and baculoviruses, have been introduced over two decades ago (Summers & Smith 1987). During these years, production of recombinant proteins has become relatively standardized (Fernandez & Hoeffler 1999). Even though the purification processes of recombinant proteins have broadly been studied, time and money consuming improvements can still be carried out.

The aims and the goals of the experimental work in this thesis were to find alternatives to the purification processes of several recombinant proteins. Recombinant proteins were produced with a diagnostic prospective as well as evaluated. Therefore, several recombinant proteins were produced in insect cells by using genetically modified baculoviruses. Starting with the firefly luciferase enzyme (I), then the Epstein-Barr virus nuclear antigen-1 (III) and finally the viral proteins 1 and 2 (VP1 and VP2) of B19 (II, IV). Proteins were purified by using affinity tag (I, III and IV) or by centrifugation (II). They were also characterized by using standard techniques like gel electrophoresis, enzymatic activity (I) or enzyme linked immunosorbent assay (II, III and IV). Fluorescence correlation spectroscopy (Ehrenberg & Rigler 1974, Ehrenberg & Rigler 1976, Magde et al. 1974) was used for monitoring the antibody-virus interactions for the detection of antibody classes (II).

The review of the literature in this thesis describes a general overview of the production of recombinant proteins in biotechnology, as well as their

purification, especially the use of immobilized metal-ion affinity chromatography (IMAC) (Porath et al. 1975) and expanded-bed adsorption chromatography (Draeger & Chase 1991). The reader will also be introduced to the biology of the firefly luciferase enzyme of *Photinus pyralis* (Green & McElroy 1956) as well as the biology of several viruses such as baculovirus (Summers & Smith 1987), Epstein-Barr virus (Epstein et al. 1964) and human parvovirus B19 (Cossart et al. 1975).

2 REVIEW OF THE LITERATURE

2.1 Recombinant protein production

During the past decades, advances in recombinant DNA (rDNA) technology have given several tools to modify and improve the production of recombinant proteins. Production will be successful, when using the cell machinery properly. Some examples of recombinant proteins, which are produced nowadays are monoclonal antibodies, vaccines (Smith 2001) and also antigens and enzymes as developed further in this thesis. Recombinant proteins are mainly produced by using cells of either prokaryotic or eukaryotic origin such as bacterial, yeast, insect or mammalian cells. Some characteristics and the post translational modifications of the previously mentioned organisms are shown in Table 1 (Fernandez & Hoeffler 1999).

TABLE 1 General characteristics and post translational modifications of organisms used in recombinant protein production (modified from Fernandez & Hoeffler 1999).

Characteristics	Expression system			
	Bacteria	Yeast	Insect	Mammalian
Cell growth	Rapid	Rapid	Slow	Slow
Complexity of growth media	Low	Low	High	High
Cost of growth media	Low	Low	High	High
Expression level	High	Low to high	Low to high	Low to moderate
Post translational modifications				
Protein folding	Refolding usually required	Refolding may required	Proper folding	Proper folding
N-linked glycosylation	None	High mannose	Simple, no scialic acid	Complex
O-linked glycosylation	No	Yes	Yes	Yes
Phosphorylation	No	Yes	Yes	Yes
Acetylation	No	Yes	Yes	Yes
Acylation	No	Yes	Yes	Yes
γ-carboxylation	No	No	No	Yes

2.2 Purification of recombinant proteins in biotechnology

After the recombinant protein production or upstream process, the downstream part of the process is initiated. Before starting the downstream process (DSP) of recombinant proteins, it should be considered whether the product is intracellular or extracellular. When the product is mainly kept inside the cells, cells should be collected usually by centrifugation or flotation and further disrupted. After the concentration and purification phases, formulation and characterization of the final product are conducted (Hatti-Kaul & Mattiasson 2001). Cell disruption could be performed by using chemical, enzymatic, mechanical or physical methods (Hatti-Kaul & Mattiasson 2001). Chemical methods are applied by modifying the composition of extraction buffer. Depending on the protein to be purify, several alternatives are possible. These include changing of pH, ionic strength or isotonicity. Moreover, the addition of detergent to solubilize the lipid bilayer cell membrane is also possible. When solubilizing insect cells, for example, mild and non denaturing detergent, such as Triton X-100 or Nonidet P40, could be used (Ersson et al. 1998). Several chemical agents can be utilized to concentrate proteins. Agents like, salt (ammonium sulfate), solvent (acetone, ethanol) or polymer (polyethylene glycol or PEG) are often employed (Ersson et al. 1998). Protein concentration occurs through a diminution of the working volume by removing solvent around the proteins (Hatti-Kaul & Mattiasson 2001). The purification phase, aiming for protein separation, is usually performed by using chromatographic techniques. The components are separated in a column, usually vertical, where a stationary phase or column matrix is packed. Around the matrix, a mobile phase or buffer is pumped through the column (Janson & Jönsson 1998). Depending on the proteins to be separated, there are many methods with different separation principles that can be used (Table 2) (Ersson et al. 1998, Hatti-Kaul & Mattiasson 2001, Janson & Jönsson 1998). General aspects of affinity chromatography and immobilized metal-ion affinity chromatography are presented in the following chapters. From the beginning to the end of the protein purification, the composition of the used buffer should be made with great attention. This with the purpose of stabilizing the final product and minimizing its destruction (Wong & Parasrampuria 1997).

TABLE 2 Separation principles of various chromatography methods (modified from Ersson et al. 1998, Hatti-Kaul & Mattiasson 2001, Janson & Jönsson 1998).

Separation principle	Chromatography
Size and shape	Gel filtration
Net charge	Ion-exchange chromatography
Isoelectric point	Chromatofocusing
Hydrophobicity interaction	Hydrophobic interaction chromatography
Biospecific affinity, molecular recognition	Affinity chromatography
Metal-ion binding	Immobilized metal-ion affinity chromatography
Content of free thiol groups	Covalent chromatography

2.2.1 Affinity chromatography

Affinity chromatography is based on the reversible affinity between ligand (attached on an insoluble matrix) and a protein. The overall process goes as follows: Firstly, binding of the ligand to the matrix (column material). Secondly, binding of the protein to the ligand and washing out the non adsorbed proteins. Thirdly, elution of the protein is done by either adding a ligand-like molecule or a non-specific elution by changing the buffer composition. The principal aims are: Firstly, to have a highly specific binding between the ligand and the target molecule. Secondly, to have a possibility to elute easily the target molecule (Wilson 2000).

There are many fusion partners available to facilitate detection, isolation and purification of heterogeneous recombinant proteins. Some of the commonly used systems and recent selected target proteins are presented in Table 3.

TABLE 3 Recent examples of selected target proteins and peptide tags used for the purification of recombinant fusion proteins.

Affinity tag	Abbreviated name	Target protein, examples	Reference
Biotin, biotin carboxyl carrier protein	BCCP	p53 protein; Phosphoroproteome protein	(Cressey et al. 2008); (Kwon et al. 2007)
FLAG™		Eukaryotic initiation factor 2B complexes, subcomplexes, and fragments	(Mohammad -Qureshi et al. 2007)
Glutathione-S-transferase	GST	Polyomavirus VP1 protein	(Lipin et al. 2008)
Immobilized metal-ion affinity chromatography	IMAC	Enhanced green fluorescent protein	(Chiang et al. 2008)
Maltose binding protein	MBP	Tumstatin, 28-kDa C-terminal fragment of collagen IV	(Luo et al. 2008)
Protein A		Antibody, protein microbicide to prevent HIV transmission	(Ramessar et al. 2008)
Protein G		Anti-SARS-CoV antibodies	(Bhatnagar et al. 2008)

2.2.2 Immobilized metal-ion affinity chromatography

Immobilized metal-ion affinity chromatography (IMAC) or also called metal-chelate affinity chromatography (MCAC) was first described in 1975 by Porath et al. (Porath et al. 1975). This chromatography method is based on the ability of certain molecules to act as electron donors and form reversible bonds with transition-metal ions that have been immobilized by a chelating group,

covalently bound to a solid support also called an affinity matrix. Molecules such as imidazole (Fig. 1A) (Sjöberg 1997) or amino acids which contain cyclopentadiene (histidine) (Gaberc-Porekar & Menart 2001), or an aromatic group (tryptophan, tyrosine, or phenylalanine) (Sulkowski 1989) (Fig. 1B-E) could be used.

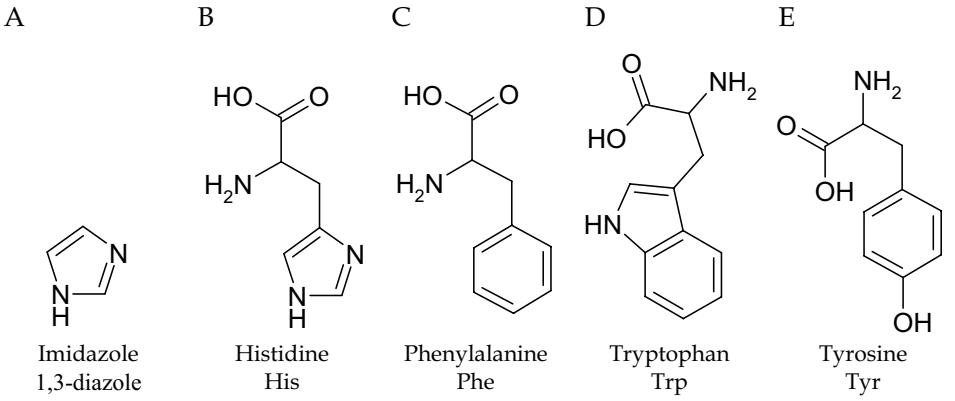


FIGURE 1 A molecular representation of imidazole (1,3-diazole) (A) and amino acids histidine (B), phenylalanine (C), tryptophan (D), tyrosine (E) which have an affinity with transition metal ions (molecules drawn with MDL ISIS™/Draw 2.5, according to Nelson & Cox 2000).

Imidazole molecules have a high affinity with transition metal cations (Sjöberg 1997). Within the amino acids shown above (Fig. 1B-E), histidine is the only one to have on its side chain an imidazole group (Fig. 1A). Consequently, it will bind to a metal cation with a higher affinity than phenylalanine, tryptophan or tyrosine. The affinity order has been specified to be in a decreasing order at pH 7: His > Trp > Tyr > Phe (Porath & Olin 1983). Even though, some proteins might have, within their structure, many histidine residues, and thus have a high affinity with metal-ions (Fig. 2A) it is preferable to design the recombinant proteins to contain short sequence of six to ten residues of histidine. Histidine residues should be added particularly in exposed surfaces, like amino or carboxyl termini (Fig. 2B) (Hemdan et al. 1989). The stability of the formed complex between metal-ion and imidazole or histidine is in agreement with the Irving-Williams sequence where: Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II) (Sjöberg 1997). Due to the Irving-Williams sequence, the affinity order is firstly Cu(II), secondly Ni(II), thirdly Zn(II), fourthly Co(II), fifthly Fe(II) and finally Mn(II). Except for iron and manganese, the other ions could be used with IMAC (Müller et al. 1998) in which copper and nickel ions have the highest affinity for histidine (Yip et al. 1989). There are many potential compounds to chelate metal ions for their immobilization in IMAC, such as iminodiacetic acid (IDA), nitrilotriacetic acid (NTA) or carboxymethylated aspartic acid (CM-Asp) (Gaberc-Porekar & Menart 2001). IDA is suitable to chelate a wide range of ions such as Cu(II), Ni(II), Zn(II) or Co(II) (Porath et al. 1975, Porath & Olin 1983).

The IDA ligand is coupled to cross-linked agarose or dextran via a spacer arm (Gaberc-Porekar & Menart 2001).

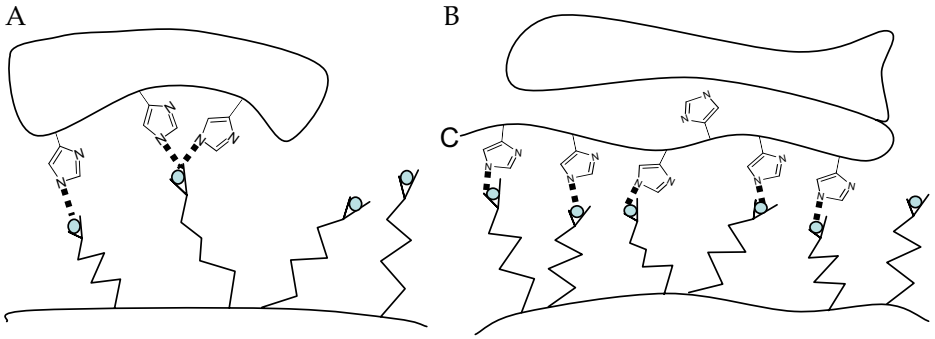


FIGURE 2 A schematic representation of the binding of histidine to IMAC matrix. A binding of native or engineered histidine on the surface of the protein is presented (A). A binding of a recombinant protein on which a polyhistidine tag (six consecutive histidine residues) is designed to the C-terminus (B) (the figure is adapted from Gaberc-Porekar & Menart 2001).

The high affinity of the polyhistidine tag with IDA is the result of the binding of the imidazole side chains of the histidine residue with the chelated metal (Fig. 2A). For better binding, the pH should be ≥7.0, where the imidazole side chain is deprotonated with a net negative charge. At the pH 5.97 (the pK of the imidazole side chain of histidine), 50% of histidines are protonated and at the pH ≤4.5, almost all of the histidines are protonated and do not interact with the matrix (Petty 2001). Thus, there are three main methods to undo the histidine/chelated metal binding. The first method uses buffers with a decreasing pH to elute the histidine tag, which is not preferable for pH sensitive proteins. The second method uses increasing concentration of imidazole at a constant pH to displace the histidine tag from the ligand, which provides a specific elution and a gentle treatment for the protein. The third one is to chelate the metal ions out of the IMAC matrix with a chelating agent such as EDTA. This provides an elution of the protein, but also an elution of the binding metal (Gaberc-Porekar & Menart 2001, Petty 2001).

2.2.3 Expanded bed adsorption chromatography

Traditional chromatography is usually executed by using a column with a packed bed. This method necessitates a clarification of the crude extract feed before the application to the chromatography column. The clarification techniques for the crude extract are usually centrifugation and/or microfiltration. The clarification steps are done to remove cells and/or cell debris. The efficiency of a centrifugation step depends on the size of the particle, the difference of density between the particles and the surrounding liquid, and the viscosity of the solution (Griffiths 2000). When handling cell lysate after the expression of recombinants, it is difficult to obtain an absolute

particle-free solution by centrifugation. The presence of particles contributes to the formation of a plug of trapped solids near the bed inlet. The plug may increase the pressure in the column and even stop the buffer flow in the column (Chase 1994). To avoid the difficulties with a packed bed column, an alternative is to use an expanded bed adsorption (EBA), a method which enables a purification with particles (Chase 1994).

In 1990, Draeger and Chase were the first to create a prototype of a stable fluidized (expanded) bed, with chromatographic characteristics similar to the conventional packed bed (Draeger & Chase 1991). In 1993, Pharmacia Biotech (Pharmacia Biotech, Uppsala, Sweden, nowadays GE Healthcare Bio-Sciences AB) introduced the commercial version of an expanded bed. That was the introduction of new types of chromatographic adsorbents and a column called Streamline™. The purification by expanded bed adsorption is a possible and preferable solution for a single-step recovery of protein (Hansson et al. 1994). When using EBA the column material should be specific to it. As an example for IMAC, the Streamline™ Chelating (GE Healthcare Bio-Sciences AB) is based on a highly cross-linked 6% agarose which has been modified by including an inert quartz core to give an optimal density to the particles (Chase 1994).

The different steps forming the purification of recombinant with EBA are schematically illustrated in Figure 3 and described in detail below (Chase 1994). Without a flow the bed is lying on the column's lower adapter. The upper adapter is positioned in the upper part of the column and the entire volume of the column is fed with buffer (Fig. 3A). The matrix expands and stabilizes when applying an upward liquid flow (Fig. 3B). The cell crude or the cell lysate could be partially purified or unclarified. Then it is applied to the expanded bed with the same liquid flow. Target proteins are bound to the adsorbent while cell debris and contaminants pass through unhindered (Fig. 3C). Weakly bound materials, such as contaminating enzymes, cell debris are washed out from the expanded bed by using an upward liquid flow (Fig. 3D). When all the impurities have been washed out of the matrix, the liquid flow is stopped and the adsorbent is sedimented in the column. The upper adapter is positioned on the top surface of the sedimented matrix. The liquid flow is reversed and the bounded proteins are eluted with appropriate buffers. The elution is done in packed bed so as not to increase the elution volume and therefore to dilute the eluted protein (Hjorth 1999). The proteins are collected in the eluted fractions (Fig. 3E). Finally, the bed is regenerated. When using IMAC, the chelated metals are removed. This step confirms that all proteins, which are not removed during the elution phase, will be removed. Removing the metal causes a change to a lighter color in the matrix (Fig. 3F).

EBA coupled with IMAC has been used in many cases such as the following selected cases: The feed stock purification for biotherapeutics (Tolner et al. 2006), the *Ld*ARL-1 protein purification (Sahin et al. 2005), the purification of intracellular diphtheria protein and the purification of malaria vaccine candidate (Noronha et al. 1999), the kinesin or motor protein purification (Gibert et al. 2000), the purification of dimeric single-chain variable fragment

antibody against Salmonella enterica serotype Paratyphi B (Makvandi-Nejad et al. 2005), the unclarified *E.coli* homogenate purification (Clemmitt & Chase 2000) and finally, the removal of polyhistidine tag from recombinant proteins (Abdullah & Chase 2005). EBA is advantageous when a large-scale is needed, but at laboratory scale its use should be carefully considered (Gaberc-Porekar & Menart 2001, Lütkemeyer et al. 2001).

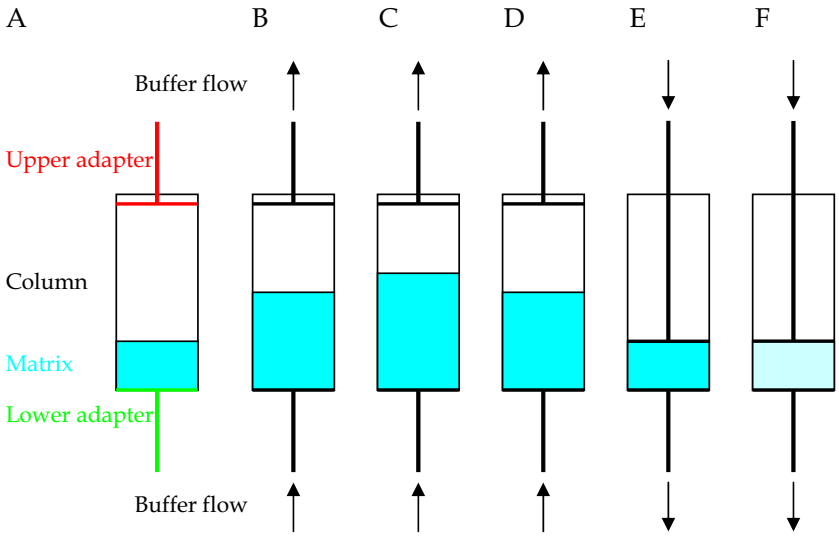


FIGURE 3 A schematic representation of the different steps passed when using the expanded bed adsorption chromatography. The details of the steps (A-F) are presented in the previous text (the figure is adapted from Chase 1994).

2.3 Characterization methods of recombinant proteins

After or during protein purification some biochemical methods are used for protein characterization and protein structure determination. The UV-absorbance measurement is an unspecific but commonly used in purification monitoring process (A at λ 280 nm). In this process, the aromatic amino acids, such as tryptophan (Fig. 1D) and tyrosine (Fig. 1E) have a stronger absorbance of UV light than phenylalanine (Fig. 1C) (Nelson & Cox 2000). Beside UV-absorbance, electrical conductivity or pH measurement could also be used when monitoring downstream processing events. All these three techniques have a rather low sensitivity without any selectivity, but with a relatively fast response time (Hatti-Kaul & Mattiasson 2001, Walker 2000). However, these analyses are usually not enough to prove the results; therefore the recovered proteins can be characterized in many ways. The relative molecular mass can be seen by SDS-PAGE (Laemmli 1970), followed by western blotting or immunoblotting (Towbin et al. 1979). Molecular exclusion chromatography or gel filtration as well as mass spectrometry can also asses the total molecular

weight of the protein (Walker 2000). In addition, the total protein amounts are usually measured (Bradford 1976). The primary structure of the protein can be determined by the cleavage (enzymic or chemical) of the protein to form peptides. The cleavage of the protein is done when the peptide mass fingerprint is determined (Henzel et al. 1993). The use of mass spectrometry and the hydrophobicity profile determination can also give information on the amino acid sequence (Walker 2000). The glycoprotein analysis or the N- and C-terminal sequencing of the proteins are also potential tools for protein identification (Walker 2000). For viruses or virus-like particles, the particles are often visualized with electron microscopy (EM). Moreover, the proteins could be characterized or monitored with high sensitivity by using techniques, such as fluorescence correlation spectroscopy (FCS), as well as enzyme linked immunosorbent assay (ELISA) as described below.

2.3.1 Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) is a spectroscopic technique for studying the size and the number of the molecules, or biomolecular interactions in solution. To follow the interaction, the basic need for the molecules is that one molecule must be much bigger than the other one and also the smaller one of the two should be fluorescent (Swift & Cramb 2008). FCS monitors the random movements of fluorescently labeled molecules moving in or out of a defined volume excited by a focused laser beam (Fig. 4A). The laser excitation light is focused on a volume of less than 0.25×10^{-15} liter (femtoliter, fl) and emission bursts are detected as fluorescent molecules are driven in and out by stochastic diffusion through this volume. In other words, the molecules are moving randomly by Brownian motion through the sample volume causing fluorescence intensity fluctuation (Schwille et al. 1997) (Fig. 4A). From the fluctuations of fluorescence, the mean number of fluorescent particles present in the detection volume (Fig. 4B) (Chen et al. 1999, Schwille et al. 1997, Sengupta et al. 2002), as well as their diffusion coefficients can be derived mathematically (Fig. 4C).

Since the diffusion properties of a particle are directly dependent on the particle's own mass, any increase in the mass of a biomolecule, for example, as a result of a binding interaction with a second molecule, is detected as an increase in the particle's diffusion time. Since the intensity fluctuation of fluorescent particles is dependent on the time they are spending in the observation volume, the particle size and concentration can be obtained simultaneously. With FCS, measurements of very dilute samples are possible (Hess et al. 2002), and it has been claimed that detection can be done at a single molecule level (Bark et al. 1999, Dittrich et al. 2001, Földes-Papp et al. 2002b). The conceptual basis of this method was developed about thirty years ago (Ehrenberg & Rigler 1974, Ehrenberg & Rigler 1976, Magde et al. 1974). However, instruments capable of accurate measurements have been introduced quite recently (Eigen & Rigler 1994, Widengren & Rigler 1997).

FCS has been used for a broad range of studies including several cases, such as the detection of fibrin polymerization (Bark et al. 1999), intracellular investigations (Braun et al. 2002, Dittrich et al. 2001, Schwille 2001, Sengupta et al. 2002), the utilization of FCS in PCR (Rigler et al. 1998), monitoring of VLPs (Gilbert et al. 2006), characterization of properties of baculovirus particles displaying GFP (Toivola et al. 2002), investigation of endocytosis (Yoshida et al. 2001) and for diagnosis of Alzheimer's disease (Funke et al. 2008). (Schrof et al. 1998) have demonstrated the application possibilities of FCS in industrial research. FCS techniques are also reviewed by (Földes-Papp et al. 2002a, Hess et al. 2002).

FCS measurements rely on mathematical and physical models. Reports concerning the background of the autocorrelation theory can be found elsewhere, see (Edman 2000, Ehrenberg & Rigler 1974, Eigen & Rigler 1994, Elson & Madge 1974, Hess et al. 2002, Huertas de la Torre et al. 2001, Kask et al. 1997, Madge 1976, Rigler 1995, Rigler et al. 1998).

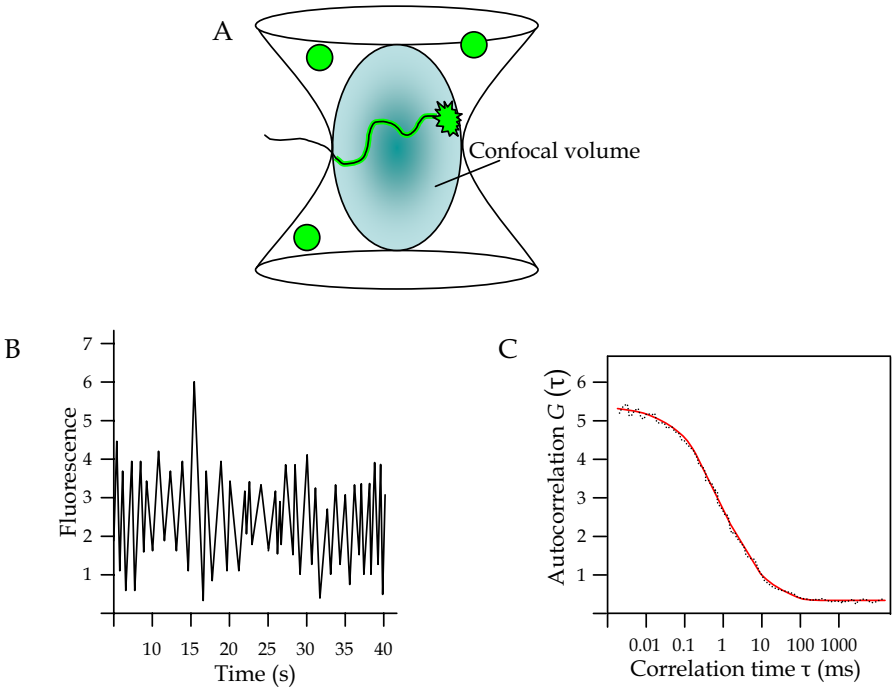


FIGURE 4 Confocal volume (blue) within which the sample's particles (green dots) are illuminated in the observation volume (blue) (A). A fluorescence signal, as a function of time, measured for fluorochrome (B). Measured G (τ) describing the fluorescence fluctuation of fluorochrome molecules as observed by FCS. Note the fit curve (solid red) and measured curve (black dot) (C) (adapted from Hess et al. 2002).

2.3.2 Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) is one type of immunoassay. The core aim of this technique is to detect either quantitatively or qualitatively the presence of an antigen or an antibody in a sample. ELISA is nowadays widely used as a routine diagnostic tool or in the prognostic of diseases starting from allergy (van Ree et al. 2008), HIV (Roberts et al. 2007), cancer (Schiffer 2007), prion molecules (Sakudo et al. 2007), food industry (Abubakar et al. 2007) to viruses as will be shown further. ELISA allows a high sample throughput as well as an ease for automation (Thorpe & Thorpe 2000).

Generally, for performing ELISA an unknown amount of antigen is coated or fixed to a solid support (usually a polystyrene microtiterplate). Then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is usually linked to an enzyme, and in the final step a substance is added that the linked enzyme can convert to some detectable signal (Thorpe & Thorpe 2000). In the first ELISA report, which was a radioimmunoassay (RIA), a radioactive label was used instead of an linked enzyme (Yalow & Berson 1960). The use of radioactive label was more frequent in a time period between 1980 and 1990 (Lequin 2005).

They are three main types of set-up for performing ELISA: the indirect ELISA, the sandwich ELISA or the competitive ELISA. The ELISA could be performed in a qualitative or quantitative set-up. Qualitative results are usually positive or negative for a sample. The cutoff between positive and negative is usually statistically determined. The qualitative analysis could be used when the determination of past or acute infection of a virus. For quantitative ELISA, a standard curve with a serial dilution of the target is usually added in the assay from which the quantification is interpolated (Thorpe & Thorpe 2000).

2.4 Firefly luciferase

Fireflies, click beetles and glowworm beetles are bioluminescent eukaryotic organisms (Wilson 2000). In nature, fireflies are members of the order *Coleoptera* and consist of the family *Lampyridae*. *Photinus pyralis* is one of over 1.800 species of fireflies (Gould & Subramani 1988). Here the main focus will be on the coleopteran North American firefly *Photinus pyralis*, of which luciferase enzyme was produced (article I). It is probably one of the most studied bioluminescent enzymes. Native firefly luciferase was first crystallized in 1956 (Green & McElroy 1956). One milligram of luciferase enzyme can be extracted from about 190 fireflies (De Luca & Mc Elroy 1978) and one milligram of luciferin substrate and can be extracted from about 1,700 fireflies (Bitler & McElroy 1957). The *luc* (Luc) gene coding for firefly luciferase was first cloned in 1985 (de Wet et al. 1985). Since that the enzyme has been expressed in a wide variety of hosts, as will be shown later on.

Firefly luciferase is a 62 kDa protein (de Wet et al. 1985, Wood et al. 1984). The enzyme's bioluminescent reaction is directly proportional to the ATP present in the reaction mixture. The firefly luciferase catalyzes bioluminescent reaction as shown in Fig. 5A (Fraga 2008, Koo et al. 1978). The achievement of the reaction is, however, dependent on the presence of oxygen and an excess of substrate D-luciferin (Fig. 5B). In order to produce carbon dioxide, AMP, oxyluciferin (Fig. 5D) and light via an intermediate molecule D-luciferyl-AMP (Fig. 5C) (Fraga 2008). Light quanta (photons) are produced during the luciferase enzyme reaction, and are not accumulated as end-products of many enzyme reactions are (Virta et al. 1995). The firefly appears to be able to control the flow of oxygen to the lantern, thus being able to make rapid and brilliant flashes of light (Carlson & Copeland 1985) which is the signal between a male and a female in courtship (Michaelidis et al. 2006). Normally, the light produced by *Photinus pyralis* emits a yellow-green light at 562 nm (Brasier et al. 1989). However, the light emission depends on several factors such as pH, temperature (>+ 20^{o}C) and heavy metals concentrations. The optimal pH is at 7.9, at pH 8 the λ_{max} is 548 nm and at pH 6 the λ_{max} is 610 nm (Viviani et al. 2008). By cloning different parts of the firefly genes the light wavelength could be anticipated: Starting from green (546 nm), yellow-green (560 nm), yellow (578 nm) to orange (593 nm) (Lindqvist et al. 1994).

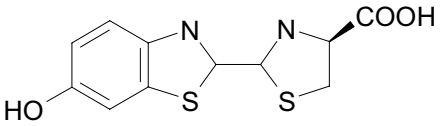
A

$$Luc + ATP \cdot Mg^{2+} + D\text{-}LH_2 \rightleftharpoons Luc \cdot D\text{-}LH_2\text{-}AMP + PP_i \cdot Mg^{2+} \quad (1)$$

$$Luc \cdot D\text{-}LH_2\text{-}AMP + O_2 \longrightarrow Luc + CO_2 + AMP + oxyluciferin + photon \quad (2)$$

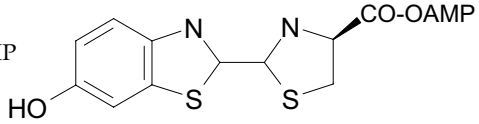
B

D-luciferin
(D-LH_2)



C

D-luciferyl-AMP
(D-LH_2-AMP)



D

oxyluciferin

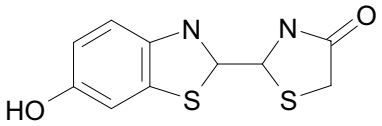


FIGURE 5 The reactions catalyzed by the firefly luciferase (Luc) with luciferin as the substrate (A). The reaction is conducted in two separate reactions (A1 and A2). The first reaction (A1) occurs independently of the presence of O_2. In this reaction, ATP· Mg^{2+} and D-LH_2 (B) react to form an intermediate product, Luc · D-LH_2-AMP (C) and pyrophosphopate- ·Mg^{2+} (PP_i ·Mg^{2+}). After this, there is an aerobic reaction in which the firefly allows the readmission of oxygen to make possible the second reaction (A2). In which the intermediate product Luc· D-LH_2-AMP (C) is oxidized by O_2 to produce CO_2, AMP, oxyluciferin (D) and light (molecules drawn with MDL ISIS™/Draw 2.5, according to Fraga 2008).

2.4.1 Use of firefly luciferase in biotechnology / diagnostic

The concentration of ATP can be measured with luciferase enzyme (Fig. 5). Therefore, the applications of firefly luciferase enzyme in ATP measurement are diverse, especially when monitoring biomass, enzyme and metabolites (Beigi et al. 1999, Kricka 1988, Lundin 2000, Sakakibara et al. 1997, Webster et al. 1988). However, the luciferase used in ATP measurements should be free of contaminants (i.e. nucleoside diphosphokinase, adenylate kinase or creatine kinase). Contaminants could interfere the ATP measurement by enabling the rephosphorylation of adenylic acid or ADP to form ATP (Rajgopal & Vijayalakshmi 1984). Otherwise the bioluminescent-based methods have several advantages over commonly used assays, such as speed, simplicity and a high sensitivity. However, the different applications of firefly luciferase are more used as reporter gene rather than the pure enzyme.

The luciferase gene (Luc) of the firefly *Photinus pyralis* has been proven to be a useful reporter gene in several organisms. Firefly luciferase has been used in many cases, as the following selected examples, in bacteria cells (Ahmed et

al. 2007, Brader et al. 2008, Sanz et al. 2008, Tomimori et al. 2007, Wood et al. 1984, Zhang et al. 2008a), on bacteria cell surface (Beigi et al. 1999), in bacterial biosensor for toxic residues (Anko et al. 2002, Hakkila et al. 2002, Ivask et al. 2004, Ivask et al. 2001, Korpela et al. 1998, Kurittu et al. 2000a, Kurittu et al. 2000b, Tauriainen et al. 1998, Tauriainen et al. 1999, Virta et al. 1995), in yeast cells (Kanjou et al. 2007, Leskinen et al. 2003, Michelini et al. 2005), in insect cells (Hasnain & Nakhai 1990, Isaac et al. 2007, Karp et al. 1996, Karp & Oker-Blom 1999, Lindqvist et al. 1994, Qin et al. 2007, Zhang et al. 2008b), in mammalian cells (Brasier et al. 1989, de Wet et al. 1987, Fan et al. 2008, Gould & Subramani 1988, Kirn et al. 2007, Medvedev et al. 2008, Morancho et al. 2008, Ray & Gambhir 2007) and in plants (Chang et al. 2007, Chung & Sano 2007, Ow et al. 1986). Luciferase reporter assays combine extreme sensitivity and high linearity with a rather simple analysis procedure. In addition, these assays are relatively inexpensive and are a non-isotopic alternative to other reporter gene assays (Bonin et al. 1994).

2.5 Viruses

In the nineteenth century, viruses were not known to be responsible for diseases in animals, plants or insects. At that time, the term *viro*, a Latin word for poisonous material, was used to describe any disease-causing substance. Since then, it has been shown that viruses are fundamentally different from any living organisms. Viruses are infectious small particles (from 18 nm to 300 nm) made of nucleic acids, either DNA or RNA, surrounded by a nucleocapsid. A nucleocapsid is composed of several copies of one or more similar structural protein subunits that could be arranged in icosahedral form. Viruses may also possess a lipid envelope, but viruses do not have a cytoplasm or a specific metabolism machinery. Their existence is dependent on host cells to proliferate (Stainer et al. 1987). Viruses must penetrate their specific host cells and divert the replication system in their favor in order to replicate (Dimitrov 2004).

Usually, the infection ends in the death of the host cell. Although the infection procedure may vary between different viruses, it follows some general rules. Firstly, the viruses deliver their genetic material into the receiver organism. Secondly, the virus mobilizes the cell machinery in order to replicate, transcribe and translate its nucleic acid. Thirdly, the freshly made protein elements assemble into particles, and finally, viral particles leave the cells by a defined route to propagate into other cells. However, the infected organisms try to avoid the expansion of viruses by using enzymes or the immune system. Therefore, the infection process can occur for long periods of time, and it can be latent (Flint et al. 2000).

2.6 Baculoviruses

The *Baculoviridae* family forms a wide group of more than 500 known viruses (Friesen & Miller 2001). *Baculum* is Latin word for rod or stick, which describes the virus. Baculoviruses are large rod-shaped (approximately 250 to 300 nm long and 30 to 60 nm in diameter) (Fig. 6A), enveloped viruses that contain circular double-stranded DNA (dsDNA) genomes (90 to 160 kb) (Stainer et al. 1987). The *Baculoviridae* family is divided into two genera: Firstly, granulovirus or granulosis virus (GV) and secondly, nucleopolyhedrovirus or nuclear polyhedrosis virus (NPV). Both genus includes a single nuclear polyhedrosis virus (SNPV) as well as multiple nucleopolyhedrovirus (MNPV) (Friesen & Miller 2001, ICTV 2008a). The prototype members are multiple nucleopolyhedroviruses (MNPVs), such as *Autographa californica* MNPV (*Ac*MNPV) and *Bombyx mori* MNPV (*Bm*MNPV). *Ac*MNPV is the most widely used in the baculovirus expression vector system (BEVS) (Thiem 1997).

Baculoviruses are lytic antropod-specific viruses with a preference to infect lepidopteran (moth and butterfly) species. Usually, baculoviruses are highly specific and have a narrow and distinct host range. However, certain baculoviruses including *Ac*MNPV have broader host ranges, infecting more than 30 different lepidopteron species (Friesen & Miller 2001). Baculoviruses are an integral part of the nature's ecosystem. They play an important role in the regulation of the size of insect populations. Baculoviruses are safe for humans and other vertebrates. Therefore, baculoviruses could be used as potential biological control agents of insect pests in agriculture and forestry as alternative to indiscriminant chemical insecticides (Bonning & Nusawardani 2007, van Beek & Davis 2007). Unfortunately, the effective use of baculoviruses as a bio-insecticide is disadvantaged by the long time required to kill the host. This can take several days or weeks, during which time insects continue to feed (Li & Bonning 2007, Thiem 1997). Also, baculoviruses have a limited shelf life (Inceoglu et al. 2006). However, modifications, such as insertion of insect specific genes for toxins, hormones or enzymes into the virus genome, have been shown to cause the insects to stop feeding (Bonning & Hammock 1996, Inceoglu et al. 2006, Miller 1995, Summers 2006).

Baculoviruses can be divided into two distinct morphological forms of infectious particles: Firstly, the occluded virus (OV), comprising enveloped virions packed within a matrix of protein. Secondly, the budded virus (BV), comprising a single virion enveloped by a membrane (Friesen & Miller 2001). Within GVs, a single virion is embedded in each ovicylindrical occluded virus (OV) particle. When particles are packed together they have a "granular" appearance under the light microscope. On the other hand, NPVs have numerous (>20) virions per polyhedral occluded virus (OV) particle (polyhedra). OV particles have a diameter of several microns and they could be easily visualized by light microscope (Fig. 6B). The polyhedra occlusion is made predominantly of a single protein, the polyhedrin protein (29 kDa). The protein is vital for the natural propagation of the virus (Murphy et al. 1997). However,

polyhedrin protein is not needed when using viruses *in vitro*. OV generally appears after the loss of the nuclear membrane in GV-infected cells, whereas in NPV-infected cells OV is produced in the nucleus (Friesen & Miller 2001). Within *in vitro* recombinant protein production the BV of NPVs is used as presented further.

A B

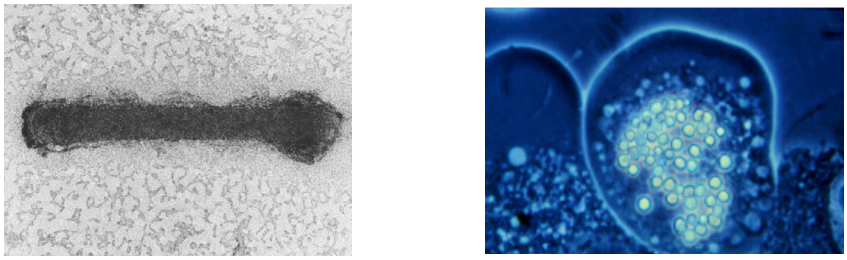


FIGURE 6 A negatively stained preparation of budded virus of *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) under EM. The particle is of 40 nm in diameter and approximately 270 nm in length (Grabherr et al. 2001 with permission from Elsevier) (A). Infected insect cell with polyhedral occluded virus particles (light green points) (B) (Kindly provided by Vail, P.; USDA Agricultural Research Service, Horticultural Crops Res. Lab., Fresno, CA, USA.)

2.6.1 Life cycle and proteins of baculovirus

The baculovirus life cycle is divided temporally into several phases: early phase (6- 9 h p.i.), late phase (6- 24 h p.i.) and very late phase (18- 72 h p.i.). Viruses enter the cell by receptor-mediated endocytosis and move to the nucleus, where their DNA is released. DNA replication starts about 6 hours after infection and is followed by viral assembly in the nucleus. Extracellular virus is released from the cell by budding (Fraser 1986). The virus production starts at approximately 12 h post infection (p.i.) (Grabherr et al. 2001, Murphy et al. 1997). During the late and very late phases glycoprotein 64 (gp64) is expressed. The gp64 is further acquired by the virus while budding out of the cell (Grabherr et al. 2001) (Fig. 7). Virus DNA is associated with the protein p6.9 to from the nucleoprotein core, which is additionally encapsidated with the major capsid protein p39 (Blissard 1996).

The BEVS is an eukaryotic expression system that has a machinery suitable for abundant (up to 500 mg per liter of cell suspension) production of soluble and correctly folded complex eukaryotic proteins (Crossen & Gruenwald 1998, Luckow & Summers 1988, Miller 1988, Summers & Smith 1987). One possible method for the production of recombinant baculoviruses is the use of commercially available Bac-to-Bac™ system (GibcoBRL, Life Technologies; Rockville, MD, USA). The system is based on the transposon-mediated insertion of the foreign gene into the baculovirus genome under the transcriptional regulation of the polyhedrin gene promoter (Luckow et al. 1993). Foreign genes could be added into the genome with an unknown limit of

maximal size of insert. In addition, the *Ac*MNPV can be propagated in insect cells adapted for growth in monolayer or suspension culture (Murphy et al. 1997). The most frequently used insect cell lines are *Spodoptera frugiperda* (*Sf*-9 and *Sf*-21) and *Trichoplusia ni* (*Tn*-368 or High-Five™, Gibco BRL, Invitrogen, Carlsbad, CA). *Trichoplusia ni* cells are more efficient for the production of secreted proteins (Hu 2005). However, insect cells viability after baculovirus infection is relatively low. Therefore, when producing secreted recombinant proteins other insect cell lines such as *Drosophila* could be taken into an account as they are not affected by induction (Bernard et al. 1994). Healthy insect cell divide every 18-24 h at +28 °C to a density of about 3 to 4 x 10^6 cells/ml (Murphy et al. 1997). When infected the cells become uniformly round, swollen, develop enlarged nuclei, as shown in Fig. 5B and they stop dividing and lysis (Crossen & Gruenwald 1998). By using a perfusion system cells densities over 30 x 10^6 cells/ml can be reached (Zhang et al. 1998). Fed-batch culture or specific nutrient supplementation could also be taken into consideration if higher cell densities would be needed (Ikonomou et al. 2003).

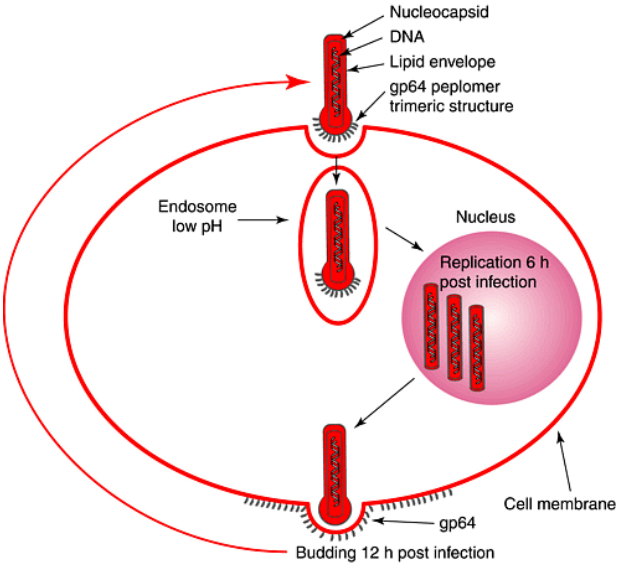


FIGURE 7 Life cycle and structure of budded baculovirus (BV) as found in insect cells and BEVS. The virion nucleocapsid is rod-shaped, enveloped with lipids. It contains a circular double-stranded DNA (dsDNA) genome. The virus enters insect cells by endocytosis, and is further released to the insect cell nucleus. Replication of virions occurs about 6 h post infection (p.i). At 12 h p.i. budding of nucleocapsid takes place and pick up gp64 in a trimer form at one end of the virus (from Grabherr et al. 2001 with permission from Elsevier).

2.6.2 Use of baculoviruses in biotechnology and gene therapy

The first protein expressed with baculovirus and insect cells was the human beta interferon in 1983 (Smith et al. 1983). Since that, baculovirus has shown to be an efficient tool for production of thousands of recombinant proteins in insect cells and acting as biological insecticide (Fath-Goodin et al. 2006, Inceoglu et al. 2006, Luckow & Summers 1988, Summers 2006, van Oers 2006). Baculovirus and insect cells have together the potential to express intricate proteins from human, animal and viral sources. The protein folding and subunit assembly as well as post translational modifications are efficiently processed (Grabherr et al. 2001). One recent example of recombinant protein is the production of hydrophobin as a fusion partner to facilitate the recombinant protein purification in one step (Lahtinen et al. 2008).

Baculovirus is non pathogenic for human, however, the virus enters mammalian cells and its DNA reach the nucleus, but do not replicate (Tjia et al. 1983). It has also been shown that baculoviruses can be internalized in human carcinoma cells (Volkman & Goldsmith 1983) which made it an attractive tool in cancer gene therapy. Moreover, the expression of foreign proteins by using baculovirus in mammalian cells has been done. One of the first experiments was the addition in the baculovirus genome of a mammalian promoter extracted from the cytomegalovirus (CMV). This was coupled to the gene expressing the luciferase enzyme of *Photinus pyralis*. The virus was then used to transduce mammalian cells where the proteins were efficiently expressed in mammalian cells (Hofmann et al. 1995). Green fluorescent protein (GFP) was also expressed, using the CMV promoter, stably in several mammalian cells lines (Condreay et al. 1999). Besides, the gene expression of baculovirus vector was compared to adenovirus vector by using the same expression cassette. It was demonstrated that the expression levels and efficiency of both vectors were similar in mammalian cells (Shoji et al. 1997). Since then, the use of baculovirus as potential tool for gene therapy vector was confirmed (Possee 1997). Cell types where baculovirus have been efficiently transduced is relatively broad, including human, rodent, fish, rabbit, monkey, porcine and bovine cells (Ghosh et al. 2002, Hu 2006). Beside CMV promoter, other promoters have been used like the Rous sarcoma virus-long terminal repeat (RSV-LTR), the chicken β-actin promoter (CAG), the simian virus 40 promoter (SV40) and the hepatitis B virus promoter (HBV) (Ghosh et al. 2002). Together, the baculovirus and its expression cassette can lead to an efficient vector *in vivo* (Kost & Condreay 2002).

One other possible application is to display proteins or peptides in a eukaryotic environment on the baculovirus surface. As the gp64 of baculovirus is essential for the virus entry into insect cells (Hofmann & Strauss 1998, Matilainen et al. 2005) it is an ideal plateau for diverse proteins presentation (Hu 2006). It should be pointed out that the fusion protein produced may be lower than the total amount of wild-type gp64. It was measured that an average of only 3.2 gp64-GFP fusion proteins were present at the surface of the virion (Toivola et al. 2002). Nevertheless, in one of the first studies, the major

glycoprotein (gp120) of the human immunodeficiency virus type 1 (HIV-1) fused to gp64 was bound to its original ligand (CD4) (Boublik et al. 1995). After which, from the same virus (HIV-1), the ectodomain of the envelope protein gp41 was fused to gp64 and to the membrane anchor sequence of gp64 (Grabherr et al. 1997). Further, small peptides and the epitope sequence of HIV-1 gp41, specific for neutralizing human monoclonal antibodies, were inserted into the coding region for gp64 (Ernst et al. 2000). Also, on the gp64 of baculovirus several proteins such as GFP, rubella virus envelope proteins E1 and E2 have been displayed. Those proteins were mainly detected by using immunoblotting techniques against the FLAG epitope. The FLAG epitope was expressed at the amino terminus of the fusion proteins (Mottershead et al. 1997). Foreign proteins have also been displayed on the major capsid protein vp39, of baculovirus (Oker-Blom et al. 2003). On the N- or C- terminus of the vp39 protein, enhanced green fluorescent protein (EGFP) was displayed with successful virus capsid formation (Kukkonen et al. 2003). Together, all those techniques have lead today to an efficient technology named baculovirus display vector system (BDVS). BDVS has numerous applications in drug delivery, in gene therapy vector and in molecule screening (Mäkelä & Oker-Blom 2008).

2.7 Epstein-Barr virus

The *Herpesviridae* family as shown in Table 6 is divided into three subfamilies (*Alphaherpesvirinae*, *Betaherpesvirinae*, *Gammaherpesvirinae*). The division is made on the basis of their biological properties (Cann 2001). The Epstein-Barr virus (EBV) or human herpes virus 4 (HHV-4) is a member of the *Gammaherpesvirinae* subfamily and *Lymphocryptovirus* genus (ICTV 2008b) and induces chronic infections in lymphoblastoid cells (Khanna et al. 1995). EBV is an enveloped icosahedral virus with a protein tegument between the capsid and the envelope with external glycoprotein spikes (Khanna et al. 1995). The EBV virus has a linear, double-stranded DNA genome of 172 kb (Murray & Young 2002). EBV has a particle size of about 120 nm (Klein et al. 1978), where the *Herpesviridae* family size range is 120-200 nm (Dimitrov 2004). The virus entry to the cell uses a non-endocytic route by fusion at the cell surface by using potential receptors such as heparin sulphate, herpesvirus entry protein A (HveA), Nectin-1α, Nectin-1β or 3-OST-3_A (Dimitrov 2004).

EBV was found more than four decades ago by Michael Epstein, Bert Achong and Yvonne Barr (Epstein et al. 1964) in Burkitt's lymphoma cultured cells taken from children in Uganda (Burkitt & Wright 1966, O'Conor 1963). The lymphoma cancer was previously described by Dennis Burkitt (Burkitt 1958, Burkitt 1962) and others (Henle et al. 1968). After this EBV was found to be involved in several diseases such as: infectious mononucleosis (IM) (Henle et al. 1968), nasopharyngeal carcinoma (zur Hausen et al. 1970), T-cell lymphomas and Hodgkin's lymphoma (Jones et al. 1988, Mueller et al. 1989), autoimmune

disease or systematic lupus erythematosus (SLE or lupus) (Harley & James 2006), multiple sclerosis (MS) (Buljevac et al. 2005, De Lorenze et al. 2006, Lünemann et al. 2007), gastric carcinoma (Takada 2000) and in acquired immunodeficiency syndrome (AIDS) patients. EBV has also been associated with non-Hodgkin's lymphoma and oral hairy leukoplakia (Greenspan et al. 1985, Ziegler et al. 1982).

EBV is widespread and more than 90% of the world's adults are infected by the virus (Rickinson & Kieff 2001). EBV's primary infection is often asymptomatic mainly in children (Hess 2004). Infection occurs in circulating B-lymphocytes where the virus persists for life (Borza & Hutt-Fletcher 2002). Free viruses could be detected in saliva (Cohen 2000). The maintenance in B cell latency is dependent on CD40 (Kim et al. 2003). Therefore, the virus is spread mainly through saliva and could cause IM, also known as kissing disease, in case of primary infection, especially in young adults (Hess 2004). However, EBV is not the only causative pathogen of IM as it could be caused by others such as human herpesvirus type 5 or human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6), adenovirus (AdV), rubella virus (RV), human immunodeficiency virus (HIV), hepatitis A virus (HAV), influenza A and B viruses and *Toxoplasma gondii* (Hess 2004).

TABLE 6 *Herpesviridae* family taxonomy and some relevant examples. B19 is classified in the erythrovirus genus (adapted from ICTV 2008b)

Subfamily	Genus	Virus	Abbreviated name
Alphaherpesvirinae	*Simplexvirus*	Herpes simplex virus type 1	HSV-1
		Herpes simplex virus type 2	HSV-2
	Varicellovirus	Human herpesvirus 3 or Varicella-zoster virus	HHV-3 or VZV
Betaherpesvirinae	*Cytomegalovirus*	Human herpesvirus type 5 or Human cytomegalovirus	HHV-5 or HCMV
	Roseolovirus	Human herpesvirus 6	HHV-6
		Human herpesvirus 7	HHV-7
Gammaherpesvirinae	*Lymphocryptovirus*	Human herpesvirus 4 or Epstein-Barr virus	HHV-4 or EBV
	Rhadinovirus	Human herpesvirus 8 or Kaposi's sarcoma-associated herpesvirus	HHV-8 or KSHV

2.7.1 Epstein-Barr virus proteins

Briefly, there are two known strains of EBV, the type 1 and 2 or type A and B (Rickinson & Kieff 2001). Both are encoding several proteins with some differences, where the most important ones are the nuclear antigens for the virus latency (EBNAs) EBNA-1, -2, -3A, -3B and -LP and three latent membrane proteins (LMPs) LMP-1, -2A and -2B and small polyadenylated RNAs (EBERs)

EBER-1 and -2 (Kieff & Rickinson 2001, Murray & Young 2002, Rickinson & Kieff 2001). The LMP-1 has been located (Liebowitz et al. 1986, Vazirabadi et al. 2003) and its immunosuppressive effects studied (Dukers et al. 2000, Keryer-Bibens et al. 2006). The genome of EBV has been completely sequenced (Murray & Young 2002). The most studied strain is the B95-8 isolate (Baer et al. 1984, Skare et al. 1982). The EBNA-1 of EBV is the only viral protein replicated during latency. EBNA-1 is a DNA binding protein essential for EBV episomal maintenance (Cohen 2000, Münz 2004) and as it binds to the latent origin of replication *cis* element (*oriP*) (Frappier & O'Donnell 1991a, Frappier & O'Donnell 1992, Sears et al. 2003). EBNA-1 has a *trans*-acting gene to allow the function of *oriP* (Yates et al. 1985). The role of EBNA-1 in the replication process has been studied (Ermakova et al. 1996, Kapoor et al. 2001, Laine & Frappier 1995, Nonkwelo et al. 1997, Ruf et al. 1999, Shah et al. 1992, Shire et al. 1999, Wu et al. 2000). The binding of *oriP* to DNA has been studied by several researchers (Aiyar & Sugden 1998, Ambinder et al. 1991, Avolio-Hunter et al. 2001). Furthermore, EBNA-1 might have a role in oncogenesis (Murray & Young 2002).

EBNA1 from prototypical B95 EBV stain consists of 641 amino acids (Baer et al. 1984, Skare et al. 1982). The protein is composed of four major components, from the amino to the carboxyl terminus: 1) an amino-terminal of 89 amino acids which is glycine/ arginine (Gly-Arg) rich (aa 33-53) (Shire et al. 2006), 2) the amino acids 90 to 325 which are glycine/ alanine (Gly-Ala) repeat (Goldsmith et al. 1993), 3) the amino acids 328 to 386 which are glycine/ arginine (Gly-Arg) rich (aa 325-376) (Shire et al. 2006) and include a nuclear localization sequence at amino acids 379 to 386 (Goldsmith et al. 1993), 4) at carboxyl terminus the amino acids 387 to 641 that bind DNA and dimerize (Goldsmith et al. 1993). The EBNA-1 native protein has a molecular weight in a range of 55 to 97 kDa, this is depending on the number of glycine/ alanine repeats within the protein (Snudden et al. 1994) and about 80 kDa for the B95-8 isolate (Falk et al. 1995). Without the glycine/ alanine repeats the molecular weight is about 55 kDa (Frappier & O'Donnell 1991b). This may avoid nonspecific reaction with DNA (Baboonian et al. 1991, Fachiroh et al. 2006, Mathew et al. 1994). It has been suggested that the glycine/ alanine repeats have an proteolytic protective effect for EBNA-1 (Levitskaya et al. 1997, Mukherjee et al. 1998).

EBNA-1 has been studied as protein-reporter with extrachromosomal maintenance (Shioda et al. 2000) or gene therapy vector (Shan et al. 2006). It has been purified in complete or truncated forms from several sources such as mammalian cells (Baron & Strominger 1978, Durocher et al. 2002, Fischer et al. 1984), insect cells (Frappier & O'Donnell 1991b, Hearing et al. 1992, Hille et al. 1993, Meij et al. 2000) and bacteria cells (Duellman & Burgess 2006). Beside EBNAs, EBV major antigens are the viral capsid antigen (VCA), the membrane antigen (MA) and early antigen (EA) (Gulley 2001). VCA has been produced in insect cells (Sánchez-Martínez et al. 1995). Immunoglobulin amounts for VCA, EBNA-1 and EA are measured when EBV diagnosis is needed, as developed below.

2.7.2 Diagnosis of Epstein-Barr virus

In EBV infection, there are several types of latency in which viral proteins are expressed and are linked with diseases (Savard & Gosselin 2006). EBNA-1 is one of the rare types to be expressed efficiently in most of the stages of latency. In type I latency occurs in healthy seropositive carrier or in Burkitt's lymphoma and gastric carcinoma cases. Type II latency is characteristic of nasopharyngeal carcinoma, Hodgkin's disease, and T cell lymphoma. Finally, in type III latency several diseases could be found, such as lymphoproliferative disease and infectious mononucleosis (Cohen 2000, Gulley 2001).

Therefore, antibodies against EBNA-1 IgG could be markers of a disease as well as antibodies against VCA IgM and VCA IgG. Table 7 presents the interpretation of results to speculate if there is no-, acute- or past-infection. Moreover, the antibody levels of VCA IgM, VCA IgG, EBNA-1 IgG and EA-IgG after acute primary infection, past infection as well as after reactivation are shown in Figure 8. Together those antibody measurements are the basis of detection of primary infection of EBV with an emphasis on EBNA-1, where antibodies IgG to EBNA-1 are the most reliable ones to prove the past presence of EBV (Bruu et al. 2000, Cohen 2000, Gärtner et al. 2003, Robertson et al. 2003).

TABLE 7 An interpretation of antibody (VCA IgM, IgG and EBNA-1 IgG) profiles for diagnosis of EBV infection. * Further testing recommended after EIA or ELSIA by avidity testing of VCA IgG, western blot analysis or PCR (modified from Hess 2004).

VCA IgM	VCA IgG	EBNA-1 IgG	Interpretation
-	-	-	No infection
+	+	-	Acute infection
-	+	+	Past infection
+	-	-	Indeterminate *
-	+	+	Indeterminate *
+	+	+	Indeterminate *
-	-	+	Not plausible

The preliminary and relatively reliable detection in serology is usually made by measurement from human sera sample by EIA and/or ELISA. The method is rapid, highly sensitive and suitable for automation (Hess 2004). Those methods are usually compared to the gold standard technique, the immunofluorescence assay (IFA). IFA is sensitive but not suitable for large sample amounts (Kleines et al. 2006). Results coming from both methods are often confirmed by immunoblotting (Hess 2004). On the other hand, the diagnosis of EBV, when EBV-associated tumors occurs, could be further proved or screened by PCR or in situ hybridization (Hess 2004). For more details read (Gulley 2001).

Diagnostic methods for EBV detection have been extensively tested as follows. Several EIA commercial tests were mainly evaluated for VCA IgM, VCA IgG and EBNA-1 IgG antibodies (Bruu et al. 2000) and similarly with an addition of IFA (Gärtner et al. 2003). For the same antibodies a combination of EIA and immunoblot have been reported for IM suspicion (Nystad & Myrmel

2007). The sensitivity of IFA for pediatric patients was suggested as limited (Kleines et al. 2006) as the technique was previously introduced (Hille et al. 1993). The antibodies against EBNA-1 were also titrated with ELISA (Inoue et al. 1992). For nasopharyngeal carcinoma a combination of ELISA and immunoblot have been used (Fachiroh et al. 2006). For EBV and risk of lymphoma detection, a screening by immunoblot analysis was carried out (de Sanjosé et al. 2007). The confirmation of acute EBV infection has been proved by immunoblotting (Schubert et al. 1998). For the identification of EBV isolates, a fragment length polymorphism and PCR have been reported (Falk et al. 1995). PCR was also used to find the evidence of non-EBV association in Hodkin disease (Gallagher et al. 2002) and Burkitt's lymphoma (Hassan et al. 2006). However, the limitation of PCR for acute detection of EBV was reported (She et al. 2007). Moreover, no apparent correlation between EBNA-1 and EBV viral DNA amount was found (Sternås et al. 1990). There is a minor correlation between viral load and EIA results (Gärtner et al. 2000). Recently, chromogenic in situ hybridization was used for the detection of EBV in cases of extra-hepatic biliary atresia (Mahjoub et al. 2008). The visualization of episomal and integrated EBV DNA have been done by fluorescence in situ hybridization (Reisinger et al. 2006).

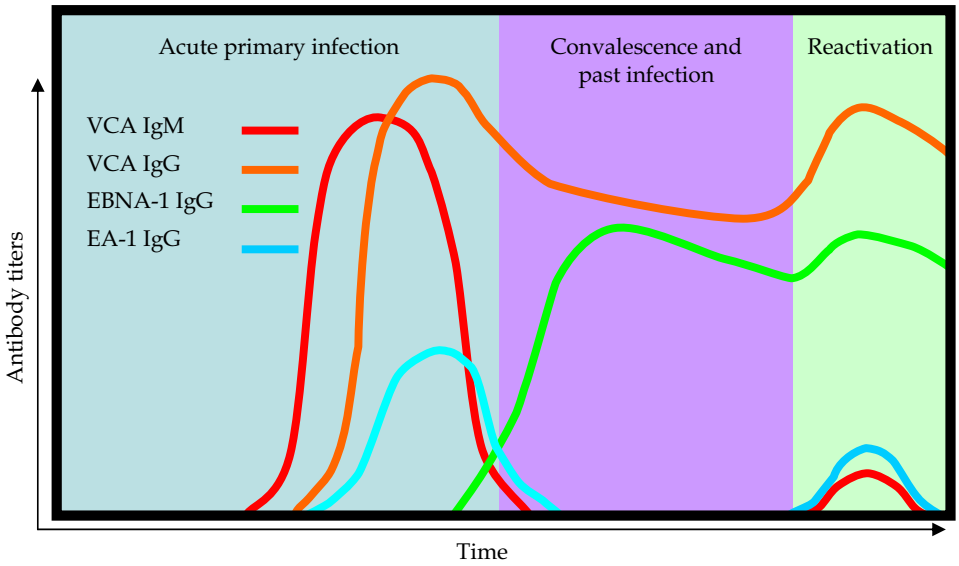


FIGURE 8 Primary EBV infection in healthy hosts causes several serological responses. First, IgM antibody against viral capsid antigen (VCA) amount rises. Then, antibodies against Epstein-Barr nuclear antigen-1 (EBNA-1) emerge at least 1 month post infection. Antigens are detectable together with IgG anti-VCA. VCA IgG and EBNA-1 IgG are markers of prior infection and indicators of EBV reactivation. In a minor level, titers against early antigen (EA) rise on primary infection, and are also reactivated in case of EBV reactivation (figure modified from Gulley 2001, Vainionpää et al. 2000).

2.8 Parvoviruses

The *Parvoviridae* family forms a wide group of viruses that are common animal pathogens. *Parvum* is Latin word for small and parvoviruses are among the smallest viruses with a diameter of 18-26 nm as measured with electron microscope (Agbandje et al. 1994, Agbandje et al. 1991, Casal 2001, Kajigaya et al. 1991, Kajigaya et al. 1989). Parvoviruses are non-enveloped, single stranded DNA (ssDNA) viruses (Muzyczka & Berns 2001). The native particle is composed of proteins and DNA (Mori et al. 1987). The parvovirus virion can be composed of a mixture of viral proteins (VP), VP1, VP2, VP3 and VP4 which encapsidate the DNA (Simpson et al. 2002). Human parvovirus B19 (B19) capsid is composed of only two proteins VP1 and VP2 (Young 1995). The virus particle has an icosahedral symmetry and comprises 60 copies of structural protein monomer (Kerr 2000a). The *Parvoviridae* family contains two subfamilies: the *Parvovirinae*, which infect vertebrates, and the *Densovirinae*, which infect invertebrates. Because of the non-vertebrate host range of the *Densovirinae*, only the *Parvovirinae* group will be focused on here (for further inquiry see (Muzyczka & Berns 2001). The five genera of the subfamily *Parvovirinae* are (Table 4): *Amdovirus*, *Bocavirus*, *Dependovirus*, *Erythrovirus* and *Parvovirus*. *Amdovirus*, *Bocavirus* and *Parvovirus* members replicate autonomously, *Dependovirus* members usually require the co-infection of a helper virus, such as adenovirus or herpesvirus, for successful replication and finally *Erythrovirus* members replicate autonomously in an extremely limited tissue host range, like erythroid progenitor cells (Kerr 2000a, Muzyczka & Berns 2001).

TABLE 4 *Parvovirinae* subfamily taxonomy and some relevant examples. B19 is classified in the erythrovirus genus (adapted from ICTV 2008c, Muzyczka & Berns 2001).

Genus	Virus	Abbreviated name	Host
Amdovirus	Aleutian mink disease parvovirus	AMDV	Mink
Bocavirus	Bovine parvovirus	BPV	Cattle
	Canine minute virus	CnMV	Dog
	Human bocavirus	HBoV	Human
Dependovirus	Adeno-associated virus, serotypes 1-8	AAV-1-8	Human
	Muscovy duck parvovirus	MDPV	Bird
	Goose parvovirus	GPV	Bird
Erythrovirus	Human parvovirus B19	B19	Human
	Simian parvovirus	SPV	Monkey
Parvovirus	Feline panleukopenia virus	FPLV	Feline
	Canine parvovirus	CPV	Dog
	Mink enteritis virus	MEV	Mink
	H-1 parvovirus	H-1	Rodent
	LuIII virus	LuIIIV	Human
	Minute virus of mice	MVM	Mice
	Porcine parvovirus	PPV	Pig

The human parvovirus B19 belongs to *Erythrovirus* genus. The particular name of B19 has its origin in the discovery of the virus in 1974 (Cossart et al. 1975). Yvonne Cossart and others noted false positive results in an assay for hepatitis B virus when evaluating different tests on blood sera. B19 originates from the coding of that patient's sample as specimen 19 in panel B. After examination of the sera with electron microscope (EM), particles of about 23 nm resembling parvovirus were seen, and the virus was subsequently named B19.

B19 is one the few parvovirus so far known to be pathogenic to humans (Desselberger 2000, Kieff & Rickinson 2001). B19 tends to infect rapidly dividing tissues, such as the human erythroid progenitor cells or haematopoetic system and the fetus (Kerr 2000a). Particularly it binds those cells on their cellular receptor, the P antigen (Weigel-Kelley et al. 2001). It has been identified to be responsible for several diseases such as a common childhood illness known as "fifth disease" or erythema infectiosum resulting in mild fever and rash (Anderson et al. 1983). It has also been involved in inducing hematologic disorders such as anemia in immunocompromised and transient aplastic crisis in sickle cell-diseased suffering patients (Kurtzman et al. 1987, Serjeant et al. 1981). Anemia in kidney, liver or bone marrow in thoracic organ (Calvet et al. 1999, Wicki et al. 1997) or in liver transplanted patients (Liang et al. 2007) have also been reported. In addition, B19 has been shown to have an effect in various rheumatic diseases (Kerr 2000b, Lehmann et al. 2002, Narvaez Garcia et al. 2001, Takahashi et al. 1998) and it has been associated with autoimmune diseases (Lehmann et al. 2003, Meyer 2003) and heart failures (Seishima et al. 2008). B19 has also been associated in hydrops fetalis, an early fetal loss or fetal death after maternal infection during pregnancy (Brown et al. 1984, Cubel et al. 1996, Nyman et al. 2002, Savarese et al. 2008, Tolfvenstam et al. 2001, von Kaisenberg & Jonat 2001). B19 manifestations and clinical aspects have been reviewed by several authors (Broliden et al. 2006, Heegaard & Brown 2002, Sabella & Goldfarb 1999).

2.8.1 Human parvovirus proteins

B19 is a non-enveloped virus with a diameter (as determined with electron microscopy) of between 18 and 26 nm (Agbandje et al. 1994, Agbandje et al. 1991). Mature virus particles have a boyant density in cesium chloride of 1.41 g/ml (Kerr 2000a). The B19 virus genome is composed of a linear single-stranded DNA molecule (5.6 kb) (Shade et al. 1986). The genome encodes two structural proteins VP1 and VP2. The region encoding VP1 has 781 amino acid residues. VP2 is included within VP1; it has 554 amino acid residues but is missing 227 amino acid residues from the N-terminus. Moreover, the genome encodes three non-structural proteins (NSPs) (Table 5) (Raab et al. 2002, St Amand et al. 1991, Umene & Nunoue 2002, Young 1995). The native capsid is composed of approximately 95% of VP2, the major capsid element, and of approximately 5% of VP1 (Agbandje et al. 1994, Kawase et al. 1995). It has been shown that NSPs are required for parvovirus replication and apoptosis (Young 1995). A direct interaction between NSPs with some DNA sequences influences

the viral gene expression and promoter activity (Raab et al. 2002). It has also been suggested that NSP induce apoptosis in erythroid lineage cells (Moffatt et al. 1998, Ozawa et al. 1988) with an unknown mechanism (Morita et al. 2001, Pillet & Morinet 2002). Others have noticed that the VP1 unique region is also needed (not only VP2) for productive infection (Dorsch et al. 2001). VP1 also has a constituent enzymatic activity possibly involved in entry (Dorsch et al. 2002). For its entry, B19 binds specifically to red blood cell's P antigen or globoside (Gb4) (Kerr 2000a). Its entry route is by endocytosis (Dimitrov 2004). This feature has been exploited in constructing a vector system for gene therapy in applications for diseases affecting the human haematopoetic system (Ponnazhagan et al. 1998, Weigel-Kelley & Srivastava 2002). It has been discovered that the P antigen is necessary, but not sufficient for successful transduction (Weigel-Kelley et al. 2001).

TABLE 5 B19 translational products (St Amand et al. 1991).

Protein	Size (kDa)	No. aa	Function	
7.5 kDa	7.5	72	Unknown	Non-structural
11.5 kDa	11.5	94	Unknown	Non-structural
NSP	78	671	Replication	Non-structural
VP1	84	781	Virus capsid	Structural
VP2	58	554	Virus capsid	Structural

The VP2 of B19, as well as the corresponding nucleocapsid proteins of other parvoviruses, is self-assembled into virus-like particles (VLPs). Those capsid-like structures are composed of viral proteins only. They resemble closely to native virus but are devoid of native DNA. Within the *Parvovirinae* family many VLPs have been successfully expressed in insect or mammalian cells such as AMDV (Wu et al. 1994), AAV (Anderson et al. 2000), B19 (Agbandje et al. 1994, Kajigaya et al. 1989), CPV (Wu & Rossmann 1993), MEV (Casal 2001), MVM (Agbandje-McKenna et al. 1998, Hernando et al. 2000), PPV (Rueda et al. 2000).

2.8.2 Parvovirus and diagnostic

The infection of B19 could be almost asymptomatic; however B19 is involved in many diseases (Kerr 2000a, Laub & Strengers 2002, Sabella & Goldfarb 1999). The seroprevalance is very high by the age of 15, 50% have detectable IgG and up to 85% at the age of 70 (Cohen & Buckley 1988, Kerr 2000a, Young 1995). B19 DNA has been found to persist in certain tissues for decades (Hokynar et al. 2000, Söderlund-Venermo et al. 2002). B19 occurrences are likely to affect children or day-care personnel (Young 1995), and hospital staff (Miyamoto et al. 2000). Rules for the control of B19 infection in healthcare workers and the population have been introduced (Crowcroft et al. 1999). B19 is generally transmitted through the respiratory tract (Kerr 2000a), infrequently through blood products (Laub & Strengers 2002), and evidently not through feces or urine (Young 1995). However, it has been pointed out that blood products should be screened (Wong & Brown 2006). The antibody response to B19 in

patients starts with an increasing of IgM 10-12 days post infection of the primary infection. IgM antibodies persist for about two months (Brown et al. 1989, Cohen et al. 1983). After which IgG concentration increases 15-17 days post infection and persists (Anderson et al. 1985). The immunological process of the virus infection has also been explained elsewhere (Klenerman et al. 2002, Modrow & Dorsch 2002, Palmer et al. 1996). The accurate detection of IgM in sera during pregnancy is crucial to anticipate fetal hydrops (Enders et al. 2007, Enders et al. 2006). Usually, the presence of IgM in sera is predicative of B19 DNA (Corcoran et al. 2007). Therefore, accurate detection of IgM and IgG against B19 is suitable.

For the diagnostic procedures of B19 infection, many techniques could be used. For antibodies detection techniques like enzyme immunoassay (EIA) or blot immunoassays are usually utilized and for the detection of virus DNA, PCR based techniques are also used (Zerbini et al. 2002). Some selected reports using EIA (Corcoran et al. 2007, Enders et al. 2007, Jordan 2000, Kock 1995, Pickering et al. 1998, Rayment et al. 1990, Salimans et al. 1992), EIA and western blot (Manaresi et al. 2004, Manaresi et al. 2001, Pfrepper et al. 2005), EIA and immunofluorescence assay as well as western blot (Kerr et al. 1999), PCR (Aberham et al. 2001, Calvet et al. 1999, Wong & Brown 2006), EIA and PCR (Enders et al. 2006, Hoebe et al. 2002, Mendonca et al. 2005, Us et al. 2007, Wermelinger et al. 2002) have been reported.

The detection of B19 specific immunoglobulin is often directed against conformational and linear epitope of the viral capsid as well as against the non structural protein (Ennis et al. 2001, Kerr et al. 1999, Pfrepper et al. 2005, Söderlund et al. 1995b). Conformational B19 epitopes are a source of choice for EIA as linear epitopes are gradually lost after infection (Jordan 2000, Kerr et al. 1999). Recombinant virus-like particles produced in eukaryotic systems such as baculovirus based (Enders et al. 2007, Kerr et al. 1999, Kock 1995, Mendonca et al. 2005, Salimans et al. 1992, Wermelinger et al. 2002) or yeast (Lowin et al. 2005) are efficient supplies of conformational epitopes. However, linear epitopes produced in *Escherichia coli* (Manaresi et al. 2004, Manaresi et al. 1999, Manaresi et al. 2001, Pfrepper et al. 2005, Söderlund et al. 1995a, Söderlund et al. 1995b, Söderlund et al. 1992) or insect cells (Kaikkonen et al. 1999) have been used for EIA, as option to conformational epitopes. To conclude, EIA have proven to be one of the most important tools in the majority of cases, for accurate diagnosis of IgM or IgG of B19. In other selected clinical cases, the detection of B19 infection can be complemented by PCR based techniques (de Jong et al. 2006, Peterlana et al. 2006).

3 AIMS OF THE STUDY

Usually recombinant proteins are expressed *in vivo* and if they are not secreted, they need to be purified from their host cells. When a large amount of functional proteins is needed, it is of great importance to develop alternatives to traditional purification processes. In addition, one should assess that the purified proteins are correctly folded and functional.

The aims of this study were to express important and diagnostically relevant recombinant proteins in insect cells. The proteins with or without an affinity tag were then purified from their host cells. Their potential in adenosine triphosphate measurement as well as in Epstein-Barr virus and human parvovirus diagnostics were also evaluated.

The specific aims of the study were:

1. To express several recombinant proteins in insect cells using genetically modified baculoviruses in order to add a polyhistidine tag on the N- or C-terminus of the protein to facilitate the purification process.

2. To purify the recombinant proteins from their host cells by using alternative processes to traditional techniques. This was carried out by using a potentially upscalable technique, IMAC.

3. To assess that the untagged recombinant VLPs of B19 are able to classify acute and past-immunity serum samples.

4. To assess that the polyhistidine-tagged recombinants, the firefly luciferase of *Photinus pyralis*, the EBNA-1 of EBV and the VP1 of B19 as well as the VP2 VLPs of B19 have comparable abilities in diagnostics compared to the untagged or native counterparts.

4 SUMMARY OF MATERIALS AND METHODS

Full details are found in the original publications (I-IV).

4.1 Constructions of recombinant baculoviruses

In order to add a polyhistidine tag on the firefly luciferase (Luc) of *Photinus pyralis*; the gene was amplified from pUHC 131-1 vector as a template (kindly provided by Matti Karp, University of Technology, Tampere). The *Bam*HI/*Eco*RI digested fragment containing the firefly luciferase coding sequence was further cloned into the corresponding sites of the vector pFastBacI (Gibco BRL, Invitrogen, Carlsbad, CA) (I). The B19 VP2 construct has been described previously (Kaikkonen et al. 1999) (II). The CPV VP2 construct was amplified from pBI517 vector as template. The resultant product was digested with *Kpn*I and *Eco*RI and cloned into pSP73 plasmid (Promega, Madison, WI) and further isolated from the plasmid and cloned into the corresponding sites of the vector pFastBacI (Gibco BRL, Invitrogen) (II). The construct for His-EBNA-1 was modified from (Frappier & O'Donnell 1991b) (kindly provided by Lori Frappier, Medical Sciences Building, #4180 Toronto, Ontario) (III). For generation of His-B19-VP1 and His-B19-VP2, the sequence encoding VP1 of B19 and VP2 of B19 was amplified by PCR using the B19 full-length clone pB19-FL (GenBank #AY504945), as a template digested and inserted into the *Xba*I/*Xho*I sites of pFastBacHTA (Invitrogen) (IV). All used restriction enzymes were from MBI Fermentas (Vilnius, Lithuania), see Table 8 for primers.

TABLE 8 Sense and antisense primers used in the PCR reactions. Restriction sites are bold* start codon, stop codon or polyhistidine tag codon underlined¤.

	Gene construct	Bold*	Underlined¤	Sense primer
I	Luc-His	*Bam*HI	Start codon	5′-CGC A**GG ATC C**GC CGC CAA CCA TGG AAG ACG CC-3′
II	CPV-VP2	*Kpn*I	Start codon	5′-TT **GGT ACC** ATG AGT GAT GGA GCA GTT CAA-3′
IV	His-B19-VP1	*Xba*I	Start codon	5′- AAA **TCT AGA** ATG AGT AAA GAA AGT GGC AAA TGG -3
IV	His-B19-VP2	*Xba*I	Start codon	5′- AAA **TCT AGA** ATG ACT TCA GTT AAT TCT GCA GAA G -3′
	Gene construct	**Bold***	**Underlined¤**	**Antisense primer**
I	Luc-His	*Eco*RI	Stop codon, poly His tag	5′-TCC T**GA ATT C**TT AAT GAT GAT GAT GAT GAT GCA ATT TGG ACT TTC C-3′
II	CPV-VP2	*Eco*RI	Stop codon	5′-CGA GGC **GAA TT**C TTA ATA TAA TTT TCT AGG TGC-3′
IV	His-B19-VP1/VP2	*Xho*I	Stop codon	5′- AAA **CTC GAG** TTA CAA TGG GTG CAC ACG G -3′

4.2 Cell lines, plasmids and generated viruses

For the construction of plasmids to create recombinant baculoviruses prior to expression and purification of recombinant proteins competent *E.coli* JM 109 and DH10Bac cells were used (I, II, IV). Recombinant polyhistidine-tagged luciferase enzyme (I), empty human parvovirus B19 and CPV VLPs (II), polyhistidine-tagged EBNA-1 (III) or polyhisitidine-tagged VP1 and VP2 of B19 (IV) were propagated in *Spodoptera frugiperda* 9 (*Sf*9) (I-IV) insect cells with the baculovirus system as follows. Insect cells were used to propagate recombinant baculoviruses in several serum-free insect cell culture media such as, *Sf-900 SFM* (GibcoBRL, Invitrogen, Carlsbad, CA) (I), HyQ SFX (HyClone Inc, Logan, UT) (II), or Insect-Xpress medium (BioWhittaker, Lonza, Walkersville, MD) (III-IV). pFastBacI plasmid was used to amplify the genes of interest (Luc-His, B19-VP2 or CPV-VP2) resulting in plasmids pLuc-HisFastBac (I), pB19-VP2FastBac and pCPV-VP2FastBac (II). pFastBacHTA plasmid was used to amplify the genes of interest (His-B19-VP1 or His-B19-VP2) resulting in plasmids pHis-B19-VP1FastBac and pHis-B19-VP2FastBac (IV). These vectors were used to produce recombinant baculoviruses with the Bac-to-Bac™ system (Gibco BRL, Invitrogen). The Bac-to-Bac™ system uses site-specific transposition for insertion of a foreign gene into a baculovirus genome in *E.coli* DH10Bac cells, which allows the generation of recombinant baculoviruses (rBV). The resulting rBVs were designated as *Ac*Luc-His (I), *Ac*B19-VP2 and *Ac*CPV-VP2 (II), *Ac*His-B19-VP1 and *Ac*His-B19-VP2 (IV). Viral stocks were produced either in monolayer or suspension cultures (130 rpm/min., 28 ºC) and further used for recombinant protein production.

4.3 Production of recombinant proteins

For the recombinant protein production two types of insect cells *Trichoplusia ni* (*Tn368*, High-Five™) (I) and *Spodoptera frugiperda* 9 (*Sf*9) (II-IV) were infected at a multiplicity of infection (MOI) of 2 to 3 (I, II, IV) or 10 (III) plaque forming units (PFU) per cell. Cells were harvested at 72 h post infection (p.i.) (I, II, IV) and at 96 h p.i. (III) before the purification of the recombinants.

4.4 Purification of recombinant proteins

4.4.1 Cytoplasmic proteins

The following recombinants proteins Luc-His (I); B19-VP2, CPV-VP2 (II); His-B19-VP1 and His-B19-VP2 (IV) were expressed in the cytoplasm of the insect cells and the overall extraction was as follows. Cells were collected by low centrifugation 4.1 l growing volume 100 x g, 10 min., 4 ºC (I); 100 ml growing volume 1,000 x g, 10 min., 4 ºC (II); 300 ml growing volume 3,500 x g, 15 min., 4 ºC (IV). Then the pelleted cells were solubilized with an ice-cold buffer containing detergent [20 mM Hepes, 10% (v/v) glycerol, 1.1% (v/v) Triton X-100, 20 mM imidazole, pH 7.8; 10 x 10^6 cells/ml] (I), [20 mM Tris, 1% (v/v) Triton X-100, 0.3 M NaCl, pH 7.4; 25 x 10^6 cells/ml] (II), [20 mM Tris, 0.3 M NaCl, 1.0% (v/v) Triton X-100, pH 7.4; 20 x 10^6 cells/ml] (IV) on ice for 15 min. with a gentle mixing. This was followed by a clarification of the lysate by a high speed centrifugation 7,000 x g, 45 min., 4 ºC (I); 10,000 x g, 60 min., 4 ºC (II); 23,400 x g, 20 min., 4 ºC (IV). After that, the recombinant proteins were purified from the lysate by IMAC (I, IV) or by ultracentrifugation at 100,000 x g, 60 min., 4 ºC for B19-VP2 and CPV-VP2. B19-VP2 and CPV-VP2 containing pellets were gently re-suspended in 1 ml of ice-cold PBS containing 0.2 M NaCl and then additionally characterized (II).

4.4.2 Nuclear proteins

His-EBNA-1 recombinant protein was expressed in the nucleus of the insect cells and purified as follows (recombinants were used in the experiments in publication III, but the protocol is unpublished). First, cell low centrifugation 1,000 x g, 15 min., 4 ºC was done, and the cell membrane was then lysed with hypotonic buffer [20 mM Hepes, 1 mM $MgCl_2$, pH 7.5] by gentle mixing. This was followed by centrifugation 2,000 x g, 10 min., 4 ºC, and then lysing in hypotonic buffer and centrifugation 2,000 x g, 10 min., 4 ºC was repeated. This continued with the nuclear pellet suspension in extraction buffer [20 mM Hepes, 1 M NaCl, 1 % NP-40 Nonidet P40, 10 % Glycerol, 1 mM $MgCl_2$, pH 7.5], after which nuclear extract was further mixed mechanically using a Dounce homogenizer. This was followed by centrifugation of contaminating DNA by

ultra- centrifugation 80,000 x g, 30 min., 4 ºC and dilution of lysate [20 mM Hepes, 750 M NaCl, 10% Glycerol, 5 mM Imidazole, pH 7.5] before application to IMAC column.

4.4.3 Affinity tags, IMAC

The following recombinant proteins, Luc-His (I), His-EBNA-1 (III), His-B19-VP1 and His-B19-VP2 (IV) were designed with a polyhistidine tag (6 times histidine) in order to enable purification with immobilized metal-ion affinity chromatography. The purification of Luc-His was executed by using a specific application of affinity chromatography as described further. Shortly the protocol used for His-EBNA-1 was the following (III): the ultracentrifuged diluted lysate was applied to IMAC (Ni^{2+}) 2.5 - 5 ml (either Streamline™ Chelating Gel or prepacked HiTrap IMAC FF, both from GE Healthcare Bio-Sciences AB, Uppsala, Sweden) column which was equilibrated with buffer [50 mM HEPES, 750 mM NaC1, 10% glycerol, 5 mM imidazole, 1 mM PMSF, pH 7.5] followed by column washing with buffer [50 mM HEPES, 750 mM NaC1, 10% glycerol, 20 mM imidazole, 1 mM PMSF, pH 7.5] and eluted from the column by increasing imidazole concentration to 400 mM. EDTA (1mM) and DTT (1 mM) was added in order to stabilize proteins before characterization and ELISA. The purification of His-B19-VP1 and His-B19-VP2 (IV) was executed by separately application of the recovered proteins to 3 ml of IMAC (Ni^{2+}) matrix (Streamline™ Chelating Gel, GE Healthcare Bio-Sciences AB) on a rotating wheel for 16 h at 4 ºC. This continued with matrix washing [20 mM Tris, 0.3 M NaCl, 20 mM imidazole, pH 7.4] and packaging of the matrix to a chromatography column. After this recombinant His-B19-VP1 and His-B19-VP2, were eluted with an increasing concentration of imidazole [20 mM Tris, 0.3 M NaCl, 500 mM imidazole, pH 7.4] before characterization of the recovered proteins.

4.4.4 Expanded bed adsorption chromatography

As the cell lysate volume was more consequent (750 ml), the Luc-His recombinant was purified by using a larger scale technique, the expanded bed adsorption chromatography (EBA) or Streamline ™ (GE Healthcare Bio-Sciences AB). This was done with 130 ml of Cu^{2+} Streamline™ Chelating Gel in a Streamline™ 25 column (GE Healthcare Bio-Sciences AB) which was expanded with buffer [0.1 M Hepes, 10%(v/v) glycerol, 0.1% (v/v) Triton X-100, 20 mM imidazole, pH 7.8]. After which the clarified cell lysate was applied and washed with buffer before the elution step from the packed bed. The elution was done with an increasing linear gradient of 20 to 500 mM imidazole. This was done by using a GradiFrac™ or ÄKTA™prime apparatus (GE Healthcare Bio-Sciences AB). Finally, in order to stabilize the eluted proteins, glycerol was added to a final concentration of 50% (v/v) before characterization.

4.5 Characterization of produced recombinants

4.5.1 SDS-PAGE and western blotting

The purified protein samples after IMAC (I, III, IV) or after ultracentrifugation (II) were analyzed by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and by western blotting (Towbin et al. 1979). The samples were boiled (5 min.) and reduced in Laemmli sample buffer (Laemmli 1970) prior to electrophoresis. The SDS-PAGE electrophoresis was done using 10 % (I, II), 12 % (IV) and 15 % (III) 12% slab gels (Bio-Rad Laboratories, Hercules, CA). Then visualization of the proteins was done by Coomassie blue staining (GelCode® Blue Stain Reagent; Pierce, Rockford, IL) (I), (Bio-Safe- Coomassie, Bio-Rad) (II), (PageBlue protein staining solution; Fermentas life sciences, Vilnius, Lithuania) (IV). The identification of the proteins was completed with specific antibodies after western blotting. The electrophoretically separated proteins were transferred on nitrocellulose sheets (Protran®; Schleicher& Schnell, Dassel, Germany). In order to avoid unspecific binding, sheets were blocked with skimmed milk solution. Primary antibodies (see Table 9 for details) were then applied and this was followed by secondary antibodies (see Table 10 for details) before enzymatic detection. Polyhistidine-tagged proteins, Luc-His, His-EBNA-1, His-B19-VP1 and His-B19-VP2 were identified with monoclonal antibodies against the polyhistidine tag and further detected with alkaline phosphatase (AP) conjugated secondary antibody (I, III, IV). On the other hand, acute, past and negative immunity human sera against B19 were detected with secondary antibody by using either horseradish peroxidase (HRP) conjugated antibody (II) or AP conjugated antibody (II, IV respectively). B19-VP2 VLPs were detected with antibody against either the whole capsid (anti-parvovirus B19 antibody) or only VP2 (anti-VP2, MAB8292) AP-conjugated antibodies against the primary antibodies were used (II). Finally, the CPV-VP2 VLPs were identified with polyclonal anti-CPV followed by labeling with AP-conjugated secondary antibody (II).

TABLE 9 Primary antibodies used in this thesis.

	Primary antibody	Target	Provider or reference	Source
I	His-Tag antibody	polyhistidine tag	Dianova GmbH, Hamburg, Germany	mouse
III	His-Tag antibody	polyhistidine tag	Clontech, Palo Alto, CA	mouse
IV	His-Tag antibody	polyhistidine tag	Immunology Consultant Laboratory, Newberg, OR	rabbit
II, IV	Acute, past-immunity and negative serum	B19 virus	Dr. Klaus Hedman, (Kaikkonen et al. 1999, Söderlund et al. 1995a)	human
II	Anti-parvovirus B19	B19 virus	Dako, Glostrup, Denmark	rabbit
II	Anti-VP2, MAB8292	aa 446-466 of B19 VP2	Chemicon, Temecula, CA	mouse
II	Anti-VP, Cornell#2	Structural proteins of CPV	Dr. Colin Parrish	rabbit

For Dot Blot analysis His-B19-VP2 proteins were transferred to nitrocellulose transfer membrane (Protran®; Schleicher& Schnell).These were probed with a polyhistidine antibody and the immunocomplexes were identified by AP conjugated goat anti-rabbit IgG monoclonal antibodies (see Table 9 and Table 10 respectively) (IV). The detection of AP-conjugated secondary antibodies was performed by using an application of a solution containing BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium) (Promega, Madison, WI) (I), (Sigma-Aldrich, St. Louis, MO) (II-IV) on the nitrocellulase sheet. The detection of HRP was done using 3,3′-diaminobenzidine tetrahydrochloride (DAB; ICN Biomedicals, Costa Mesa, CA, USA) and hydrogen peroxide as substrates (II). Molecular weights were estimated using protein standards (Protein Marker Broad Range, New England BioLabs, Inc., Berverly, MA) (I), (MBI Fermentas) (II-IV).

TABLE 10 Secondary antibodies used in this thesis.

	Secondary antibody	Substrate	Provider or reference	Source
I, III	AP-conjugated goat anti mouse IgG	NBT, BCIP	Bio-Rad Laboratories, Hercules, CA	goat
IV	AP-conjugated goat anti rabbit IgG	NBT, BCIP	Promega, Madison, WI	goat
II	HRP-conjugated rabbit anti human IgG	DAB	Dako, Glostrup, Denmark	rabbit
IV	AP-conjugated goat anti-human IgG	NBT, BCIP	Promega, Madison, WI	goat
II	AP-conjugated swine anti-rabbit IgG	NBT, BCIP	Dako, Glostrup, Denmark	swine
II	AP-conjugated goat anti-mouse IgG	NBT, BCIP	Promega, Madison, WI	goat
II	AP-conjugated goat anti-rabbit IgG	NBT, BCIP	Promega, Madison, WI	goat

4.5.2 Electron microscopy

The formation or non-formation of VLPs was visualized by electron microscopy (EM). Shortly, recovered proteins from ultracentrifuged samples of B19-VP2, CPV-VP2 (II) and His-B19-VP1, His-B19-VP2 (IV) were applied to a metal grid and incubated for 1 -2 h at RT for negative staining. Excess liquid was blotted away using Whatman 3MM filter paper and the proteins fixed in 4% PFA/PBS and 0.1% glutaraldehyde (Sigma-Aldrich) for 15 min. at RT. Then, negative stain (2% potassium phospho tungstate, pH 6) was added, and excess removed after which after the grids were allowed to dry at RT. The protein specimens were examined by transmission EM (JEM- 1200 EX; Jeol; Tokyo, Japan) (II, IV).

4.5.3 Enzyme-linked immunosorbent assay

Using the purified proteins B19-VP2 (II), His-EBNA-1 (III), His-B19-VP1 and His-B19-VP2 (IV) enzyme-linked immunosorbent assays (ELISAs) were set up. The purified proteins were used as solid phase antigens. For B19-VP2 (II) the protocol is explained in more detail in (Kaikkonen et al. 1999). For His-B19-VP1 and His-B19-VP2 (IV) the proteins were diluted with PBS to a final concentration of 2 µg/ml. Dilutions were used for coating 96-well plates (Nunc Polysorp, Cat no 469078; Nalge Nunc International, Rochester, NY) over night at room temperature (100 µl/well). The coated wells were further incubated with PBS containing 0.05% Tween-20 (PBST) for 10-30 min. according to standard procedures. The ELISA measurements were performed by sera dilution 1:100 in PBST, incubated for 60 min. at 37°C and washed 3x with PBST. Immunocomplexes were detected with polyclonal rabbit anti-human IgG (Dako, Glostrup, Denmark) this was followed by adding substrate OPD tablets (Dako). The reaction was stopped by adding 100 µl 0.5 M sulfuric acid. The absorbance data was collected at a wavelength of 492 nm with a microplate photometer (Multiskan; Thermo Fisher Scientific, Waltham, MA). The results were compared to an in-house assay where the VP1 of B19 expressed in bacteria was used as control antigen (Kaikkonen et al. 1999, Söderlund et al. 1992).

For His-EBNA-1 (III), the performance of the ELISA was tested (now commercially available kit *EBV EBNA-1-IgG-ELISA PKS medac*; Medac GmbH, Hamburg, Germany) by using a serum panel of 304 samples for performance testing and 29 serum samples for precision testing. The assay was set up in order to have quantitative results in arbitrary units (AU/ml). Results were obtained without calibration curve by a single-point quantitation. The investigation of the diagnostic performance of the kit was done by using pre-defined sera of which 141 anti EBV seronegative sera, 110 with acute EBV infection and 52 with past EBV infection. On the other hand, to evaluate the precision performance of the kit several samples such as a positive control and 7 samples with different reactivity to EBNA-1 were measured. This was done in 22 determinations in one test run (intra assay) and 11 independent test run (inter assay). This was done either manually or by using an automated device. To investigate the dilution linearity, of the kit 10 reactive sera were titrated in 1:2 dilution steps and compared to the determined vs. expected values. Single-point quantitation (SPQ) was performed by measuring the antibody concentration of 89 sera. This was calculated by SPQ and by using an internal calibration curve (6.25-200 AU/ml) in one test run. The precision of the kit was also investigated by measuring the person-to-person variation. Finally, the suitability for automation was studied by parallel test runs which were performed manually and compared to the use of automated devices. For more details see III.

4.5.4 Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) was carried out using a fluorescence correlation microscope ConfoCor2 (Carl Zeiss, Jena, Germany) (II). An Argon-ion laser was used at excitation wavelength of 480 nm and the pinhole diameter was adjusted to 70 µm. To collect the emission light photons from the excitation photons, a band pass emission filter (530-600 nm) was used. B19-VP2 VLPs were labeled with Oregon Green 488 succinimidylester (Molecular Probes, Eugene, OR), purified by Sephadex G50 columns and diluted in PBS before FCS measurements in LabTek® II 8-well borosilicate glass plates (Nalge Nunc International). Each analysis was repeated six times. Diffusion times and the normalized autocorrelations G (t) was calculated using the ConfoCor2 software. The labeled B19-VP2 VLPs only and its interaction between acute, past-immunity, and seronegative human serum samples with the B19-VP2 VLPs were analyzed by FCS. The statistical data behind the autocorrelation theory can be read elsewhere (Ehrenberg & Rigler 1974, Eigen & Rigler 1994, Elson & Madge 1974, Madge 1976, Rigler 1995, Rigler et al. 1998, Yoshida et al. 2001).

5 REVIEW OF THE RESULTS

The production of recombinant proteins using baculoviruses and insect cells such as *Trichoplusia ni* (*Tn368*, High-Five™) and *Spodoptera frugiperda* 9 (*Sf9*) relies on relatively standardized protocols. However, the efficient purification of the recombinants is often delicate. This thesis was written to provide alternative techniques and protocols to recover recombinant proteins and to test their abilities in several applications.

5.1 Characterization of firefly luciferase, EBNA-1, CPV and B19 viral proteins by standard techniques

Insect cells (High-Five™ or *Sf9*) were grown in suspension at +28 °C, and infected with recombinant baculoviruses Luc-His, CPV-VP2, B19-VP2, His-EBNA-1, His-B19-VP1 and His-B19-VP2 encoding the polyhistidine-tagged firefly luciferase enzyme, the VP2 of CPV, the VP2 of B19, the polyhistidine-tagged EBNA-1, the polyhistidine-tagged VP1 of B19 and the polyhistidine-tagged VP2 of B19, respectively. Infected cells were collected at 72-96 h p.i. and additionally solubilized by detergent incubation or different buffers. The lysates were finally clarified by centrifugation and further purified by using high scalable technique, expanded bed adsorption chromatography (Streamline™) and/or IMAC or ultracentrifugation.

For polyhisitidine-tagged proteins (I, III and IV), after IMAC matrix washing with low imidazole (20 mM) the proteins were eluted with imidazole 500 mM from the column material and the fractions containing the recombinants were additionally characterized. The eluted fractions were analyzed with SDS-PAGE. This was followed by Coomassie brilliant blue gel staining and/or immunoblotting as well as protein amount measurements (I, Fig. 1A and B) (III, unpublished results presented in this thesis) (IV, Fig. 2). For Luc-His (I) a major band can be seen at about 62 kDa. The presence of polyhistidine at the C-terminus of the protein was confirmed by immunoblot

analysis (I, Fig. 2). The IMAC purified luciferase (Fig. 9, lane 1) can be compared to the non-chromatographically purified (De Luca & Mc Elroy 1978) native enzyme (Fig. 9, lane 2) (data not published). For His-EBNA-1 a major band can be seen at about 55 kDa in Figure 9, lane 3 for infected cells and lane 5 for purified EBNA-1 pool (data not published). The presence of the polyhistidine at the N-terminus of the protein was confirmed by western blotting (data not published, Fig. 9, lane 4 for infected cells and lanes 6 for purified EBNA-1 pool).

For non-tagged proteins (II), after recovery with ultracentrifugation, the VPLs of CPV and B19 were further analyzed with Coomassie brilliant blue gel staining and immunoblotting with several antibodies (II, Fig. 1).

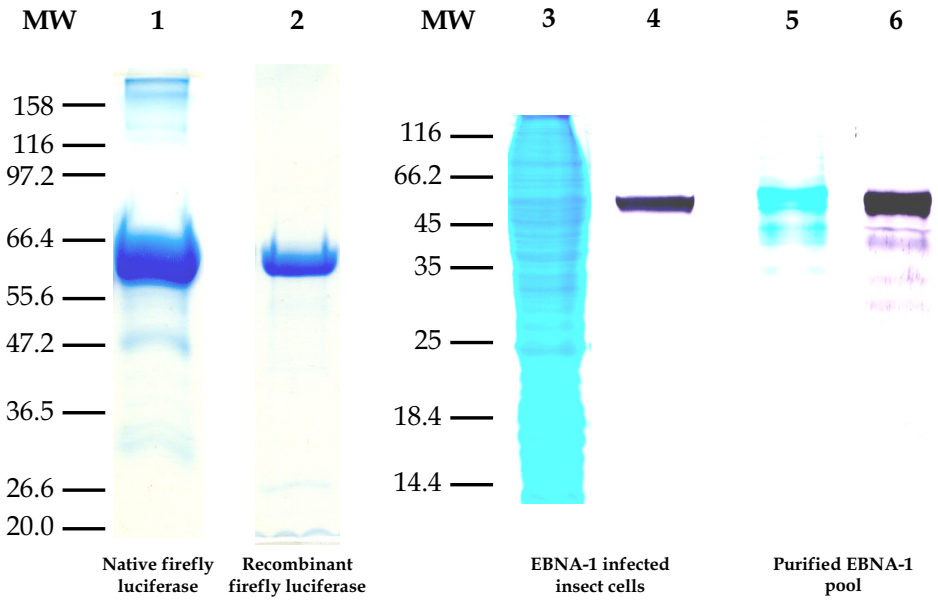


FIGURE 9 SDS-PAGE analysis of firefly luciferase and EBNA-1. The proteins were detected by Coomassie brilliant blue staining followed by immunoblotting for EBNA-1. The native enzyme of *Photinus pyralis* after extraction by ammonium sulfate precipitation followed by separation by isoelectric focusing (De Luca & Mc Elroy 1978) (kindly provided by Susanna Jussila) (lane 1). The polyhistidine-tagged recombinant firefly luciferase purified with IMAC (lane 2). The infected insect cells expressing EBNA-1 (lane 3) and the purified EBNA-1 pool (lane 5). The immunoblotting of EBNA-1 of infected insect cells (lane 4) and the pool of the IMAC purified EBNA-1 of EBV (lane 6). The molecular weight standards are shown in kDa (MW).

5.2 Enzymatic activity of recombinant luciferase

The proper bioluminescent activity of luciferase enzyme is proportional to the amount of ATP present in the surrounding solution. The activity was measured (I, Fig. 3). Beside the enzymatic activity, the principal interfering enzymes levels or activities were measured and compared to the purified recombinant enzyme (I, Table 1).

5.3 Electron microscopy of virus-like particles

The proper assembling of the parvovirus CPV-VP2 and B19-VP2 (II), His-B19-VP1 and (IV) into VLPs was verified by electron microscopy. The non-fused proteins CPV-VP2 and B19-VP2 (II, Fig. 2A and B), and the fused proteins His-B19-VP2 (IV, Fig 3) assembled into roughly spherical particles. The His-B19-VP1 (IV) did not form VLPs. The diameter of the B19-VP2 particles (II) was approximately 19 nm (Table 11) (Michel 2002).

TABLE 11 The diameter of B19 VLPs detected by EM (II) (unpublished data adapted from Michel 2002).

EM diameter (nm) B19-VP2				
Mean	Median	Std deviation	Min	Max
18.75	18.94	1.80	14.13	21.93

5.4 ELISA with EBNA-1 and B19 viral proteins 1 and 2

The immunological properties of the recovered proteins His-EBNA-1, His-B19-VP1 and His-B19-VP2 after purification with IMAC were analyzed with ELISA by coating microtiter plates with either His-EBNA-1 (III), His-B19-VP1 (IV) or His-B19-VP2 VP1 (IV). For His-EBNA-1 serum samples with EBV IgM and IgG negative, IgM positive, IgG positive were pre-tested with a commercially available kit (EIA Anti-EBV EBNA IgG ELISA, Biotest, Dreieich, Germany) and the results were compared with the kit which was made with His-EBNA-1 (*EBV EBNA-1-IgG-ELISA PKS medac*, Medac GmbH) as a solid phase antigen (III, Table 1) (Table 12). For His-B19-VP1 and His-B19-VP2 VP1 serum samples with B19 IgM and IgG negative, IgM positive, IgG positive were pre-tested with an in-house kit (Kaikkonen et al. 1999, Söderlund et al. 1995a, Söderlund et al. 1995b, Söderlund et al. 1992) and the results were compared with the ELISA microtiter plates coated with His-B19-VP1 or His-B19-VP2 VP1 as solid phase antigens (IV, Table 1) (Table 12).

TABLE 12 Comparison of the ELISA results with polyhistidine-tagged antigens to ELISA results with well established methods using untagged recombinant antigens. Several human serum samples were measured by ELISA with polyhistidine-tagged recombinant antigens His-EBNA-1 (III), His-B19-VP1 (IV) or His-B19-VP2 (IV). The same samples were also measured by ELISA with either a commercially available kit (EBV) or an in-house kit (B19). For both the results can be seen in the columns as well as the number of samples having the expected results.

ELISA set-up for the detection of:	Serum samples	Recombinant solid phase antigens	ELISA with our antigens		ELISA with commercial (EBV) or in-house (B19) antigens	
			Results	Samples number	Results	Samples number
EBV	IgM, IgG negative	His-EBNA-1	-	141	-	141
	IgM positive, acute infection		-	109	-	110
	IgG positive, past infection		+	52	+	52
B19	IgM, IgG negative pool	His-B19-VP1 and His-B19-VP2	-	1	-	1
	IgM positive, acute infection pool		+	1	+	1
	IgG positive, past infection pool		+	1	+	1
	IgM, IgG positive		+	4	+	4

5.5 Monitoring of the binding of B19 VLPs with human antibodies by FCS

The binding in solution of immunoglobulin M and G was monitored with FCS by using B19-VP2 VLPs (II). First, the VLPs were labeled with Oregon Green 488 and the diffusion time was measured by FCS. According to the diffusion time, a hydrodynamic radius of 14 nm was calculated (II, Table 1). Moreover, negative, acute and past-immunity serum samples from different persons were measured. A clear shift of the autocorrelation curve to the right was seen for acute (IgM positive) and past immunity (IgG positive) serum samples (II, Fig. 4). For the acute-phase serum, a mean τ_{diff}-value of 6.2 ms was obtained to correspond to D of 1.5 x 10^{-12} m^2s^{-1} and a hydrodynamic radius of approximately 160 nm (II, Table 1). For the past-immunity serum a mean τ_{diff}-value of 2.7 ms was obtained to correspond to a diffusion coefficient of 3.5 x 10^{-12} m^2s^{-1} and a hydrodynamic radius of approximately 70 nm (II, Table 1). No specific binding was observed in the presence of a control sample devoid of B19 VP2 specific antibodies, as can clearly be seen from the normalized autocorrelation (II, Fig. 4A).

6 DISCUSSION

Biotechnology is using living cells for processes, which are not possible by using traditional synthesis methods. One area of biotechnology is the production *in vivo* of recombinant proteins. The baculovirus expression vector system (BEVS) is a popular system for production of diverse recombinant proteins. Those proteins could be used in several fields e.g. disease diagnostics, enzyme technologies, vaccines or as research tools. However, in order to use the proteins, they usually need to be purified from their host cells. Improvements in the purification processes can be done by designing alternative techniques to traditional processes. Some alternatives should be found as to minimize the number of purification steps and to have scalable processes. Good alternatives are found when time and money are saved in the processes. After the purification step, before the final intended use, the recombinant should also be characterized.

The main goal of the research presented in this thesis was to set up options to conventional purification protocols, for the following proteins produced in insect cells: The firefly luciferase enzyme of *Photinus pyralis* (Luc-His, I), the EBNA-1 of EBV (His-EBNA-1, III) as well as the VP1 (His-B19-VP1, IV) and VP2 (His-B19-VP2, IV) of B19. After purification, the proteins were principally tested, for their suitability for: Measurement of adenosine triphosphate (ATP) for Luc-His (I), enzyme linked immunosorbent assay (ELISA) for His-EBNA-1 (III), His-B19-VP1 and His-B19-VP2 (IV). These purposes are potential final uses of the respective recombinant proteins. Additionally, the VP2 (B19-VP2, II) of B19 was used to monitor its binding to antibodies with fluorescence correlation spectroscopy (FCS).

6.1 Production of the recombinant proteins

Several recombinant baculoviruses were generated by using transposon-mediated insertion of the corresponding genetic construct under the polyhedrin promoter of the *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) (Luckow et al. 1993). Insect cells, *Trichoplusia ni* (High-Five™) (I) or *Spodoptera frugiperda* (*Sf*9) (II-IV) were infected with a recombinant virus at a MOI of 2-3 (I, II, IV) or 10 (III) PFU:s per cell according to standard procedures (Summers 2006). After infection, cells were harvested at 72 h p.i. (I, II, IV) and at 96 h p.i. (III) and further purified and characterized. The unusual infection time, of 96 h p.i., for His-EBNA-1 is in concordance with (Meij et al. 2000). Meij and others noticed that even the *Sf*9 cell viability is rather low (about 30%) the expression level of EBNA-1 was rather high.

The choice of BEVS, compared to other potential organisms (bacteria, yeast, and mammalian cells) for the production of recombinants, was quite evident. As shown in Table 1, insect cells have nearly the same abilities for post translational modifications as mammalian cells (Fernandez & Hoeffler 1999). One of the main things lacking is the uncompleted N-linked glycosylation in insect cells. However, the baculovirus genome can be modified, if needed, to overcome this limited ability of insect cells in N-linked glycosylation (Harrison & Jarvis 2006). In addition, the baculovirus genome can be adapted to affinity chromatography by adding an affinity tag, such as a polyhistidine tag (Porath et al. 1975). Moreover, the use of the BEVS gives a possibility of relatively high expression levels of recombinant proteins. Additionally, insect cells can grow in monolayer as well as in suspension (Murhammer 1991). This relative ease of growth in suspension opens the possibility of larger scale production (Ikonomou et al. 2003, Schlaeppi et al. 2006, Zhang et al. 1998).

The firefly luciferase has been expressed in many organisms as presented in the section 2.4.1. However, the expression and purification of the enzyme, to be used as a reagent for ATP measurement, has not been widely published. Still, Wang and colleagues expressed in bacteria cells a luciferase enzyme fused with a polyhistidine tag (six residues of histidine) and biotin carrier protein (BCCP) domain at its amino terminus (Wang et al. 1997). On the other hand, production of luciferase, fused with a polyhistidine tag (ten residues of histidine) at its amino terminus, in bacteria was also done. In this study mutant and non-mutant form of luciferase were designed (Law et al. 2006). The comparison of the data shown in article I and the studies of Wang et al. (1997) and Law et al. (2006) are developed in the following parts.

EBNA-1 of EBV is a preferable source for the diagnosis of IgG against Epstein-Barr virus (III). EBNA-1 protein is found in rather low amounts in EBV infected cells (Duellman & Burgess 2006). Therefore, the EBNA-1 should be preferably be produced *in vitro*. The recombinant EBNA-1 has been produced in many organisms such as mammalian cells, insect cells or bacteria cells, as previously mentioned. In most of the cases, the EBNA-1 is produced in truncated form. Those recombinants are usually produced to observe the

EBNA-1 binding to DNA and the related processes (Bochkarev et al. 1996, Bochkarev et al. 1995, Ceccarelli & Frappier 2000, Frappier & O'Donnell 1991a, Kapoor & Frappier 2003, Kapoor et al. 2005, Kirchmaier & Sugden 1997, Leight & Sugden 2000, Lu et al. 2004, Oddo et al. 2006, Wang et al. 2006). On the other hand, for ELISA purposes, the EBNA-1 protein has been produced using the BEVS (Frappier & O'Donnell 1991b). By using insect cells, the post-translational modifications occur. The EBNA-1 is a phosphorylated protein (Polvino-Bodnar et al. 1988). Therefore higher organisms should be used for the recombinant production. However, the EBNA-1 of EBV has been produced recently and successfully in bacteria (Duellman & Burgess 2006). The bacteria cell line used for this production included plasmids coding for rare codons. The baculovirus construct used in this thesis, coding for the EBNA-1 was lacking the Gly-Ala repeat sequence (aa. 90-325) (III) (Frappier & O'Donnell 1991b). Moreover, a polyhistidine tag (six residues of histidine) was added to the N-terminus of the amino acid sequence. Its production and purification has not been published as such, however a similar construct has been produced and used for studying its regulation ability (Shire et al. 2006) as well as its interaction ability with certain proteases (Holowaty et al. 2003).

The human parvovirus B19 is composed of two structural proteins VP1 and VP2. The virus capsid is mainly composed of VP2 (95%) (Ozawa & Young 1987). When produced in eukaryotic organisms the VP2 of B19 can assemble into virus-like particles (VLPs) on its own (Kajigaya et al. 1991, Kajigaya et al. 1989) where as the VP1 does not assemble into VLPs (Casal 2001). The VP1's inability to self-assemble is probably due to the extra amino acids which interfere the empty capsid formation (Rosenfeld et al. 1994). The VLPs composed of VP2 of B19 (II, IV) as well as the VP1 of B19 (IV) are the selected reagents for many purposes as developed in section 2.7.2. In the previously mentioned cases, the viral proteins have been essentially produced using insect cells. In contrast, denaturated and truncated forms of VP1 and VP2 have been successfully produced in *Escherichia coli* resulting in linear epitopes (Söderlund et al. 1995b, Söderlund et al. 1992). The production of B19 structural proteins using insect cells results in conformational proteins. Those conformational proteins have a slightly better ability to detect immunoglobulin than the *Escherichia coli* linear epitopes (Jordan 2000). Moreover, the conformational proteins of B19 have been used in many cases such as the structural study of B19 (Kaufmann et al. 2004) or the reactivity of genotype-specific recombinant viral proteins with blood plasma (Parsyan et al. 2006). It has also been demonstrated that the VLP formation, for VP2, is not affected by the display of foreign molecules (Gilbert et al. 2005, Toivola et al. 2005) or deletion of the first 25 amino acids on the N-terminus (Kawase et al. 1995). According to these studies, a polyhistidine tag was added to the N-terminus of the amino acid sequence of VP1 and VP2 (IV). The eukaryotic systems are usually used for the production of VLPs. Using *Spodoptera frugiperda* larvae is an alternative to cell culture, as it has been done for the production of rotavirus VLPs (Molinari et al. 2008). However, a recent study showed that an alternative to eukaryotic cells can be exploited. The MS2 bacteriophage coat protein and the hepatitis B core

protein have been packaged *in vitro* to form VLPs. This was accomplished using an *Escherichia coli* based cell-free synthesis protocol (Bundy et al. 2008). This technique is relatively recent. Still, it should be taken into account when, for example, cell originating contaminants are affecting the final use of the proteins. It should be mentioned, that VLPs are good candidates as vaccines for hepatitis B (HBV), human papillomavirus (HPV) (Ludwig & Wagner 2007) and B19 (Casal 1999, Franssila et al. 2005, Franssila & Hedman 2004, Franssila et al. 2001).

In this study, several recombinant proteins, the firefly luciferase enzyme of *Photinus pyralis* (Luc-His, I), the EBNA-1 of EBV (His-EBNA-1, III) and the VP1 and VP2 of B19 (B19-VP2 II, His-B19-VP1 IV, His-B19-VP2, IV) were successfully expressed using insect cells and baculovirus. The purification process which was used to recover the recombinants and their final uses will be developed in the following sections.

6.2 Purification of the recombinant proteins

After recombinant protein expression in insect cells, cells were collected and solubilized with detergent (Ersson et al. 1998) and finally the recombinant proteins were purified by using different techniques. The immobilized metal-ion affinity chromatography (IMAC) technique was used in articles I, III and IV. The expanded bed adsorption chromatography (EBA) was coupled to IMAC in article I. In article II the differences in protein physical properties were exploited by using centrifugation. In addition, the recovered proteins were further characterized.

The addition of a polyhistidine tag (six residues of histidine) to the N- or C-terminus of the protein enables relatively specific purification with IMAC (Porath et al. 1975). Moreover, the addition of polyhistidine tag does not usually affect the protein activity (Gaberc-Porekar & Menart 2001). Therefore, most of the proteins reported in this thesis, were expressed in insect cells and designed to have a polyhistidine tag, and thus they were purified with IMAC. The affinity tag was added to the C-terminus for the luciferase enzyme (I) or to the N-terminus for the EBNA-1 of EBV (III) as well as for the VP1 and VP2 of B19 (IV).

For the luciferase enzyme (I), the scale-up ability of the purification process was shown by using the EBA technique (Draeger & Chase 1991). The eluted proteins were quantified as well as analyzed by electrophoresis (I, Fig. 1). The presence of the polyhistidine tag was confirmed by immunoblotting analysis (I, Fig. 2). The firefly luciferase enzyme of *Photinus pyralis* (Luc-His) was estimated to have a molecular weight of about 62 kDa (I, Fig. 1A) as expected (Gould & Subramani 1988). There is also a similarity of the IMAC purified versus the non-chromatographic purified native enzyme (De Luca & Mc Elroy 1978) (this thesis Fig. 9 lanes 1 and 2). The total yield was calculated to be 23 mg of luciferase per one liter of insect cell culture (I). In comparison, the

BCCP luciferase purified after bacterial expression yielded 9.1 mg per 200 ml of bacterial cell culture (Wang et al. 1997). It can be then calculated that Wang and colleagues yielded 45.5 mg luciferase from one liter of bacterial cell culture. However, the BCCP-domain weight is about 9 kDa. Thus the percentage of the luciferase in their construct is about 87%. Therefore, the yield of only luciferase can be estimated to be about 40 mg from one liter cell culture. Nevertheless, the final yield is higher; there are differences in bioluminescence activity as developed in the following section.

The His-EBNA-1 was produced using insect cells and baculovirus (III). Proteins were extracted form the cell nucleus using mechanical and physical techniques followed by IMAC purification. Before purification and after elution the recombinant proteins were analyzed by SDS-PAGE as well as by immunoblotting. A major band was seen at about 55 kDa (Fig. 9, lane 3 for infected cells and lane 5 for purified EBNA-1 pool) as it is the predictable molecular weight (MW) of the His-EBNA-1 of EBV (Frappier & O'Donnell 1991b). The presence of the polyhistidine tag was confirmed by immunoblotting (Fig. 9, lane 4 for infected cells and lane 6 for purified EBNA-1 pool). There are studies (Holowaty et al. 2003, Shire et al. 2006) which have applied a similar construct as this one. The proteins were purified by using two chromatographical steps. At first, there was an IMAC purification which was followed by a heparin-agarose column purification. One of the first studies, where EBNA-1 was expressed and purified from insect cells, was slightly different. The nuclear extract was applied first to a heparin-agarose column, then further purified by using DNA affinity column, and finally the proteins were concentrated by using MonoQ chromatography (Frappier & O'Donnell 1991b). Frappier and O'Donnell (1991b) yielded 1 mg of EBNA-1 from 9 x 10^8 insect cells, i.e. a 450 ml cell culture at a density of 2 x 10^6 cells/ml. For the bioreactor-scale production, the EBNA-1 recombinant was purified with DNA affinity using biotinylated PCR-amplified family of repeat domains which were immobilized to streptavidin agarose (Meij et al. 2000). They yielded 0.8 mg from 6 x 10^7 insect cells, i.e. a 30 ml cell culture at a density of 2 x 10^6 cells/ml. By using the purification protocol presented in this thesis, the final His-EBNA-1 yield is about 10 mg from 300 ml cell culture at a density of 2 x 10^6 cells/ml. By comparing the final yield of EBNA-1 of the different purification protocols, it can be clearly seen that the presence of a polyhistidine tag on the N-terminus of the protein sequence did not affect the protein function as an efficient antigen for the detection of IgG against EBV. The use of the protocol presented here reaches to a relatively high protein recovery.

The VP1 and VP2 of B19 are traditionally purified by using sucrose or cesium chloride gradient and ultracentrifugation as presented in article IV. Those techniques are time consuming and not scalable. The use of polyethylene glycol (PEG) precipitation, ion-exchange chromatography or ultracentrifugation are potential alternatives for purifing the VP2 VLPs of B19 (Michel 2002). Except for the ultracentrifugation method, the results were described as not very conclusive. The use of PEG precipitation was efficient to recover VLPs, but such VLP precipitate was rather difficult to solubilize. The use of ion-exchange

chromatography resulted in a relatively low yield of VP2 VLPs. Here, in article II ultracentrifugation was used to recover the untagged VP2 VLPs from clarified insect cell lysate. In article IV, affinity chromatography was used to recover polyhistidine-tagged VP1 and VP2. The recovered proteins were then analyzed by SDS-PAGE followed by Coomassie staining and/or immunoblotting. For VP1, proteins of expected size, 84 kDa (Ozawa & Young 1987), were seen by immunoblotting. Human convalescent serum antibodies confirmed the identity of parvovirus B19 VP1 proteins (IV, Fig. 2 lane 5). The identity of parvovirus B19 VP1 was, in addition, confirmed by using acute human serum antibody (IV, Fig. 2 lane 4). In addition, the presence of polyhistidine tag was confirmed by immunoblotting (IV, Fig. 2, lane 1 and 2). For VP2, proteins of expected size, 58 kDa (Kaufmann et al. 2004), were seen by Coomassie blue staining (II, Fig. 1, lanes 2). Human convalescent serum antibodies confirmed the identity of the parvovirus B19 VP2 proteins (II, Fig. 1, lanes 4; IV, Fig. 2 lane 10). The identity of parvovirus B19 VP2 was, in addition, confirmed by using monoclonal anti-parvovirus B19 antibodies (II, Fig. 1, lane 3) or acute human serum antibody (IV, Fig. 2 lane 9). In addition, the presence of the polyhistidine tag was confirmed by immunoblotting (IV, Fig. 2, lane 6 and 7). As expected, the constructs expressing the VP2 (II, Fig. 2A; IV, Fig. 3B), self assembled into VLPs (Casal 2001, Kajigaya et al. 1991, Kajigaya et al. 1989, Kawase et al. 1995). The particles, as seen with electron microscopy, were very similar in size and shape when compared to authentic B19 virus particles (Agbandje et al. 1994, Agbandje et al. 1991, Casal 2001). The VP1 and VP2 were similar (molecular weight for VP1 and VP2 as well as size and shape of VLPs for VP2) when compared to findings in previous studies. Still, it is important to compare the yield of the proteins purified in article IV and the untagged proteins purified by using gradient centrifugation. After the production in insect cells and SDS-PAGE analysis the following amounts were estimated from the gel (Brown et al. 1991). For VP1, 40 mg were recovered from 10^9 insect cells, i.e. approximately 24 mg from 300 ml cell culture at a density of 2 x 10^6 cells/ml. For VP2, 50 mg were purified from 10^9 insect cells i.e. approximately 30 mg from 300 ml cell culture at a density of 2 x 10^6 cells/ml. The purification was done by using first sucrose cushion (40%) (2.5 h, 100,000 x g, +4 ºC) and then sucrose gradient (15 to 30%) (2.5 h, 110,000 x g, +4 ºC). This process yielded in 10 mg of VP2 VLPs from 10^9 insect cells i.e. an estimation of 6 mg from 300 ml cell culture at a density of 2 x 10^6 cells/ml. Lowin et al. (2005) obtained similar yield as Brown et al. (1991) when viral proteins of B19 were produced in yeast. Another report uses slightly the same protocol as Brown et al. (1991), except for the last purification step where cesium chloride gradient centrifugation was used (Salimans et al. 1992). They yielded about 0.5 mg from 10^8 insect cells meaning approximately 3 mg from 300 ml cell culture at a density of 2 x 10^6 cells/ml. Interestingly, in several publications (Agbandje et al. 1994, Agbandje et al. 1991, Kajigaya et al. 1991), parvovirus capsid proteins have been expressed in insect cells. The proteins were purified by using sucrose cushion and this was followed by cesium chloride gradient centrifugation. Unfortunately, they do not clearly mention their final protein yields. When purifying the polyhistidine-tagged VP1 and

VP2 VLPs the protein yield was about 30-35 mg of proteins from 300 ml cell culture at a density of 2 x 10^6 cells/ml (IV). Finally, the results presented here undoubtedly show that the use of the protocol advanced in article IV reaches to a higher yield of B19 capsid proteins (VP1 and VP2) than other previously mentioned reports. Moreover, the use of ultracentrifugation for VP2 VLPs of B19 allows a rapid way to isolate VLPs from a crude cell lysate (II). The isolated empty virus particles were pure enough to be monitored with the sensitive FCS apparatus as described below.

Together, several recombinant proteins, the firefly luciferase enzyme of *Photinus pyralis* (Luc-His, I), EBNA-1 of EBV (His-EBNA-1, III) and VP1 and VP2 of B19 (B19-VP2 II, His-B19-VP1 IV, His-B19-VP2, IV) were effectively purified from insect cells. The purification processes that were used enabled a relatively high yield of recombinants in an active form and in conformation similar to the authentic counterparts. Moreover, the use of the recovered recombinant proteins in diagnostics was proved as developed below.

6.3 Use of the recombinant proteins in diagnostics

6.3.1 Firefly luciferase enzyme of *Photinus pyralis*

Adenosine triphosphate (ATP) is found in all living organisms (Kassi & Papavassiliou 2008). The amount of ATP present in living cells, is subsequently diminished after cell death (Fan & Wood 2007). Luciferase enzyme catalyses the reaction between ATP and its substrate, the luciferin, to produce bioluminescence (De Luca & Mc Elroy 1978, Fraga 2008, Gould & Subramani 1988, Green & McElroy 1956). The enzyme can be used for several purposes such as: Biomass measurement (Lundin 2000, Vanhaecke & Pijck 1988), microbial activity in compost (Horiuchi et al. 2003), hygiene monitoring (Costa et al. 2006, Davidson et al. 1999, Larson et al. 2003), antibiotic activity on filamentous cells (Hattori et al. 1998), tumor cell viability in apoptosis research (Wang et al. 2008), food processing equipment validation (Oulahal-Lagsir et al. 2000), microbial metabolism in aquatic samples (Björkman & Karl 2001).

For these and other purposes, several companies are providing commercially available kits or tests. They are made for ATP measurement using luciferase enzyme and its substrate, luciferin, for bioluminescence reaction. Some companies or manufacturers (*in italic*), provide these kind of tests (test names are written in parenthesis), references are either cases were the related tests have been used or have been referred to by the manufacturers: *Biaffin GmbH & Co KG*, proteinkinase.de (ATP determination kit) Kassel, Germany (Kricka 1988), *BioThema AB* (ATP Hygiene Kit HS) Haninge, Sweden (Lundin 2000), *Biotium Inc* (ATP-Glo™ Bioluminetric Cell viability Assay Kit) Hayward, CA (Lundin 2000), *Biotrace International Plc* (Clean-Trace®) Bridgend, United Kingdom (Davidson et al. 1999, Simpson et al. 2006), *BioVision Research Products* (ApoSENSOR™ Cell Viability Assay Kit) Mountain View, CA (Burnett et al.

2006, Varma et al. 2007), *Cambrex Bio Science Rockland Inc* (ApoGlow® Assay Kit) Rockland, ME (Kim-Choi et al. 2006), *Hygiena* (Ultrasnap™) Camarillo, CA (Simpson et al. 2006), *Kinkkoman Corporation* (Lucipac-W) Chiba, Japan (Hattori et al. 1998, Horiuchi et al. 2003), *LuminUltra*™ (QGA™ Quench–Gone, Aqueous Formulation) New Brunswick, Canada (Whalen et al. 2006), *PerkinElmer Life Sciences* (ATPlite™ Luminescence ATP Detection Assay System), Boston, MA (Wang et al. 2008), *Promega* (BacTiter-Glo™ Microbial Cell Viability Assay), Madison, WI (Fan & Wood 2007), *PromoKine, PromoCell GmbH* (PromoKine Bioluminescent Cell Viability Kit), Heidelberg, Germany and *Roche Diagnostics GmbH* (ATP Bioluminescence Assay kit) Mannheim, Germany (Lundin 2000).

The luciferase enzyme was produced by using BEVS and it was purified with IMAC and EBA (I). The enzyme was electrophoretically analyzed and then it was proved to be similar to the native one. The ability of the luciferase to be used as a possible diagnostic reagent was then established (I). The Luc-His bioluminescent activity can be seen in Figure 3 (I). Moreover, the usually found contaminants (Rajgopal & Vijayalakshmi 1984) that could interfere the proper enzyme activity, were measured. A comparison between the native and the purified recombinant enzyme was also done (I, Table 1). It should be noted that the concentration of purified Luc-His was measured to be at 0.4 mg/ml for an ATP activity of 116,500 RLU. For the Luc-control (native luciferase) a concentration at 0.7 mg/ml for an ATP activity of 142,700 RLU was measured. When the luciferase amount increases, the ATP activity also increases relatively linearly, as it can be seen when comparing Figure 1A and Figure 3 (I). This correlation is also agreed on by Fan & Wood (2007). Therefore, it can be estimated that, if there is one mg/ml for Luc-His, the ATP activity is about 290,000 RLU, and in the control, the ATP activity is about 200,000 RLU. Therefore, for the Luc-His which was produced in insect cells, the ATP activity could be estimated to be about +45% more efficient than the ATP activity of the control. This contradicts the claim that Luc-His and the control had similar ATP activities as stated in article I.

It is rather difficult to compare ATP activity measured with different luminometers as the bioluminescence results are often given in relative light units. However, when measurements are done in the same conditions, with the same added reagents, the comparison between samples is possible. Especially when the following parameters are constant in luciferin amount, ATP amount, temperature, pH, etc. Wang et al. (1997) measured an ATP activity of 1509 for the control luciferase (Sigma-Aldrich, St. Louis, MO) and 1602 for the, polyhistidine-tagged-BCCP domain fusion luciferase which was expressed in bacteria. The calculated result is +6% ATP activity for the polyhistidine-tagged-BCCP domain fusion luciferase. When calculating the difference in percent for the results measured by Law et al. (2006) the difference is negative. They had an ATP activity of 3.25 for the control luciferase (Promega, Madison, WI) and an ATP activity 2.27 for the nonmutant polyhistidine-tagged luciferase which was expressed in bacteria. The calculated result is then -30% ATP activity for the non-mutant polyhistidine-tagged luciferase.

In contrast, the yield of bacterial expressed, polyhistidine-tagged-BCCP domain fusion luciferase of Wang et al. (1997) was estimated to be about 40 mg from one liter cell culture and the yield for, insect cell produced, Luc-His was estimated to be about 23 mg from one liter cell culture. Even though yield is estimated to be higher for the bacterial expressed luciferase (Wang et al. 1997), the bioluminescent activity is estimated as lower. This means that in the bacterial expressed purified proteins, there could be enzyme molecules with inefficient bioluminescent activity. One possible answer to these results is that, due to its insect origin (*Photinus pyralis*), the insect cell produced enzyme have better bioluminescent activity. Interestingly, Law et al. (2006) mentioned that polyhistidine-tagged luciferase has a lower bioluminescent activity than the other wild type or untagged luciferase recombinant. Together, the data shown here demonstrates clearly that the insect cell produced Luc-His enzyme is efficient and could be used in ATP monitoring and possibly commercialized by the companies mentioned above.

However, its chemical and physical resistance should be confirmed. There are recent publications of modifications in the enzyme that improve its resistance to temperature, to pH changes (Law et al. 2006) and to chemicals (Kim-Choi et al. 2006). Changes in sequence also allowed more efficiently high-throughput drug screening (Fan & Wood 2007). On the other hand, the immobilization or attachment on a carrier opened other possible applications. This has been done previously, on a glass strip, with luciferase extracted from firefly lantern extract (Ribeiro et al. 1998). BCCP domain has been fused with enzyme that has been produced in bacteria cells. This allowed the immobilization on avidin coated surfaces (Wang et al. 1997). The same enzyme was also immobilized on polystyrene beads (Ho et al. 1998). More recently luciferase was immobilized on a monolayer surface on the hydrophobic surface of graphite (Palomba et al. 2006). The immobilization of the Luc-His (I) on chelated metal-ions should also be evaluated in future studies.

6.3.2 Epstein-Barr nuclear antigen-1 of Epstein-Barr virus

The Epstein-Barr virus (EBV) is a gamma herpes virus, which isenveloped and it has a double-stranded DNA genome (Rickinson & Kieff 2001). EBV is the causative of many diseases such as infectious mononucleosis (IM) in immunocompetent individuals (Hess 2004) and is also associated with others malignancies (Leight & Sugden 2000). Moreover, EBV has been shown to be responsible for high mortality in immunocompromised individuals by causing malignant lymphomas (Hess 2004). EBV is capable of infecting B and T lymphocytes and others cells and the virus persist for life (Gulley 2001). During latent infection the virus is still transcriptionally active. Many different genes are expressed during the latency (Middeldorp et al. 2003). The Epstein-Barr nuclear antigen-1 (EBNA-1) is a DNA binding protein and has an essential role in EBV latency (Kieff & Rickinson 2001, Wang et al. 2006). The EBNA-1 gene products can be derived from four diverse promoters (Middeldorp et al. 2003). Therefore, the EBNA-1 is one of the few proteins to be transcripted in all four

latency phases the EBV goes through an infection (Middeldorp et al. 2003). Additionally, the EBNA-1 is essential for the maintenance of the EBV genome in dividing host cell and can be found in all EBV-associated diseases (Leight & Sugden 2000).

As developed in section 2.8.2, the diagnosis of EBV infection should be tested first by measuring the antibodies IgG against EBNA-1 and then by measuring the antibodies IgM and IgG against VCA. Some reports where antibodies against EBV were measured have been published. In those reports, several antigens have been used. These are, for example, recombinant antigens, purified native antigens or epitope peptides (Bruu et al. 2000, Buisson et al. 1999, Dardari et al. 2001, Fachiroh et al. 2006, Färber et al. 2001, Gorgievski-Hrisoho et al. 1990, Gärtner et al. 2003, Hille et al. 1993, Inoue et al. 1992, Kleines et al. 2006, Korhonen et al. 1999, Lang et al. 2001, Linderholm et al. 1994, Nystad & Myrmel 2007, Paramita et al. 2007, Pitiriga et al. 2003, Pratesi et al. 2006, Rea et al. 2002, Robertson et al. 2003, Schubert et al. 1998, Svahn et al. 1997, Tellam et al. 2004).

To measure the EBV infection state, many companies are offering kits for the detection of EBV infection. Some manufacturers are using as a solid phase antigen the nuclear antigen of EBNA-1 peptide p72 (reading frame, BKRF1). The p72 is coded by 42.7% of the C-terminal part of the gene coding for EBNA-1. The molecular weight of the expressed protein is about 46 kDa. The BKRF1 has been expressed in *Escherichia coli*, and the yield was about 6 mg from a 6 liter growing volume (Gorgievski-Hrisoho et al. 1990).

Bruu et al. (2000) compared in detail the products of several companies. In their kits the following companies are using for the VCA detection the peptides p23 and p18, for the EA detection the peptides p54 and p138 and for the EBNA detection the peptide p72. The names of the manufacturer are written *in italic* and the detected antibodies are in brackets: *Behringwerke*, *Dade Behring now Siemens Healthcare Diagnostics*, Marburg, Germany (combination of VCA, EA, EBNA), *Biotest Diagnostics*, Dreieich, Germany (EA, EBNA), *Centocor Inc*, Horsham, PA (VCA, EBNA), *Gull Laboratories*, Salt Lake City, UT (VCA, EBNA), *Institute Virion Ltd*, Rüschlikon, Switzerland (VCA and combination of VCA, EA, EBNA), *Organon Teknika*, Dublin, Ireland (VCA, EBNA). According to their results, Bruu et al. reported sensitivities of 95-100% and specificities from 86 to 100%.

Other companies are also providing tests using p72 recombinant antigen for EBNA IgG detection such as GA GENERIC ASSAYS GmbH (Dahlewitz, Germany). They claim that their ELISA test have a sensitivity of over 95% and a specificity of 89.5% (Anon 2005, Färber et al. 1993). Furthermore, the manufacturer DiaSorin (Saluggia, Vercelli, Italy) is providing ELISA tests for EBNA-1 IgG with a synthetic peptide from EBV EBNA-1 protein (Gärtner et al. 2003). Additionally, Wampole, Inverness Medical Professional Diagnostics (Princeton, NJ), which does not mention their solid phase antigen, is offering ELISA tests for EBNA-1 IgG (Anon 2008e).

In addition to the companies mentioned above, some others are also providing EIA or ELISA test for EBNA-1 IgG. Due to the manufacturing confidentiality, some of the companies are not mentioning their source of antigen or the reference is rather short. Besides, the sensitivity and specificity data is often not revealed. The following tests are reported to have recombinant EBNA-1 as a solid phase antigen. In the following list, the manufacturer is mentioned first (*in italic*), then there is the commercial name of the test and this is followed by the antigen origin (if known it is in brackets). Finally, there are the test sensitivity % and specificity % are reported in brackets: *Diagnostic Automation Inc*, Calabasas, CA, EBV EBNA-1 IgG ELISA (Anon 2006), *GenBio, ImmunoWELL*, San Diego, CA, ImmunoWELL™ EBNA IgG TEST, same as Novitec, (93%, 100%, n=86/8) (Anon 2008a, Gärtner et al. 2003), *Genzyme Virotech*, Rüsselsheim, Germany, EBV EBNA Virotech, (full length EBNA-1 recombinant, origin?) (Dardari et al. 2001, Gärtner et al. 2003), *Gull Laboratories*, Salt Lake City, UT, EBV IgG EBNA ELISA, (recombinant EBNA-1 without glycine-alanine copolymer, origin?) (95%, 100%, n=203) (Svahn et al. 1997), *Meridian Bioscience Inc*, Cincinnati, OH, Premier EBNA-1 IgG EIA (94.4%, 98.9, n=179/87) (Anon 2008b), *NovaTec Immundiagnostica GmbH*, Dietzenbach, Germany, EBV (EBNA) IgG. (98.5%, 89.5%, n=12) (Anon 2007), *Novitec, HiSS Diagnostics GmbH*, Freiburg, Germany, NOVITEC® EBV EBNA-1 IgG, (Full length EBNA-1 recombinant) (Gärtner et al. 2003, Nystad & Myrmel 2007), *Panbio Inc*, Columbia, MD, EBNA IgG, E-EBV02G (97.1%, 94.4%) (Anon 2008d), *R-Biopharm Ag*, Darmstadt, Germany, RIDASCREEN ® EBV EBNA IgG (82.8%, 97.8%) (Anon 2004, Gorgievski-Hrisoho et al. 1990).

In addition to EIA or ELISA techniques, one manufacturer is providing flow cytometry based test for the antibody detection Institut Virion, Serion Immundiagnostica GmbH (Würzburg, Germany). When compared to ELISA test, they claim a sensitivity of 96.6% and a specificity of 96.8% (n=98) (Degen et al. 2006). Line assay and western blot assays are provided by Mikrogen GmbH (Martinsried, Germany) for the detection of EBV antibodies (using the EBV nuclear antigen EBNA-1, p72, recombinant peptide) (Anon 2008c).

When compared to the other results, the data of the ELISA made with the Epstein-Barr nuclear antigen 1 (EBNA-1) of the Epstein-Barr virus (EBV), the His-EBNA-1 (III) gave specificity of 99.6% and sensitivity of 100%. This, even though the Gly-Ala repeat has been reported as the major epitope of EBNA-1 (Rumpold et al. 1987). This repeat sequence is not present in our construct. When referring the results of other commercially available tests, no one reported as good results as the EBV EBNA-1-IgG-ELISA PKS medac (Medac GmbH, Hamburg, Germany). In article III, our EBNA-1 ELISA set-up was compared to the one made by Biotest (Dreieich, Germany). The Biotest kit was one of the most efficient as compared to the reference method (Gärtner et al. 2003). Moreover, the set-up presented here, allows a single-point quantification (SQP) as well as a good automation possibility. Together, the data presented here clearly demonstrates that the baculovirus expressed polyhistidine-tagged EBNA-1 protein is an excellent source for the detection of EBV IgG antibodies by ELISA.

6.3.3 Virus protein 1 and 2 of human parvovirus B19

The human parvovirus B19 (B19) is a member of the *Erythrovirus* genus. B19 is a small, icosahedral shaped and non-enveloped virus. The major capsid element is VP2 (95%) and the minor element is VP1 (5%). The VP2 can only self assemble into VLPs (Ozawa & Young 1987). The virus is associated with many diseases such as erythema infectiosum or fifth disease, hematologic disorders, rheumatic diseases or hydrops fetalis (Broliden et al. 2006, Heegaard & Brown 2002, Parsyan & Candotti 2007). B19 infections are often asymptomatic (Parsyan & Candotti 2007).

The detection of immunoglobulin M (IgM) and IgG antibodies by using EIA or ELISA is a sensitive approach to detect B19 infection (Anderson et al. 1986). The IgM antibodies appear a few days after virus infection, before the physical manifestation of the virus (Parsyan & Candotti 2007). The acute antibody (IgM) level declines in about one to two months after infection (Anderson et al. 1986). The past antibodies (IgG) are detectable about three weeks after virus infection (Anderson et al. 1986). Therefore, for accurate diagnostic of B19, the used test set-up should be able to see the difference between IgM and IgG or, at least, to detect both. The detection of IgM is important, especially in pregnant woman where their presence could confirm a recent B19 infection. Testing could prevent hydrops fetalis (Servey et al. 2007). The IgM antibodies can be found in 37-81% of the cases when the clinical diagnosis of fetal hydrops is made (Zerbini et al. 2002).

The immunoglobulin M forms a pentamer of five Ig units and it has ten binding sites (Wiersma et al. 1998). The immunoglobulin G is formed of one basic Ig unit and it has two binding sites (Gergely & Sarmay 1990). This variation in conformation explains the difference in size of the antibody-antigen complex between IgM, IgG and B19 VLPs as seen with FCS (II, Table 1). The VLP-IgG had a measured average radius of 69 nm and the VLP-IgM 157 nm. The dilution of the human serum containing the antibodies for the FCS measurement was 1:30,000. The use of serum so dilute and the accuracy of the results as shown in article II demonstrated clearly that the used FCS set-up enables accurate measurement, in solution, of the formation of antibody-antigen complex.

Other more often used methods for the measurement of B19 antibodies are ELISA or EIA. Several manufacturers are providing kits for that purpose. Biotrin international (Dublin, Ireland) is providing Parvovirus B19 IgM or IgG EIA. In both separate kits, recombinant baculovirus produced VP2 VLPs are used. For IgG, the VLPs are coated on the microtiterplate. For IgM detection, the microtiterplate is coated with a rabbit anti-human IgM antibody. All the IgM antibodies present in the patient serum bind to the solid phase. Thereafter this a biotinylated VP2 VLP of B19 is added and bound to any B19 IgM present in the well, this is followed by the detection of the formed complex. Some of Biotrin's kits are FDA approved and they have been used in many reports where B19 antibodies detection was needed (Azzi et al. 2004, Beersma et al. 2005, Butchko & Jordan 2004, Corcoran et al. 2007, Ekman et al. 2007, Enders et

al. 2007, Enders et al. 2006, Freitas et al. 2008, Hoebe et al. 2002, Isa et al. 2006, Jordan 2000, Kerr et al. 1999, Manaresi et al. 2004, Mendonca et al. 2005, Parsyan et al. 2006, Pfrepper et al. 2005, Regaya et al. 2007, Us et al. 2007). In addition to Biotrin, other manufactures are providing enzymatic assays for B19 antibody detection such as DAKO, MRL Diagnostics, Genzyme Virotech, Rüsselsheim, Germany (Bruu & Nordbo 1995, Jordan 2000, Kaikkonen et al. 1999, Wermelinger et al. 2002), Gull Laboratories, Salt Lake City, UT (Pickering et al. 1998), Hillcrest Biologicals, Cypress, CA (Wermelinger et al. 2002), IBL Immunobiological laboratories, Hamburg, Germany (Pickering et al. 1998), Medac GmbH, Hamburg, Germany (Butchko & Jordan 2004, Manaresi et al. 2004, Manaresi et al. 2001) and Mikrogen, Munich, Germany (Butchko & Jordan 2004, Enders et al. 2007, Pfrepper et al. 2005).

Immunoblot technique has also been used for the detection of B19 antibodies. Arnika, Milan, Italy (Manaresi et al. 1999), Biotrin international, Dublin, Ireland (Beersma et al. 2005, Manaresi et al. 1999, Manaresi et al. 2001) and Mikrogen, Munich, Germany (Hemauer et al. 1999) provide immunoblot test for the B19 antibody detection.

In all these reports and the related tests, there are differences in the antigen used. Some are using undenaturated and conformational recombinant proteins VP1 and/or VP2 produced in insect cells (Peterlana et al. 2006). Others are using denaturated linear epitopes usually produced in prokaryotic system (Peterlana et al. 2006). Zerbini and others (2002) mentioned that a more efficient method is to use conformational antigen for the detection of B19 immune response. This statement was confirmed by others (Kerr et al. 1999, Manaresi et al. 2004). More recently, Enders et al. (2007) proposed that when having equivocal or weakly positive B19 IgM results after EIA, the results should be confirmed by a PCR assay.

Altogether, the undenaturated and conformational proteins VP1 and VP2 of B19 are the preferable sources as antigen for EIA. Therefore, the recombinant proteins VP1 and VP2 of B19 (His-B19-VP1 IV, His-B19-VP2, IV) produced in insect cells could be used in the tests for the diagnostic of B19. However, more testing than the ones presented in Table 1 (IV) should be done. Nevertheless, the Biotrin Company, as a provider of the mainly used B19 diagnostic kit, has awakened interest in the recombinant produced in Article IV (Sarantos 2006).

7 CONCLUSIONS

Different procedures can be applied for purification of recombinant proteins. The purification processes are case dependent. Alternative processes, by the addition of an affinity tag gave possibilities to obtain functional recombinant proteins in easier ways.

Specific conclusions of this study were as follows:

1. An affinity polyhistidine tag was successfully added at the C-terminus of the firefly luciferase of *Photinus pyralis*, at the N-terminus of the EBNA-1 of EBV and at the VP1 of B19 as well as on the VP2 VLPs of B19. This appeared not to affect correct protein folding and furthermore, enabled purification by using IMAC.

2. The polyhistidine-tagged proteins, the firefly luciferase of *Photinus pyralis*, the EBNA-1 of EBV and the VP1 of B19 as well as the VP2 VLPs of B19 were effectively purified by using IMAC. In addition, a larger scale one-step purification process, the EBA, was evaluated for the firefly luciferase.

3. The antigenic untagged VP2 VLPs of B19 were able to bind IgM and IgG antibodies essentially present in acute and past-immunity serum samples, respectively. The antigen-antibody complex formation was monitored by FCS.

4. The potential of polyhistidine-tagged recombinant proteins in diagnostics were shown. The firefly luciferase of *Photinus pyralis*, had sustained its bioluminescent activity. The EBNA-1 of EBV, the VP1 of B19 and the VP2 VLPs of B19 were all shown to be potential sources as antigens for ELISA in diagnostics.

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YHTEENVETO (Résumé in Finnish)

Diagnostisesti tärkeiden proteiinien tuotto hyönteissolussa sekä niiden puhdistus ja karakterisointi

Yksi biotekniikan päätavoitteista on hyödyntää eläviä organismeja solutehtaina rekombinanttiproteiineja tuotettaessa. Bioteknologiset prosessit sisältävät erilaisia puhdistusvaiheita, jotta haluttu proteiini tai peptidi voidaan eristää elävästä ja siksi monimutkaisesta lähtömateriaalista. Tuottoprosessissa käydään läpi ainakin seuraavat vaiheet: tuotto-organismin kasvatus, rekombinanttiproteiinien tuotto isäntäsolussa induktiovaiheen jälkeen ja lopputuotteen jälkikäsittely. Jälkikäsittelyvaiheen jälkeen rekombinanttiproteiineja voidaan käyttää mm. entsyymiteknologiassa ja erilaisten tautien diagnostiikassa.

Tämän väitöskirjan tutkimuksessa käytettiin lähinnä hyönteissoluja ja niille spesifisiä bakulovirusvektoreita. Lyhyesti sanoen, *Baculoviridae*-virusperhe muodostaa laajan ryhmän, johon kuuluu yli 500 tunnettua virusta. Bakulovirusryhmästä tunnetuin ja käytetyin on *Ac*MNPV (engl. *Autographa californica* multiple nucleopolyhedrovirus), jota käytetään bakuloviruksekspressiosysteemissä (engl. baculovirus expression vector system, BEVS). Bakuloviruksesta ja hyönteissoluista on tullut maailmanlaajuisesti hyvin suosittuja rekombinanttiproteiinien tuotossa. Ongelmattomasti kasvavat hyönteissolut mahdollistavat proteiinien translaation jälkeiset muutokset, joita ei tapahdu esimerkiksi bakteereissa. Tämän lisäksi bakuloviruksen genomi on riittävän suuri vastaanottamaan isoja, vieraita DNA-inserttejä. Työssä kehitettiin geneettisten modifikaatioiden avulla erilaisia viruskonstrukteja, jotta lopputuotteen puhdistus olisi optimaalinen. Proteiinien vaihtoehtoiset puhdistusprosessit evaluoitiin, jotta löydettiin menetelmiä mahdolliseen isomman skaalan tuottoprosessiin. Rekombinanttiproteiinien tuottoprosessissa oli vaatimuksena mm. korkea tuottotaso ja puhtausaste sekä biologinen toimivuus ja aktiivisuus. Vaatimuksena oli saada aikaan solun sisällä tuotettaville proteiineille oikeat post-translaation modifikaatiot, jotka kliiniseltä ja diagnostiselta kannalta toimivat ja joita puhtaat proteiinit edellyttävät. Tämä tarkoittaa sitä, että vain eukaryoottisolut pystyvät tähän mutta halvemmat bakteerisolutuottojärjestelmät eivät. Tässä väitöskirjaprojektissa on ollut pitkäjänteisenä tavoitteena kehittää toimivia ja kliinisesti käyttökelpoisia rekombinanttiproteiinien puhdistusmenetelmiä, joissa proteiinit on tuotettu autenttisessa muodossa hyönteiseukaryoottisoluissa. Autenttisten rekombinanttiproteiinien tuotanto ja oikeanlainen puhdistus ovat kliinisen tutkimuksen sekä tuotekehityksen lähtökohtia.

Tässä työssä avainasemassa oli proteiinien kloonauksessa käytetty tekniikka, jossa proteiinien amino- tai karboksyyliterminukseen lisättiin polyhistidiinihäntä (kuusi kertaa histidiiniaminohappoa), joka ei yleensä vaikuta lopputuotteen biologiseen aktiivisuuteen. Tekniikka mahdollisti puhdistuksen, jossa oli vain muutama vaihe. Ison skaalan puhdistukseen käytettiin mm. leijupetiadsorptiokromatografiatekniikkaa (engl. expanded bed adsorption, EBA).

Tuoton ja puhdistuksen lisäksi oli tärkeää, että biologinen aktiivisuus pystyttiin osoittamaan nopeasti ja kvalitatiivisesti. Siihen käytettiin luminometriaa, ELISAa (engl. enzyme linked immunosorbent assay), elektronimikroskopiaa (EM) ja yksittäisten molekyylien karakterisoimiseksi fluoresenssikorrelaatiospektroskopiaa (engl. fluorescence correlation spectroscopy, FCS).

Tämän väitöskirjan osatutkimuksissa (I-IV) kohteena olivat tulikärpäsen lusiferaasientsyymi, Epstein-Barr-viruksesta (EBV) peräisin olevan Epstein-Barr-tuma-antigeeni (engl. Epstein-Barr nuclear antigen, EBNA-1) sekä ihmisen parvorokkoviruksen B19 viruskapsidiproteiinit VP1 ja VP2. Yhdessä osatyössä tuotettiin onnistuneesti tulikärpäsen lusiferaasia. Tämä entsyymi osoitettiin käyttökelpoiseksi mm. ATP-määrityksessä tai elävien organismien määrityksessä (I). Toisena aiheena oli ei-infektiivisten ihmisen parvoviruspartikkelien (B19) tuotto, puhdistus ja karakterisointi. Osana karakterisointia käytetiin fluoresenssikorrelaatiospektroskopiaa. Tämä oli keskeinen työkalu B19 parvorokkoviruksen immunokompleksien havaitsemiseen. Sillä pystyttiin erottamaan potilaiden seerumista infektion jälkeen akuutti immuniteetti tai jälkiimmuniteetti (II). Tätä osatutkimusta jatkettiin lisäämällä polyhistidiinihäntä B19 viruksen rakenneproteiineihin (VP1 ja VP2), jotta parvorokkoviruksen partikkelien puhdistus olisi helpompaa. Kyseisten proteiinien toimivuus varmistettiin mm. ELISA:n avulla, ja rakenne varmistettiin elektronimikroskopialla (IV). Lisäksi yksi osaprojekti liittyi Epstein-Barr-viruksesta peräisin olevan EBNA-1-proteiinin tuottoon, puhdistukseen ja sen käyttöön diagnostisissa tarkoituksissa. Tutkimuksessa käytettiin ja testattiin yhteistyössä alan yrityksen kanssa metallioniaffiniteettikromatografialla puhdistettua proteiinia kaupallisen kitin valmistukseen (III).

Tämän väitöskirjan osatutkimuksissa keskeisimmät tavoitteet olivat mm. tuottaa rekombinanttiproteiineja hyönteissolussa ja puhdistaa niitä sekä karakterisoida niiden biologista toimivuutta. Tavoitteena oli myös kehittää prosesseja siten, että puhdistukseen käytettävän ajan lyheneminen ja työvaiheiden väheneminen johtavat resurssien säästöön sekä ympäristöystävällisempiin prosesseihin. Tavoitteena oli sen lisäksi käyttää lopputuotteita erilaisissa diagnostiikan tutkimuksissa.

Tutkimustyöni on osa laajempaa pitkäjänteistä tutkimustyötä, joka osaltaan auttaa sekä tiedemaailman että Suomen bioteknisen liiketalouden kehitystä. Tämän kaltaisen tutkimustyön ansiosta proteiineja pystytään modifioimaan geneettisesti ja analysoimaan molekyylitasolla. Niitä voidaan tuottaa ja puhdistaa niin tieteellistä tutkimuskäyttöä varten kuin myös kaupallisiin tarkoituksiin.

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