Production of Recombinant Allergen Birch Pollen Betv1a Modified with Hydrophobic Moieties in the Host *Escherichia coli*

Michael Bensoussan
Department of Microbiology and Immunology
McGill University, Montreal

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements of the
degree of Master of Science (M.Sc.)

August 2001

Your file Votre référence
ISBN: 0-612-85919-3
Our file Notre référence
ISBN: 0-612-85919-3

ACKNOWLEDGMENTS

I thank Dr. Clément Rioux, supervisor of this project, for his good support, enthusiasm, and sustained interest in the design and completion of this research project.

I also wish to extend my gratitude towards the National Sciences and Engineering Research Council of Canada (NSERC) / Conseil de recherches en sciences naturelles et en génie du Canada (CRSNG), for the financial help provided during my time of study and research as a Master's student.

RÉSUMÉ

Le traitement des allergies par l'immunothérapie spécifique (ITS) est l'administration contrôlée d'un allergène spécifique à un patient allergique vers l'allégement ou la remédiation de la maladie. Une immunothérapie de succès est associée à un changement dans la réaction immunitaire du phénotype de type 2 vers celui de type 1 et minimise la production d'IgE. Cependant, les obstacles principaux de l'ITS incluent le besoin pour une source d'allergène standardisée et pour un système adjuvant et de livraison qui promouvrait l'immunité de type 1 aux allergènes. Ce travail se concentre sur l'expression recombinante de l'allergène principal du pollen du bouleau européen (*Betula verrucosa*), Betv1a, avec des modifications covalentes par des structures hydrophobes ajoutées pour augmenter les qualités immunogènes de l'antigène et pour faciliter le processus de complexation aux Protéosomes hydrophobes qui forment un système adjuvant et de livraison. Les Protéosomes sont constitués de protéines de membranes externes extraites de *Neisseria meningitidis*, que l'on reconnaît pour favoriser l'immunité de type 1 aux antigènes. Six plasmides, dérivés du vecteur d'expression commercial pKK233-2, ont été conçus et construits pour coder pour Betv1a avec un ajout de décahistidine (His) seul (His-Betv1a et Betv1a-His), ou modifié d'un pied hydrophobe décapeptide à l'extrémité carboxyl ou amino de la protéine dans la famille vectorielle pKLY2; et Betv1a modifié à l'extrémité amino avec une queue d'acide gras (lipo-Betv1a-His et lipo-His-Betv1a) avec la famille vectorielle pKLY3. Les produits de pKLY2 ont été produits à de faibles niveaux dans le cytoplasme d'*Escherichia coli* DH5αF'IQ. La lipo-His-Betv1a, produite à des niveaux significativement plus élevés que la lipo-Betv1a-His, a été modifiée par des lipides dans *E. coli*, comme démontré par l'incorporation métabolique de l'acide palmitique gras radioactif et par l'identification des précurseurs prolipoprotéines par le traitement des cellules hôtes avec la globomycine, un inhibiteur de l'enzyme peptidase II. Les produits d'expression des lipoprotéines, qui ont été localisés exclusivement dans le compartiment de membrane cellulaire interne, tel que révélé par le fractionnement sous-cellulaire, a aussi diminué la croissance cellulaire après l'induction. La protéine recombinante Betv1a modifiée avec des lipides a été purifiée en une étape par chromatographie d'affinité sur des colonnes de nickel-acide nitriloacétique après une étape d'enrichissement avec de l'urée et par solubilization avec le détergent Empigen® BB.

ABSTRACT

Specific immunotherapy (SIT) treatment of allergies is the controlled administration of a specific allergen to an allergic patient towards the alleviation or remediation of the disease. Successful immunotherapy is associated with a shift in the immune response from type 2 to type 1 phenotype away from IgE production. But the main obstacles in SIT include the need for a standardized source for the allergen and for an adjuvant/delivery system that would promote type 1 immunity to allergens. This work focuses on the recombinant expression of European birch (*Betula verrucosa*) pollen's main allergen Betv1a with covalent modifications with hydrophobic structures whose additions were made to increase immunogenicity and to facilitate the process of complexing to the hydrophobic Proteosome adjuvant/delivery system. Proteosomes consist of outer membrane proteins extracted from *Neisseria meningitidis* which are known to favor type 1 immunity to antigens. Six plasmids, derived from the commercial expression vector pKK233-2, were designed and constructed to encode for Betv1a with a decahistidine tag (His) alone (His-Betv1a and Betv1a-His), or modified with a hydrophobic foot decapeptide at the carboxyl or amino-terminus of the protein with the pKLY2 vector family; and Betv1a modified with a N-terminal fatty acid tail (lipo-Betv1a-His and lipo-His-Betv1a) with the pKLY3 vector family. The products of pKLY2 were produced at low levels in the cytoplasm of *Escherichia coli* DH5αF'IQ. Lipo-His-Betv1a, which was produced at significantly higher levels than Lipo-Betv1a-His, was shown to be lipid-modified in *E. coli*, as demonstrated by the metabolic incorporation of the radiolabelled fatty acid palmitate and by identification of precursor prolipoproteins upon treatment of the host cells with globomycin, an inhibitor of signal peptidase II. Lipoprotein expression products, which were exclusively localized to the cell inner membrane compartment, as revealed by subcellular fractionation, also inhibited cell growth upon induction. Lipid-modified recombinant Betv1a was purified by single-step affinity chromatography on nickel-nitriloacetic acid columns following an enrichment step with urea and solubilization by Empigen® BB detergent.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

^{14}C	Carbon-14
^{3}H	Tritium
α	Alpha
β	Beta
ε	Epsilon
λ	Lambda (wavelength)
μ	Micro
aa	Amino acid
AmpR	Ampicillin resistance
ATP	Adenosine triphosphate
Betv1a/*betv1a*	*Betula verrucosa* birch pollen-derived protein/Betv1a-encoding gene
Bis-Tris	Bix-(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane
bp	Base pair
cal	Calorie
cDNA	Complementary deoxyribonucleic acid
Ci	Curie
CMC	Critical micelle concentration
COOH (or C)	Carboxyl
CT	Cholera toxin
Da	Dalton
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
EBB	Empigen® BB
EL	Eluate
ER	EBB resuspension
E. coli	*Escherichia coli*
EtBr	Ethidium bromide
g	Gravity
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GuHCl	Guanidine hydrochloride
Hft	Hydrophobic foot
His	Histidine
I	Induced

I/G	Induced, with globomycin added
Ig	Immunoglobulin
IL	Interleukin
IM	Inner membrane
IMAC	Immobilized-metal affinity chromatography
INF-γ	Interferon-gamma
IPTG	Isopropyl-β-D-thiogalactopyranoside
k	Kilo
l	Liter
LacI/*lacI*	Promoter-repressor protein/LacI-encoding gene
LB	Luria Bertani (growth medium)
Lipo	Lipid-bearing; lipidated; fatty-acylated
LPS	Lipopolysaccharide
m	Milli
M	Molar
mAb	Monoclonal antibody
mg	Milligram
MW	Molecular weight
N/A	Not applicable
NH_2 (or N)	Amino
NI	Non-induced
Ni-NTA	Nickel-nitrilotriacetic
nm	Nanometer
NMR	Nuclear magnetic resonance
OD	Optical density
OM	Outer membrane
Ori	Origin of replication
p	Plasmid designation; pico
PCR	Polymerase chain reaction
PhoA	Alkaline phosphatase
pI	Isoelectric point
psi	Pound per square inch
Ptr	Promoter (transcriptional)
r	Recombinant
RecA/*recA*	Protein involved in DNA recombination/RecA-encoding gene
RNA	Ribonucleic acid
rpm	Rotation per minute
rRNA	Ribosomal RNA
rrnB T1/T2	Terminator sites (transcriptional) T1 and T2
S	Soluble
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sec	Secretory
SIS	Signal sequence
SIT	Specific immunotherapy

SPase II	Signal peptidase II
Spp	Species
Taq	*Thermus aquaticus* (polymerase)
TCA	Trichloroacetic acid
T_H	T helper (lymphocyte)
Tm	Melting temperature (DNA)
TNF-α	Tumor necrosis factor-alpha
Tris	Tris(hydroxymethyl)aminomethane
u	Unit (enzymatic)
UV	Ultra-violet
V,v	Volt, volume
w	Weight
WC	Whole cell

LIST OF FIGURES and TABLES

CHAPTER I

INTRODUCTION

1.1 The basis of allergic reactions

As much as one third of the US population suffers from some form of allergy (American College of Allergy, Asthma, and Immunology; http://allergy.mcg.edu), a disease prevalent in developed, industrialized societies such as North America and Western Europe. Various animals (mites, pets), plants (trees, grasses, weeds), and other organisms (also foods, drugs, venoms...) may in fact be responsible for the clinical symptoms associated with type I hypersentivity: rhinitis, rhinoconjunctivitis, and even bronchial asthma, in severe, chronic cases. Proteins, more rarely sugars, are a cause underlying atopic diseases and are found in such structures as the pollen of numerous trees and grasses, in the case of pollen allergies (commonly, summer hay fever). In such cases, the allergen is usually a low molecular weight, hydrophilic protein that enters the body at very low doses through the respiratory mucosal surfaces in the nose and lungs and triggers the release of inflammatory mediators (histamine, heparin), cytokines (IL-3, IL-4, IL-5, GM-CSF, TNF-α), chemokines, and lipid mediators (leukotrienes) from mast cells by cross-linking their cell surface receptor FCε. Two other important players are eosinophils and basophils, which also contribute to the inflammation and tissue damage associated with allergic reactions in a late phase process, especially in the case of atopic asthma (Janeway et al., 1999; Menz, 1998).

Strong allergic reactions to an allergen require previous exposure(s) to the same allergen since immediate cell-mediated reactions to allergens are dependent upon the presence of IgE in circulation in the body. IgE antibodies bind directly to the FCε receptor on mast cells by their

heavy chains, and serve to stimulate mast cell activity when the cross-linking of these IgE-bound FCε receptors occurs in the presence of allergen molecules to which IgE has specificity.
It is not fully known why allergens initially favor the production of IgE versus other antibody isotypes (e.g. IgG, or IgA), but evidence points to a combination of genetic and environmental factors (Cookson, 1998). IgE plays an essential role in the pathology of atopic diseases, and B lymphocytes are responsible for its synthesis (following an isotypic switch from IgM) under the control of T helper (T_H) lymphocytes; this implies that immunological reactions to allergens are T_H2-mediated or of type 2. This comes in contrast with a T_H1- mediated or type 1 immune response, which favors primarily the activation of macrophages (e.g. following a bacterial or viral infection), producing an inflammation, and a different isotypic pattern (mostly IgG2a and IgG2b, in mice) of antibody production (Janeway et al., 1999). Immunotherapy therefore consists in providing an effective antibody class switch from IgE to IgA, a mucosal antibody, and/or IgG, a serum antibody, which would inhibit the effects of the specific allergen at the mucosa or inside the body, respectively.

1.2 Specific immunotherapy: aims and immunological mechanisms

Most often, prophylactic treatment of allergies includes allergen avoidance, use of histamine blockers, corticosteroids, and other pharmaceutical drugs that interfere with the cellular processes associated with allergic reactions. These measures are useful at providing fast relief especially during episodes of an allergic reaction, but such effects are short- lived and do not provide a cure (O'Byrne et al., 1998). Specific immunotherapy (SIT), which consists in the therapeutic administration of increasing doses of a purified allergen to an allergic patient over a prolonged period of time, aims at redirecting the immune system's response to the innocuous allergen by inducing a shift from type 2 to type 1 in the overall immunological mechanism responsible for the response to the specific allergen. Many studies involving patients suffering from allergies have correlated the immune switch from type 2 to type 1 to a decrease in IL-4 and IL-5 production, and an increase in INF-γ following SIT (Wiedermann et al., 1998 and 1999a). In turn, this switch is associated with a long-lasting (months to years) reduction in the clinical symptoms and immunological reactions (e.g. mast cell and eosinophil count) associated with allergy. The state of immune tolerance, or lack of immune responsiveness to the allergen,

induced by the administration of the allergen may be beneficial to individuals who seek long-term, possibly permanent relief from specific allergies.

Recently, the effectiveness of SIT was questioned in its application for pediatric asthma patients and allergic or asthmatic children, and statistical analysis of the results presented in various studies revealed that, in certain cases, SIT failed to provide evidence of therapeutic benefit (Wahn, 1999). This revealed the lack of a) a standardized source for the allergen (in some studies, crude allergen mixtures were used; see also Van Ree et al., 1999) and b) the necessity for an adjuvant/delivery vehicle that will help in establishing tolerance to the allergen (Wiedermann et al., 1999b).

1.3 The major birch pollen allergen Betv1a: a model system in the development of Proteosome-based vaccines

1.3.1 Rationale for the selection of Birch pollen Betv1a

SIT has been successful for the treatment of many allergies (Bousquet et al., 2001; Pichler et al., 2001; Ross et al., 2000) including allergies to Betv1a, one of the isoforms of the protein allergen isolated from the pollen of the European birch tree *Betula verrucosa* (order *Fagales*). In addition to its demonstrated value in SIT, Betv1a was also selected as a model for this study because of the fact that:

- The genetic sequence of the encoding gene *betv1a* is known; as determined by cDNA cloning of *betv1a* from a cDNA library whose expression products were immuno-screened with specific IgE anti-Betv1a from the serum of allergic patients (Figure 1; Breiteneder et al., 1989).
- Molecular cloning of Betv1a has been accomplished successfully in the heterologous host *Escherichia coli*, which produced high levels of this protein estimated at 17.4 kDa (Hoffmann-Sommergruber et al, 1997; Spangfort et al., 1996; Weiss et al., 1996), with characteristics identical to the natural protein counterpart found in birch pollen (Godnic-Cvar et al., 1997).
- The conformation and hyperstructure of Betv1a is known through NMR and X-ray analysis and reveals a basket-shaped structure, formed by seven β-strands, supported

by a rigid backbone constituted of a α-helix spanning the molecule's length (Figure 2; Gajhede et al., 1996). This essentially makes the molecule a structurally stable monomer, mostly hydrophilic throughout its surface.

This work reports on the molecular modifications of Betv1a, accomplished with the addition of hydrophobic moieties to the COOH or NH_2-terminal of rBetv1a. Such modifications will provide two main advantages:

a) an enhancement of the recombinant protein's immunogenicity by Hft (Lowell et al., 1988a, 1988b) or lipid modification of Betv1a. Indeed, the lipid moiety of lipoproteins of Gram-negative cells have been shown, in murein models, to be potent B cell mitogens, inducers of immunoglobulin synthesis (Bessler et al., 1985), and activators of inflammatory cytokines by macrophages (Melchers et al., 1975); and

b) the creation of hydrophobic anchors to allow for the efficient complexing of Betv1a with Proteosomes (Lowell et al., 1989).

To this end, two vector families, pKLY2 and pKLY3, were designed:

- **pKLY2** derivatives, for the production of the unmodified, His-tagged allergen, His-Betv1a and Betv1a-His, and for its modification with the hydrophobic foot (Hft) decapeptide NH_2-Gly-Gly-Tyr-Cys-Phe-Val-Ala-Leu-Leu-Phe-COOH in His-Betv1a-Hft, and with the reverse Hft sequence NH2-Phe-Leu-Leu-Ala-Val-Phe-Cys-Tyr-Gly-Gly-COOH in Hft-Betv1a-His
- **pKLY3** derivatives, for the production of the allergen modified post-translation with a lipid moiety at the amino-terminus (as explained in more details in section 1.4.1), Lipo-His-Betv1a and Lipo-Betv1a-His (Figure 3).

1.3.2 Proteosomes and Betv1a: vaccines for allergen immunization

Proteosomes are an adjuvant/delivery system made of hydrophobic outer membranes proteins of *Neisseria meningitidis*. A Proteosome-based vaccine therefore requires that the allergen ideally carry hydrophobic moieties for complexing to occur. The Proteosome-antigen complex size, which has been shown to vary between 60 and 100nm (Johnson et al., 2000), should provide for

potential enhancement in the mechanism of mucosal administration of a vaccine (Levine et al., 1997; Kiyono et al., 1996). In addition, the presence of Proteosomes should also help induce a state of immunotolerance following vaccine administration. In mice, previous work with formulations of antigens has shown that some purified antigens (e.g. flu virus hemagglutinin), which typically induce a type 2 immune response when administered alone and intranasally, produced a distinctive type 1 response when complexed to Proteosomes for the same type of immunization (Plante et al., 2001). Similar results were reported on the use of the cholera toxin (CT) adjuvant with respect to Betv1 intranasal immunization in a murine model (Wiedermann et al., 1998), and also with genetic immunization with CpG-bearing plasmids that encode for Betv1 (Hartl et al., 1999a, b; Jahn-Schmid et al., 1999). But whereas CT toxicity hampers the use of this adjuvant in humans, and genetic vaccines remain controversial, Proteosomes have been demonstrated as safe. Thus, comparative analytic and animal immunization studies should ultimately help determine which of the Hft or lipid-modified Betv1a to Proteosome complexing formulation(s) will provide the vaccine most appropriate for the proper immuno-modulation required in the treatment of allergies.

1.4 Covalent modification of Betv1a with lipids: a fate common to bacterial lipoproteins

1.4.1 Lipoproteins in prokaryotes

The fact that many prokaryotic cellular proteins undergo post-translational modifications is well documented (Inouye, 1987). Following the discovery of murein or Braun's lipoprotein in *Escherichia coli* (Braun and Rehn, 1969), many other lipoproteins were characterized in this organism (e.g. ColE, ColA lysis proteins), but also in numerous other Gram-negative (e.g. Lpp in *Shigella dysenteriae or Salmonella typhimurium*), and Gram-positive prokaryotic species (e.g. MalX in *Streptococcus pneumoniae*).

Mature lipoproteins share common structural characteristics which include the presence of:

- an N-terminal cysteine residue

- two ester-linked fatty acids, whose composition is similar to membrane phospholipids, making up a diacylglycerol molecule linked at the sulfhydryl group of the above terminal cysteine
- an N-terminal, amide-linked, fatty acid, usually palmitate (Ghuysen and Hakenbeck, 1994).

Protein modification with lipids requires that a prolipoprotein precursor carry a tripartite signal sequence (SIS) essential to its downstream processing in cells. This signal sequence may be divided in a) a positively charged N-terminal , b) a mid-stretch of 10-16 hydrophobic residues, and c) a C-terminal lipobox, composed of three amino acids (a.a.; Leu-Ala/Ser-Gly/Ala at positions -3, -2, and -1 with respect to the first residue, cysteine (+1), of the mature lipoprotein; von Heijne, 1989), which is the specific region recognized by the prolipoprotein glyceryl transferase and by the cleaving enzyme leader peptidase SPase II (more details in section 1.4.3). Additionally, the presence of a β-turn structure adjacent to +1 cysteine, at positions +2 to +5, seems essential for the complete processing of the precursor protein (Inouye et al., 1986).

1.4.2 pKLY3: genetic elements encoding for lipoprotein synthesis

pKLY3 is a plasmid vector designed to encode for the 5'end of the *celB* (in the colE2 operon) signal sequence (Cole et al., 1985), and enables the in-frame fusion of this sequence with DNA coding for a recombinant protein via the blunt-end cloning site *Sna*BI (Figure 4; Rioux et al., 1992). This 19 a.a. signal sequence, MKK ITGIILLLLAVIILSA, conforms with the sequence structure described above in that it contains a positively charged MK^+K^+ at the N-terminal, followed by a 13 a.a.-long hydrophobic stretch, ending with the LSA lipobox. The presence of the cysteine residue immediately following this signal sequence (+1) is necessary to the process of covalent modification of the recombinant protein by lipidation. Lack of cysteine at this position in the protein molecule will result in the transfer of a protein out of the cytoplasm and into another cellular or extra-cellular compartment, depending on the additional structural information the mature protein itself may contain, but without any lipidation. Examples of such proteins include the non-lipidated, periplasmic enzyme alkaline phosphatase (PhoA), whose encoding gene provides for all the elements of a SIS with a contiguous arginine, instead of cysteine a.a. (Chang et al., 1986; Karamyshev et al., 1998), or the mutant CelB protein whose

cysteine residue was replaced by arginine by site directed mutagenesis in the experiment described by Pugsley and Cole, 1987. Finally, the predicted β–structure, formed by QANY, is also present to insure efficient protein processing, and remains as an integral part of the mature lipoprotein.

1.4.3 Molecular details of lipoprotein biosynthesis

Lipoprotein synthesis in Gram-negative cells is laborious (Figure 5; Ghuysen and Hakenbeck, 1994). Prolipoproteins, carrying the signal sequence in the molecular format described above, are initially synthesized in the cell cytoplasm, and subsequently undergo a sequence of modifications by specific cellular enzymes. A diglyceride moiety is created by a glyceryl transferase at the N-terminal cysteine by addition of a glyceryl residue to the sulfhydryl group, and followed by the attachment of two, ester-linked, fatty acids, as catalyzed by at least one O-acyl transferase. An apolipoprotein is obtained following the removal of the signal sequence by SPaseII, which recognizes the diglyceride moiety and the lipobox sequence on the modified lipoprotein in order to cleave the protein at the N-terminus of cysteine (Tjalsma et al., 1999). Proteolytic cleavage by SPase II exposes the amino group at the N-terminal of the apolipoprotein, making it available for acylation by an N-acylase. This last acylation, usually by a palmitic acid molecule, represents the final step of lipoprotein synthesis. As is shown in figure 5, some proteins may undergo additional processing, as is the case for Braun's lipoprotein, by covalent attachment of the C-terminal lysine residue to a meso-diaminopimelic acid in the murein structure. However, in the case of Betv1a, lack of a C-terminal lysine makes the molecule unavailable for further modification. It is expected that Betv1a will undergo lipidation according to the scheme described above, and that it should remain membrane-bound via its hydrophobic moiety, within the cell.

Because all the enzymes that play a role in protein lipidation are located usually on the trans side of the cytoplasmic membrane (Inouye, 1987), any prolipoprotein synthesized in the cytoplasm therefore requires translocation to this compartment in order to be processed efficiently. The export of prolipoproteins is dependent upon the functionality of a group of secretory or Sec proteins involved in general protein secretion (Economou, 1998; Pugsley, 1993). Sec B is a chaperone-like protein that binds reversibly to the signal sequence of a nascent prolipoprotein (or

any protein that requires export), and insures that the proper conformation of the signal sequence is maintained at the protein's N-terminal. A large complex, or translocase, found in association with the inner membrane, constitutes the motor (i.e. SecA, a peripheral ATPase that shoves SIS-bearing proteins through the inner membrane), and the pore (SecY, SecE, SecG, and others) through which protein translocation occurs (Arkowitz and Bassilana, 1994; Driessen et al., 1998). No lipoproteins may be produced in the cell if the process of protein translocation is deficient (Hayashi and Wu, 1985), or if the export protein is defective in the presentation of a SIS to the translocase apparatus (e.g. Michaelis et al., 1983).

The overall process of protein modification by lipids is intricate since it requires both efficient translocation and a series of specific molecular modifications. Recombinant lipo-PhoA expression has already been successfully accomplished using the pKLY3 expression vector, and produced a functional, lipidated PhoA enzyme in association with the outer membrane of cells (Rioux et al., 1992).

One main aspect of this work will be to investigate lipid modification, but also protein processing, and compartmentalization of Betv1a in order to provide for an efficient and practical method by which recombinant lipidated Betv1a may be produced and purified.

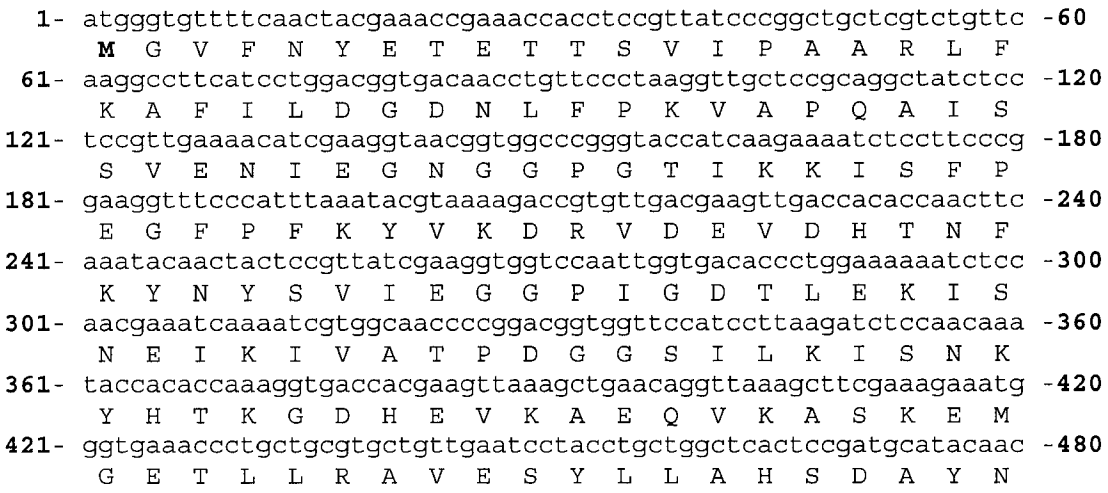
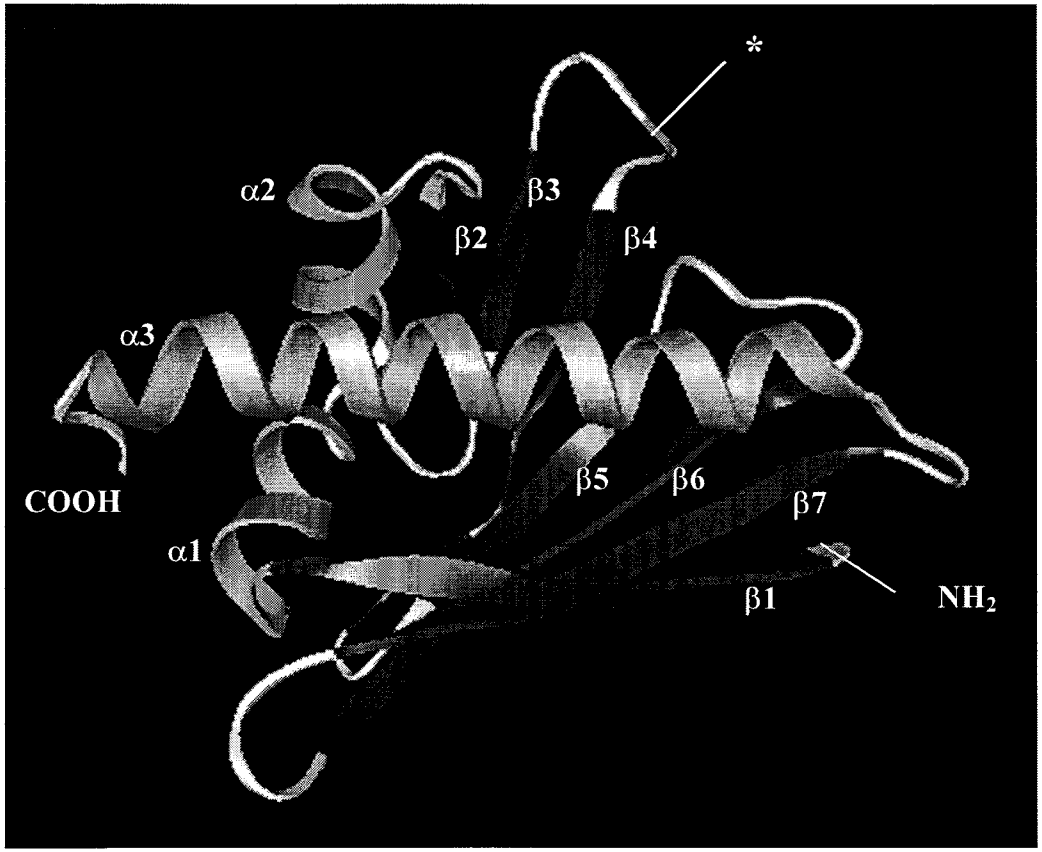


FIGURE 1. The *betv1a* cloning sequence. Codon bias is eliminated to maximize recombinant protein production in *E. coli* by replacement of the rare codons from the encoding sequence found in the corresponding eukaryotic genetic sequence (GenBank access #X15877; Breiteneder et al., 1989), and adapted to match the preferential codon usage in *E. coli* (Boehm and Rösch, 1997; Nakamura et al., 2000).



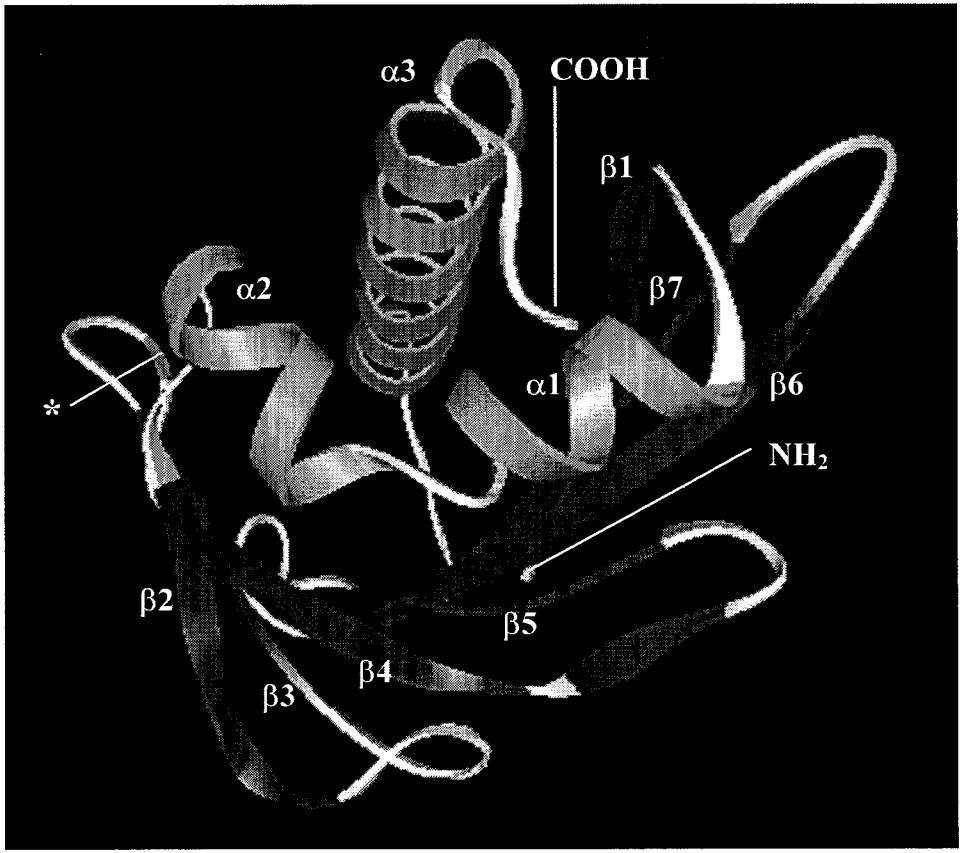


FIGURE 2. X-ray structure of recombinant protein Betv1a. The molecule displays a total of 3 alpha (α) chains and 7 beta (β) strands. A striking structural element is the α3 chain that spans the entire length of the molecule. The asterisk (*) indicates an a.a. change –Phe 63 to Leu- between the above molecule sequence and the sequence used in this work. The molecule structural data was obtained from the Research Collaboratory for Structural Bioinformatics (RCSB; http://www.rcsb.org/index.html) protein databank.

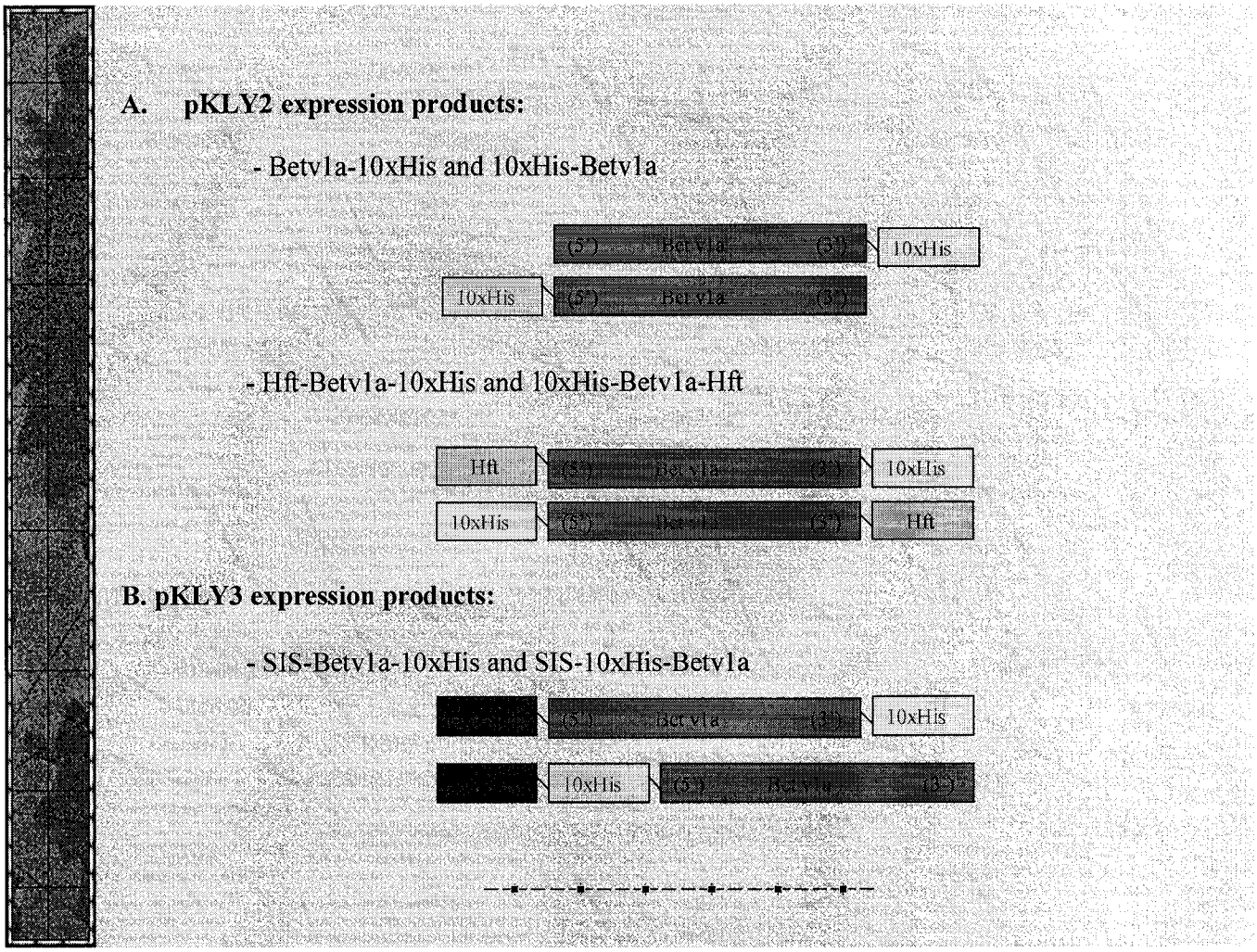


FIGURE 3. Expected expression products of pKLY2 and pKLY3 plasmid vectors following induction in the heterologous host *Escherichia coli* transformed with the corresponding vectors.

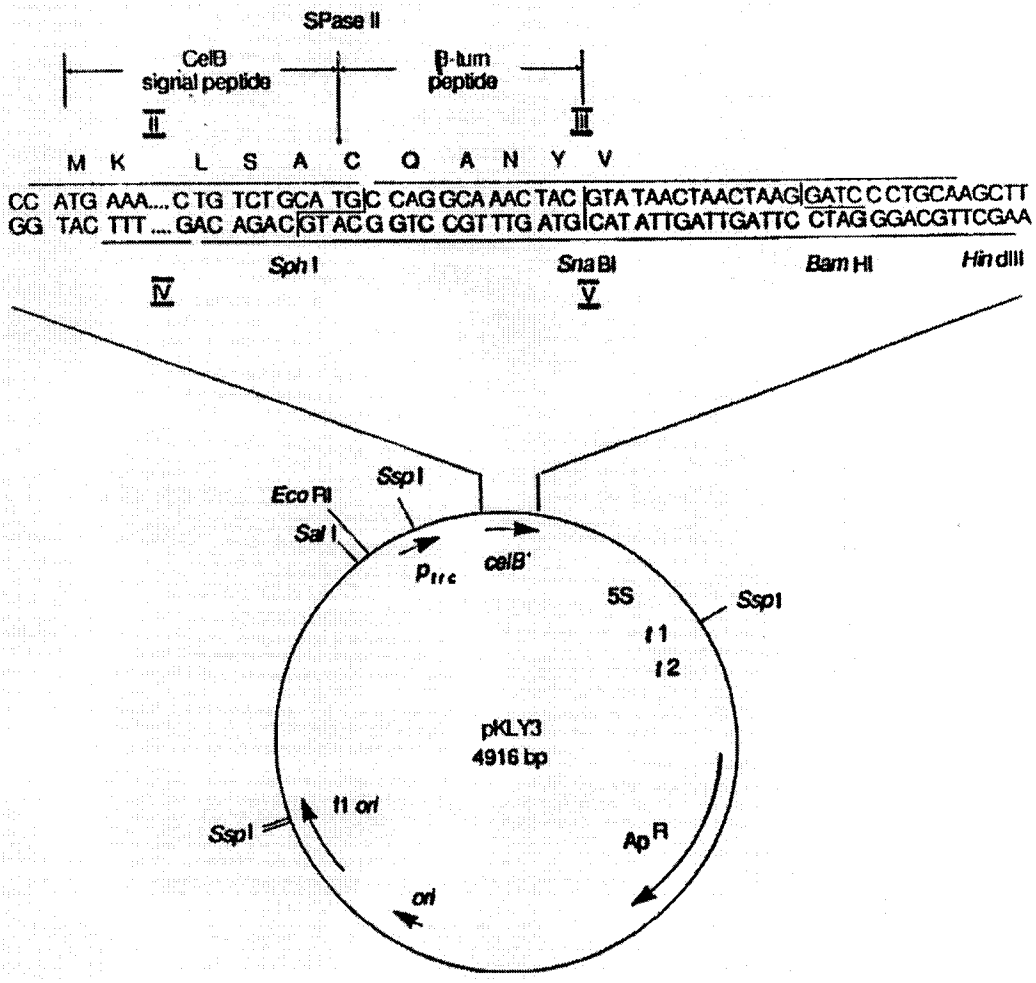


FIGURE 4. pKLY3, a plasmid vector for the synthesis of SIS-carrying prolipoproteins in *E. coli*. All essential elements for lipoprotein recognition and processing by the cell are present: the tripartite, 19 a.a.-long SIS, which includes the LSA lipobox directly adjacent to a cysteine residue for covalent, N-terminal modification of the protein, and the QANY β-turn. SPase II, the proteolytic enzyme cleaves the molecule at the junction of the lipobox sequence, LSA, and the cysteine residue (from Rioux et al., 1992).

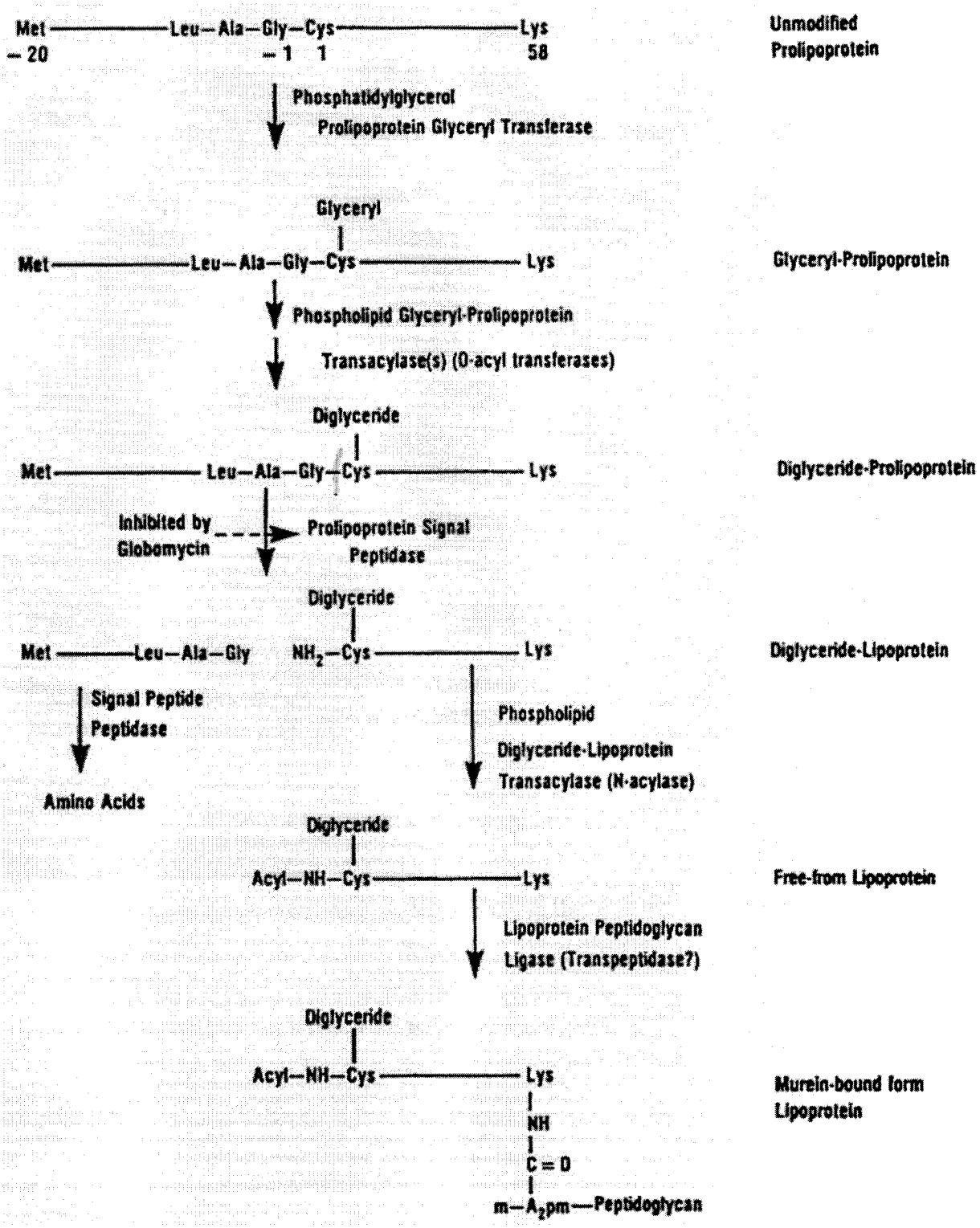


FIGURE 5. In *Escherichia coli*, a prolipoprotein displaying an N-terminal signal sequence is processed through a multi-enzymatic pathway into a diglyceride prolipoprotein (or apolipoprotein) and lipoprotein (from Inouye, 1987).

CHAPTER II

DESIGN AND CONSTRUCTION OF PLASMIDS pKLY2 AND pKLY3

2.1 Origin of vectors pKLY2 and pKLY3 and specification of the *E. coli* host strain

Parent plasmid pKLY3 was obtained from Dr. Clément Rioux, and is derived from vector pKK233-2 (Amersham, Canada; NCBI access #U02439).

The pKLY2 parent vector was created through recombination of the .29 kbp promoter- containing region of pKK233-2 -5' *Eco*RI/ HindIII 3'- with the 4.54 kbp plasmid DNA digest product of pKLY3 5' *Eco*RI/ HindIII 3'.

Escherichia coli DH5αF'IQ (Lifetech, USA) was chosen in this study as the main strain for transformation with pKLY2 and pKLY3 plasmids and for the expression of all modified recombinant proteins of Betv1a. DH5αF'IQ is RecA deficient (*recA*$^-$), which ensures genetic stability of the plasmid constructs introduced into this cell type. Control of the activity of the trc promoter in the plasmid vectors is due to the repressive activity of LacI, expressed constitutively in *E. coli* DH5αF'IQ (*lacI*^{q} genotype). Induction of promoter activity was usually carried out with 1-2mM isopropyl-β-D-thiogalactopyranoside (IPTG). DH5αF'IQ also carries the marker gene for antibiotic resistance to kanamycin (episomal gene). Resistance to the antibiotic ampicillin (AmpR) is imparted by all pKK233-2 derived vectors and was used as a marker for selection of transformed cells. DH5αF'IQ was made competent by calcium chloride treatment, and transformation with plasmid DNA enabled by heat shock (Maniatis et al., 1982). Cells were grown in rich liquid medium (LB broth base, Lennox L; Gibco BRL), in aerobic conditions, with single (75μg/ml ampicillin) or double (50μg/ml ampicillin, 40μg/ml kanamycin) antibiotic selection. All experiments were performed using aseptic techniques.

2.2 Design and preparation of DNA inserts for the plasmid vectors

2.2.1 General characteristics of pKLY2 and pKLY3 DNA-modifying inserts

- *Specific 5' and 3' restriction sites for the incorporation of the new synthetic double-stranded DNA into the target vector.* Inserts of pKLY2 vectors carry a 5' *Nco*I site (CCATGG), and inserts to pKLY3, a blunt-ended 5' *Sna*BI site (TACGTA). All inserts display at their 3'end the *Hin*dIII-kill sequence 5' (T)AGCT**A** 3' which, upon cloning, eliminates the vector-encoded *Hin*dIII restriction site 5' AAGCT**T** 3' by introduction of a single base change in the recombinant DNA. Elimination of the unique *Hin*dIII site was necessary to accommodate for the subsequent insertion of the gene *betv1a*, which also carries a *Hin*dIII restriction site.
- *Unique restriction sites for the incorporation of the PCR-amplified, recombinant gene betv1a*: *Bam*HI, 5' to the gene, and *Bsr*G1, 3' to the gene.
- *A decahistidine or hydrophobic peptide-encoding region* located 5' or 3' to *betv1a,* and flanked by two sets of unique restriction sites: 5' *Nhe*I/*Sac*I 3', at the 5' or 3' end of the betv1a insertion region in the pKLY3 vectors, or 5' *Pfl*23II/*Bcu*I 3' and 5' *Nhe*I/*Sac*I 3', respectively at the 5' and 3' end of betv1a in the pKLY2 Hft-carrying vectors. This should allow for the removal of or change in the decahistidine sequence (e.g. to a hexahistidine sequence) or hydrophobic peptide region, if needed. The presence of a histidine tag is essential to the process of purification by Ni-NTA technology (Sulkowski, 1985).

2.2.2 Preparation of synthesized DNA inserts

Individual oligonucleotides were synthesized at the Sheldon Biotechnology Center, McGill University. Each oligonucleotide was reconstituted to a concentration of 50pmol/µl in ddH_2O, and its actual relative concentration and quality evaluated on 2% agarose gel containing the detection dye ethidium bromide (EtBr) under a long-wavelength UV lamp (λ=260nm).

Oligonucleotides were mixed in pair in an equimolar ratio to produce DNA inserts OINT (27-28) and (29-30). For OINT (35-38), (39-42), (43-46), and (47-50) four oligonucleotides were needed per construct, also equimolarly. For the latter, the single-stranded oligonucleotide located at the 3'-end of each strand of the insert required phosphorylation by T4 polynucleotide kinase at the 5'-terminus (forward enzymatic reaction; Maniatis et al., 1982) since all synthetic DNA strands were provided unphosphorylated at this end. Thus, the following synthetic DNA molecules individually required phosphorylation prior to their incorporation into the corresponding inserts: OINT36, 37, 40, 41, 44, 45, 48, and 49. Briefly, DNA phosphorylation of these inserts was accomplished by incubation of 300pmoles of each oligonucleotide with 10u of T4 polynucleotide kinase (MBI Fermentas Inc., Canada) in a buffered solution (50mM Tris, pH=7.5; 10mM $MgCl_2$; 5mM DTT) that included 1mM ATP, at 37°C, for 1 hour. Incubation at 65°C for 10 minutes followed and resulted in T4 enzyme inactivation. Paired oligonucleotides, phosphorylated or not, were mixed (50pmoles, each), in the same buffered solution described above (no ATP), heated to 80°C for 5minutes in a heating block, then removed from the heat source along with the block in order to allow for slow temperature drop of the DNA mixture, i.e. efficient DNA annealing. All double-stranded DNA inserts thus obtained were analyzed on 2% agarose/EtBr gels to assess for the homogeneity of each product.

2.2.3 Sequences of vector-modifying DNA inserts for pKLY2 and pKLY3

2.2.3.1 Betv1a-His expression in pKLY2:
insert (*Pfl*23II-*Bcu*I)-(*Bam*HI-*Bsr*G1)-10xHis

*Nco*I (CCATGG) *Bam*HI (GGATCC) *Bsr*GI (TGTACA)
*Pfl*23II (CGTACG) *Bcu*I (ACTAGT) *Nhe*I (GCTAGC)

```
    M   A   A   Y   G   T   S   G   I   L   L   Y   I   A   S
CC ATG GCT GCG TAC GGT ACT AGT GGG ATC CTG CTG TAC ATC GCT AGC
GG TAC CGA CGC ATG CCA TGA TCA CCC TAG GAC GAC ATG TAG CGA TCG
```

*Hind*III killed (TAGCTT)
Sac I (GAGCTC)

H H H H H H H H H H G A L *
CAC CAT CAC CAT CAC CAT CAC CAT CAC CAC GGA GCT CTG TAGCTT
GTG GTA GTG GTA GTG GTA GTG GTA GTG GTG CCT CGA GAC ATCGAA

*stop codon

Actual oligonucleotide strands synthesized:

Upper DNA strands: OINT39 [36 bases]: 5' CATG--------TGTA 3'
OINT41 [50 bases]: 5' CATC--------CTGT 3'
Lower DNA strands: OINT40 [45 bases]: 5' GTGG-------CAGC 3'
OINT42 [41 bases]: 5' AGCT-------GATG 3'

The resulting double-stranded DNA insert is termed OINT (39-42).

Underlined DNA bases represent strand complementarity regions found only on the target vector pKLY2 or pKLY3; synthetic DNA appears with no underline or in color.

2.2.3.2 His-Betv1a expression in pKLY2:
insert 10xHis -(*Bam*HI-*Bsr*G1)-(*Nhe*I-*Sac*I)

*Nco*I (CCATGG)
*Pfl*23II (CGTACG) *Bcu*I (ACTAGT)

M A A Y G H H H H H H H H H H T S
CC ATG GCT GCG TAC GGT CAC CAT CAC CAT CAC CAT CAC CAT CAC CAC ACT AGT
GG TAC CGA CGC ATG CCA GTG GTA GTG GTA GTG GTA GTG GTA GTG GTG TGA TCA

*Bam*HI (GGATCC) *Bsr*GI (TGTACA) *Hind*III killed (TAGCTT)
*Nhe*I (GCTAGC) *Sac* I (GAGCTC)

G I L L Y I A S I G A L *
GGG ATC CTG CTG TAC ATC GCT AGC ATC GGA GCT CTG TAGCTT
CCC TAG GAC GAC ATG TAG CGA TCG TAG CCT CGA GAC ATCGAA

Actual oligonucleotide strands synthesized:

Upper DNA strands: OINT35 [43 bases]: 5' CATG--------TCAC 3'
OINT37 [46 bases]: 5' CACA--------CTGT 3'
Lower DNA strands: OINT36 [54 bases]: 5' GATC--------CAGC 3'
OINT38 [35 bases]: 5' AGCT--------GCAG 3'

The resulting double-stranded DNA insert is termed OINT (35-38).

2.2.3.3 His-Betv1a-Hft expression in pKLY2:
10xHis -(*Bam*HI-*Bsr*G1)- hydrophobic peptide
at the COOH-end of Betv1a

*Nco*I (CCATGG)
*Pfl*23II (CGTACG) *Bcu*I (ACTAGT)

M A A Y G **H H H H H H H H H H** T S
CC ATG GCT GCG TAC GGT CAC CAT CAC CAT CAC CAT CAC CAT CAC CAC ACT AGT
GG TAC CGA CGC ATG CCA GTG GTA GTG GTA GTG GTA GTG GTA GTG GTG TGA TCA

*Bam*HI (GGATCC) *Bsr*GI (TGTACA)
*Nhe*I (GCTAGC)
G I L L Y I A S **G G Y C F V A L L F**
GGG ATC CTG CTG TAC ATC GCT AGC GGT GGT TAC TGT TTC GTT GCT CTG CTG TTC
CCC TAG GAC GAC ATG TAG CGA TCG CCA CCA ATG ACA AAG CAA CGA GAC GAC AAG

*Hin*dIII killed (TAGCTT)
Sac I (GAGCTC)
G A L *
GGA GCT CTG TAGCTT
CCT CGA GAC ATCGAA

Actual oligonucleotide strands synthesized:

Upper DNA strands: OINT43 [51 bases]: 5' CATG--------CTAG 3'
OINT45 [65 bases]: 5' TGGG--------CTGT 3'
Lower DNA strands: OINT44 [60 bases]: 5' CAGC--------CAGC 3'
OINT46 [56 bases]: 5' AGCT---------TGTA 3'

The resulting double-stranded DNA insert is termed OINT (43-46).

2.2.3.4 Hft-Betv1a-His expression in pKLY2:
hydrophobic peptide at the NH2-end of
Betv1a-(*Bam*HI-*Bsr*G1)-10xHis

*Nco*I (CCATGG)
*Pfl*23II (CGTACG) *Bcu*I (ACTAGT)

M A A Y G **F L L A V F C Y G G** T S
CC ATG GCT GCG TAC GGT TTC CTG CTG GCT GTT TTC TGT TAC GGT GGT ACT AGT
GG TAC CGA CGC ATG CCA AAG GAC GAC CGA CAA AAG ACA ATG CCA CCA TGA TCA

*Bam*HI (GGATCC) *Bsr*GI (TGTACA)
*Nhe*I (GCTAGC)
G I L L Y I A S **H H H H H H H H**
GGG ATC CTG CTG TAC ATC GCT AGC CAC CAT CAC CAT CAC CAT CAC CAT
CCC TAG GAC GAC ATG TAG CGA TCG GTG GTA GTG GTA GTG GTA GTG GTA

*Hin*dIII killed (TAGCTT)
Sac I (GAGCTC)
H H G A L *
CAC CAC GGA GCT CTG TAGCTT
GTG GTG CCT CGA GAC ATCGAA

Actual oligonucleotide strands synthesized:

Upper DNA strands: OINT47 [51 bases]: 5' CATG--------CTAG 3'
OINT49 [65 bases]: 5' TGGG--------CTGT 3'
Lower DNA strands: OINT48 [60 bases]: 5' CAGC--------CAGC 3'
OINT50 [56 bases]: 5' AGCT---------TGTA 3'

The resulting double-stranded DNA insert is termed OINT (47-50).

2.2.3.5 SIS-His-Betv1a expression in pKLY3:
10xHis at the NH2-end of Betv1a

*Sna*BI (TACGTA)
*Nhe*I (GCTAGC)
Y V A S **H H H H H H H H H H**
TAC GTA GCT AGC CAC CAT CAC CAT CAC CAT CAC CAT CAC CAC
ATG CAT CGA TCG GTG GTA GTG GTA GTG GTA GTG GTA GTG GTG

Sac I (GAGCTC) *Hin*dIII killed (TAGCTT)
*Bam*HI (GGATCC) *Bsr*GI (TGTACA)
G A L G I L L Y I *
GGA GCT CTG GGG ATC CTG CTG TAC ATC TAGCTT
CCT CGA GAC CCC TAG GAC GAC ATG TAG ATCGAA

Actual oligonucleotide strands synthesized:

Upper DNA strand: OINT27 [67 bases]: 5' GTAG--------ATCT 3'

Lower DNA strand: OINT28 [71 bases]: 5' AGCT--------CTAC 3'

The resulting double-stranded DNA insert is termed OINT (27-28).

2.2.3.6 SIS-Betv1a-His expression in pKLY3:
10xHis at the COOH-end of Betv1a

*Sna*BI (TACGTA) *Bsr*GI (TGTACA)
*Bam*HI (GGATCC) *Nhe*I (GCTAGC)

Y	V	G	I	L	L	Y	I	A	S	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
TAC	GTA	GGG	ATC	CTG	CTG	TAC	ATC	GCT	AGC	CAC	CAT	CAC	CAT	CAC	CAT	CAC	CAT
ATG	CAT	CCC	TAG	GAC	GAC	ATG	TAG	CGA	TCG	GTG	GTA	GTG	GTA	GTG	GTA	GTG	GTA

Sac I (GAGCTC) *Hin*dIII killed (TAGCTT)

H	**H**	G	A	L	*
CAC	CAC	GGA	GCT	CTG	TAGCTT
GTG	GTG	CCT	CGA	GAC	ATCGAA

Actual oligonucleotide strands synthesized:

Upper DNA strand: OINT29 [67 bases]: 5' GTAG--------CTGT 3'

Lower DNA strand: OINT30 [71 bases]: 5' AGCT--------CTAC 3'

The resulting double-stranded DNA insert is termed OINT (29-30).

2.3 Recombination of vectors pKLY2 and pKLY3 with prepared DNA inserts

2.3.1 First generation vectors

Purified parent plasmids pKLY2 and pKLY3 were quantified on 1% agarose/EtBr gel using a quantifying molecular weight (MW) standard (Massruler™ DNA ladder; Fermentas, MD). Each plasmid was enzymatically treated with two DNA restriction endonucleases: pKLY2 with *Nco*I/*Hin*dIII and pKLY3 with *Sna*BI/*Hin*dIII. The products of the enzymatic digest were

separated on 1% agarose gel/EtBr. The bands corresponding to a MW of approximately 5 kbp were excised from the gel and the DNA extracted using one of two methods: phenol/freeze (Maniatis et al., 1982), or agarose hydrolysis followed by DNA purification on silicate columns (Concert Gel Extraction System; Life Technologies, Canada). After quantitation on 1% agarose/EtBr gel, pKLY2 and pKLY3 were mixed at a ratio of 1:5 to 1:10 (mol/mol) with one of the DNA insert molecules. Ligation of the doubly-digested plasmids with the corresponding double-stranded synthetic DNA molecules was mediated by T4 DNA ligase activity. The ligation reaction was performed in buffer (660mM Tris, pH=7.5; 66mM $MgCl_2 \cdot 6H_2O$; 100mM DTT; 10mM ATP), and allowed to proceed overnight at 16°C. Approximately 800u of T4 DNA ligase (New England Biolabs, Canada) was used per reaction. Typically, DNA concentrations for either plasmids or DNA inserts varied between .25 and 5ng/ul in the reaction vessel. The positive control consisted of the purified *Hin*dIII-digested pKLY3 parent plasmid to test for ligation reaction efficiency. A negative control, in which only T4 DNA ligase was omitted, was set up for each plasmid, and served as a background check to estimate the proportion of non-digested plasmids in each ligation reaction. *E. coli* DH5αF'IQ was transformed with 20 to 80ng of the resulting recombinant products. Transformants were plated on LB agar (Lennox agar, Gibco BRL) with single or double antibiotic selection. Selected colonies (20-30/vector) were plated a second time to ensure homogeneity of each clone selected. Each clone's plasmid was extracted using an alkaline lysis method (Maniatis et al., 1982) from an overnight culture growth in LB medium. Identity of a transformant carrying the vector modified by an insert was revealed by restriction digest analysis. Recombination of a vector with a synthetic insert introduces many new unique restriction sites in the plasmid sequence (e.g. *Nhe*I, *Sac*I, *Bam*HI, *Bsr*GI); a positive digestion by an endonuclease in one of these sites therefore enabled differentiation, on 1% agarose/EtBr gel, between a recombinant (linearized by digestion) and a non-recombinant (non-linearized) vector.

Substantial amounts of purified plasmid DNA (20-50μg) were produced for each clone using standard DNA extraction kits (Life Technology's Concert™ Purity Plasmid Midiprep System or Concert™ Rapid PCR Purification System (technique modified and adapted); or Qiagen's Plasmid Mini Kit). Each of the selected clones was sequence-validated by automatic DNA sequencing of the purified DNA using the Taq dye deoxy terminator cycle sequencing technique (Applied Biosystems device, CA); all works were performed at the Sheldon Biotechnology Center, McGill.

Experimentally determined sequences were found to match with the designed theoretical sequences upon sequence comparison analysis. The single-stranded forward and reverse DNA primers used for the automated sequencing of the insert-modified, first generation vectors are:
5' ATGTGTGGAATTGTGAGCGG 3' (PRSLV1), and
5' ATCTGTATCAGGCTG AAAATCTTC 3' (PRPKKR), respectively (Rioux et al., 1992).

2.3.2 Strategy for the cloning of *betv1a* from vector pBlue-Bet-Wt

pBlue-Bet-Wt, the plasmid bearing the gene *betv1a*, was obtained from Dr. Paul Rösch (Boehm and Rösch, 1997). Amplification by polymerase chain reaction (PCR) of the gene required the design of sense and antisense primers which introduced sequence modifications at the 5' and 3'end of the target gene. The theoretical characteristics of the primers (see below) were calculated using Vector NTI 6.0 (Informax Inc., MD), which includes a PCR primer analysis program.

Forward primer (OINT5):
5' CG TAG GCG TGG ATC CTG **ATG** GGT GTT TTC AAC TAC GAA ACC 3'
(the codon in bold indicates the first codon of the target gene)
Length: 41 bases
Theoretical Tm: 64.4 °C
%GC content: 51.2
dH: -322.5 kcal/mol
dS: -821.6 cal/mol
dG: -75.8 kcal/mol

Reverse primer (OINT6):
5' C CAG AGC TGT GTA CAC **GTT** GTA TGC ATC GGA GTG AGC C 3'
(the codon in bold indicates the last complementary codon of the target gene)
Length: 38 bases
Theoretical Tm: 64.8 °C
%GC content: 55.3
dH: -281.7 kcal/mol
dS: -714.2 cal/mol
dG: -67 kcal/mol

Tm Difference: .4 (not significant)
GC Difference: 4.0 (not significant)

The OINT 5 forward primer enables replication of the DNA sense strand and introduces the *Bam*HI restriction site (GGATCC) 5' to the target gene. The OINT 6 reverse primer enables

replication of the DNA anti-sense strand and introduces the *Bsr*GI (TGTACA) restriction site 3' to the target gene. The length of the product after PCR is 513 bp. Introduction of two new restriction sites, one upstream and the other downstream to the gene *betv1a*, enables its cloning into vectors pKLY2 and pKLY3 by in-frame fusions.

PCR parameters and set up:

- *PCR molecular components:*

pBlue-Bet-Wt: 2.5pg plasmid DNA per PCR
primer OINT5: 50 pmol per PCR
primer OINT6: 50 pmol per PCR
Pfu DNA polymerase (Stratagene, CA): 2.5 units
dNTP: 2mM per PCR (final concentration)

The reaction medium was buffered with the Stratagene's 10x buffer; the working concentration being 1x. The final volume per reaction tube was adjusted with ddH_2O to 48 µl.

- *PCR cycling parameters:*

All components, except for the DNA polymerase Pfu, were present in the reaction mixture at step a, below.

a. DNA denaturing: 94 °C, 4 min
b. Addition of Pfu DNA polymerase: 2.5 units; 80 °C, 2min
c. First generation primer extension: 94 °C, 1min
   50 °C, 1min
   72 °C, 1min
   → 5 cycles
d. Second generation primer extension: 94 °C, 1min
   50 °C, 1min
   72 °C, 1min
   → 25 cycles
e. Sustain DNA polymerization reaction: 72 °C, 10min
f. Terminate: 4 °C, indefinite

Automated thermal cycling was programmed and performed on a GeneAmp®–PCR System 9700 instrument (PE Applied Biosystems). Negative controls consisted of pBlue-Bet-Wt plasmid DNA in a buffered/dNTP solution (with no Pfu polymerase enzyme), alone, with primer OINT5, or with primer OINT6. Additionally, to test for the presence of amplifiable contaminating DNA, a

complete reaction mixture (except for the pBlue-Bet-Wt plasmid DNA) was also included. All negative controls yielded no detectable products as assessed on 2% agarose/EtBr gel. Visual inspection of the PCR products on a 2% agarose/EtBr gel revealed that a single DNA product was obtained at a MW of approximately .5kbp (Figure 6).

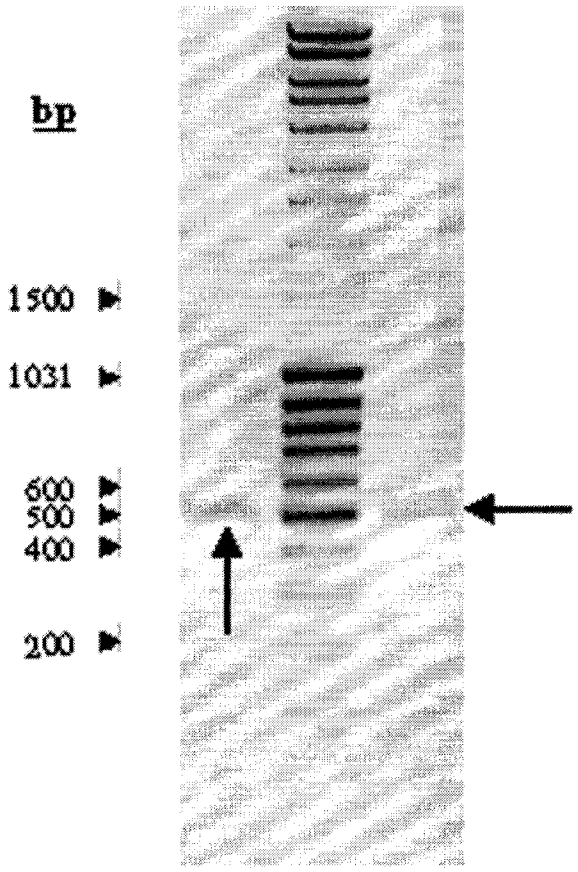


FIGURE 6. A single PCR product (in duplicate; arrows) at a MW of approximately 500 bp is obtained following PCR amplification of the gene *betv1a* from vector pBlue-Bet-Wt.

2.3.3 Second generation vectors: final pKLY2 and pKLY3 constructs

Intermediate or first generation plasmid constructs all required the in-frame addition of the gene *betv1a* flanked by restriction enzyme sequences 5' *Bam*HI and 3' *Bsr*GI. Plasmid vectors pKLY3

and the 513 bp PCR product were treated individually with endonucleases *Bam*HI and *Bsr*GI. All DNA products were purified on agarose gel/EtBr and extracted using the standard methods of phenol freeze or DNA extraction kits (Life Technology). After quantification on agarose gel (with the Massruler^{TM} MW standard), endonuclease-treated vectors and gene *betv1a* were allowed to recombine by co-incubation in a ratio of about 1:10 (mol/mol), i.e. with gene *betv1a* DNA in excess in the ligation reaction medium. The method for recombination by DNA ligation is described in the preceding paragraph. The products of recombination served for transformation into *E. coli* DH5αF'IQ under the same set up and conditions as described above. Extraction of plasmid DNA for each clone selected from the transformant pool was used in two ways to screen out recombinant from non-recombinant clone plasmids. New unique DNA restriction sites were introduced by the gene *betv1a* (e.g. *Bgl*II, *Bsp*119I, *Hin*dIII; Figure 9A, B, C) and represented markers of DNA recombination; in addition, recombinant plasmids undergo a significant increase in molecular size, approximately .5kbp, which can readily be assessed on agarose/EtBr gels with linearized plasmids (treated with a single endonuclease). Either of the two methods allowed for rapid first-stage screening of the clones selected for each target vector prior to submission for DNA sequencing (Sheldon Biotechnology Center).

A number of second generation recombinant pKLY3 vectors/clones were sequenced using forward DNA primers PRSLV1 and 5' CCTTCCCGGAAGGTTTCCC 3' (OINT 7), and reverse primers PRPKKR and 5' TGGACCACCTTC GATAACGG 3' (OINT 8). One of the sequence-validated pKLY3 clones was selected and the 5' *Bam*HI-*betv1a*-*Bsr*GI 3' sequence region of its plasmid used to introduce the target gene into the first generation pKLY2 vectors by DNA recombination and ligation. Recombinant pKLY2 plasmids were checked for the correct in-frame insertion of the sequenced gene *betv1a* into each vector by digestion at the DNA restriction sites *Bam*HI and *Bsr*GI. Figures 7 and 8 recapitulate the overall scheme involved in the design of the derivatives of plasmids pKLY2 and pKLY3.

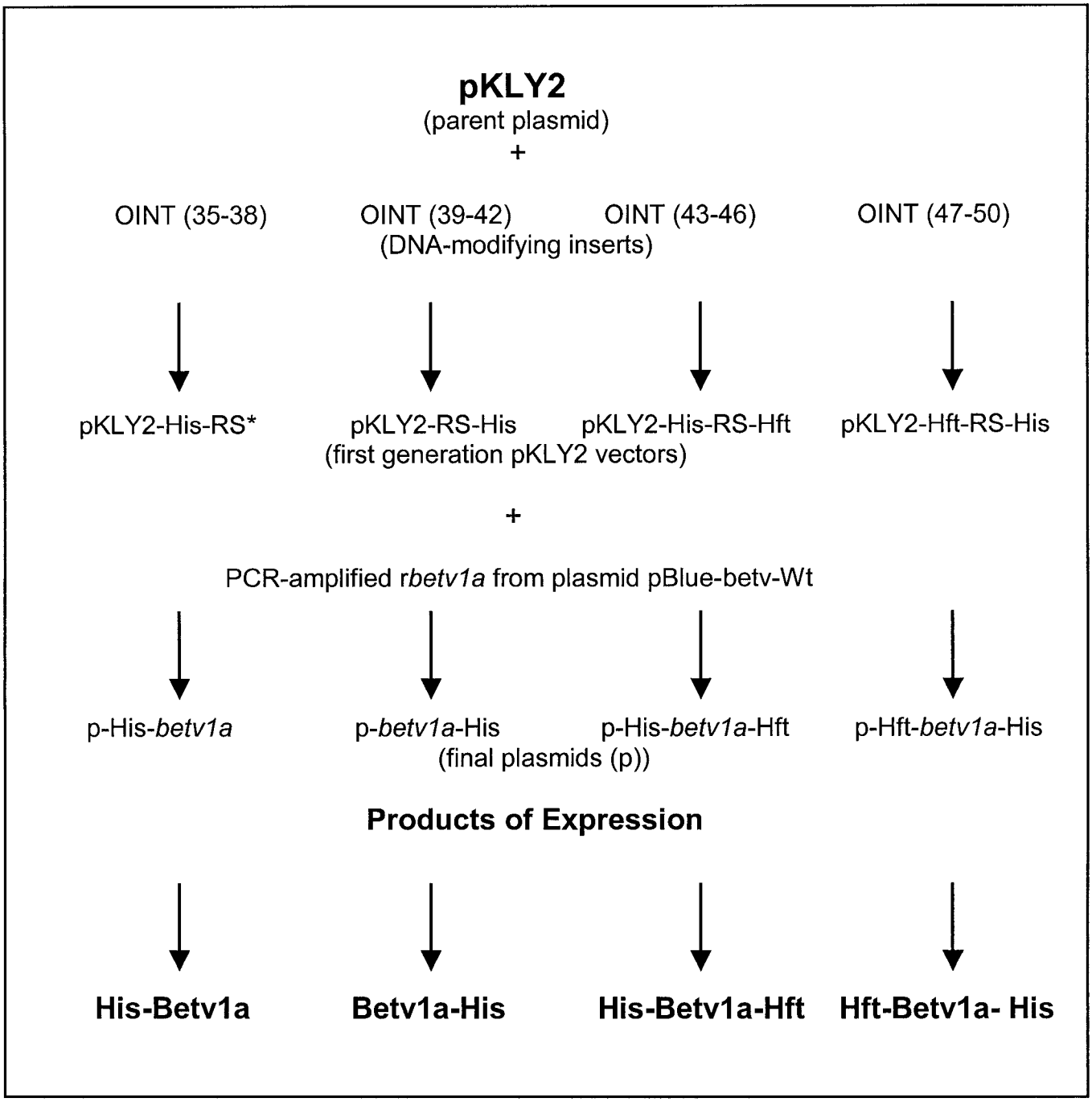


FIGURE 7. Summary of the strategy for the design and construction of the pKLY2 vector derivatives. The host strain for all intermediate and final plasmids is *E. coli* DH5αF'IQ.
* RS is the plasmid DNA region where gene *betv1a* is incorporated after treatment by specific endonucleases and DNA recombination.

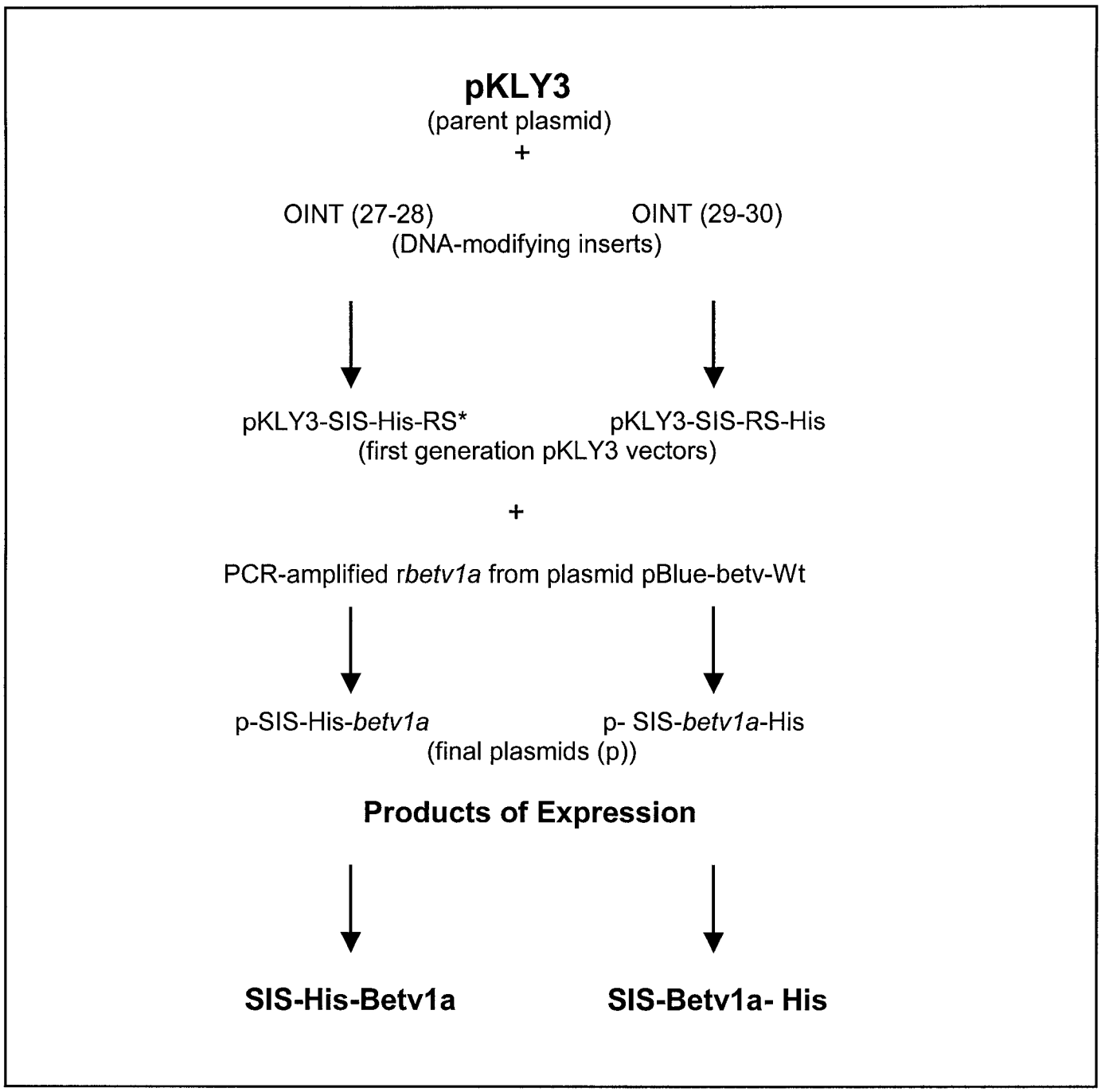
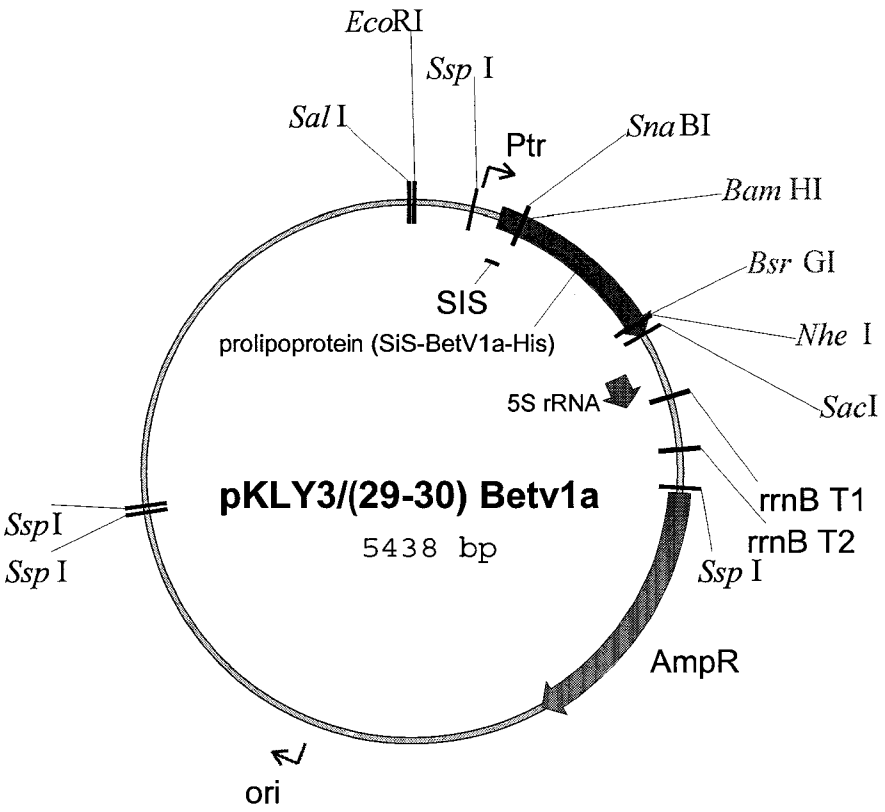


FIGURE 8. Summary of the strategy for the design and construction of the pKLY3 vector derivatives. The host strain for all intermediate and final plasmids is *E. coli* DH5αF'IQ.
* RS is the plasmid DNA region where gene *betv1a* is incorporated after treatment by specific endonucleases and DNA recombination.



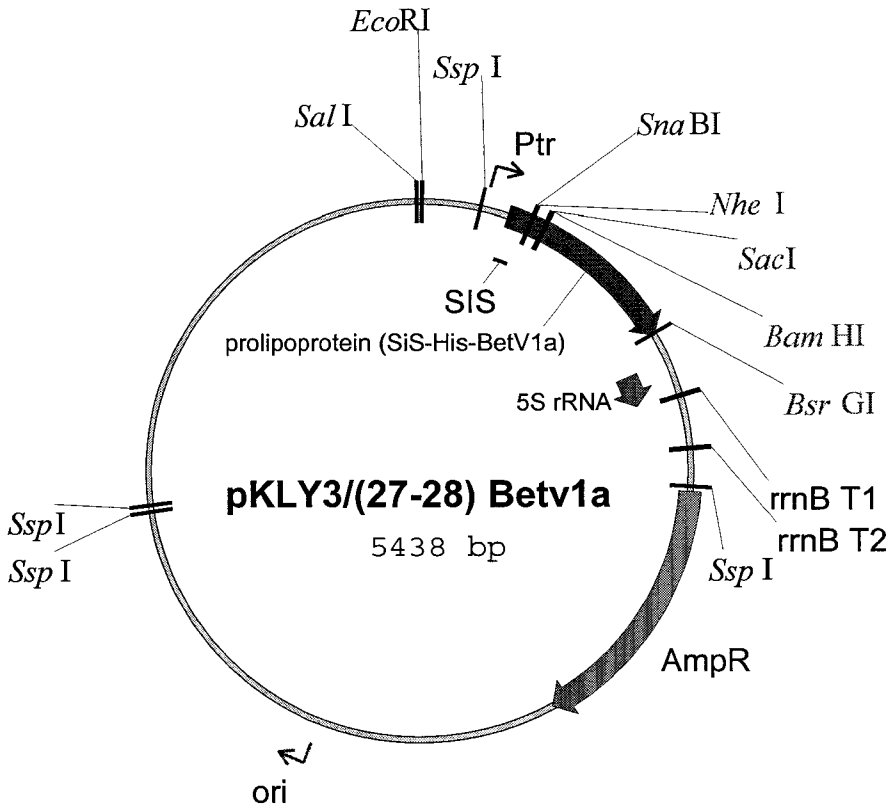
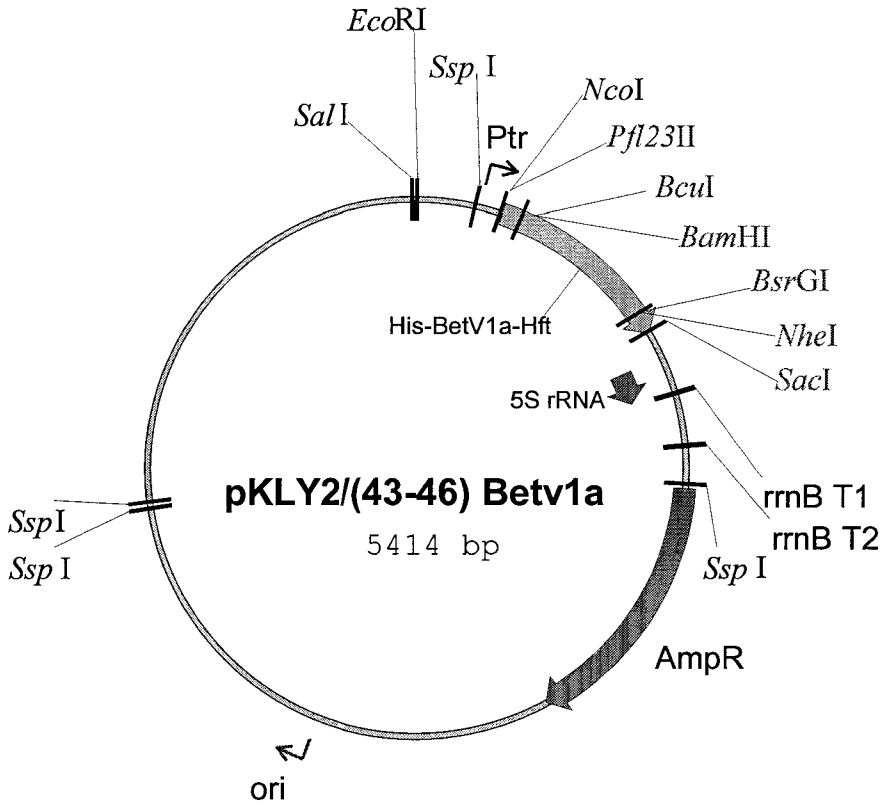


FIGURE 9A. pKLY3 vectors designed for the expression of recombinant proliproteins SIS-10xHis-Betv1a (right) and SIS-Betv1a-10xHis (left). SIS comprises the 57 bp (19 a.a.) sequence encoding for 16 hydrophobic a.a. and the 3 a.a.-signal peptidase sequence. Notable restriction sites: *Bam*HI/*Bsr*GI (green), flanking the Betv1a encoding region; *Nhe*I/*Sac*I (red) for the 10xHis encoding region; and *Sna*BI (blue), the 5' insertion site of DNA OINT (27-28) or (29-30).



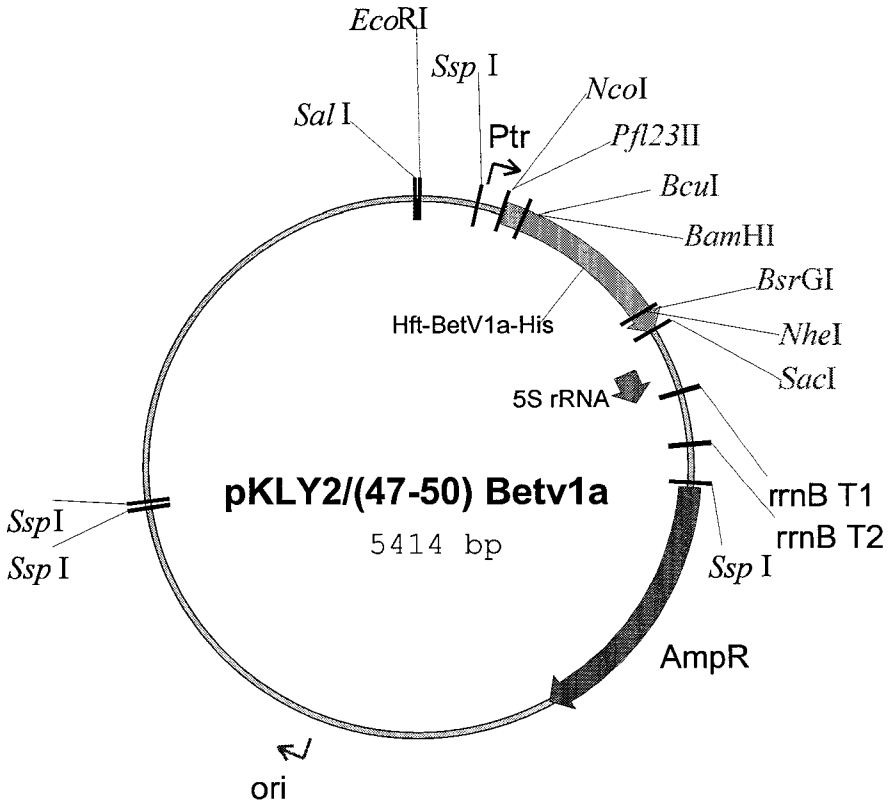


FIGURE 9B. pKLY2 vectors for the expression of recombinant proteins Hft-Betv1a-10xHis (right) and 10xHis-Betv1a-Hft. Notable DNA restriction sites: *Bam*HI/*Bsr*GI (green), flanking the Betv1a encoding region; *Pfl*23II/*Bcu*I (red) for the 10xHis- or Hft-encoding region at the 5'end of *betv1a*; *Nhe*I/*Sac*I (indigo) for the 10xHis or Hft sequence at the 3'end of *betv1a*; and *Nco*I (blue), the 5' insertion site of DNA OINT (43-46) or (47-50).

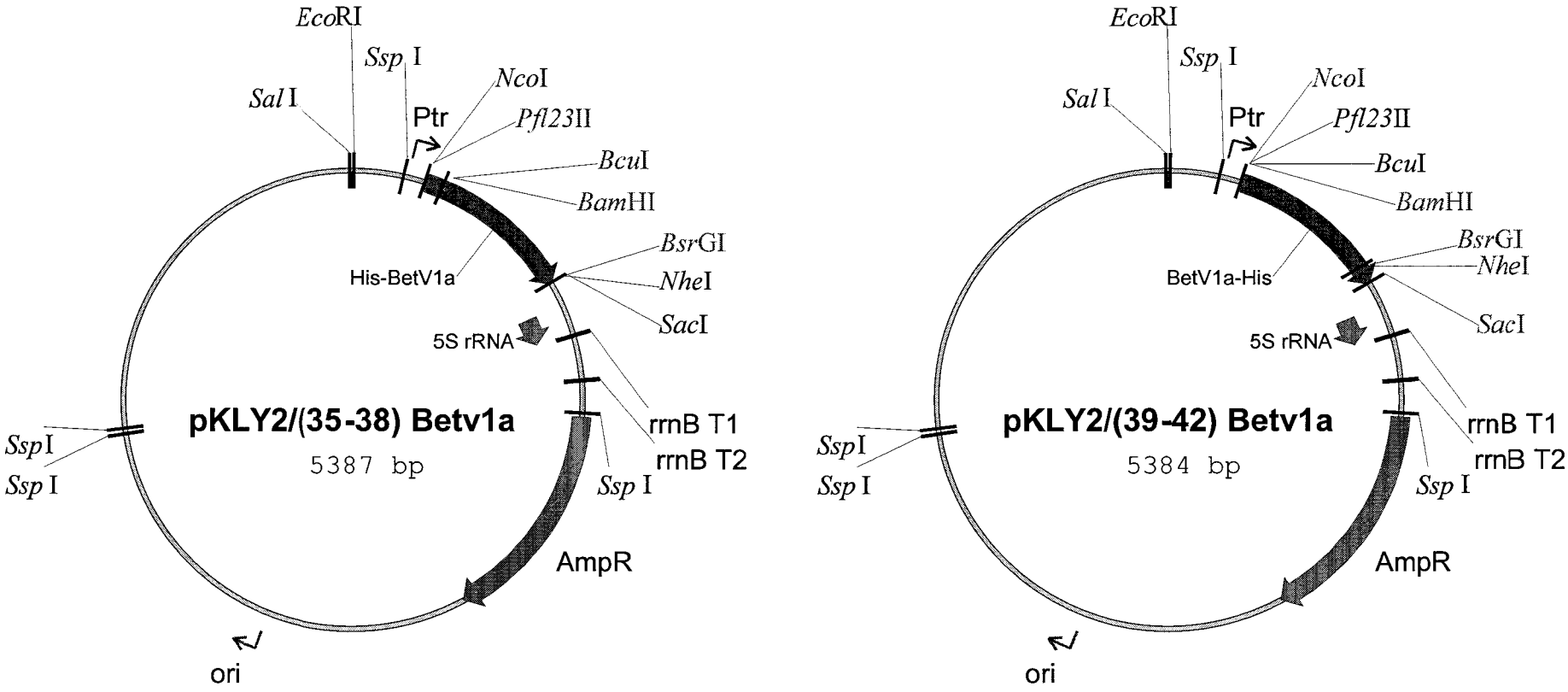


FIGURE 9C. pKLY2 vectors designed for the expression of recombinant proteins Betv1a-10xHis (right) and 10xHis-Betv1a (left). Notable DNA restriction sites: *Bam*HI/*Bsr*GI (green), flanking the Betv1a encoding region; *Pfl*23II/*Bcu*I or *Nhe*I/*Sac*I (red) for the 10xHis encoding region at the 5' or 3' end of *betv1a*, respectively; and *Nco*I (blue), the 5' insertion site of DNA OINT (35-38) or (39-42).

CHAPTER III

MOLECULAR CHARACTERIZATION OF RECOMBINANT EXPRESSION PRODUCTS

3.1 General method of protein expression and extraction

Standard methods were used in the overexpression of rBetv1a proteins in the *E. coli* DH5α F'IQ host cell (Maniatis et al., 1982, Sambrook et al., 1989). Clones carrying final vectors pKLY2 or pKLY3 derivatives were grown overnight at 37°C, under continuous agitation at 250 rpm, in single or double antibiotic LB, and used to inoculate a fresh volume of LB. The cells were grown to exponential phase (optical density, OD_{600}=. 6) prior to induction with 1-2mM IPTG. Growth was allowed to continue from 1 to 6 hours, or overnight, depending on the clone used or on the experiment. The induced cells were harvested by centrifugation, and resuspended in various chaotropic or detergent (buffered) solvents: 8M urea, 6M guanidine hydrochloride (GuHCl), or 2.5% (w/v) of the zwittergent Empigen® BB (EBB).

3.2 Detection and quantification of the pKLY2 and pKLY3 expression products from induced whole cells

Direct detection of the rBetv1a expression with an antibody specific to this molecule was not possible initially for lack of anti-Betv1a antibody. Commercially available monoclonal anti-

6xHis antibody (Clontech, CA) was therefore selected as the primary means for the detection and quantification of the pKLY3 expression products in dot or Western blot assays.

Detection of products of expression of both pKLY3-derived vectors in induced whole cells by SDS-PAGE, (12 % polyacrylamide / bis-tris buffering system, reducing conditions; Laemmli, 1970) was not possible using standard Coomassie Blue staining procedure (Allen and Budowle, 1999) since these products represent only a small percentage of total cell proteins loaded onto the gel. Transfer of whole cell samples onto nitrocellulose membranes was therefore performed by western blotting. Detection with anti-6xHis mAb demonstrated the existence of two main expression products for each pKLY3-derived constructs (Figure 10). In both constructs the two main products detected displayed molecular weights 1,100 apart. For the construct SIS-His-Betv1a, the relative molecular weights of the products corresponded to 23,500 and 22,400, whereas for construct SIS-Betv1a-His molecular weights of 23,400 and 24,500 were calculated. For comparison, purified His-Betv1a (provided by Intellivax International Inc., research laboratories; Montreal) migrated on the same gel midway to the first construct's two products with an apparent molecular weight of 23,000. His-Betv1a's molecular weight was significantly different from the predicted theoretical mass of 20.8 kDa (Table 1); this is probably due to the presence of the decahistidine tag which shifts Betv1a's natural pI of 5.4 to a pI of 6.3, hence affecting electrophoretic migration in the gel. It may also be noted that Betv1a molecules underwent dimerization as can be assessed from the presence of a 47 kDa band on Coomassie Blue stained gels (not shown) or on western blots (Figure 10). No product was detectable in non-induced cells for both pKLY3-based constructs using anti-His mAb detection, which demonstrates the efficient repression of recombinant protein expression by lacI in this *E. coli* DH5αF'IQ expression system.

Western blotting also enabled quantitation of the pKLY3 derivative protein expression products using a densitometer (Molecular Dynamics, CA; model 375) and the image analysis software ImageQuant (version 5.1). Quantitation of products in SIS-His-Betv1a with respect to the 6xHis standard on western blots was evaluated at .8 mg/l of culture for the 22.4 kDa protein product and 1.6mg/l for the 23.5 kDa product. Products of SIS-Betv1a-His were estimated to .1mg/l of culture for the 23.4 kDa product while the 23.5 kDa product was present at .033mg/l in induced cultures.

pKLY2 expression levels were estimated on dot blot (using rabbit polyclonal anti-Betv1a detection) with serial dilution of induced whole cells against a negative control, and by Coomassie Blue staining of sample proteins in a 12% SDS-PAGE gel. The expression levels, which were similar among all pKLY2 vectors, were estimated at 5mg/l of culture after 4 hours of induction.

Expression Vector	Molecule	Mass (kDa)	Total a.a.	pI
pKLY2	His-Betv1a	20.8	189	6.25
	Betv1a-His	20.7	188	6.25
	His-Betv1a-Hft	21.7	198	6.25
	Hft-Betv1a-His	21.7	198	6.25
pKLY3	SIS-His-Betv1a	22.7	206	6.52
	SIS-Betv1a-His	22.7	206	6.52
N/A	SIS	2.0	19	10
N/A	Betv1a	17.6	160	5.39

Table 1: Predicted molecular masses of the protein products. Protein characterization performed with the World Wide Web accessible PeptideMass tool (http://www.expasy.ch/tools/peptide-mass.html).

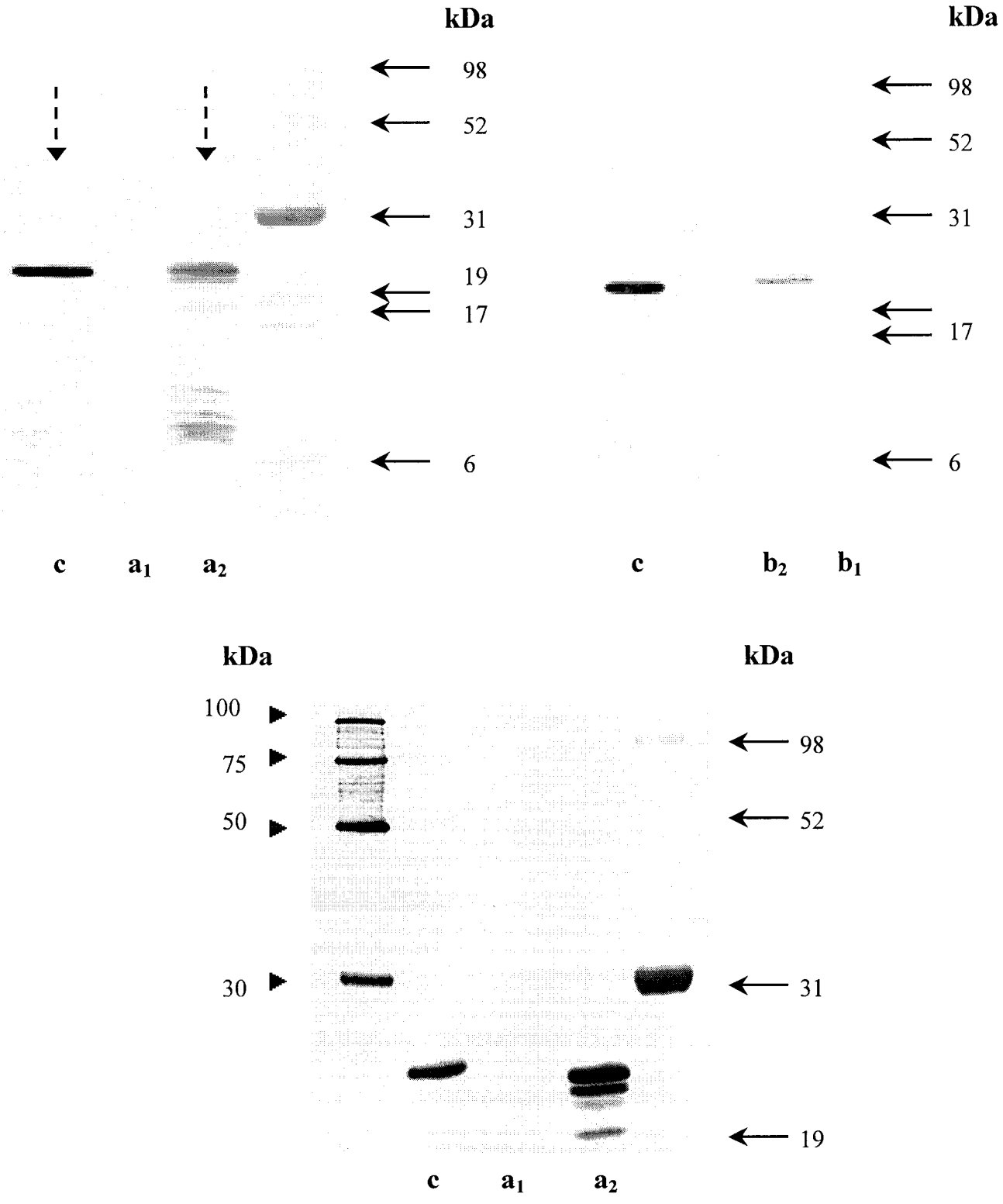


FIGURE 10. Western blot detection of the main products of expression in induced DH5αF'IQ cells transformed with the vectors pKLY3-derivatives using an anti-histidine tag mAb. Legend: (a_1) non-induced, and (a_2) induced (for 4 hours) SIS-His-Betv1a expressing cells; (b_1) and (b_2) are the same as (a_1) and (a_2), respectively, except that the cells

express SIS-Betv1a-His; (c) purified His-Betv1a. The protein MW standard (Multimark™, Novex) is indicated by arrows, and the 6xHis protein ladder (Qiagen) serves as a protein MW standard and positive control for the anti-His mAb (shown on the left of the bottom gel). Dashed arrows indicate a probable dimer of Betv1a. Top gels were run for 50 minutes at 200V, whereas the bottom gel was run for 90 minutes to provide for a more accurate evaluation of the MW of the protein products. Sample volumes loaded (a_1, a_2, b_1, b_2) on gels are equivalent to .1ml of cell culture.

3.3 Extraction of pKLY3 expression products from whole cells

Protein extraction of SIS-His-Betv1a and SIS-Betv1a-His expression products from induced whole cells resisted extraction treatment by buffered solution (pH=8.0) of 8M urea or 6M GuHCl. In general, chaotropic solutions readily solubilize a wide range of recombinant proteins regardless of protein localization within the cell. Most *E. coli* proteins, whether located in the cytoplasm, periplasm or membranes, are usually solubilized and denatured by 8M urea or 6M GuHCl solvents (Bollag et al., 1996). However, for the recombinant protein products, solubilization was not possible, regardless of the volume of urea or GuHCl used, the period of incubation of whole cells with the denaturing solutions (overnight or 1 hour), or the temperature of the medium (on ice or at room temperature). In all cases, the induced whole cells, which were collected as pellets out of induced liquid cultures, were fully resuspended with either solvent so as to obtain a homogeneous mixture, and cell resuspensions were centrifuged at 10,000g, 4°C for 30 minutes. Coomassie staining of solubilized cell samples run on SDS-PAGE gel or western blotting using the anti-His or anti-Betv1a antibody did not detect any significant amounts of Betv1a proteins in the supernatant of urea or GuHCl resuspension of whole cells (data not shown). Both urea and GuHCl chaotropic agents were therefore inappropriate for the direct extraction of Betv1a proteins since these products remained associated exclusively with the pellet obtained from the centrifugation of whole cell resuspension in urea or GuHCl solvents. Whereas such products were not detectable by Coomassie staining in whole cells, they were easily detected by the same method in the pellet obtained from treatment with urea or GuHCl. The pellet was therefore effectively enriched with the proteins expressed from the pKLY3-derived vectors. Removal of cytoplasmic, periplasmic, or membrane-bound cell proteins along with cellular DNA and RNA (combined with a lack of any significant recombinant protein loss) is beneficial to the process of purification since many cell constituents interfere with the process on purification on Ni-NTA columns (Janknecht et al., 1991; Schmitt et al., 1993). Multiple resuspension of the pellet with urea or GuHCl solvents also did not solubilize these recombinant proteins.

3.4 Selection of a suitable detergent for the process of protein purification

3.4.1 General considerations

Ni-NTA technology requires that a recombinant protein carrying a histidine-tag be solubilized and preferentially denatured to allow for its purification by immobilized-metal affinity chromatography (IMAC). Solubilization of lipoproteins was therefore essential to subsequent steps leading to their purification. A number of detergents –ionic, non-ionic, and zwitterionic- were considered to achieve this goal. Screening for a suitable detergent effective at extracting target proteins from the urea or GuHCl solubilization pellet meant that it should be:

- Compatible with Ni-NTA columns, that is, non-damaging to the columns used for the purification of His-tagged proteins; for example, many ionic and non-ionic detergents will strip out essential nickel ions from the column even at low detergent concentrations
- Not a competitor or inhibitor in Nickel-protein His-tag interaction
- Useful over a wide range of concentrations
- Relatively easy to dialyze out since removal of the detergent from the solvent may be required in subsequent analytical or immunological testing
- Compatible with the Proteosome technology in the process of complexing purified recombinant proteins with this adjuvant system

3.4.2 Assessment of the use of Empigen® BB in the process of lipoprotein purification

Overall, few detergents are compatible with the Ni-NTA technology at concentrations exceeding 1% (Qiagen, 1999; Sanders et al., 1996). The zwittergent Empigen® BB (n-dodecyl-N, N-dimethylglycine; Calbiochem, CA) was determined to be the most appropriate for the extraction of the overexpressed lipoproteins in this *E. coli* expression system. Empigen® BB at a concentration of 2.5% (w/v) was effective at extracting out the partially purified lipoproteins out of the pellet obtained from urea or GuHCl solubilizations (Figure 18). This relatively high

concentration of detergent was selected to ensure that critical micelle concentration (CMC; .04 - .06% (w/v) for EBB) was achieved over a wide range of protein concentrations in the mixture of proteins to be purified and to maximize protein dispersion in solution. In practice, higher CMC's are advantageous to the process of detergent dialysis (Neugebauer, 1990). Because the use of urea or GuHCl is not compatible with the use of detergents in general since these tend to raise significantly the CMC of a detergent in solution, chaotropic agents were excluded from all detergent extraction buffers. Empigen® BB also did not noticeably degrade recombinant proteins, based on Coomassie or Western analysis, was safe to the columns as assessed visually by monitoring changes in Ni-NTA agarose column color (a direct indicator of nickel ion presence within the column), did not interfere with purification of the target proteins (demonstrated below), and could be readily dialyzed out of purified protein samples, if desired. Proteosomes, because of their hydrophobic nature as outer membrane integral proteins of the Gram-negative *N. meningitidis*, require the presence of high concentrations of the Empigen® BB detergent (typically, 1%) to remain soluble in watery solvents. Thus, Empigen® BB is compatible with the Proteosome adjuvant technology and is an effective agent in the extraction and purification of the recombinant lipoproteins from induced whole cells.

3.5 Study of the effects of recombinant SIS-His-Betv1a and SIS-Betv1a-His expression in the system host DH5αF'IQ

In *E. coli*, recombinant protein expression may be affected by the ability of the cell to tolerate an overexpressed foreign protein, especially one of eukaryotic origin, as is the case for rBetv1a. Cell growth, reflected by direct OD_{600} measurements, is an important indicator of potential toxicity of the protein once it is expressed in the cell. There have been no reports of toxicity associated with the overexpression of Betv1a in *E. coli* to this date, but expression of lipid-modified, His-tagged Betv1a was never investigated. Additionally, studies of the kinetics of protein expression in the cell system DH5αF'IQ was performed in order to assess the level and stability (i.e. possible degradation by cellular proteases) of recombinant proteins produced within the cell.

Figure 11 displays growth curves of LB culture of DH5αF'IQ transformed with several plasmids whose OD_{600}'s were monitored over time following induction with 1mM IPTG. The

clones tested were carriers of the plasmids pKLY3 (27-28), pKLY3 (29-30), pKLY3/(27-28) Betv1a, pKLY3/(29-30) Betv1a, and pKLY2/(35-38) Betv1a, and encoded respectively for SIS-QANYVAS-10xHis (or SIS-His), SIS-QANYVGILLYIAS-10xHis (or SIS---His), SIS-10xHis-Betv1a, SIS-Betv1a-10xHis, and 10xHis-Betv1a. As control, non-transformed DH5αF'IQ was included. The culture medium was identical for all clones (rich liquid LB broth, ampicillin at 75μg/ml, aerobic conditions), except for the non-transformed DH5αF'IQ whose culture medium contained the antibiotic kanamycin (50μg/ml), instead of ampicillin, given that the F' plasmid in DH5αF'IQ carries the gene for kanamycin resistance.

At the exception of His-Betv1a and SIS-His expressing cells, all other induced clones exhibited significantly impaired culture growth upon induction with IPTG. For example, after six hours of induction, all pKLY3-derived clones, except for pKLY3 (29-30) expressing SIS---His, displayed increased OD_{600}'s only by as much as 25% with respect to starting OD_{600} at induction, compared to an increase in OD_{600} of above 100% for the control clone DH5αF'IQ and for the His-Betv1a producing clone. Within .5 to 1 hour following induction, the effects of lipoprotein expression were noticeable within cultures. However, it is unlikely that the recombinant protein products were lethal to cells since all cultures, at the exception of SIS-Betv1a-His-producing cultures, achieved high OD_{600}'s after overnight incubation.

Protein expression was also investigated for clone pKLY3/(27-28) Betv1a, which expresses the lipoprotein lipo-His-Betv1a. A time study for protein expression following induction was performed. Partially purified recombinant protein products, obtained from induced whole cells by the 8M urea extraction method described in section 3.3, were loaded onto 12% polyacrylamide gel (Figure 12). The negative control consisted of the SIS-His expressing culture of DH5αF'IQ carrying pKLY3 (27-28), and was subjected to the same growth conditions as the above culture. Results revealed that protein production within the culture reached its peak after .5 to 1 hour of induction and that the two main recombinant protein products (22.4 and 23.5 kDa) did not continue to accumulate in the cells subsequently. Cells in culture seem to behave as if recombinant protein production became significantly repressed after the first hour of induction. Recombinant protein production is under the control of the Ptrc plasmid promoter; thus, protein synthesis resulting from induction with IPTG cannot, in theory, be repressed by the cell. However, it is possible for the cell to stall protein production at the RNA translation level for example. Gram-negative organisms are sensitive to the degree of occupancy of their membranes

by various proteins (Boyd and Holland, 1979; Fralick and Diedrich, 1982), and possess numerous mechanisms by which to control membrane protein expression. The fact that general protein synthesis may be inhibited within the cell would explain the poor culture growth observed for cultures that express the lipid-modified recombinant proteins. If the argument that the pKLY3/(27-28) Betv1a clone produces proteins that incorporate into the membrane(s) holds true (see next section for evidence on this), then recombinant protein production or yield is proportional to the available surface area of membrane present within each cell and to the total number of viable cells present within an induced culture. The idea that protein degradation by cell proteases may influence the intracellular recombinant protein concentration, and is keeping protein levels constant in the cell is not likely to be the case here since no significant protein bands of degradation, in the MW range of 17 to 6 kDa, could be detected by Coomassie on gels (Figure 12), or even with more sensitive detection by western blotting (not shown).

Finally, whether the effects of lipid-modified Betv1a are specific to the DH5αF'IQ expression system remains to be investigated. JM105, for example, is an *E. coli* strain that is commonly used for recombinant protein overproduction, and has been successfully used in combination with other homologous pKLY3 vectors in the past (Rioux et al., 1992). Additional insight may be gained on understanding the effects of lipoprotein production in *E coli* or on improving levels of protein production in culture by the use of this strain.

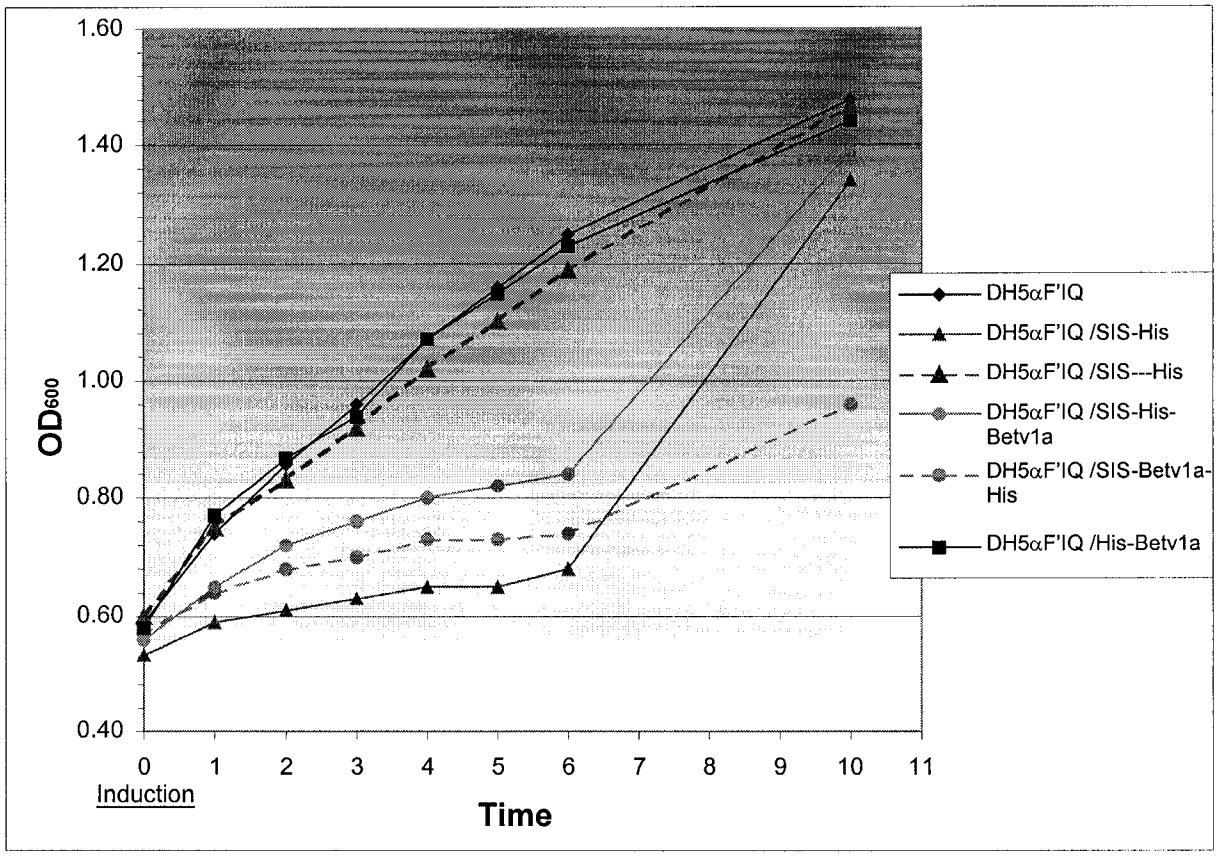


FIGURE 11. The effects of prolipoprotein expression on the growth of *E. coli* DH5αF'IQ in LB culture medium. All cell cultures were induced with 1mM IPTG at OD_{600}~. 60. Growth was monitored hourly by spectrophotometric measurement, and allowed to continue overnight. Culture conditions were identical for all transformants.

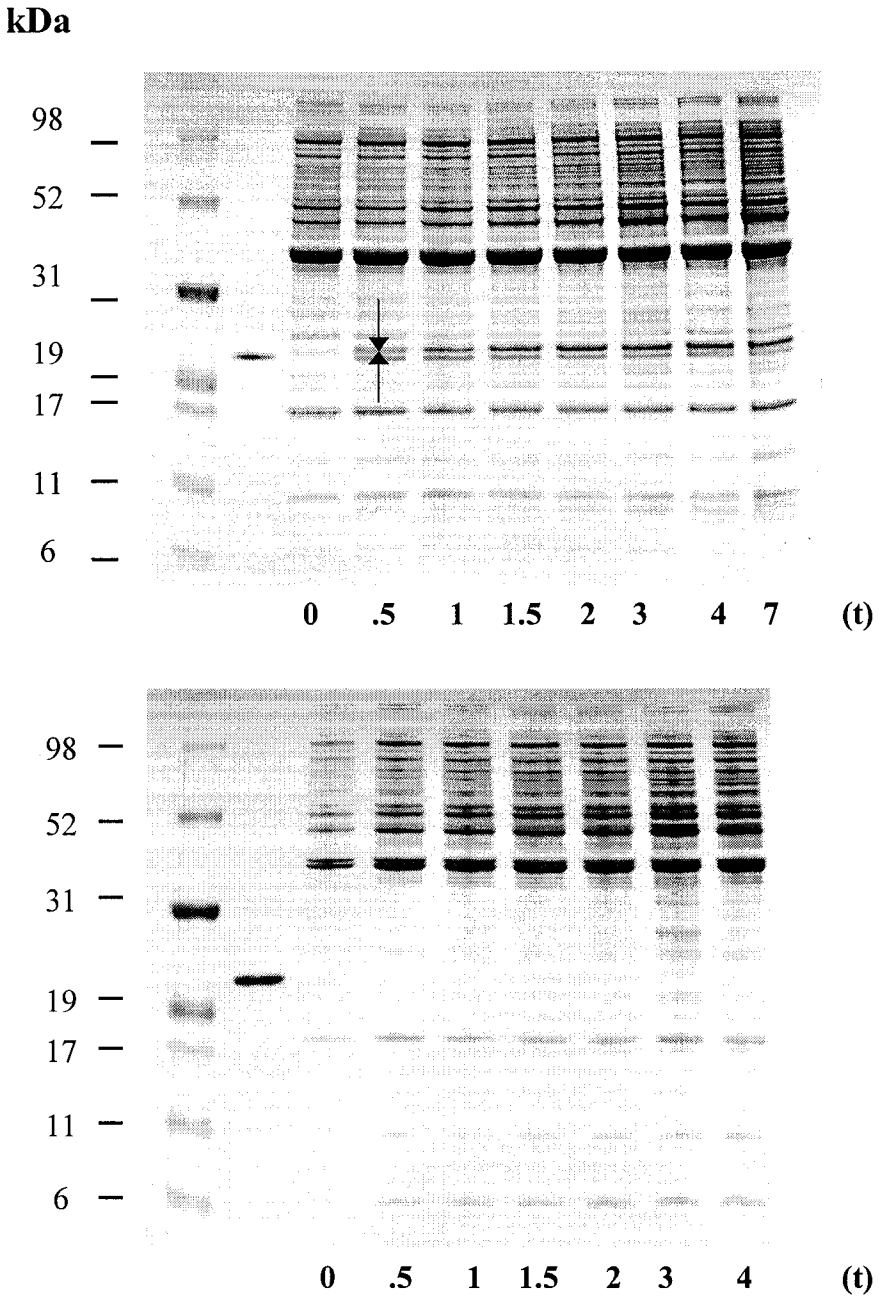


FIGURE 12. Study of SIS-His-Betv1a protein expression in DH5αF'IQ. Enriched fractions of recombinant proteins from clone harboring pKLY3/(27-28) Betv1a (arrows show the products), obtained as pellet after solubilization of whole induced cells with 8M urea, were loaded for 12% SDS-PAGE and proteins stained with Coomassie Blue (top gel). Purified His-Betv1a is shown in the second lane from the left on both gels. Negative control clone pKLY3 (27-28), which produces SIS-His, is displayed as the bottom gel. Culture sample collection time points are indicated at the bottom of each gel (the 7 hour collection point for the control clone was not done). Total protein loaded on gel for each sample is approximately the same for all collections and correspond to 225µl of culture at OD_{600}~.60.

3.6 Subcellular localization of pKLY3 expression products

3.6.1 Rationale and methodology

Establishing the subcellular localization of the expression products of the pKLY3 vectors was important in order to assess the efficiency of the cell to process the prolipoprotein into an apolipoprotein and lipoprotein since lipid modification of proteins occurs through the enzymes localized in the cytoplasmic membrane (Inouye, 1987). For example, finding that the recombinant product is exclusively restricted to the cell cytoplasm indicates that the protein expressed is an unmodified prolipoprotein that remains unprocessed (not transferred /structurally modified) by the cell. On the other hand, localization of the same protein product to the cytoplasmic or outer membrane usually indicates the presence of a mature lipoprotein molecule. Additionally, recombinant protein localization may facilitate the process of extraction from induced cells by better adaptation of extraction methods.

E. coli's three main cellular compartments –soluble (cytoplasm and periplasm; S), inner (IM), and outer (OM) membrane- were separated using the methodology of Filip et al. (1973), after modifications. Highlights of the technique are as follows: culture of clones producing SIS-His-Betv1a and SIS-Betv1a-His (pKLY3/(27-28) BetV1a and pKLY3/(29-30) Betv1a, respectively) were induced for 2 hours, with 1mM IPTG, under the usual pre-established conditions (LB medium, aerobic conditions). The induced cells were collected by low speed centrifugation and resuspended in a buffered solution of .01M $NaPO_4$ containing a protease inhibitor cocktail (.02μM aprotinin, 8.7μM bestatin, .09μM E-64, .07μM leupeptin; Sigma, Missouri). The cell lysate obtained by passing twice the resuspended culture through a French press (EmulsiFlex-C5; Avestin, Canada) at 18,000 psi was cleared of unlysed cells by rapid centrifugation at 12,000g. Separation of total cellular membranes from the soluble fraction was achieved by high-speed centrifugation of the clear lysate: 40 minutes at 100,000g, 4°C. Total proteins in the supernatant fraction were precipitated with 10% trichloroacetic acid (TCA), and washed with methanol prior to resuspension in electrophoresis loading buffer. The total membrane pellet, obtained from the high-speed centrifugation, was resuspended in the same buffered solution as above and solubilized with .5% sodium sarcosinate (N-lauroyl sarcosine) detergent, which is known to solubilize preferentially the inner membrane of Gram-negative cells (Reid, 1973). Since the

cellular outer membrane resists resuspension by this detergent, it was collected as a pellet following a second high-speed centrifugation of the resuspended membranes, whereas the detergent-solubilized inner membrane's components were collected in the supernatant fraction, and its constituent proteins TCA precipitated, methanol washed, and reconstituted in loading buffer. Outer membrane proteins were solubilized in SDS/reducing loading buffer. All three samples were loaded unto 12% SDS-PAGE; sample protein detection was performed directly on the gel by Coomassie Blue staining (Figure 13) or by western transfer from the gel onto a nitrocellulose membrane with detection with anti-His mAb or with polyclonal rabbit anti-His-Betv1a antibody (provided by Intellivax International Inc., research laboratories, Montreal; Figure 14).

All manipulations were performed at 4°C or on ice.

3.6.2 Expression products of the pKLY3 vectors are confined to a single cell compartment

Detection on a Western blot of the protein products of lipid-modified BetV1a indicated that most of the target proteins were located within the cytoplasmic membrane of cells. Detection with anti-His mAb revealed only minimal or trace amounts of recombinant proteins for both vectors in the outer membrane of induced cells, and no detectable protein products in the soluble fraction (Figure 14, top). More sensitive detection with polyclonal rabbit anti-Betv1a also gave the same results (Figure 14, bottom), except for some protein products that were detected in the soluble and OM cellular fractions for both vectors. These results were confirmed by Coomassie staining of the same samples on 12% SDS-PAGE (Figure 13).

3.6.3 Confirming identities and assessment in potential cross-contamination of cell fractions

Subcellular fractionation of Gram-negative cells calls for evidence on the proper identification of the fractions obtained by the method described in the preceding section. The outer membrane of *E. coli* and other Gram-negative cells invariably display a major protein of MW 42-44 kDa that

represents as much as 70% of the cell's outer membrane protein weight (Schnaitman, 1970a,b). Another protein band typical of the outer envelope is found at approximately 17 kDa (Hancock and Nikaido, 1978). These two bands were easily observed in the OM fraction of both pKLY3 transformants on Coomassie stained gels (Figure 13, arrows 1 and 2, respectively), and are uniquely found with such abundance in this cell fraction. Inner membrane characterization is less straightforward since this compartment contains a greater variety of proteins with less disproportionate protein weight distribution as is observed for the outer membrane. A hallmark of inner membrane identity is found in the protein double-band located at a MW~43-45kDa (Hancock and Nikaido, 1978; Figure 13, arrow 3).

Subcellular fractions were also characterized using the commercially available polyclonal goat IgG anti-lipid A antibody (Maine Biotechnology Services Inc., ME) which cross-reacts with a number of strains (e.g. *Escherichia*, *Salmonella*, and *Enterobacter* spp.) that share a common lipid A structure in their lipopolysaccharides (LPS). Lipid A is a structural component of bacterial LPS and is exclusively associated with the cell's outer membrane (Hammond et al., 1984). Thus, LPS is a marker that easily discriminates the OM from the other cell fractions and serves as an indicator of contamination among these fractions.

Samples collected from subcellular fractions were assessed for their reactivity to the above antibody in a dot blot assay with two-fold serial dilutions of the samples (not shown). The protein amount used on the blot was the same for all the samples tested and was calculated at 10μg by Lowry protein assay (Lowry et al., 1951) for the first dots. Specific binding of the primary anti-lipid A antibody was revealed by the secondary antibody alkaline-phosphatase conjugated anti-goat IgG. Relative signal intensities permitted to establish the relative amounts of lipid A antigen in each cellular fraction and hence the degree of cross-contamination between the fractions. For both pKLY3-derivative expression products, the samples representing the cell outer membrane was found to contain at least 130 times more lipid A antigen compared to the inner membrane or soluble fraction; the latter two samples displayed relatively weak signals. Identity of the outer membrane was therefore confirmed with this assay, and levels of cross-contaminations between the fractions estimated as minimal.

3.6.4 Recombinant protein localization as an indication of structural modification

Recombinant Betv1a prolipoprotein SIS-His-Betv1a and SIS-Betv1a-His synthesis in the cytoplasm of the cell is followed by two distinct steps. Initially, protein translocation occurs, with an apparent high efficiency since little prolipoprotein product remains in the cytoplasm; this is then followed by the incorporation of the proliproteins into the IM of the cell. All enzymes necessary to the conversion of the prolipoproteins into mature lipoproteins of Betv1a are also found within the IM. Based on the above data, it is therefore likely that prolipoprotein production of Betv1a results in the production of an apolipoprotein or lipoprotein. On the other hand, it is also possible that no further recombinant protein processing occurs within the membrane, which may be linked to the lack of transfer of the membrane-imbedded prolipoproteins out of the IM compartment. Past experience with pKLY3 in the expression of lipo-PhoA (Rioux et al., 1992) has revealed that the mature lipoprotein is found mostly in the OM of cells. However, the production of a mature lipoprotein is not contingent on the transfer of this molecule to the OM. Prolipoproteins of Gram-negative cells mutated at the signal peptide have been shown to export out of the IM without any structural modification (Inouye et al., 1977). It is not known precisely what directs a lipoprotein to the cell periplasm or outer membrane, but structural determinants of the mature protein itself influence the ultimate location of the recombinant protein in the cell (see for instance Gennity et al., 1992). Such structural determinant(s) may be present in PhoA, normally a periplasmic protein of Gram-negative cells, but may be lacking in the eukaryotic protein Betv1a.

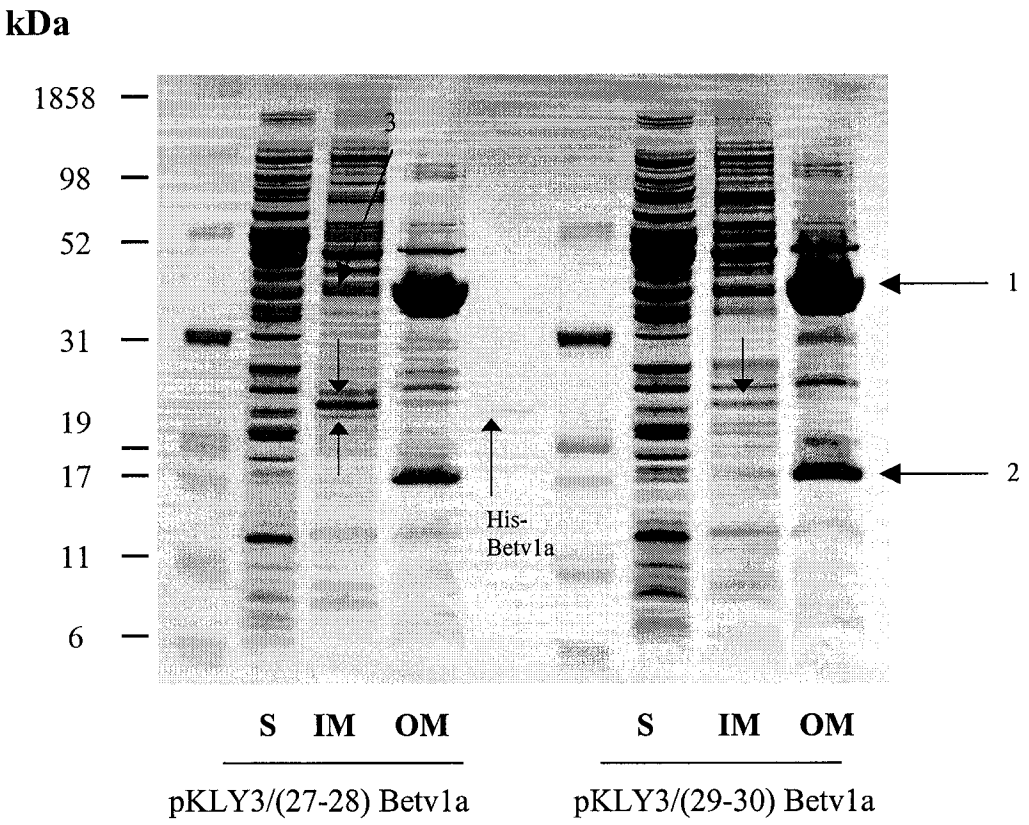


FIGURE 13. Detection by Coomassie staining on 12% SDS-PAGE of total proteins in the samples obtained from subcellular fractionation of Gram-negative *E. coli* DH5αF'IQ transformants. The soluble (S), inner membrane (IM), and outer membrane (OM) fractions were prepared for both *E. coli* clones carrying the pKLY3-derivatives: pKLY3/(27-28) Betv1a producing SIS-His-BetV1a and pKLY3/(29-30) Betv1a producing SIS-Betv1a-His. Equal total protein amounts (40μg) were loaded for all samples, which correspond, in terms of culture volume (OD_{600}~.8), to .3ml for S, 4ml for IM, and 3ml for OM samples. Arrows indicate the position of the overexpressed products, respectively to each vector. The middle lane contains the reference protein His-Betv1a. Numbered arrows point to proteins bands described in details in the text.

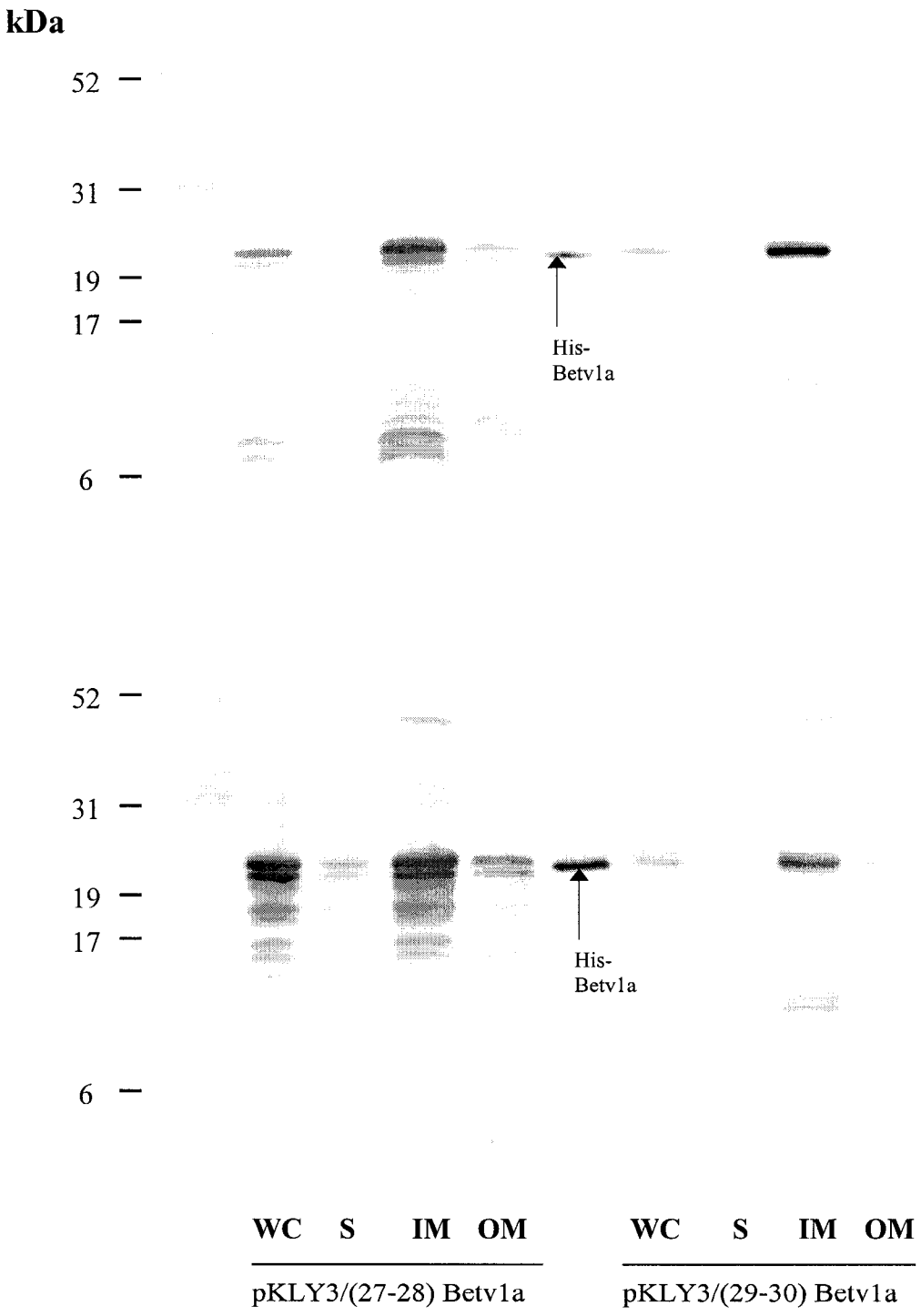


FIGURE 14. Detection of recombinant SIS-His-Betv1a (from clone pKLY3/(27-28) Betv1a) and SIS-Betv1a-His (from clone pKLY3/(29-30) Betv1a) protein products with mAb anti-His (top) and rabbit polyclonal anti-Betv1a (bottom) in Western blot assays. Samples were identical for both top and bottom membranes and consisted of the subcellular fractions from the pKLY3 induced cell cultures of DH5αF'IQ carrying the pKLY3-derivative plasmids (S, IM, OM). Whole cell samples (WC) and the reference protein His-Betv1a (sixth lane from left) were also

included. All samples were loaded with equal total protein quantities: 24μg for the mAb anti-His detection system, and 8μg for the more sensitive detection with rabbit anti-Betv1a.

3.7 Radiolabelling of pKLY3 expression products

3.7.1 Methodology

Lipid modification of BetV1a was investigated with the use of the radiolabelled palmitate ([9,10(n)-^{3}H] palmitic acid, Amersham Pharmacia, Canada). DH5αF'IQ cells carrying pKLY3 derivative plasmids were grown aerobically to exponential phase (OD_{600}~.5 to .6), at 37°C, in glycerol rich (.5% (v/v)) defined medium containing casamino acids (2% (w/v); Difco-Becton Dickinson, MD), 1μg/ml vitamin B1 (thiamine), and essential salts (2μM $FeSO_4.7H_2O$, 100mM KH_2PO_4, 1mM $MgSO_4$, .7mM $(NH_4)_2SO_4$). Antibiotics were included in the growth medium at 50μg/ml ampicillin and 40μg/ml kanamycin. Cells were supplied with .1mCi of ^{3}H palmitate per ml of culture, corresponding to .5μg of palmitate, solubilized in n-propanol. When needed, the antibiotic globomycin (a generous gift of Dr. Masayori Inouye; Department of Biochemistry, Robert Wood Johnson Medical School, Piscataway, NY), dissolved in dimethyl sulfoxide, was added to cultures to a final concentration of 100μg/ml, 10 minutes prior to the addition of ^{3}H palmitic acid. After 5 minutes of incubation with the radiolabelled palmitate, cultures were induced for 1 hour with 1.8mM IPTG. For each pKLY3 clone, the exponentially growing culture was split in two prior to the addition of globomycin or palmitate; one half of the culture was induced as described above, the other remained uninduced. The cells were collected by centrifugation and total cellular proteins obtained by TCA (10% (w/v)) precipitation. Alternatively, the cells were solubilized in 8M urea, pH=8.0 (1.2ml for each ml of culture), and proteins collected by a single centrifugation at 10,000g at room temperature. The precipitated proteins were washed twice with methanol for the removal of free palmitic acid. Samples were left to dry overnight, at room temperature, and were resuspended in SDS-PAGE loading buffer prior to being loaded for 12% SDS-PAGE (50 minutes; 200 V, constant). The vacuum-dried gels (Biorad Gel Dryer 583) were apposed against high-sensitivity photographic film (Kodak Biomax™ MS; Kodak, NY) at –70°C temperature. Beta-particle emission from tritium-labelled palmitate was detected with the energy-converter, signal-intensifying screen Biomax™

Transcreen LE (Kodak, NY) by autoradiographic exposure of the film to radiolabelled proteins. Films were developed by hand using Kodak GBX developer and fixer solutions.

3.7.2 Evidence of fatty-acylation in the pKLY3-derivative expression products

In vivo labelling of induced DH5αF'IQ cells carrying the pKLY3-derivative plasmids revealed lipid modification of the products of SIS-His-Betv1a expression (Figure 15). However, SIS-Betv1a-His product expression and labelling, under the same conditions, failed to reveal any significant protein lipidation.

In the case of SIS-His-Betv1a expression, two significant product bands were detected, at molecular weights matching with the previously calculated protein products on Coomassie stained gels or western blots (see section 3.2). Such bands were missing in the non-induced cells of the SIS-His-Betv1a-expressing transformant, and in the non-induced and induced negative control pKLY3 (27-28) transformant (producer of SIS-His), thereby indicating that the radiolabelled product obtained specifically resulted from SIS-His-Betv1a expression following induction of the respective clone culture. These observations thus provide support for evidence of at least partial lipidation of Betv1a towards the production of Lipo-His-Betv1a in the form of an apolipoprotein or some other intermediate lipidated protein product. Conversely, palmitoylation of SIS-His-Betv1a was undetectable and could indicate very low levels or lack of cellular processing of this prolipoprotein.

Treatment of cells with globomycin, the cyclic peptide antibiotic that inhibits prolipoprotein processing by Spase II (Dev et al., 1985; Inukai et al., 1978), was performed on an exponentially growing, ^{3}H palmitate labelled culture of Lipo-His-Betv1a. The equivalent experiment was not performed for the SIS-Betv1a-His culture because no palmitate-labelled product was detected, as noted above. Because globomycin interferes with the proteolytic cleavage, by Spase II, of the N-terminal SIS peptide sequence, it was expected that an apparent increase of as much as 2kDa, based on theoretical SIS MW calculation (Table 1), would be observed for a given mature lipoprotein. Comparison of globomycin-treated versus globomycin-free induced cultures of Lipo-His-Betv1a demonstrated that the lipidated recombinant products each undergo an increase

of approximately 1kDa due to the addition of globomycin to the medium (Figure 16). This finding is correlated by an identical MW shift, also 1kDa, in the 14 kDa endogenous lipoprotein of the host cell (Ichihara et al., 1981). Thus, both the 23.5 kDa and the 22.4 kDa lipoprotein products of lipo-His-Betv1a expression are mature lipoproteins, apparently fully processed by the cell at both the sulfhydryl residue and at the N-terminal with fatty acid moieties.

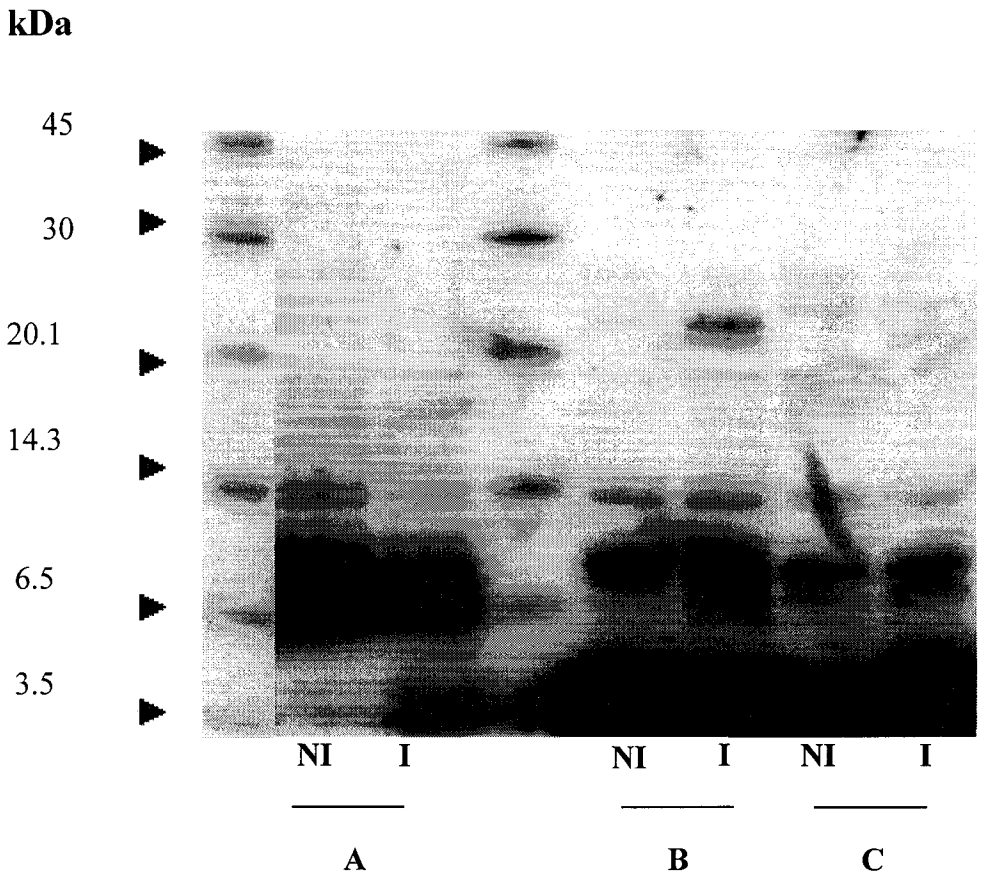


FIGURE 15. ^{3}H palmitate labelling of the expression products of SIS-His-Betv1a and SIS-Betv1a-His. pKLY3/(27-28) Betv1a and pKLY3/(29-30) Betv1a transformants of DH5αF'IQ were cultured and labelled according to the protocol described in the text. The equivalent of .35ml culture was loaded on gel for each sample. The standard (first and fourth lane from left) is Rainbow™ [^{14}C] methylated protein molecular weight marker (Amersham Pharmacia). Legend: transformants A, pKLY3 (27-28); B, pKLY3/(27-28) Betv1a; C, pKLY3/(29-30) Betv1a for SIS-His, SIS-His-Betv1a, and SIS-Betv1a-His expression, respectively. NI indicates no induction, and I, induction of cultured cells A, B, or C.

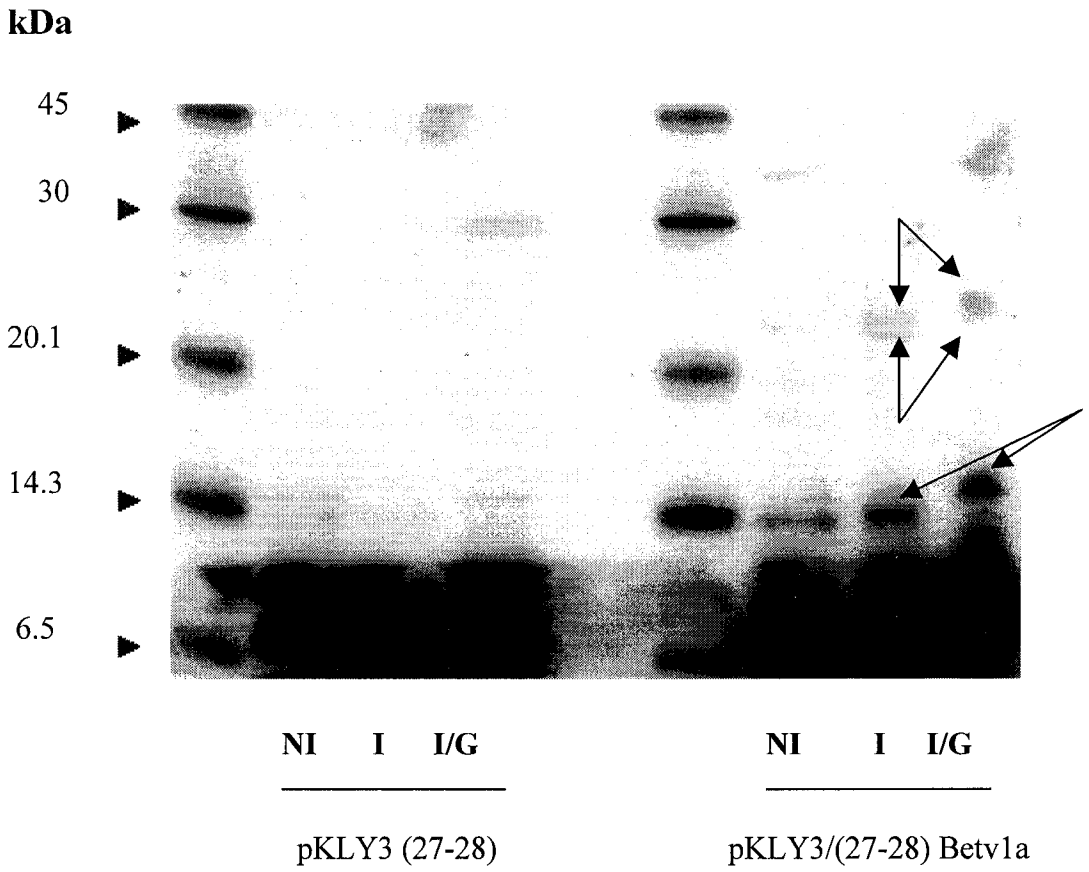


FIGURE 16. Investigation of the effects of the antibiotic globomycin on the production of lipidated Betv1a. All cell cultures were labelled with ^{3}H palmitic acid, then left to incubate non-induced (NI), or induced with (I/G) or without (I) the addition of globomycin to the medium. Arrows display the shift in MW of the top (23.5kDa) and bottom (22.4kDa) labelled lipo-His-Betv1a bands (resulting from pKLY3/(27-28) Betv1a culture induction). The bottom set of arrows indicates a 1kDa shift in the 14kDa *E. coli* endogenous lipoprotein, identical to the MW shift observed for the recombinant lipoprotein products. Negative control corresponds to the SIS-His-expressing pKLY3 (27-28) culture, and was subjected to the same conditions (NI, I, and I/G) as the SIS-His-Betv1a expressing culture.

CHAPTER IV

LIPOPROTEIN LIPO-HIS-BETV1A PURIFICATION SCHEME

The experimental data obtained in previous experiments enables the design of a protocol well-adapted for the efficient purification of lipidated Betv1a bearing the decahistidine tag. Complete purification of lipidated Betv1a is divided in three steps (Figure 17):

- Enrichment of lipidated Betv1a by treatment of induced whole cells with 8M urea, pH=8.0
- Extraction of the target proteins from the resulting pellet (also termed urea pellet) using a buffered solution of 2.5% (w/v) EBB detergent
- High-efficiency purification of the product by IMAC

4.1 Partial purification

DH5αF'IQ cells were induced for 1 to 2 hours with 1mM IPTG when the cells were well into the exponential phase (OD_{600}~.80). The cells were grown aerobically in LB containing 75μg/ml ampicillin. The induced cells were harvested by centrifugation at 12,000g for 15min, and resuspended to homogeneity in the resuspension buffer 8M urea, pH=8.0, 5mM imidazole, .5M NaCl. The volume of the urea solution used for resuspension was not critical (see section 3.3); cell resuspension was usually easily achieved with at least 12ml of resuspension buffer per gram of cell. The homogenous resuspension was centrifuged at 10,000g for 20 minutes at room

temperature or at 4°C. As demonstrated earlier, all lipidated products of Betv1a remained segregated to the pellet obtained from this centrifugation (also termed urea pellet).

4.2 Lipoprotein extraction

The lipoBetv1a-enriched pellet obtained was first resuspended in a buffered solution (50mM Tris-HCl, .5M NaCl, 5mM imidazole), by hand or with the help of a homogenizer, using 20ml of buffer per liter of induced culture. The detergent EBB was then added to the resuspension to a final concentration of 2.5% (w/v). The homogeneous sample obtained was centrifuged at 10,000g for 10 min at 4°C. The clear supernatant fluid, which contained the proteins of interest, could be directly purified on Ni-NTA spin columns. Extraction of the proteins from the urea pellet required multiple detergent treatments. It was calculated that each detergent resuspension extracted about 50% of lipo-Betv1a proteins out of the urea pellet (data not shown). For all practical purposes, two detergent resuspensions were sufficient to extract at least 75% of all recombinant proteins found in the pellet, but additional resuspension may be required, especially if the culture volume treated for protein extraction is important.

4.3 Purification by metal affinity chromatography

The final step in the purification of lipidated Betv1a takes advantage of the high affinity of the decahistidine tag for the nickel ion present on Ni-NTA columns. The clear detergent resuspension sample was passed through an Ni-NTA spin column (Qiagen, CA). Following two column washes (2.5% (w/v) EBB, 50mM Tris-HCl, .5M NaCl, 40mM imidazole; pH=8.0), the column-bound proteins were eluted with the elution buffer (2.5% (w/v) EBB, 50mM Tris-HCl, .5M NaCl, .5M imidazole; pH=8.0). At least 50% of the recombinant proteins were purified out of cultures using this purification process, as calculated by the quantification of protein bands on Coomassie stained gels by densitometric analysis (same method as in section 3.2).

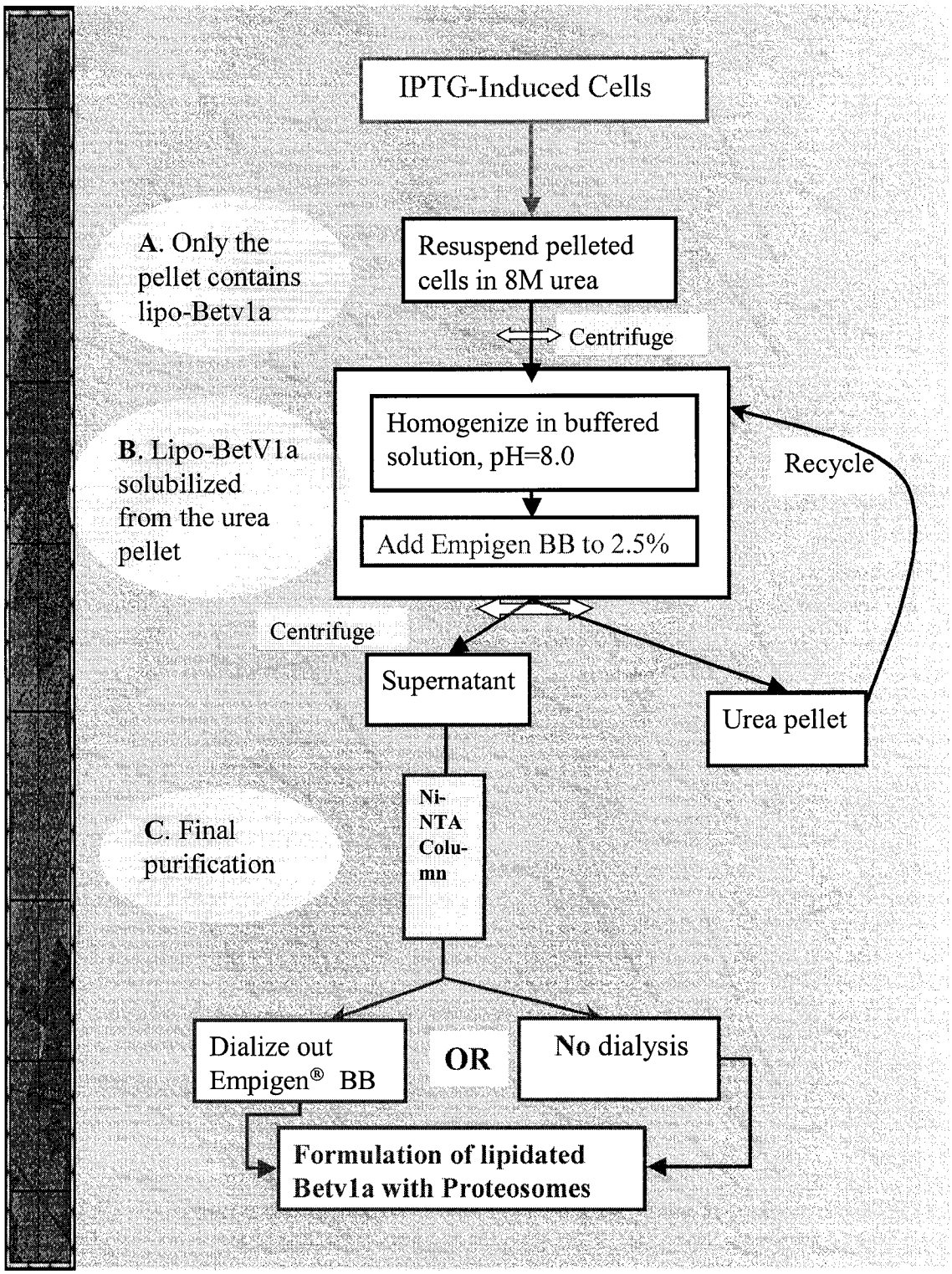


FIGURE 17. Extraction and purification procedure for the recombinant lipidated Betv1a protein expression products. Extraction is a two-step process that combines treatment of induced whole cells with 8M urea, pH=8.0, followed by the resuspension of the resulting urea pellet with 2.5% (w/v) EBB. Purification is achieved by passage of the cleared EBB pellet resuspension through a Ni-NTA column. The high concentration of EBB detergent in the purified protein sample may be dialyzed out, or the purified proteins may be directly combined with Proteosomes for complexing due to buffer and detergent compatibility between the Proteosomes and the purified lipidated Betv1a sample.

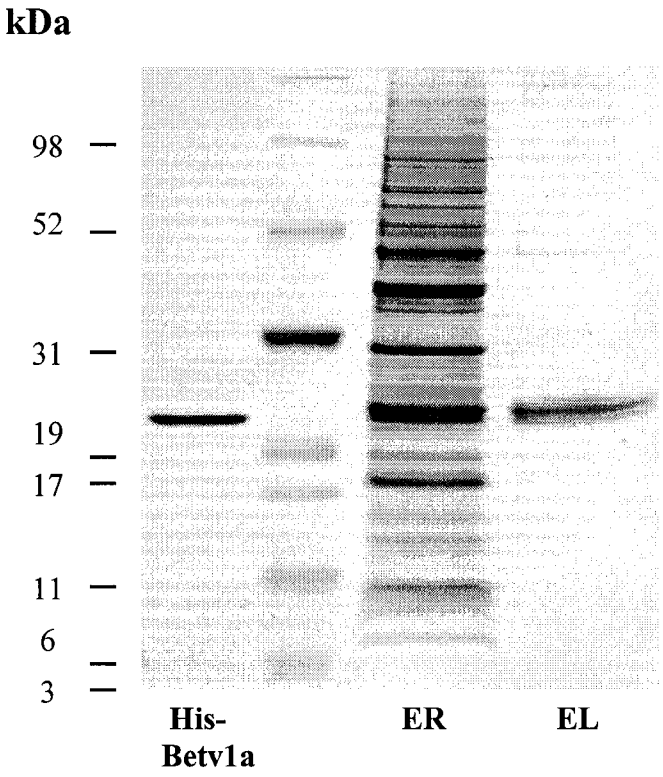


FIGURE 18. Purification of lipo-His-Betv1a on a Ni-NTA spin column following lipoprotein extraction with a 2.5% (w/v) Empigen® BB detergent extraction. Resuspension of the urea pellet, which was enriched with the recombinant lipoprotein, with the buffered Empigen® BB solution was cleared by centrifugation at 10,000g for 10min, 4°C. The equivalent of .3ml induced culture was loaded for 12% SDS-PAGE for the cleared detergent resuspension (ER) sample, and an equivalent of 1ml culture for the purified lipoprotein in the column eluate (EL). The reference protein His-Betv1a enables unambiguous identification of the products in the ER and EL.

CHAPTER V

CONCLUSION and GENERAL DISCUSSION

Production of Betv1a with hydrophobic peptides (Hft) and of lipid-modified Betv1a was accomplished successfully using vectors pKLY2 and pKLY3, respectively, in *Escherichia coli* host strain DH5αF'IQ. All Betv1a derivatives contained a His-tag to facilitate the purification on Ni-NTA columns. pKLY2 and pKLY3-derived plasmids share in common the pKK233-2 vector promoter trc repressed by LacI and induced by IPTG. However, despite structural similarities in the expression system, the protein production levels obtained from these vectors varied with the nature of the proteins produced. Production of His-tagged Betv1a and Betv1a modified with the Hft yielded similar and low to moderate amounts of proteins. In other studies, it was also reported that *betv1a* expression, using the pKK233-2 vector system, was not very successful in achieving high-level production of the unmodified Betv1a protein (Ferreira et al., 1993). But, while production levels of the recombinant protein Betv1a using pKLY2 and pKLY3-derived vectors was dependent upon promoter strength, the production levels of mature lipoproteins using pKLY3-derived vectors was additionally dependent upon the ability of the cell to process the prolipoprotein. Thus, the requirements for extensive structural modifications of the Betv1a prolipoprotein and the effects that the prolipoprotein and lipoprotein may have on cellular functions led to protein expression with the pKLY3-derived vectors that was even lower than for the pKLY2 expression vectors.

pKLY2 vectors may not be suitable for efficient, high-level production of Betv1a and of Hft-modified proteins. The use of pET vectors, which include the tightly regulated and stronger bacteriophage T7 promoter (Rosenberg et al., 1987), has been used successfully in Intellivax's laboratories to produce high levels of Betv1a derivatives with His-tag and

hydrophobic peptides. The use of stronger promoter than trc, such as theT7 promoter, may not, however, allow greater overall lipoprotein production compared to the pKLY3 vectors. The production of lipoprotein is a multi step process requiring various enzymes localized in the cytoplasmic membrane of the cell . If any of the cellular enzymes or processes along the path of prolipoprotein synthesis (Figure 5) is rate-limiting, then providing for greater levels of prolipoprotein production in the cell with the use of a strong promoter will probably result in a bottleneck effect at the translocation or protein modification level. Use of a strong promoter may also result in the IM accumulation of the lipoprotein, probably to levels similar to those reported in this work (assuming similar experimental conditions are used). Prolipoprotein levels, however, may accumulate in the cell cytoplasm and remain unprocessed or may become targeted for degradation by cellular proteases. Enhancing lipoprotein production levels in cells is therefore a complex undertaking since little is known about the efficiency or the rate of the biosynthetic reactions that leads to apolipoprotein and lipoprotein production *in vivo*. On the other hand, a more useful effect may be obtained by replacing the promoter currently used in the pKLY3 system by a weaker one, because lowering prolipoprotein production in cells may help prevent premature saturation of the IM (and thus cellular shutdown as described in section 3.5) while contributing to culture progression towards higher population density, which should directly translates into higher lipoprotein yields. Alternatively, simply reducing the amounts of IPTG for culture induction (e.g. less than .5mM) may help achieve the same results. Another factor may be considered, such as temperature of the incubation medium. Temperature affects the physical and biological properties of membranes (Jin et al., 1999; Morein et al., 1996). Since lipo-His-Betv1a is associated with the IM, it may be helpful to investigate the effects of temperature variation on the cellular concentration of this lipoprotein. It may also be useful to direct the lipoprotein to the periplasm or to the OM to prevent occurrence of the negative effects on cellular growth observed with pKLY3-derived expressions; however, this will require redesigning the pKLY3 vectors to include a sequence known to direct proteins to these other cellular compartments. Finally, replacing the *E. coli* DH5αF'IQ strain with one (e.g. JM105, BL21) that may better tolerate the lipoprotein products may also help significantly improve on lipoprotein production levels. In this work, it was reported that overexpression of SIS-His-Betv1a resulted in the production of a structurally stable, notwithstanding limited, of lipo-His-Betv1a. But, SIS-Betv1a-His expression resulted in poorer protein production, and

despite its apparently efficient translocation to the IM, no detectable lipidation was observed, as assessed by ^{3}H palmitate labelling. This finding is intriguing because the prolipoprotein SIS-Betv1a-His, is identical to the prolipoprotein SIS-His-Betv1a in all aspects (MW, number of a.a., and pI; see Table 1), except for the C-terminal position of the decahistidine tag. It may be assumed that this tag, which is polar, interferes with the protein-modifying role of membrane-bound cellular enzymes (whether through prolipoprotein conformational changes or through physical interaction of the tag with the enzymes, for example), and thus with the downstream processing of this prolipoprotein in the cell. To eliminate or alleviate these possible effects, the decahistidine tag at the carboxyl end of the molecule could either be replaced by a hexahistidine tag or removed altogether by modification of the plasmid encoding sequence (section 2.2.3.6 describes the restriction sites that frame the decahistidine tag).

An efficient method for the purification of these membrane-bound lipo-Betv1a was developed. It involved the enrichment of lipidated Betv1a into an insoluble fraction upon treatment of whole induced cells with urea followed by lipoprotein extraction with the detergent Empigen® BB. Methods of extraction by mechanical (e.g. with a French press, or sonicator) and enzymatic (e.g. lysozyme) means, and the usual requirement for protease inhibitors were not needed with the purification scheme provided here.

It was also indicated that lipidation by radiolabelled palmitate occurred at the glyceryl group bound to the cysteine sulfhydryl, and at the N-terminal group by amide linkage by treatment of the host cell with the antibiotic globomycin, a SPase II inhibitor. These results imply that at least two fatty acid molecules (out of three possible) are incorporated for each molecule of mature Lipo-His-Betv1a.

Thus, the lipidated Betv1a molecule characterized in this work may represent, at this time, the first His-tagged Betv1a allergen molecule to be produced, using the heterologous host *E. coli*, with a lipid tail and effectively purified. Even though low levels of lipidated Betv1a were produced, it should be possible to provide sufficient quantities of this purified lipoprotein for a study of its immunogenicity when administered alone or complexed to the Proteosme adjuvant system in animals.

REFERENCES

Allen RC, and Budowle B. 1999. Protein Staining. In *Protein Staining and Identification Techniques* (Biotechniques Molecular Laboratory Methods Series, number 3). Eaton Publishing. USA, pp.1-17.

Arkowitz RA, and Bassilana M. 1994. Protein translocation in *Escherichia coli*. Biochim Biophys Acta. 1197(3): 311-343.

Bessler WG, Cox M, Lex A, Suhr B, Wiesmuller KH, and Jung G. 1985. Synthetic lipopeptide analogs of bacterial lipoprotein are potent polyclonal activators for murine B lymphocytes. J Immunol. 135(3): 1900-1905.

Boehm M, and Rösch P. 1997. Expression in *Escherichia coli*, purification, and spectroscopic characterization of two mutant Betv1 proteins. Biol Chem. 378(7): 687-95.

Bollag DM, Rozycki MD, and Edelstein SJ. 1996. Protein Methods, second edition. Wiley-Liss. NY, NY.

Bousquet J, Demoly P, and Michel FB. 2001. Specific immunotherapy in rhinitis and asthma. Ann Allergy Asthma Immunol. 87(1Suppl 1): 38-42.

Boyd A, and Holland IB. 1979. Regulation of the synthesis of surface protein in the cell cycle of *E. coli* B/r. Cell. 18(2): 287-296.

Braun V, and Rehn K. 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the *E. coli* cell wall. The specific effect of trypsin on the membrane structure. Eur J Biochem. 10(3): 426-438.

Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, and Breitenbach M. 1989. The gene coding for the major birch pollen allergen Betv1, is highly homologous to a pea disease resistance response gene. EMBO J. 8(7): 1935-1938.

Chang CN, Kuang WJ, and Chen EY. 1986. Nucleotide sequence of the alkaline phosphatase gene of *Escherichia coli*. Gene. 44(1): 121-125.

Cole ST, Saint-Joanis B, and Pugsley AP. 1985. Molecular characterisation of the colicin E2 operon and identification of its products. Mol Gen Genet. 198(3): 465-72.

Cookson WO. 1998. Genetic aspects of atopic allergy. Allergy. 53(45 Suppl): 9-14.

Dev IK, Harvey RJ, and Ray PH. 1985. Inhibition of prolipoprotein signal peptidase by globomycin. J Biol Chem. 260(10): 5891-5894.

Driessen AJ, Fekkes P, and van der Wolk JP. 1998. The Sec system. Curr Opin Microbiol. 1(2): 216-22.

Ebner C, Siemann U, Bohle B, Willheim M, Wiedermann U, Schenk S, Klotz F, Ebner H, Kraft D, and Scheiner O. 1997. Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. Clin Exp Allergy. 27(9): 1007-15.

Economou A. 1998. Bacterial preprotein translocase: mechanism and conformational dynamics of a processive enzyme. Mol Microbiol. 27(3): 511-518.

Ferreira FD, Hoffmann-Sommergruber K, Breiteneder H, Pettenburger K, Ebner C, Sommergruber W, Steiner R, Bohle B, Sperr WR, Valent P, Kungl AJ, Breitenbach M, Kraft D, and Scheiner O. 1993. Purification and characterization of recombinant Bet v I, the

major birch pollen allergen. Immunological equivalence to natural Bet v I. J Biol Chem. 268(26): 19574-19580.

Filip C, Fletcher G, Wulff JL, and Earhart CF. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J Bacteriol. 115(3): 717-22.

Fralick JA, and Diedrich DL. 1982. Studies on the expression of outer membrane protein 2 in *Escherichia coli*. Mol Gen Genet. 188(1): 139-142.

Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, Joost van Neerven RJ, Schou C, Lowenstein H, and Spangfort MD. 1996. X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. Nat Struct Biol. 3(12): 1040-1045.

Gennity JM, Kim H, and Inouye M. 1992. Structural determinants in addition to the amino-terminal sorting sequence influence membrane localization of *Escherichia coli* lipoproteins. J Bacteriol. 174(7): 2095-2101.

Ghuysen JM, and Hakenbeck R. 1994. Lipoproteins, structure, function, biosynthesis and model for protein export. In *Bacterial Cell Wall*. Elsevier Science. NY, pp. 319-341.

Godnic-Cvar J, Susani M, Breiteneder H, Berger A, Havelec L, Waldhor T, Hirschwehr R, Valenta R, Scheiner O, Rudiger H, Kraft D, and Ebner C. 1997. Recombinant Bet v 1, the major birch pollen allergen, induces hypersensitivity reactions equal to those induced by natural Bet v 1 in the airways of patients allergic to tree pollen. J Allergy Clin Immunol. 99(3): 354-359.

Hammond SM, Lambert PA, and Rycroft A. 1984. The Envelope of Gram-Negative Bacteria. In *The Bacterial Cell Surface*. Kapitan Szabo Publishers. Washington, DC, pp.57-118.

Hancock RE, and Nikaido H. 1978. Outer membranes of Gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and definition of the permeability barrier. J Bacteriol. 136(1): 381-90.

Hartl A, Kiesslich J, Weiss R, Bernhaupt A, Mostbock S, Scheiblhofer S, Ebner C, Ferreira F, and Thalhamer J. 1999a. Immune responses after immunization with plasmid DNA encoding Bet v1, the major allergen of birch pollen. J Allergy Clin Immunol. 103(1 Pt 1): 107-113.

Hartl A, Kiesslich J, Weiss R, Bernhaupt A, Mostbock S, Scheiblhofer S, Flockner H, Sippl M, Ebner C, Ferreira F, and Thalhamer J. 1999b. Isoforms of the major allergen of birch pollen induce different immune responses after genetic immunization. Int Arch Allergy Immunol. 120(1): 17-29.

Hayashi S, and Wu HC. 1985. Accumulation of prolipoprotein in *Escherichia coli* mutants defective in protein secretion. J Bacteriol. 161(3): 949-954.

Hoffmann-Sommergruber K, Susani M, Ferreira F, Jertschin P, Ahorn H, Steiner R, Kraft D, Scheiner O, and Breiteneder H. 1997. High-level expression and purification of the major birch pollen allergen, Bet v 1. Protein Expr Purif. 9(1): 33-39.

Ichihara S, Hussain M, and Mizushima S. 1981. Characterization of new membrane lipoproteins and their precursors of *Escherichia coli*. J Biol Chem. 256(6): 3125-3129.

Inouye M. 1987. Lipoproteins in Bacteria. In *Bacterial Outer Membranes as Model Systems*. Wiley-Interscience Publication. NY, pp. 451-471.

Inouye S, Duffaud G, and Inouye M. 1986. Structural requirement at the cleavage site for efficient processing of the lipoprotein secretory precursor of *Escherichia coli*. J Biol Chem. 261(24): 10970-10975.

Inouye S, Wang S, Sekizawa J, Halegoua S, and Inouye M. 1977. Amino acid sequence for the peptide extension on the prolipoprotein of the *Escherichia coli* outer membrane. Proc Natl Acad Sci U S A. 74(3): 1004-1008.

Inukai M, Takeuchi M, Shimizu K, and Arai M. 1978. Mechanism of action of globomycin. J Antibiot (Tokyo). 31(11): 1203-1205.

Jahn-Schmid B, Wiedermann U, Bohle B, Repa A, Kraft D, and Ebner C. 1999. Oligodeoxynucleotides containing CpG motifs modulate the allergic T_H2 response of BALB/c mice to Bet v1, the major birch pollen allergen. J Allergy Clin Immunol. 104(5): 1015-23.

Janeway CA, Travers P, and Walport M. 1999. Immunobiology-The Immune System in Health and Disease. Fourth edition. Garland Publishing, NY.

Janknecht R, de Martynoff G, Lou J, Hipskind RA, Nordheim A, and Stunnenberg HG. 1991. Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. Proc Natl Acad Sci U S A. 88(20): 8972-8976.

Jin AJ, Edidin M, Nossal R, and Gershfeld NL. 1999. A singular state of membrane lipids at cell growth temperatures. Biochemistry. 38(40): 13275-13278.

Johnson E, Bergeron D, St-Félix N, Gauthier J, Williams J, Burt D, Lowell GH, and White GL. 2000. Poster presented at the meeting organized by the European Scientific Working Group on Influenza (ESWI), entitled: Options for the Control of Influenza IV. 23-28 September 2000. Hersinissos, Greece.

Karamyshev AL, Karamysheva ZN, Kajava AV, Ksenzenko VN, and Nesmeyanova MA. 1998. Processing of *Escherichia coli* alkaline phosphatase: role of the primary structure of the signal peptide cleavage region. J Mol Biol. 277(4): 859-70.

Kiyono H, Ogra PL, and McGhee JR. 1996. ISCOMS, Liposomes, and Oil-Based Vaccine Delivery Systems. In *Mucosal Vaccines*. Academic Press, Inc. CA, pp.175-186.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227(259): 680-685.

Levine MM, Woodrow GC, Kaper JB, and Cobon GS. 1997. Proteosomes for improved nasal, oral, or injectable vaccines. In *New Generation Vaccines*. New York : Marcel Dekker. Second edition, revised and expanded; pp. 193-206.

Lowell GH, Ballou WR, Smith LF, Wirtz RA, Zollinger WD, and Hockmeyer WT. 1988a. Proteosome-lipopeptide vaccines: enhancement of immunogenicity for malaria CS peptides. Science. 240(4853): 800-802.

Lowell GH, Smith LF, Seid RC, and Zollinger WD. 1988b. Peptides bound to proteosomes via hydrophobic feet become highly immunogenic without adjuvants. J Exp Med. 167(2): 658-63.

Lowell GH, Ballou WR, Smith LF, Zollinger WD, and Hockmeyer WT. 1989. Proteosome-hydrophobic 'foot' malaria peptide vaccines for *Plasmodium falciparum* and *P. vivax*. Trans R Soc Trop Med Hyg. 83 Suppl: 101-2.

Lowry OR, Rosebrough NJ, Lewis AL, and Randall RJ. 1951. Protein measurement with the folin phenol reagent. J Biol Chem. 193: 265-275.

Maniatis T, Fritsch EF, and Sambrook J. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor. NY.

Melchers F, Braun V, and Galanos C. 1975. The lipoprotein of the outer membrane of *Escherichia coli*: a B-lymphocyte mitogen. J Exp Med. 142(2): 473-82.

Menz G, Ying S, Durham SR, Corrigan CJ, Robinson DS, Hamid Q, Pfister R, Humbert M, and Kay AB. 1998. Molecular concepts of IgE-initiated inflammation in atopic and nonatopic asthma. Allergy. 53(45 Suppl): 15-21.

Michaelis S, Inouye H, Oliver D, and Beckwith J. 1983. Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. J Bacteriol. 154(1): 366-74.

Morein S, Andersson A, Rilfors L, and Lindblom G. 1996. Wild-type *Escherichia coli* cells regulate the membrane lipid composition in a "window" between gel and non-lamellar structures. J Biol Chem. 271(12): 6801-6809.

Nakamura Y, Gojobori T, and Ikemura T. 2000. Codon usage tabulated from international DNA sequence databases: status for the year 2000. Nucleic Acids Res. 28(1): 292.

Neugebauer JM. 1990. Detergents: An Overview. In *Methods in Enzymology* (vol. 182) Academic Press. NY, pp.239-253.

O'Byrne PM, Gauvreau GM, and Wood LJ. 1998. Pharmacologic modulation of airway inflammation. Allergy. 53(45 Suppl): 41-47.

Pichler CE, Helbling A, and Pichler WJ. 2001. Three years of specific immunotherapy with house-dust-mite extracts in patients with rhinitis and asthma: significant improvement of allergen-specific parameters and of nonspecific bronchial hyperreactivity. Allergy. 56(4): 301-306.

Plante M, Jones T, Allard F, Torossian K, Gauthier J, St-Félix N, White GL, Lowell GH, and Burt DS. 2001. Nasal immunization with subunit Proteosome influenza vaccines induces serum HAI, mucosal IgA and protection against influenza challenge. Vaccine. 20(1-2): 218-225.

Pugsley AP. 1993. The complete general secretory pathway in Gram-negative bacteria. Microbiol Rev. 57(1): 50-108.

Pugsley AP, and Cole ST. 1987. An unmodified form of the ColE2 lysis protein, an envelope lipoprotein, retains reduced ability to promote colicin E2 release and lysis of producing cells. J Gen Microbiol. 133 (Pt 9): 2411-2420.

Qiagen. 1999. The QIAexpressionist™-A handbook for high-level expression and purification of 6xHis-tagged proteins. Third edition. Qiagen, USA.

Reid E. 1973. Advances in Zonal Rotors. Method. Dev. Biochem. 3: 21.

Rioux CR, Bergeron H, Lin L, Grothe S, O'Connor-McCourt M, and Lau PC. 1992. A fusion plasmid for the synthesis of lipopeptide-antigen chimeras in *Escherichia coli*. Gene. 116(1): 13-20.

Rosenberg AH, Lade BN, Chui DS, Lin SW, Dunn JJ, and Studier FW. 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. 56(1): 125-135.

Ross RN, Nelson HS, and Finegold I. 2000. Effectiveness of specific immunotherapy in the treatment of hymenoptera venom hypersensitivity: a meta-analysis. Clin Ther. 22(3): 351-358.

Sambrook J, Fritsch EF, and Maniatis T. 1989. Molecular Cloning. A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory. Cold Spring Harbor. NY.

Sanders CR 2nd, Czerski L, Vinogradova O, Badola P, Song D, and Smith SO. 1996. *Escherichia coli* diacylglycerol kinase is an alpha-helical polytopic membrane protein and can spontaneously insert into preformed lipid vesicles. Biochemistry. 35(26): 8610-8618.

Schmitt J, Hess H, and Stunnenberg HG. 1993. Affinity purification of histidine-tagged proteins. Mol Biol Rep. 18(3):223-230.

Schnaitman CA. 1970a. Examination of the protein composition of the cell envelope of *Escherichia coli* by polyacrylamide gel electrophoresis. J Bacteriol. 104(2): 882-889.

Schnaitman CA. 1970b. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J Bacteriol. 104(2): 890-901.

Spangfort MD, Ipsen H, Sparholt SH, Aasmul-Olsen S, Larsen MR, Mortz E, Roepstorff P, and Larsen JN. 1996. Characterization of purified recombinant Bet v 1 with authentic N-terminus, cloned in fusion with maltose-binding protein. Protein Expr Purif. 8(3): 365-373.

Sulkowski E. 1985. Purification of proteins by IMAC. Trends Biotechnol. 3: 1-7.

Tjalsma H, Zanen G, Venema G, Bron S, and van Dijl JM. 1999. The potential active site of the lipoprotein-specific (type II) signal peptidase of *Bacillus subtilis*. J Biol Chem. 274(40): 28191-28197.

Van Ree R, Van Leeuwen WA, Akkerdaas JH, and Aalberse RC. 1999. How far can we simplify *in vitro* diagnostics for Fagales tree pollen allergy? A study with three whole pollen extracts and purified natural and recombinant allergens. Clin Exp Allergy. 29(6): 848-855.

von Heijne G. 1989. The structure of signal peptides from bacterial lipoproteins. Protein Eng. 2(7): 531-534.

Wahn U. 1999. Do asthmatic children benefit from specific immunotherapy? Clin Exp Allergy. 29: 143.

Weiss C, Kramer B, Ebner C, Susani M, Briza P, Hoffmann-Sommergruber K, Breiteneder H, Kraft D, Scheiner O, Breitenbach M, and Ferreira F. 1996. High-level expression of tree pollen isoallergens in *Escherichia coli*. Int Arch Allergy Immunol. 110(3): 282-287.

Wiedermann U, Jahn-Schmid B, Bohle B, Repa A, Renz H, Kraft D, and Ebner C. 1999a. Suppression of antigen-specific T- and B-cell responses by intranasal or oral administration of recombinant bet v 1, the major birch pollen allergen, in a murine model of type I allergy. J Allergy Clin Immunol. 103(6): 1202-1210.

Wiedermann U, Jahn-Schmid B, Fritsch R, Bauer L, Renz H, Kraft D, and Ebner C. 1998. Effects of adjuvants on the immune response to allergens in a murine model of allergen inhalation: cholera toxin induces a Th1-like response to Bet v 1, the major birch pollen allergen. Clin Exp Immunol. 111: 144-151.

Wiedermann U, Jahn-Schmid B, Lindblad M, Rask C, Holmgren J, Kraft D, and Ebner C. 1999b. Suppressive versus stimulatory effects of allergen/cholera toxoid (CTB) conjugates depending on the nature of the allergen in a murine model of type I allergy. Int Immunol. 11(10): 1717-1724.