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ABSTRACT

This application relates to a recombinant carrier molecule comprising a modified lichenase B polypeptide that has a modification in the loop region.

Australian Patents Act 1990 – Regulation 3.2
ORIGINAL COMPLETE SPECIFICATION
STANDARD PATENT

Invention Title:

**"Recombinant carrier molecule for expression, delivery and purification of
target polypeptides"**

The following statement is a full description of this invention, including the best method of
performing it known to us:-

P/00/011

RECOMBINANT CARRIER MOLECULE FOR EXPRESSION, DELIVERY AND PURIFICATION OF TARGET POLYPEPTIDES

5 This application is a divisional of Australian Patent Application No. 2004272972, the entire content of which is incorporated herein by reference.

FIELD OF THE INVENTION

10 The present invention is directed to the field of protein expression, purification and molecular biology. Specifically, the present invention is directed to a carrier protein expression in which a mature polypeptide of a thermostable enzyme is used as carrier molecule for production, recovery and delivery of target polypeptides. The carrier molecule is useful for the production of foreign sequences in different expression systems and hosts including plants and mammalian cell cultures.

BACKGROUND OF THE INVENTION

15 Vaccines are the most effective means for preventing and even eliminating infectious diseases. Although, there are a number of efficacious vaccines based on full pathogens, development of safer more potent and cost effective vaccines based on portions of pathogen (subunit vaccines) is important. During the last two decades several
20 approaches to the expression (bacterial, yeast, mammalian cell culture and plant) and delivery (DNA, live virus vectors, purified proteins, plant virus particles) of vaccine antigens have been developed. All these approaches have significant impact on the development and testing of newly developed candidate vaccines. However, there is a need for improving expression and delivery systems to create more efficacious but safer
25 vaccines with fewer side effects. Some of the desired features of future vaccines are (a) to be highly efficacious (stimulates both arms of immune system), (b) to have known and controlled genetic composition, (c) to have time efficiency of the system, (d) to be suitable for expression of both small size peptides and large size polypeptides, (e) to be suitable for expression in different systems (bacteria, yeast, mammalian cell cultures, live
30 virus vectors, DNA vectors, transgenic plants and transient expression vectors), and (f) to be capable of forming structures such as aggregates or virus like particles that are easy to recover and are immunogenic.

Thus, there is a need for novel carrier molecules for engineering, development and delivery of efficacious subunit vaccines. These carrier molecules should provide advantages and flexibility for: expressing commercially sufficient quantities of target polypeptide in different systems, economical recovery of target polypeptides from source material, accommodating different size (4 amino acids and higher) polypeptides, accommodating tandem repeats of target polypeptides, providing enhanced immune function, use as a high throughput screening tool, and use as a delivery tool for vaccine antigens and disease markers.

10 SUMMARY OF THE INVENTION

In the present invention, a novel recombinant protein has been discovered. It will serve as a carrier molecule for expression and recovery of useful target polypeptides for use as therapeutic or preventative agents against infectious diseases or even cancer. The carrier molecule discovered herein can accommodate polypeptides of varying sizes (4 amino acids to a 100 kD protein and higher) (target polypeptides) and can be expressed in different systems. The target polypeptides can be vaccine antigens

In a general aspect, the present invention provides a recombinant carrier molecule having a modified mature polypeptide of a thermostable enzyme lacking one or more segments of amino acids or a substantially complete mature polypeptide of the thermostable enzyme suitable for fusing to a heterologous polypeptide at each of N-terminus and C-terminus of the mature polypeptide, and optionally in the loop region. The modified mature polypeptide and substantially complete mature polypeptide retain their thermostability and/or enzyme activity. The mature polypeptide of is modified in that it lacks a loop region or has a disrupted loop region, or has at least one restriction site in the loop region not naturally present in the wild type thermostable enzyme.

In one preferred embodiment, the carrier molecule discovered herein is based on lichenase B (*licB*) gene from *Clostridium thermocellum* (accession: X63355, [gi:40697]). The inventors discovered that this thermostable bacterial enzyme can be used as a carrier molecule for producing target polypeptides. It has loop structure exposed on the surface that is located far from the active domain. It has been discovered by the present inventors that this loop structure can be used for the insertion of target polypeptides. The target polypeptides can be expressed as N or C terminal fusions or internal fusions and/or as

inserts into loop structure. Modified protein is expressed and characterized for any of the parameters such as thermostability, pH and temperature conditions for optimal activity. Engineered protein retained its pH and temperature conditions for optimal activity. It also did not change its thermostability at 65°C.

5 Accordingly, the present invention discloses a recombinant molecule derived from a thermostable enzyme for use as a carrier for various heterologous target polypeptides (e.g., vaccines, hormones, anticoagulants, immunoglobulins, interferons, interleukins, hematopoietic growth factors, etc). In specific embodiments, it discloses Rec LicB and LicKM. The carrier protein (i.e., modified or engineered rec LicB or
10 LicKM linked to one or more heterologous target polypeptides) is a fusion protein and it may be expressed in either prokaryotic or eukaryotic systems. Specifically it has been found that these carrier molecules can accommodate from small to a large size polypeptides of up to 100 kD and more, can accommodate tandem repeats of the same polypeptide, can be expressed in different systems, including bacterial, yeast,
15 baculovirus, mammalian cell cultures, plants, DNA and virus vectors, can provide economic advantages for recovery of target product due to their thermostability or capacity to form aggregates, can be used as high throughput system for screening target polypeptides; antigens, disease markers or other therapeutic polypeptides.

 The present invention also discloses a method for expressing peptides as fusion
20 proteins, by using a recombinant mature polypeptide of a thermostable enzyme as the carrier for heterologous polypeptide(s) and using the peptide expression methods described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

25 **Figure 1. A:** Schematic representation of engineering of recombinant LicKM carrier molecule. l is the loop structure. A indicates the region upstream of the loop structure. C indicates the region downstream of the loop structure. To create LicKM, the gene encoding Lic B was split at the loop region and assembled as shown. Unique cloning sites were created during engineering. The nucleic acid sequence for engineered
30 molecule LicKM (SEQ ID NO:1) is shown in part B of the figure. The split was done by PCR using specific primers. PCR resulted in 2 subclones (fig. 1A) designated as A (159 nucleotides, 364 trough 522) and C (486 nucleotides, 523 trough 1009). In final clone

fragment A was cloned downstream of fragment C preserving the original amino acid composition.

Figure 1C shows the construction of Rec LicB from the wildtype LicB. The Rec LicB consists of mature protein without cellulosome binding domain. Target sequences
5 can be fused to N and C terminus as well as into loop structure using BamHI and BglII restriction sites.

Figure 1D shows the nucleic acid sequence for engineered molecule Rec LicB (SEQ ID NO:2).

Figure 1E shows a sequence of amino acids (SEQ ID NO:3) encoded by LicKM
10 nucleic acid (SEQ ID NO:1).

Figure 1F shows a sequence of amino acids (SEQ ID NO:4) encoded by Rec LicB (SEQ ID NO:2).

Figure 1G shows the nucleic acid sequence for a variant of LicKM carrier
15 molecule (SEQ ID NO:5). It also has a KpnI restriction site created at the 5' end and XhoI restriction site created at the 3' end and BamHI/Bgl site in the loop region.

Figure 1H shows a sequence of amino acids (SEQ ID NO:6) encoded by a variant of LicKM carrier molecule (SEQ ID NO:5).

Figure 2. Schematic representation of cloning of GFP into the loop structure of
20 rec Lic B to obtain recombinant Lic B-GFP. The coding region of GFP was PCR amplified and cloned into the open reading frame of LicB.

The cloning was done in 2 steps by PCR. Using primers shown in fig. 1 legend, 2 subclones, A and C were created. Then the sequences encoding GFP were PCR amplified (during PCR at the 5' and 3' ends, BamHI and BglII restriction sites were incorporated, respectively). Later, using the introduced BamHI and BglII sites, the 3
25 fragments were ligated as A-GFP-C to obtain LicB-GFP. Primers for GFP were:

Plus: 5'gcag gga tcc atg gtg agc aag ggc gag3' (SEQ ID NO:7)

Minus: 5'gcag aga tct ctt gta cag ctc gtc cat3' (SEQ ID NO:8)

Figure 3. Zymogram of lichenase activity in bacterial and yeast extracts detected
30 in the presence of 0.1% lichenan as substrate. Proteins were separated in 12% PAGE. The gel was loaded with proteins extracted from *E. coli* strain XL-1 blue [C control, LicB (wild type), LicKM (engineered carrier molecule) and recombinant LicB-GFP (E)] and *Saccharomyces cerevisiae* strain YPH 857 (LicB-GFP (Y)).

Figure 4. Schematic representation of cloning of target polypeptides in engineered carrier molecule LicKM. DNA fragments encoding target polypeptides from respiratory syncytial virus (RSV) G protein, green fluorescent protein (GFP) from gely fish, and human interferon α (IFN α) were PCR amplified and inserted into open reading

5 frame of LicKM.

Figure 5. A is zymogram of lichenase activity in bacterial extracts detected in the presence of 0.1% lichenan as substrate. Proteins were separated in 12% PAGE. The gel was loaded with proteins extracted from *E. coli* strain XL-1 blue. C is a negative control. LicKM is engineered carrier molecule. LicKM-RSV, LicKM-GFP, and LicKM-IFN α are

10 engineered proteins containing respective target polypeptide. B shows the results of Western blot analysis. Proteins were separated in 12% PAGE, electroblotted onto nylon membrane and reacted with monoclonal antibodies specific for peptide from RSV G protein. Antibodies reacted with LicKM-RSV, RSV positive control (RSV (C+)) and plant virus coat protein containing identical peptide (RSV (plant)). Extracts from LicKM

15 that did not contain target peptide had no specificity to RSV antibodies.

Figure 6. RSV G peptide-specific serum antibody (IgG) response of mice immunized i.p. with LicKM-RSV. Serum antibody responses were measured by ELISA on plates coated with recombinant ALMV particles containing identical peptide (amino acids 171 to 191) from RSV G protein. Data represent OD₄₉₀ values obtained using

20 preimmune (LicKM-RSV Pre) and sera after third dose (LicKM-RSV Final) of antigen. Numbers 1, 2, 3, and 4 indicates individual animals.

Figure 7. Detection of LicKM-F200 enzymatically (A) and serologically (B) by Western analysis. Proteins were separated in 12% PAGE. A is zymogram of lichenase activity in plant extracts detected in the presence of 0.1% lichenan as substrate. LicKM-F200 (F200) reacted with antibodies specific to LicKM. Both methods detected protein

25 of expected size (47 kD).

Figure 8. RSV F protein-specific serum antibody (IgG) response of mice immunized i.p. with LicKM-F200. Serum antibody response was measured by ELISA using plates coated with inactivated RSV Long strain. Data represent OD₄₉₀ values

30 obtained using preimmune (LicKM-F200 Pre) and sera after third dose (LicKM-F200 Final) of antigen. Numbers 1, 2, 3, and 4 indicates pre and post-immune serum samples collected from individual animals.

Figure 9. Western blot analysis of recombinant LicKM-PAD4. Proteins were separated electrophoretically (12% SDS-polyacrylamide gel), transferred to a membrane, and reacted with different antibodies. All antibodies specific to PA, including monoclonal antibody 14B7 recognized the LicKM-PAD4 or control PA. AIMV CP or LicKM, used as negative controls, did not react with any of antibodies.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that recombinant forms of certain thermostable enzymes can be used as carriers or carrier molecules for expression, stabilization, display, purification and/or delivery of various genetically fused polypeptides of interest (target polypeptides) such as vaccine antigens, enzymes, antibodies (single chain) and therapeutic polypeptides.

The present invention discloses, among other things, (i) a variety of thermostable carrier molecules derived from thermostable enzymes and heterologous polypeptide-containing carrier proteins, (ii) nucleic acid constructs, which can encode recombinant carrier molecules and carrier proteins of the invention, and cells and organisms transformed with carrier protein expression constructs, (iii) methods for producing vaccine antigens in cells and organisms; (iv) methods for stimulating an immune response in animals and humans, the immune response being directed toward a carrier protein, specifically toward target antigen of the present invention, (v) methods for inducing humoral and cellular responses against infectious agents using a carrier fusion protein described below, and (vi) methods for producing various industrial enzymes (other than the thermostable enzymes) and therapeutic proteins.

Thermostable enzymes are polypeptides that function at or greater than 60°C. A number of thermostable enzymes that are known in the art, can be obtained from thermophilic organisms found in hot springs, volcanic regions etc. and used as carrier molecule. Lichenase B (LicB) protein. from *Clostridium thermocellum* is one such example of thermostable enzymes. The present invention encompasses recombinant carrier molecules derived from thermostable enzymes from natural sources, i.e., any microbial sources (bacteria and fungi,) or synthetic sources. Examples of such enzymes are lichenase B (Piruzian et al., 2002, Mol Genet Genomics, 266: 778-786), xylanase and xylosidase from *Bacillus thermactarantis* that are active at 80oC (Calandrelli et al., Res. Microbiol. 2004, 155(4):283-289), formiltransferase from *Methanopyrus kandleri* (Shima

et al., Biochem Soc. Trans., 2004, 32:269-272), Taq polymerase, alpha- amylase from *Aspergillus tamaris* (Moreira et al., J. Basic Microbiology, 2004, 44:29-35) or beta-glucosidase from *Thermus nonproteolyticus* (Wang et al., J. Bacteriology, 2003, 185:4248-55).]

5 The molecular structure of wild type lichenase B (LicB) gene and protein are well known to one skilled in the art (See, GenBank Accession Number X63355). The wild type LicB has 27 amino acids long signal peptide and 235 amino acids long mature peptide. Mature peptide has a catalytic domain and 12 amino acid (a.a. 82-94) loop region. LicB is member of glycosyl hydrolases (hydrolases β glucan in position 1-4) and
10 is a thermostable protein. Optimum temperature for enzymatic activity is between 65-70°C. According to 3D structure of the wild type Lic B, the N and C terminal regions of protein are co-localized in close proximity from active domain. The external loop is positioned far from active domain and exposed on the surface.

 The terms "carrier" "carrier molecule" "recombinant carrier molecule" used
15 interchangeably herein refer to a recombinant thermostable enzyme used for expression, stabilization, display, purification and/or delivery of heterologous polypeptide(s) translationally fused to the recombinant thermostable enzyme. The thermostable enzyme is recombinant in the sense that it is a modified mature polypeptide of a selected wild-type thermostable enzyme. The modified mature polypeptide lacks one or more portions
20 (or strings or segments) of amino acids but the modified mature polypeptide must retain its enzymatic activity or thermostability. For example, the mature polypeptide may lack a loop region or a string of 5 or more amino acids. Further, for example, the loop region of the mature polypeptide is disrupted (i) by introducing few amino acids coded for by at least one unique restriction site, and/or (ii) by splitting the gene at its loop region to
25 generate two portions (N and C-terminal portions) of the mature polypeptide, which two portions are then reengineered (circularly permuted) into a single reading frame from C-terminus to N-terminus. As a result, the original C-terminal portion remain fused upstream of the original N-terminal portion. During this reengineering, unique restriction site(s) may be incorporated at 5' and 3' ends as well as internally including at the site
30 corresponding to the fusion site. be recombined so that the recombined polypeptide is flanked at N and C-termini by the disrupted loop portions of or a string of 5 or more amino acids.

In the context of the present invention, the unique restriction site means the one introduced into the nucleic acid during the engineering process and it is the only site present in the engineered nucleic acid.

Alternatively, the thermostable enzyme is recombinant in the sense that it is a
5 complete or substantially complete mature polypeptide of a selected wild-type
thermostable enzyme and the encoding recombinant nucleic acid sequence has unique
restriction sites at the 5' end and at the 3' end, and optionally in the loop region for fusion
of a heterologous polypeptide at each of N-terminus and C-terminus, and in the loop
region. Upstream of the unique restriction site at the 5' end, an ATG codon is
10 incorporated. Downstream of the unique restriction site at the 3' end, a stop codon is
incorporated. One skilled in the art would know how to create a carrier molecule of the
invention by making manipulations at the nucleic acid level.

In one embodiment, the wild type licB protein is modified such that it lacks signal
peptide and cellulosome binding domain to create a recombinant licB carrier molecule
15 with unique cloning sites introduced into the loop region.

Referring to LicB shown in Figure 1C, the wild type LicB consists of a leader
peptide (27 amino acids, indicated as Lp), mature polypeptide (235 amino acids
symbolically divided into 3 regions (A, I and C), Pro-thr-box and cellulosome binding
domain designated as C-BD. Whereas the Rec LicB contains only the open reading
20 frame for mature protein (235 a. a.) that lacks sequences for Lp and C-BD. In some
embodiments, however, the C-BD is retained.

In another embodiment, the wild type licB protein is modified so that certain
regions of it are deleted together and certain regions of it are shuffled or swapped to
create a recombinant carrier molecule. Specifically, the N and C terminal regions
25 (designated herein as A and C, respectively) are circularly permuted. For example a
recombinant carrier molecule referred to herein as LicKM can be created as follows. As
described in the brief description of figure 1, sets of primers are used to obtain fragments
A and C which subsequently are ligated as C-A, fusing the fragment A into the open
reading frame of fragment C. LicKM maintains both enzymatic activity and
30 thermostability similar to that of wild type.

The carrier molecules recLicB and LicKM are merely preferred and exemplary
molecules of the enzyme. It should be readily apparent that a number of variant or
equivalent recLicB or LicKM carrier molecules (and nucleotide sequences coding for

equivalent molecules) having the same or similar or higher thermostability can be prepared by mutating these preferred carrier molecules, for example, by deletion, addition or substitution of amino acids or by directed evolution or gene shuffling of these molecules. One skilled in the art would know how to carry out such alterations to arrive at equivalent or variant LicB-based carrier molecules. A variant carrier molecule, as the term used herein, will have the same ability, like that of recLicB or LicKM, to facilitate at least one of expression, stabilization, display, purification or delivery of a heterologous polypeptide fused to the molecule.

A variant or equivalent carrier molecule will have a degree of amino acid similarity or identity with the exemplified preferred molecule (e.g., LicKM or Rec LicB). This amino acid similarity or identity will typically be greater than 60%, preferably be greater than 75%, more preferably greater than 80%, yet more preferably greater than 90%, and can be greater than 95%. The amino acid similarity or identity will be highest in critical regions of the carrier molecule that account for the molecule's thermostability or are involved in the determination of three-dimensional configuration which ultimately is responsible for its carrier function. In this regard, certain amino acid substitutions are acceptable and can be expected if these substitutions are in regions that are not critical to activity or are conservative amino acid substitutions which do not affect the three-dimensional configuration of the molecule. Conservative substitutions whereby an amino acid of one class (non-polar such as Ala, Val, Leu, Ile, Pro, Met, Phe, Trp; uncharged polar such as Gly, Ser, Thr, Cys, Tyr, Asn, Gln; basic such as Lys, Arg, His; or acidic class such as Asp, Glu) is replaced with another amino acid of the same class so long as the substitution does not materially alter the thermostability or three-dimensional configuration. In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the ability of "variant carrier molecule" to facilitate at least one of expression, stabilization, display, purification or delivery of a heterologous polypeptide.

The term "carrier fusion protein or carrier protein" as used herein generally refers to a chimeric fusion polypeptide or protein wherein one more heterologous polypeptides are fused to the carrier molecule.

The general architecture of the carrier protein can be, for example, any of the following :

NH₂-carrier molecule-heterologous polypeptide-COOH

NH₂-tag-cleavage site-carrier molecule-heterologous polypeptide-COOH

NH₂-carrier molecule-cleavage site-heterologous polypeptide-COOH

NH₂-tag-carrier molecule-cleavage site-heterologous polypeptide-COOH

NH₂-tag-cleavage site-carrier molecule-heterologous polypeptide-COOH

5 The carrier molecule may also have an internal fusion, in which case the heterologous polypeptide is flanked on either side by a segment of the recombinant carrier molecule. The carrier protein exhibits a high degree of thermotolerance (at least at about 60°C) which facilitates separation of the fusion protein from all other host cell proteins, nucleic acids, pyrogens, and the like after subjecting the lysate to heat and/or centrifugation. Fusion of heterologous polypeptide(s) either at N-terminus or C-terminus
10 or internally) of a carrier molecule may not result in loss of enzymatic activity and thermostability.

A tag may also be linked to the carrier molecule or carrier protein as a tool for purification. The tag will serve as an additional tool for purification of the carrier molecule or carrier protein. The tag may also serve as fall back tool for purification. The
15 tag refers to a peptide used for facilitating purification of a fusion protein prepared through expression by gene recombination. It is preferred that the bonding between a tag and a substance capable of binding thereto is reversible. The tag can be, for example, glutathione S-transferase with affinity for glutathione, a peptidic sequence of histidine residues where histidine has an affinity for a metal, and the like known in the art. In one
20 preferred embodiment of the invention, such a tag is His His His His His His (SEQ ID NO:2) (i.e., (His)₆). In the present invention, one more linker sequences may be positioned in the carrier protein as needed.

As used herein, the term "heterologous polypeptide or protein" refers to a polypeptide or protein of interest (for therapeutic, diagnostic or preventative use) that is
25 encoded by nucleic acid introduced into a host cell. The term heterologous polypeptide or protein does not include a thermostable enzyme or domains of a thermostable enzyme or its signal peptide. The heterologous polypeptide for purposes of this invention denotes a polypeptide of up to 100 kDa and higher and it generally refers to a polypeptide which is not endogenous to the host selected, although this definition will also include
30 endogenous peptides in cases in which overexpression of such is desired. In addition, heterologous polypeptide will also exhibit some form of useful activity, typically either antigenic activity for use in recombinant vaccines and/or immunological assays or other biological activity (for example as a peptide hormone, biological marker etc).

The heterologous polypeptides include growth factors, cytokines, ligands, receptors and inhibitors, as well as antigenic determinants and antibodies. Heterologous proteins may also include enzymes such as hydrolases including carbohydrases, and lipases. Representative polypeptides within the scope of the invention include, without
5 limitation, GFP, IFN α , antigens (or epitopes) such as from tetanus toxin, anthrax, measles virus, Mycobacterium tuberculosis, plague, and monoclonal antibodies specific for RSV, insulin, and the like.

In addition other peptides or proteins (or fragments thereof) such as epitopes from cytokines, e.g., interleukin-2 (IL-2), or granulocyte-macrophage colony stimulating factor
10 (GM-CSF) or peptides containing both T cell and B cell epitopes may also be used to recruit various effector systems of the immune system, as required. For example, based upon the available nucleotide sequences of the target pathogen, one can clone computer generated open reading frames, express the target polypeptides in an appropriate system and screen them using material from infected individuals. Target polypeptides selected
15 based on their immunoreactogenicity can be used for developing vaccine candidates, therapeutic or diagnostic reagents. The screening could provide highly time efficient and potent method and would be particularly important if one has to keep pace with emerging pathogens or disease out brakes such as SARS. Further, the carrier molecule can be used to determine appropriate vaccine antigens for developing efficacious vaccine against
20 pathogens such as SARS, tuberculosis as well as subunit vaccines (e.g., against hepatitis B using surface antigen).

One or more cleavage sites can be introduced between the carrier molecule and the heterologous polypeptide depending on the location of the heterologous polypeptide in the carrier protein. This can facilitate further purification of the target polypeptides. It
25 may also provide advantages over current protein synthesis methodologies, which result in much reactant and solvent toxic waste which must be disposed of.

For example, any of a number of prior art known cleavage sites specific to proteases or other such enzymes or chemicals useful in the efficient hydrolysis of peptide bonds may be introduced. Proteases that are active both as endo- and exopeptidases are
30 known in the art. For example, protease specific cleavage site can be introduced into a recombinant LicKM carrier protein such that the LicKM carrier molecule has at its N-terminus a poly His tag and at its C-terminus the cleavage site followed by a target

polypeptide such as an antigenic determinant and/or a therapeutic polypeptide of interest (e.g., interferon).

5 In some embodiments, for improving qualitative and quantitative parameters of target polypeptides, secretory signal sequences may be added. The use of leader sequences or secretory signal sequences are only optional, not necessary, for practicing the present invention. For example, one can construct recombinant vectors containing carrier protein with a leader sequence such as to direct the secretion of heterologous proteins into the medium used to culture various host cells.

10 Such a system would enable homogenous synthesis of the recombinant protein and the system would allow easy scaling-up and subsequent downstream processing, for example, purification. Such modifications have been made to a number of proteins known in the art.

15 The heterologous polypeptides can be fused to the carrier molecule framework as outlined above, whether at a single location or non-contiguous locations. Generally speaking, in the context of carrier proteins as vaccines, heterologous polypeptides or a sequence of amino acids containing one or more epitopes (i.e., epitope-containing segments having two or more identical or non-identical epitopes), which can stimulate an immune response that protects or prevents against an infectious disease or allergic reactions are candidate polypeptides. The use of an epitope-containing segment in which 20 two or more distinct epitopes are displayed is preferred when attempting to create bifunctional antibodies for experimental, diagnostic or therapeutic uses. The heterologous polypeptides may contain epitopes that can be B cell epitopes, T cell epitopes or a mixture of B and T cell epitopes. In some contexts, preferred epitopes are B-cell epitopes which are known to be a target for neutralizing antibodies.

25 A preferred embodiment of the present invention relates to a carrier protein having the recombinant carrier molecule fused to two or more non-contiguous epitope-containing heterologous polypeptide segments. The non-contiguous locations where fusion is appropriate are internal locations within the carrier protein moiety including the loop region, or at the N- or C-terminus of the recombinant carrier molecule.

30 It has been found in the present invention that insertions and substitutions can be made within these loop regions without disrupting the integrity of the carrier molecule or abolishing the features which make the recombinant thermostable enzymes a useful carrier for the delivery expression various polypeptides or display of epitope containing

heterologous polypeptides. Insertions and substitutions within these loop regions tend not to alter the relationships between the prominent structural features of the carrier molecule. One skilled in the art would know how to create a carrier protein of the invention by making manipulations at the nucleic acid level.

5 In some embodiments, the carrier protein will have cleavage sites such that the heterologous polypeptides fused to the C-terminus, N-terminus and/or internally of a recombinant carrier molecule of the invention can be cleaved off by specific proteases in vivo or in vitro. This allows the peptide to be administered to a cell as part of a larger fusion protein which is both easier to purify and handle as compared to free heterologous
10 polypeptide. Following cellular uptake, the heterologous polypeptide attached to the carrier molecule can be cleaved from the molecule.

One skilled in the art would know how to create a carrier protein of the invention by making manipulations at the nucleic acid level. Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and
15 restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored and religated in the form desired. Virus vectors such as plant, insect and mammalian virus vectors or bacterial plasmids can be used as vectors.

As representative examples of expression vectors can be viral particles, plasmids,
20 cosmids, bacterial artificial chromosomes, viral DNA (e.g. vaccinia, adenovirus, fowl pox virus, pseudorabies and derivatives of SV40), yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as bacteria, yeast and other fungi, plants, etc.) Thus, for example, the DNA may be included in any one of a variety of expression vectors for expressing the recombinant
25 carrier protein. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE70 (Qiagen), pBluescript SK, pBluescript KS (Stratagene); pTRC99a,, pRIT2T (Pharmacia); Eukaryotic: pWLNEO, pXT1, pSG (Stratagene) pSVK3, pSVLSV40 (Pharmacia). Any other plasmid or vector may be used as long as they are
30 replicable and viable in the host.

The recombinant DNA capable of encoding carrier protein may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art..

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) or a promoter to direct mRNA synthesis. Promoters used in the present invention can be ubiquitous or constitutive and/or tissue specific promoters from prokaryotic and eukaryotic organisms. Examples of constitutive promoters are CaMV 35S promoter, the nopaline synthase promoter, the octopine synthase promoter, the ribulose-1,5-bisphosphate carboxylase promoter, Act1, SAM synthase promoter, and Ubi promoter and the promoter of the chlorophyll a/b binding protein. Examples of tissue specific promoters are potato protease inhibitor II (pin2) gene promoter, napin gene promoter, cruciferin gene promoter, beta-conglycinin gene promoter, phaseolin gene promoter, zein gene promoter, oleosin gene promoter, acyl carrier protein stearoyl-ACP desaturase gene promoter, a fatty acid desaturase gene promoter, glycinin, Bec4 and promoters from a number of nodule genes. A number of such promoters are known in the art. Inducible promoters that specifically respond to certain chemicals (copper etc.) or heat-shock (HSP) are also contemplated. In addition, the promoters also include artificial sequences designed to function as promoters. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art. The expression vector also contain other appropriate control sequences or other regions for facilitating transcription and translation and selection.

The expression vector may be introduced into a suitable host. The host cell can be a eukaryotic cell, such as a mammalian cell, plant cell or a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Plant and animal cell cultures can also be used to produce carrier proteins of the invention. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein. Preferred host cells are plant cells and organisms are plants. Introduction of the construct into the host cell can be effected by transformation, calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation or other methods known in the art.

Depending on the host cell used, transformation is accomplished using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, which is known in the art, can be used. for prokaryotes or other cells which contain substantial cell wall barriers. Transformations into yeast are carried out according to the methods known in the art. For mammalian cells without cell walls electroporation or DNA uptake methods can be used. Insect cells known and routinely used for protein

expression purposes are also used as host cell in the present invention. Infection with *Agrobacterium tumefaciens* is used for certain plant cells. Accordingly, in the methods of the invention, plant of interest is transformed with a vector containing the carrier protein of interest to produce a transgenic plant. *Agrobacterium*-based transformation methods may be used to produce transgenic plants. Several other methods for stable transformation of plants are available in the art (see, Piruzian et al., 2002, Mol Genet Genomics 266:778-786, which is incorporated herein by reference). In the present invention, the RecLicB and LicKM constructs containing several target antigens, including RSV peptide and hepatitis B surface antigen can be expressed in plants.

10 The carrier protein of the present invention may also be expressed from a suitable viral vector after infecting a host plant with the selected viral vector. Recombinant viral vectors can be constructed by manipulating the genomic component of the wild-type viruses. Preferred viruses are RNA containing plant viruses. Although many plant viruses have RNA genomes, it is well known that organization of genetic information differs among groups. Thus, a virus can be a mono-, bi-, tri-partite virus. "Genome" refers to the total genetic material of the virus. "RNA genome" states that as present in virions (virus particles), the genome is in RNA form.

Some of the viruses which meet this requirement, and are therefore suitable, include Alfalfa Mosaic Virus (AIMV), ilarviruses, cucumoviruses such as Cucumber Green Mottle Mosaic virus (CGMMV), closteroviruses or tobamaviruses (tobacco mosaic virus group) such as Tobacco Mosaic virus (TMV), Tobacco Etch Virus (TEV), Cowpea Mosaic virus (CMV), and viruses from the brome mosaic virus group such as Brome Mosaic virus (BMV), broad bean mottle virus and cowpea chlorotic mottle virus. Additional suitable viruses include Rice Necrosis virus (RNV), and geminiviruses such as tomato golden mosaic virus (TGMV), Cassava latent virus (CLV) and maize streak virus (MSV). Each of these groups of suitable viruses are well characterized and are well known to the skilled artisans in the field. A number of recombinant viral vectors have been used by those skilled in the art to transiently express various polypeptides in plants. See, for example, U.S. Patents 5,316,931 and 6,042,832; and PCT International Publication, WO 00/46350, WO 96/12028 and WO 00/25574, the contents of which are incorporated herein by reference. Thus, the methods already known in the art can be used as a guidance to

develop recombinant viral vectors of the present invention to deliver transacting factors.

The recombinant viral vector used in the present invention can be heterologous virus vectors. The heterologous virus vectors as referred to herein are those having a recombinant genomic component of a given class of virus (for example TMV) with a movement protein encoding nucleic acid sequence of the given class of virus but coat protein (either a full-length or truncated but functional) nucleic acid sequence of a different class of virus (for example AIMV) in place of the native coat protein nucleic acid sequence of the given class of virus. Likewise, native movement protein nucleic acid sequence instead of the coat protein sequence is replaced by heterologous (i.e. not native) movement protein from another class of virus. For example, a TMV genomic component having an AIMV coat protein is one such heterologous vector. Similarly, an AIMV genomic component having a TMV coat protein is another such heterologous vector. The vectors are designed such that these vectors, upon infection, are capable of replicating in the host cell and transiently expressing the carrier protein in the host cell.

In an aspect of the invention, both viral vectors and transgenic plants are used to express the carrier proteins of the present invention in cells of a host plant by taking advantage of a transactivation system is provided. The transactivation system has two components: (i) a transgenic plant and (ii) a recombinant viral vector. The genetically transformed cells of the host plant having integrated into their nuclear genome, an inactive or silenced carrier protein encoding nucleic acid sequence, are capable of encoding the carrier protein only upon activation of the silenced sequence. To activate the silenced sequence, a recombinant RNA viral vector is used that is capable of infecting the cells of the host plant and encoding therein a factor for activating the expression of inactive or silenced carrier protein nucleic acid sequence.

The carrier protein encoding nucleic acid sequence may be silenced by placing a blocking sequence between promoter sequence and the carrier protein encoding nucleic acid sequence. The blocking sequence (e.g., a selectable marker element or any other nucleic acid sequence (stuffer) should be sufficient enough to block the promoter's ability to drive expression of the gene. The blocking sequence must be flanked on each side by a recombinase target site (e.g., "FRT" site) with a defined 5' to 3' orientation. The FRT refers to a nucleic acid sequence at which the product of

the FLP gene, i.e., FLP recombinase, can catalyze the site-specific recombination. In addition to the genomic elements necessary for infection, replication, movement and spread of the viral vectors, the vectors contain sequences encoding a recombinase (e.g., FLP) or other factor (e.g., GAL4-VP16) to activate the silenced carrier protein encoding nucleic acid sequence.

In accordance with the present invention, the host plants included within the scope of the present invention are all species of higher and lower plants of the Plant Kingdom. Mature plants, seedlings, and seeds are included in the scope of the invention. A mature plant includes a plant at any stage in development beyond the seedling. A seedling is a very young, immature plant in the early stages of development. Specifically, plants that can be used as hosts to produce foreign sequences and polypeptides include and are not limited to Angiosperms, Bryophytes such as Hepaticae (liverworts) and Musci (mosses); Pteridophytes such as ferns, horsetails, and lycopods; Gymnosperms such as conifers, cycads, Ginkgo, and Gnetales; and Algae including Chlorophyceae, Phaeophyceae, Rhodophyceae, Myxophyceae, Xanthophyceae, and Euglenophyceae.

Host plants used for the production of carrier proteins can be grown either in vivo and/or in vitro depending on the type of the selected plant and the geographic location. It is important that the selected plant is amenable to cultivation under the appropriate field conditions and/or in vitro conditions including cell culture.

Among angiosperms, the use of crop and/or crop-related members of the families are particularly contemplated. The plant members used in the present methods also include interspecific and/or intergeneric hybrids, mutagenized and/or genetically engineered plants. These families include and not limited to Leguminosae (Fabaceae) including pea, alfalfa, and soybean; Gramineae (Poaceae) including rice, corn, wheat; Solanaceae particularly of the genus *Lycopersicon*, particularly the species *esculentum* (tomato), the genus *Solanum*, particularly the species *tuberosum* (potato) and *melongena* (eggplant), the genus *Capsicum*, particularly the species *annuum* (pepper), tobacco, and the like; Umbelliferae, particularly of the genera *Daucus*, particularly the species *carota* (carrot) and *Apium*, particularly the species *graveolens dulce*, (celery) and the like; Rutaceae, particularly of the genera *Citrus* (oranges) and the like; Compositae, particularly the genus *Lactuca*, and the species *sativa* (lettuce), and the like and the Family Cruciferae, particularly of the genera

Brassica and *Sinapis*. Examples of "vegetative" crop members of the family Brassicaceae include, but are not limited to, digenomic tetraploids such as *Brassica juncea* (L.) Czern. (mustard), *B. carinata* Braun (ethopian mustard), and monogenomic diploids such as *B. oleracea* (L.) (cole crops), *B. nigra* (L.) Koch (black mustard), *B. campestris* (L.) (turnip rape) and *Raphanus sativus* (L.) (radish). Examples of "oil-seed" crop members of the family Brassicaceae include, but are not limited to, *B. napus* (L.) (rapeseed), *B. campestris* (L.), *B. juncea* (L.) Czern. and *B. tournifortii* and *Sinapis alba* (L.) (white mustard). Flax plants are also contemplated.

Particularly preferred host plants are those that can be infected by AIMV. For example, it is known in the art that alfalfa mosaic virus has full host range. Other species that are known to be susceptible to the virus are: *Abelmoschus esculentus*, *Ageratum conyzoides*, *Amaranthus caudatus*, *Amaranthus retroflexus*, *Antirrhinum majus*, *Apium graveolens*, *Apium graveolens* var. *rapaceum*, *Arachis hypogaea*, *Astragalus glycyphyllos*, *Beta vulgaris*, *Brassica campestris* ssp. *rapa*, *Calendula officinalis*, *Capsicum annuum*, *Capsicum frutescens*, *Caryopteris incana*, *Catharanthus roseus*, *Celosia argentea*, *Cheiranthus cheiri*, *Chenopodium album*, *Chenopodium amaranticol*, *Chenopodium murale*, *Chenopodium quinoa*, *Cicer arietinum*, *Cichium endiva*, *Ciandrum sativum*, *Crotalaria spectabilis*, *Cucumis melo*, *Cucumis sativus*, *Cucurbita pepo*, *Cyamopsis tetragonoloba*, *Daucus carota* (var. *sativa*), *Dianthus barbatus*, *Dianthus caryophyllus*, *Emilia sagittata*, *Fagopyrum esculentum*, *Glycine max*, *Gomphrena globosa*, *Helianthus annuus*, *Lablab purpureus*, *Lactuca sativa*, *Lathyrus odatus*, *Lens culinaris*, *Linum usitatissimum*, *Lupinus albus*, *Lycopersicon esculentum*, *Macroptilium lathyroides*, *Malva parviflora*, *Matthiola incana*, *Medicago hispida*, *Medicago sativa*, *Melilotus albus*, *Nicotiana bigelovii*, *Nicotiana clevelandii*, *Nicotiana debneyi*, *Nicotiana glutinosa*, *Nicotiana megalosiphon*, *Nicotiana rustica*, *Nicotiana sylvestris*, *Nicotiana tabacum*, *Ocimum basilicum*, *Petunia* × *hybrida*, *Phaseolus lunatus*, *Phaseolus vulgaris*, *Philadelphus*, *Physalis flidana*, *Physalis peruviana*, *Phytolacca americana*, *Pisum sativum*, *Solanum demissum*, *Solanum melongena*, *Solanum nigrum*, *Solanum nodiflum*, *Solanum rostratum*, *Solanum tuberosum*, *Sonchus oleraceus*, *Spinacia oleracea*, *Stellaria media*, *Tetragonia tetragonoides*, *Trifolium dubium*, *Trifolium hybridum*, *Trifolium incarnatum*, *Trifolium pratense*, *Trifolium repens*, *Trifolium subterraneum*,

Tropaeolum majus, *Viburnum opulus*, *Vicia faba*, *Vigna radiata*, *Vigna unguiculata*,
Vigna unguiculata ssp. sesquipedalis, and *Zinnia elegans*.

In an aspect, the present invention also includes methods for stimulating an immune response in an animal. The use of carrier protein of the invention to stimulate
5 immune response is described in more detail in the following Examples section. Specifically, the experiments demonstrate, for example, that the immunogenic heterologous polypeptides containing B-cell and T-cell epitopes in the carrier fusion protein stimulated pathogen specific immune responses. Surprisingly, the target specific immunogenicity of antigenic determinants fused to carrier molecule of the present
10 invention is significantly superior to that of antigenic determinants administered alone without the carrier molecule. Further, the experiments demonstrate that it is possible to generate a humoral immune response to an internally inserted epitope-containing polypeptide segments. Although the in vivo data reported herein were generated in experiments employing murine assays for the generation of antibodies against the carrier
15 proteins, the fundamental principles are applicable to humans as well as other animals such as rabbits, pigs, goats, monkeys and chimpanzees. Given the disclosure of the subject application and the general knowledge of one skilled in the art, it is a matter of routine experimentation to select heterologous polypeptides of interest and incorporate such polypeptides of interest into a carrier molecule for use as an immunogen. One of
20 skill in the art can identify heterologous polypeptides with B-cell epitopes which have the ability to drive a strong humoral immune response following administration to an animal. The B-cell epitope which is selected will depend upon the intended use of the carrier protein. For instance, if the carried protein is to be used as a vaccine, the heterologous polypeptides can be derived from a protein which is expressed by a virus, bacteria or
25 other infectious organism associated with causing a disease. The heterologous polypeptide, which is selected, should be one which contains epitopes which elicit strong immune responses. In general, this will include proteins found on the surface of the infectious organism which are involved in binding and to which antibodies have a high degree of access.

30 The selection of immunogenic heterologous polypeptides is not limited to proteins associated with infectious organisms. For instance, the carrier protein containing an internally (or at the N or C-terminus) inserted polypeptide from a prostate-specific antigen may be used to induce a strong immune response. One of skill in the art will

recognize that any heterologous polypeptide containing one or more B-cell or T-cell epitopes, which is capable of driving a humoral immune response can be included as part of the carrier protein of the present invention. Many such heterologous polypeptides are known and others can be determined through routine experimentation.

5 In some instances, it is desired to stimulate cytotoxic T-cells as part of a cellular immune response. In such instances, heterologous polypeptides with T-cell epitopes are fused to the carrier molecule, preferably inserted internally within the carrier. Cytotoxic T-cells play an important role in the surveillance and control of viral infections, bacterial infections, parasitic infections and cancer, for example. protocols of T-cell activation
10 allow the triggering of more selective cytotoxic T-cell responses with greater therapeutic effectiveness.

Generally, the fusion of peptides to the C-terminus of carrier molecule with a cleavage site in between, may generate a desirable construct, which is cleavable, in vivo, by the recombinant carrier protein-specific cleavage agent. The carrier protein-specific
15 cleavage agent (e.g., proteases) cleaves carrier protein fusion after a C-terminal residue thereby releasing the C-terminal peptide.

Thus, the carrier protein based vaccine can be used to drive a cellular and/or humoral immune response depending on the type of heterologous polypeptides fused to the carrier protein. The therapeutic amount of the carrier protein given to an animal
20 species will be determined as that amount deemed effective in eliciting the desired immune response. The carrier protein is administered in a pharmaceutically acceptable or compatible carrier or adjuvant. Accordingly, the present invention also encompasses pharmaceutical compositions for the administration of carrier proteins. Examples of specific diseases which can be treated in this manner include, for example, infection with
25 HIV, cancer, gastrointestinal diseases, respiratory infections etc. The pharmaceutical compositions are prepared by methods known to one of skill in the art. In general, the carrier protein is admixed with a carrier and other necessary diluents which are known in the art to aid in producing a product which is stable and administrable. Administration of the pharmaceutical composition can be accomplished by several means known to those of
30 skill in the art. These include, i.p., oral, intradermal, subcutaneous, intranasal, intravenous or intramuscular. Typically patients to be treated are dosed subcutaneously with the carrier proteins once per week for several weeks. However, dosing can also be done

orally or intranasally over a similar length of time. The result is a reduction of the allergic and/or autoimmune responses.

In addition to the conventional vaccination methods, the present invention can be used for DNA vaccination. In this method, DNA encoding the appropriate carrier protein is introduced into the cells of an organism. Within these cells, the epitope-containing carrier protein is directly expressed. Direct expression of the carrier proteins of the present invention by endogenous cells of a vaccinated animal allows for the continual stimulation of humoral and cellular immune responses over an extended period of time. Direct expression can be accomplished by introducing DNA constructs which encode the desired carrier protein into the cells of an animal. The constructs typically contain promoter elements and other transcriptional control elements which direct the expression of the carrier protein. Introduction of the DNA construct can be by any conventional means including direct injection. The preferred administration site is muscle tissue. This direct expression is in contrast to standard immunization protocols whereby the vaccine is injected at a single site one or more times. Following injection, the vaccine is disseminated to lymphoid organs where a single immune response occurs.

EXAMPLES

The examples presented below are provided as a further guide to one of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

Example 1: Construction of Carrier Molecules and Carrier proteins

This example addresses construction of the carrier protein expression vector for expression in prokaryotic and eukaryotic cells.

Shown in Figure 1 is a schematic representation of engineering of recombinant carrier molecules LicKM and recLicB. Letter "I" indicates the loop structure, A indicates the region (domain) upstream of the loop structure and C indicates the region (domain) downstream of the loop structure. To create LicKM the gene encoding a mature Lic B was split at the loop region and assembled as shown. Unique cloning sites were created during engineering. The sequence for the engineered gene (LicKM) is shown in part B of figure 1.

The LicKM was created in 2 step PCR cloning. 5 and 3' primers were used to amplify the *lic B* gene into 2 fragments designated as A (159 nucleotides of the *lic B* gene, 364 trough 522) and C (486 nucleotides of the *lic B* gene, 523 trough 1009). In the final clone, fragment A was cloned downstream of fragment C preserving the original amino acid composition.

The following are the specific primers used

Fragment C:

5' primer: 5'gga tcc ATG GGC GGT TCA TAT CCG TAT-3' (SEQ ID NO:10)

3' primer: 5'g cag aga TCT ATA TTC CCT GTC AAG GGT-3' (SEQ ID NO:11)

Fragment A:

5' primer: 5'aga tcc ATG GTG GTA AAT ACG CCT TTT-3' (SEQ ID NO:12)

3' primer: 5'g cac aga TCT ACC GTT AGG ATA GTA TTT TAC-3' (SEQ ID NO:13).

Shown in Figure 1C is a schematic of construction of rec LicB from the wildtype LicB.

Example 2: Cloning and Expression of GFP Using recLic B

The recLic B was symbolically divided into 3 regions as shown in the Figure 2; I is the loop structure. The region (domain) upstream of the loop structure is indicated as A and downstream of loop structure is indicated as C. To use the recLic B as a carrier molecule unique cloning sites (*Bam*HI and *Bgl*II) were introduced into the loop region of the gene. The gene encoding GFP (green fluorescent protein) was cloned into the loop region of recLic B to obtain recLic B-GFP (Fig. 2). The recombinant protein was expressed using both *Escherichia coli* and yeast expression system (Fig. 3). Target polypeptides can be inserted not only into the loop structure as it is shown in this example but can also be fused to the N or C terminus of carrier protein.

Example 3: Fermentation and Carrier Protein Recovery.

E. coli dH5alpha cells transformed with recLic B-GFP constructs were cultured or fermented by overnight culturing process in LB media. The fermentation was continued for 12 h and harvested at a cell density of 10^4 . Two liters of cell culture or fermentation broth were divided into 1 liter containers/ bottles and centrifuged at

10,000 rpm for 30 min in a centrifuge. The supernatant was discarded and the pellet was used to recover the carrier protein.

5 **Example 4: Cloning and Expression of Various Target Polypeptides Using the Engineered LicKM**

This example addresses the cloning and expression of the following three target polypeptides using the engineered LicKM:

- a. Peptide from G protein of respiratory syncytial virus (24 a.a.)
- b. GFP (27 kD)
- 10 c. IFN α .(19 kD)

To demonstrate the capacity of engineered LicKM as a carrier molecule, 3 constructs were created where the target sequences polypeptides (a) fragment of DNA encoding 24 amino acid peptide from respiratory syncytial virus G protein, (b) open reading frame of GFP or (c) open reading frame of human interferon α were PCR
15 amplified and cloned into the open reading frame of engineered LicKM as shown in figure 4. These three engineered target polypeptides were expressed in *E. coli* as shown in figure 5 and yeast (data not shown). Shown in Figure 5A is a zymogram of lichenase activity in bacterial extracts detected in the presence of 0.1% lichenan as substrate. Proteins were separated in 12% PAGE. The gel was loaded with proteins
20 extracted from *E. coli* strain XL-1 blue. C is a negative control. LicKM is engineered carrier molecule. LicKM-RSV, LicKM-GFP, and LicKM-IFN α are engineered proteins containing respective target polypeptide. Figure 5B shows the results of Western blot analysis. Proteins were separated in 12% PAGE, electroblotted onto nylon membrane and reacted with monoclonal antibodies specific for peptide from
25 RSV G protein. Antibodies reacted with LicKM-RSV, RSV positive control (RSV (C+)) and plant virus coat protein containing identical peptide (RSV (plant)). Extracts from LicKM that did not contain target peptide had no specificity to RSV antibodies.

30 **Example 5: Immunization of Mice with LicKM-RSV Containing 24 Amino acid Peptide from RSV G protein.**

Eight-week-old female balB/c mice were immunized with 200 μ g per dose of recombinant LicKM-RSV engineered to express the 24 amino acid (171-191 of G protein) of RSV G protein (Johnson et al., 2004, J Virol. 2004 Jun;78(11):6024-32).

Three immunizations of 0.1 ml were administered intra-peritoneally at intervals of 2 weeks (first dose with complete Freund's adjuvant (CFA) at a 1:1, vol:vol ratio, second dose with incomplete Freund's adjuvant (CFA) at a 1:1, vol:vol ratio and third dose without any adjuvant). An equal quantity of LicKM was used as a control.

- 5 Samples of pre-immune sera were collected 1 day before first dose of antigen. Twelve (12) days after each immunization serum samples were obtained from individual mice and RSV-specific antibody titers assessed. Antigen-specific antibody analysis of serum was performed using a solid phase enzyme-linked immunoabsorbant assay (ELISA). ELISA plates (Nunc Polysorp, Denmark) were coated with 100 µl per well
- 10 (1.0 µg per well) of Recombinant AlMV containing identical peptide from RSV G protein (10 µg/ml in Phosphate-buffered saline) overnight at room temperature (RT; about 25 °C). Coated plates were washed 3x with PBS-Tween (0.05%) and then blocked with 0.5 % of I-block (Tropix) in PBS-Tween at RT for at least 1 hour. A series of dilutions of sera were added to the plates (30 µl/well) for 2 to 4 hours at RT.
- 15 The plates were then washed 3x with PBS-Tween and peroxidase-conjugated secondary antibodies (goat anti-mouse IgG, either whole molecule or gamma chain specific), were added (100 µl per well) at a final dilution of 1:10,000 in PBS-Tween, for 1 hour at RT. Plates were then washed 5x with PBS-Tween and OPD (Sigma Fast™) substrate added (100 µl/well) in phosphate-citrate buffer containing urea, for
- 20 30 min at RT in the dark. The reaction was stopped with 2M H₂SO₄ (50 µl per well) and the color change resulting from bound specific antibody measured at 490 nm in an ELISA plate-reader (Spectramax Plus³⁸⁴). The results, expressed in O.D. units, are shown in Figure 6.
- 25 **Example 6: Engineering and experimental immunization of mice with LicKM-F200 containing 200 amino acid portion of RSV F protein.**

Engineering of LicKM-F200 was carried out as follows: As template DNA, plasmid DNA containing cDNAs for F, G, and M genes of RSV obtained from National Institute of Health, USA, was used (Johnson et al., 2004, J Virol. 2004 Jun;78(11):6024-32).

For cloning a portion of F gene encoding amino acids 324 to 524 was amplified using 5'- GCAC AGATCT GGGTCCAACATCTGTTTAAC -3' (SEQ ID NO:14). and 5'- GCAC AAGCTT ATTTGTGGTGGATTACCA -3' (SEQ ID NO:15). as 5' and 3' primers. PCR amplified fragment was digested and cloned into final vector using unique

restriction sites introduced during PCR reaction (*Bgl*III site at 5'- and *Hind*III at 3'-end, respectively). Target DNA was cloned into *E. coli*, agrobacterial and plant virus expression vectors. Results described in this example obtained using LicKM-F200 where target gene is cloned and expressed plant virus vector D4.

5 For expression, plants were inoculated with in vitro synthesized transcripts of LicKM-F200. Plant inoculations were carried out using the prior art known procedures. See, PCT International Publication, WO 00/46350 for guidance on infectious RNA transcripts and procedures for viral infection. Two weeks after inoculation samples were collected for analysis of target protein expression as well as recovery. Recombinant protein
10 maintained enzymatic activity (Fig. 7A) and was recognized by antibodies specific to LicKM (Fig. 7B).

For stimulating immune response, eight-week-old female balB/c mice were immunized with 200 µg per dose of recombinant LicKM-F200 engineered to express the 200 amino acids (amino acid 324 to 524 of F protein) of RSV F protein. Three
15 doses of antigen (0.1 ml/dose) were administered intra-peritoneally at intervals of 2 weeks (first dose with complete Freund's adjuvant (CFA) at a 1:1, vol:vol ratio, second dose with incomplete Freund's adjuvant (CFA) at a 1:1, vol:vol ratio and third dose without any adjuvant). An equal quantity of LicKM was used as a control. Samples of pre-immune sera were collected 1 day before first dose of antigen. Twelve
20 (12) days after each immunization serum samples were obtained from individual mice and RSV-specific antibody titers assessed. Antigen-specific antibody analysis of serum was performed using a solid phase enzyme-linked immunoabsorbant assay (ELISA). ELISA plates (Nunc Polysorp, Denmark) were coated with 100 µl per well (1.0 µg per well) of inactivated RSV Long strain (Hy Test, 10 µg/ml in Phosphate-buffered saline) overnight at room temperature (RT; about 25 °C). Coated plates were
25 washed 3x with PBS-Tween (0.05%) and then blocked with 0.5 % of I-block (Tropix) in PBS-Tween at RT for at least 1 hour. A series of dilutions of sera were added to the plates (30 µl/well) for 2 to 4 hours at RT. The plates were then washed 3x with PBS-Tween and peroxidase-conjugated secondary antibodies (goat anti-mouse IgG, either whole molecule or gamma chain specific), were added (100 µl per well) at a
30 final dilution of 1:10,000 in PBS-Tween, for 1 hour at RT. Plates were then washed 5x with PBS-Tween and OPD (Sigma Fast™) substrate added (100 µl/well) in phosphate-citrate buffer containing urea, for 30 min at RT in the dark. The reaction

was stopped with 2M H₂SO₄ (50 µl per well) and the color change resulting from bound specific antibody measured at 490 nM in an ELISA plate-reader (Spectramax Plus³⁸⁴). The results, expressed in O.D. units, are shown in Figure 8.

5 **Example 7: Engineering and experimental immunization of mice with LicKM-PAD4 containing 145 amino acid domain four of anthrax PA protein:**

Engineering of LicKM-PAD4 was carried out as follows:

As template DNA, *E. coli* plasmid DNA containing whole Domain four (amino acids 621 to 760) of anthrax protective antigen was obtained from NMRC
10 (Moayeri et al., 2004, Curr Opin Microbiol., 7(1):19-24).

For cloning Domain four encoding amino acids 621 to 760 was amplified using 5' GCACAGATCTAATATTTTAATAAGAGATAAACG 3' (SEQ ID NO:16).and 5'GCACAAGCTT TCCTATCTCATAGCCTTTTT 3' (SEQ ID NO:17).as 5' and 3' primers. PCR amplified fragment was digested and cloned into
15 final vector using unique restriction sites introduced during PCR reaction (BglII site at 5'- and HindIII at 3'-end, respectively). Target DNA was cloned into *E. coli*, agrobacterial and plant virus expression vectors. Results described in this example obtained using LicKM-PAD4 where target gene is cloned and expressed plant virus vector D4.

20 For expression, tobacco plants were inoculated with *in vitro* synthesized transcripts of LicKM-PAD4. Plant inoculations procedures remain the same as in the above example. Two weeks after inoculation tissue samples were collected for analysis of target protein expression as well as recovery. Recombinant protein was recognized by antibodies specific to protective antigen of anthrax (Fig. 9).

25 For inducing immune response, eight-week-old female balB/c mice were immunized with 200 µg per dose of recombinant LicKM-PAD4 engineered to express the 145 amino acid (amino acids 621 to 760 of PA protein) of anthrax PA protein. Three immunizations of 0.1 ml were administered intra-peritoneally at intervals of 2 weeks (first dose with complete Freund's adjuvant (CFA) at a 1:1, vol:vol ratio,
30 second dose with incomplete Freund's adjuvant (CFA) at a 1:1, vol:vol ratio and third dose without any adjuvant). An equal quantity of LicKM was used as a control. Samples of pre-immune sera were collected 1 day before first dose of antigen. Twelve (12) days after each immunization serum samples were obtained from individual mice

and RSV-specific antibody titers assessed. Antigen-specific antibody analysis of serum was performed using a solid phase enzyme-linked immunoabsorbant assay (ELISA). ELISA plates (Nunc Polysorp, Denmark) were coated with 100 μ l per well (1.0 μ g per well) of recombinant PA (10 μ g/ml in Phosphate-buffered saline) overnight at room temperature (RT; about 25 °C). Coated plates were washed 3x with PBS-Tween (0.05%) and then blocked with 0.5 % of I-block (Tropix) in PBS-Tween at RT for at least 1 hour. A series of dilutions of sera were added to the plates (30 μ l/well) for 2 to 4 hours at RT. The plates were then washed 3x with PBS-Tween and peroxidase-conjugated secondary antibodies (goat anti-mouse IgG, either whole molecule or gamma chain specific), were added (100 μ l per well) at a final dilution of 1:10,000 in PBS-Tween, for 1 hour at RT. Plates were then washed 5x with PBS-Tween and OPD (Sigma Fast) substrate added (100 μ l/well) in phosphate-citrate buffer containing urea, for 30 min at RT in the dark. The reaction was stopped with 2M H₂SO₄ (50 μ l per well) and the color change resulting from bound specific antibody measured at 490 nM in an ELISA plate-reader (Spectramax Plus³⁸⁴). The results, expressed in O.D. units, are shown in Figure 10.

LicKM-HbsAg was also expressed in plants. Tobacco plants are used to produce target antigens as fusions with carrier protein.

All publications, patents and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications, patents and patent applications referred to herein are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. While this invention has been described with a reference to specific embodiments, it will be obvious to those of ordinary skill in the art that variations in these methods and compositions may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the claims.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

2010200667 08 Aug 2012

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A recombinant polypeptide comprising a modified lichenase B polypeptide that has an amino acid sequence with at least 90% sequence identity to amino acids 14-231 of SEQ ID NO: 3, or an amino acid sequence with at least 90% sequence identity to amino acids 8-226 of SEQ ID NO: 6, wherein the modified lichenase B polypeptide is optionally fused to a heterologous polypeptide sequence that is not found in the modified lichenase B polypeptide.
2. The recombinant polypeptide of claim 1, wherein the modified lichenase B polypeptide has an amino acid sequence with at least 95% sequence identity to amino acids 14-231 of SEQ ID NO: 3, or at least 95% sequence identity to amino acids 8-226 of SEQ ID NO: 6.
3. The recombinant polypeptide of claim 1, wherein the modified lichenase B polypeptide has the sequence of amino acids 14-231 of SEQ ID NO: 3, or the amino acid sequence of amino acids 8-226 of SEQ ID NO: 6.
4. The recombinant polypeptide of claim 1, wherein the recombinant polypeptide is encoded by SEQ ID NO: 1 or 5.
5. The recombinant polypeptide of claim 1, wherein the recombinant polypeptide comprises the sequence set forth in SEQ ID NO: 3 or 6.
6. The recombinant polypeptide of claim 1, wherein the heterologous polypeptide sequence is fused to the modified lichenase B polypeptide so as to create a fusion protein.
7. The recombinant polypeptide of claim 6, wherein the heterologous polypeptide sequence is fused to the N-terminus or C-terminus of the modified lichenase B polypeptide, or is fused internally into the modified lichenase B polypeptide.
8. The recombinant polypeptide of claim 6, wherein the recombinant polypeptide is fused to two or more heterologous epitope-containing polypeptide segments so as to create a fusion protein.
9. The recombinant polypeptide of claim 1, wherein the heterologous polypeptide sequence comprises a therapeutic polypeptide or disease-associated epitope.

2010200667 30 Jan 2012

10. The recombinant polypeptide of claim 1, wherein the heterologous polypeptide comprises a B cell epitope, a T cell epitope, a growth factor, a cytokine, a ligand, a receptor, an inhibitor, an antigen, an epitope, an antibody, a hydrolase, green fluorescent protein, an interferon, an interleukin, a pathogen-associated protein, or a toxin.
11. The recombinant polypeptide of claim 1, wherein the heterologous polypeptide sequence comprises a vaccine antigen.
12. The recombinant polypeptide of claim 1, further comprising a tag usable for separation or purification, or both.
13. The recombinant polypeptide of claim 12, wherein the tag is His₆.
14. The recombinant polypeptide of claim 6, further comprising an enzymatic cleavage site.
15. The recombinant polypeptide of claim 14, wherein the cleavage site is located between the heterologous polypeptide sequence and the modified lichenase B polypeptide.
16. The recombinant polypeptide of claim 1, further comprising a tag and a cleavage site, wherein the cleavage site is located adjacent to the tag.
17. The recombinant polypeptide of claim 1, further comprising a linker.
18. A nucleic acid that encodes the recombinant polypeptide of any one of claims 1-17.
19. An expression vector comprising the nucleic acid of claim 18.
20. The expression vector of claim 19, wherein the vector is a plant virus vector.
21. A host cell comprising the expression vector of claim 20.
22. The host cell of claim 21, wherein the host cell is a plant cell.
23. A composition comprising:
 - the recombinant polypeptide of any one of claims 1-17; and
 - a pharmaceutically acceptable carrier medium or adjuvant.

2010200667 30 Jan 2012

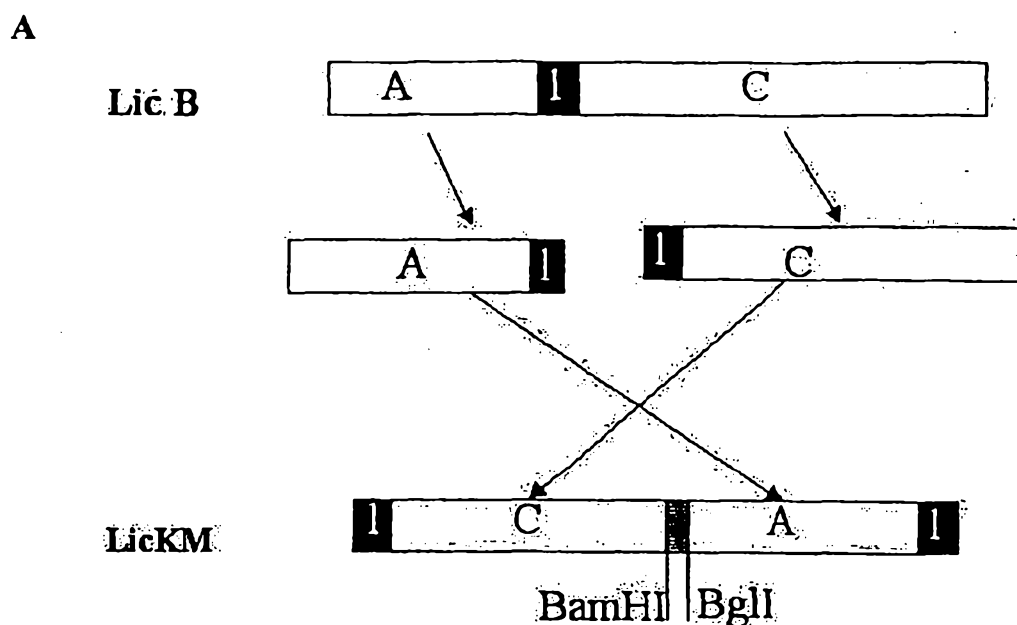
24. A method for production of a recombinant polypeptide in a plant, comprising:

- (a) providing a plant containing an expression cassette having a nucleic acid encoding the recombinant polypeptide of any one of claims 1-17 operably linked to a promoter such that expression of the cassette results in expression of the polypeptide; and
- (b) growing said plant under conditions in which the nucleic acid is expressed and the polypeptide is produced.

25. The method of claim 24, wherein the promoter is selected from the group consisting of: plant constitutive promoters, plant virus promoters, and plant tissue specific promoters.

26. The method of claim 24, wherein the recombinant polypeptide is expressed in leaf, root, or seed of the plant.

27. Use of the recombinant polypeptide of any one of claims 1-17 in the manufacture of a medicament for treating one or more diseases of an animal or for stimulating an immune response in an animal, wherein the recombinant polypeptide comprises a lichenase B polypeptide fused to a heterologous polypeptide sequence.

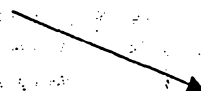


B

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 CAG TGG GAA AAA GCG GAT TGG GCG AAC GGT TCG GTG TTC AAC TGT GTT TGG AAG
 CCT TCA CAG GTG ACA TTT TCG AAC GGT AAA ATG ATT TTG ACC CTT GAC AGG GAA
 TAT aga tct3'

Figure 1

Pro-Thr-box



recLic B

Figure 1 C

D

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Figure 1

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H

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Figure 1

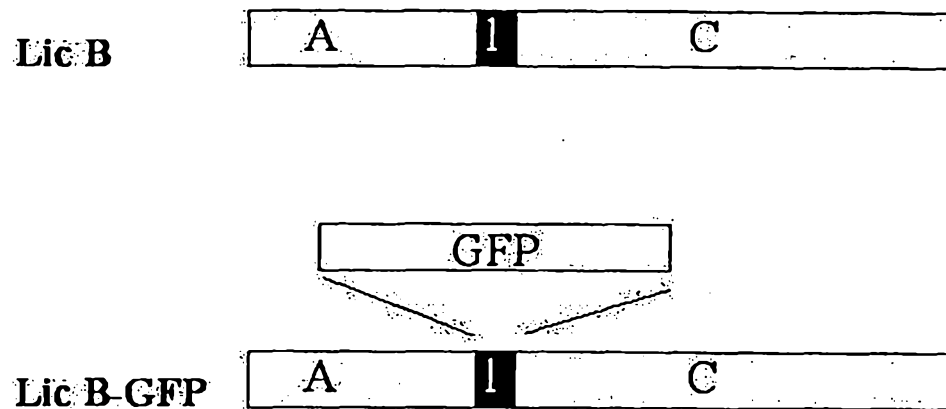


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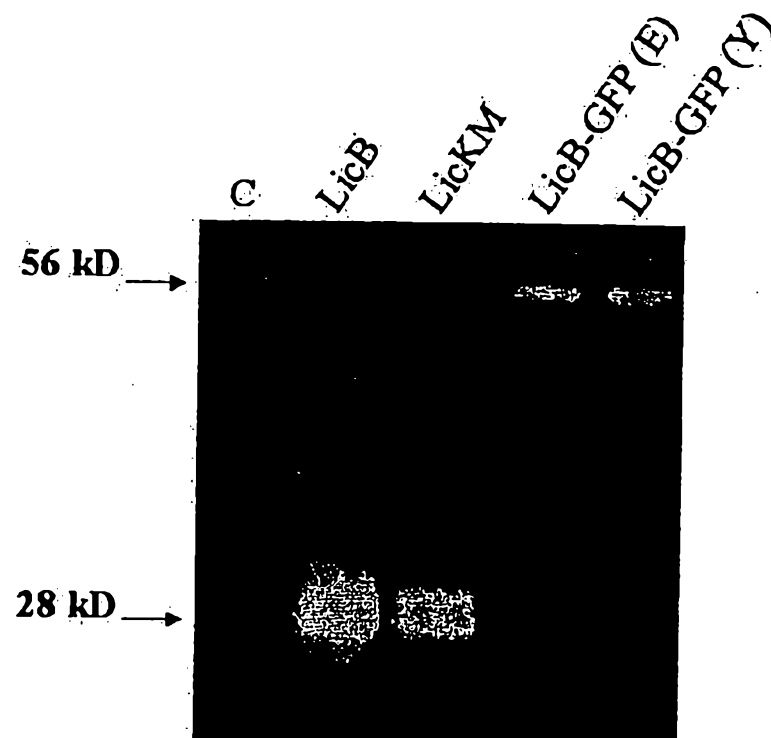


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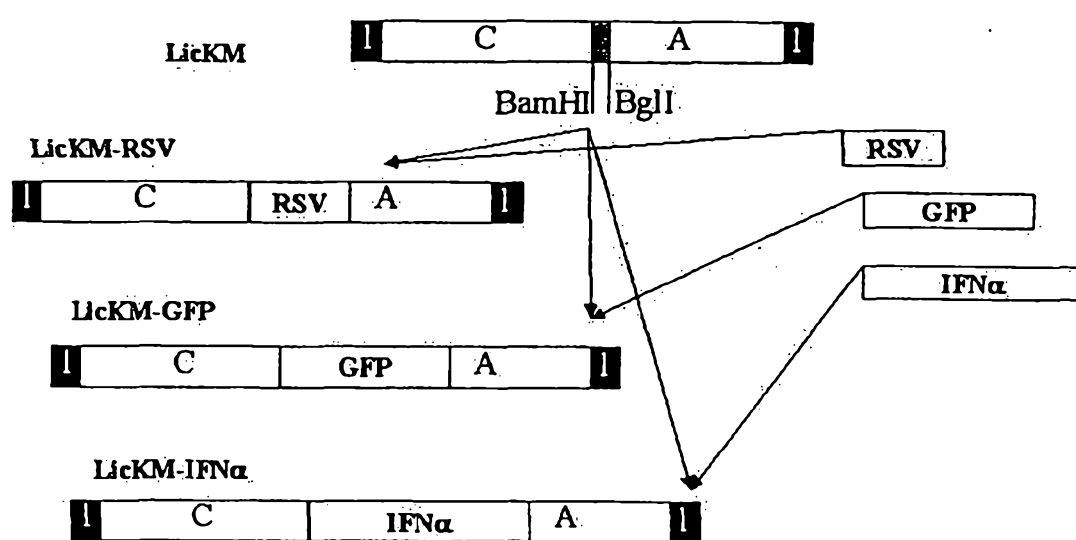
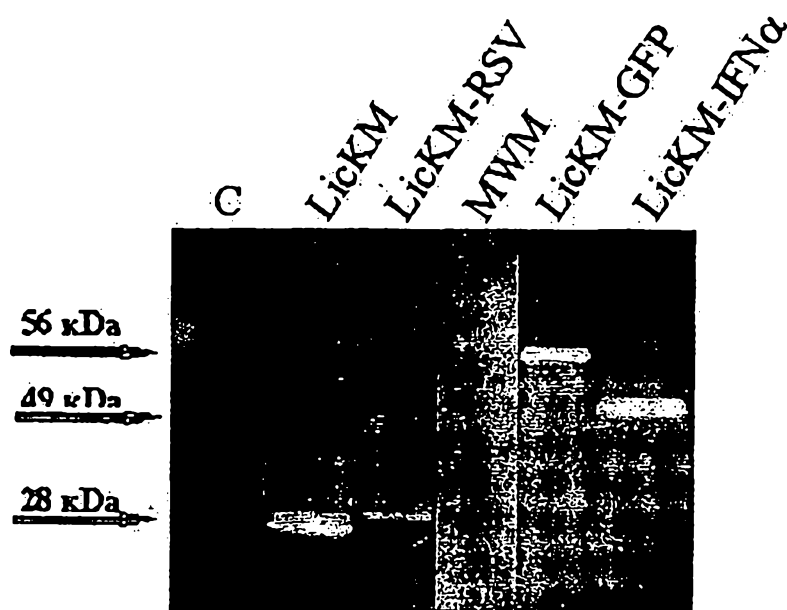


Figure 4

A



B

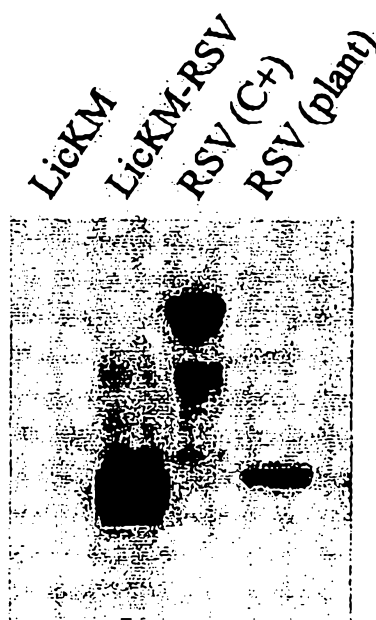


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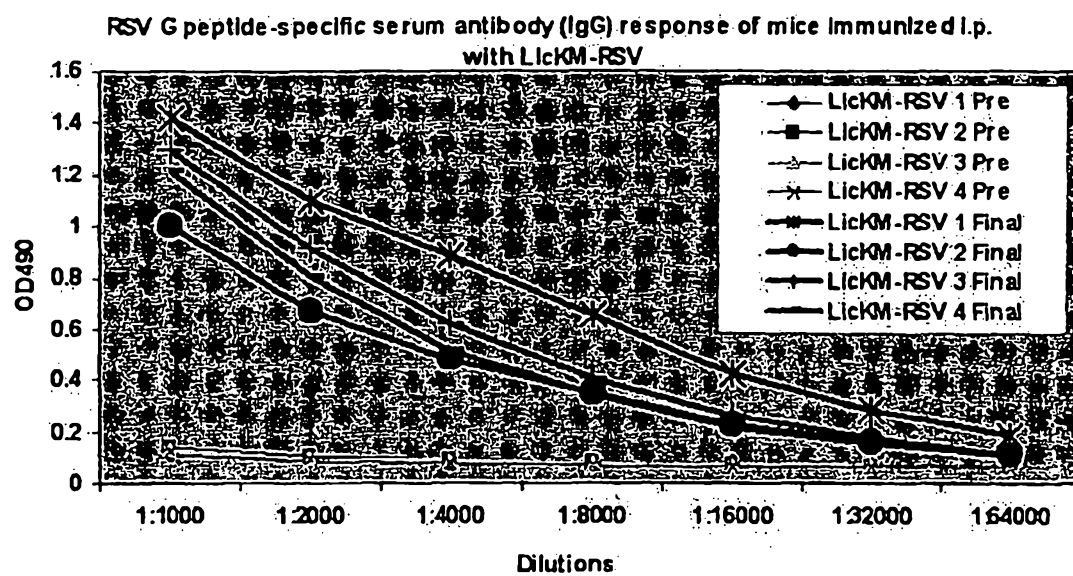


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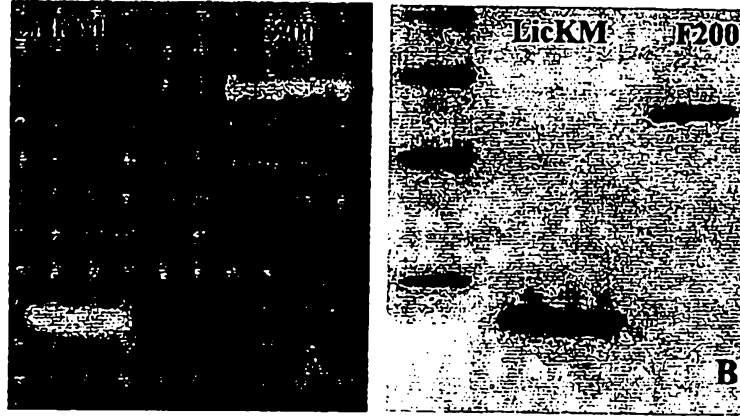


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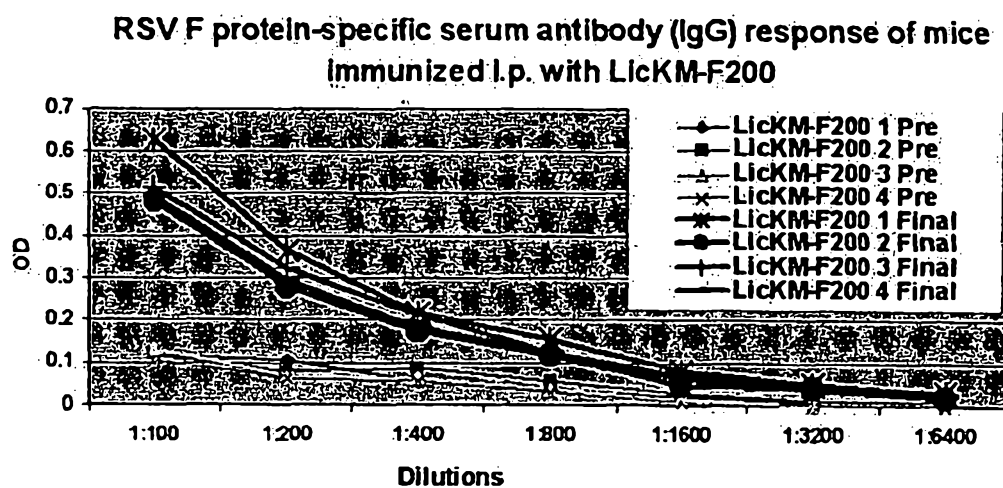


Figure 8

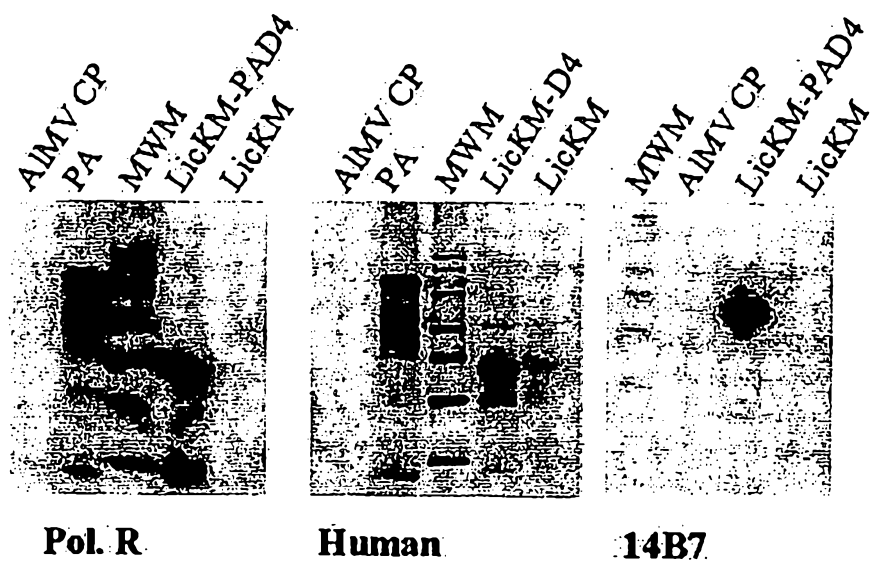


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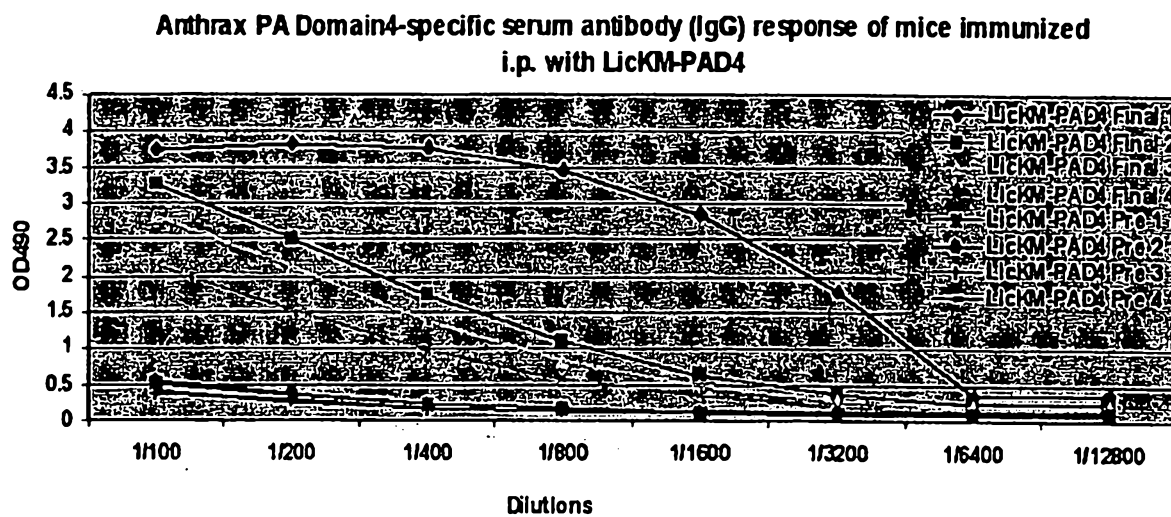


Figure 10

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<120> New Carrier Molecule for Expression, Delivery and Purification of Target Polypeptides
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2010200667 23 Feb 2010

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 35 40 45

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 Page 2

23 Feb 2010
2010200667

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2010200667 23 Feb 2010

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2010200667 23 Feb 2010

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 35 40 45

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Leu Gly Lys Asp Thr Thr Lys Val Gln Phe Asn Trp Tyr Lys Asn Gly
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Val Gly Gly Asn Glu Tyr Leu His Asn Leu Gly Phe Asp Ala Ser Gln
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Asp Phe His Thr Tyr Gly Phe Glu Trp Arg Pro Asp Tyr Ile Asp Phe
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Tyr Val Asp Gly Lys Lys Val Tyr Arg Gly Thr Arg Asn Ile Pro Val
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23 Feb 2010
2010200667

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23 Feb 2010

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2010200667

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2010200667 23 Feb 2010

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