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(54) Title: PEPTIDES FOR VACCINES AGAINST BIRCH ALLERGY

(57) Abstract: The present invention relates to compositions comprising peptides for preventing or treating allergy to birch, and in particular to optimal combinations of peptides for preventing or treating said allergy.

PEPTIDES FOR VACCINE AGAINST BIRCH ALLERGY

Field of the Invention

The present invention relates to compositions for preventing or treating allergy to birch.

Background of the Invention

T-cell antigen recognition requires antigen presenting cells (APCs) to present antigen fragments (peptides) on their cell surface in association with molecules of the major histocompatibility complex (MHC). T cells use their antigen specific T-cell receptors (TCRs) to recognise the antigen fragments presented by the APC. Such recognition acts as a trigger to the immune system to generate a range of responses to eradicate the antigen which has been recognised.

Recognition of external antigens by the immune system of an organism, such as man, can in some cases result in diseases, known as atopic conditions. Examples of the latter are the allergic diseases including asthma, atopic dermatitis and allergic rhinitis. In this group of diseases, B lymphocytes generate antibodies of the IgE class (in humans) which bind externally derived antigens, which are referred to in this context as allergens since these molecules elicit an allergic response. Production of allergen-specific IgE is dependent upon T lymphocytes which are also activated by (are specific for) the allergen. Allergen-specific IgE antibodies bind to the surface of cells such as basophils and mast cells by virtue of the expression by these cells of surface receptors for IgE.

Crosslinking of surface bound IgE molecules by allergen results in degranulation of these effector cells causing release of inflammatory mediators such as histamine, 5-hydroxtryptamine and lipid mediators such as the sulphidoleukotrienes. In addition to IgE-dependent events, certain allergic diseases such as asthma are characterised by IgE-independent events.

Allergic IgE-mediated diseases are currently treated with agents which provide symptomatic relief or prevention. Examples of such agents are anti-histamines, β2 agonists, and glucocorticosteroids. In addition, some IgE-mediated diseases are treated by desensitisation procedures that involve the periodic injection of allergen

components or extracts. Desensitisation treatments may induce an IgG response that competes with IgE for allergen, or they may induce specific suppressor T cells that block the synthesis of IgE directed against allergen. This form of treatment is not always effective and poses the risk of provoking serious side effects, particularly general anaphylactic shock. This can be fatal unless recognised immediately and treated with adrenaline. A therapeutic treatment that would decrease or eliminate the unwanted allergic-immune response to a particular allergen, without altering the immune reactivity to other foreign antigens or triggering an allergic response itself would be of great benefit to allergic individuals.

Pollen allergens are recognised as a major cause of allergic diseases in humans and animals, including asthma, allergic rhinitis and allergic dermatitis. At least 10% of the population of the USA suffers from pollen allergies at various times and to varying extents. Proteins present in tree pollen, in particular from trees of the order Fagales, for example birch, alder, hazel, hornbeam and oak, are particularly important. Of these species, birch pollen allergens are the most frequent initiators of allergic responses to tree pollen (Jarolim *et* al: Allergy 1989; 44(6):385-95). For example, approximately 25% of hayfever sufferers are responsive to birch pollen. Hayfever is the common term for a form of seasonal allergy characterised by sneezing, runny nose and itching eyes. Allergy to tree pollen is most problematic during the spring months, with the birch pollen season typically occurring around April (in the northern hemisphere). However, some related types of tree such as alder and hazel can release airborne pollen as early as January (northern hemisphere). These are followed by elm, willow and ash in March, with oak in late April and early May.

It has been calculated that for adults in the United States, hayfever is the 5th leading chronic disease and a major cause of work absenteeism, resulting in nearly 4 million missed or lost workdays each year, resulting in a total cost of more than $700 million in total lost productivity. Allergies are also the most frequently reported chronic condition in children, limiting activities for more than 40% of them. Each year, allergies account for more than 17 million outpatient office visits in the United States; seasonal allergies such as hayfever account for more than half of these allergy visits.

A therapeutic or preventative treatment would therefore be of great benefit to

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humans that suffer or are at risk of suffering from tree allergy.

Summary of the Invention

The present inventors have discovered that certain peptide fragments derived from the major allergens in the pollens of birch species are useful in desensitising individuals to these allergens. Peptide fragments derived from Bet v2, Bet v1, Bet v3, Bet v4, Bet v6 and Bet v7 of birch (family name: *Betulaceae*) are particularly useful.

The peptides of the invention were selected as MHC class II-binding T cell epitopes through use of *in silico* analysis to predict peptide-MHC interactions and MHC class II binding assays. Additional epitopes were identified by homology.

A difficulty associated with approaches to desensitisation based on peptide immunisation lies in how to select an appropriate size and region of the allergen as the basis for the peptide to be used for immunisation. The size of the peptide of choice is crucial. If the peptide is too small, the vaccine would not be effective in inducing an immunological response. If the peptides are too large, or if the whole antigen is introduced into an individual, there is the risk of inducing adverse reactions, such as anaphylaxis, which may be fatal.

The polypeptides of the invention have been selected to retain T cell specificity whilst being small enough in size to not possess significant tertiary structure that would enable them to retain the conformation of an IgE-binding epitope of the whole molecule. The polypeptides of the invention therefore do not induce significant crosslinking of adjacent specific IgE molecules on cells such as mast cells and basophils and consequently do not cause significant histamine release.

An advantage of the invention is the ability of the peptides to broadly target Major Histocompatibility Complex (MHC) molecules. T cell receptors (TCRs) are highly variable in their specificity. Variability is generated, as with antibody molecules, through gene recombination events within the cell. TCRs recognise antigen in the form of short peptides bound to molecules encoded by the genes of the Major Histocompatibility Complex (MHC). These gene products are the same molecules that give rise to "tissue types" used in transplantation and are also referred to as Human Leukocyte Antigen molecules (HLAs) which terms may be used interchangeably. Individual MHC molecules possess peptide binding grooves which, due to their shape

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and charge are only capable of binding a limited group of peptides. The peptides bound by one MHC molecule may not necessarily be bound by other MHC molecules.

When a protein molecule such as an antigen or allergen is taken up by antigen presenting cells such as B lymphocytes, dendritic cells, monocytes and macrophages, the molecule is enzymatically degraded within the cell. The process of degradation gives rise to peptide fragments of the molecule which, if they are of the appropriate size, charge and shape, may then bind within the peptide binding groove of certain MHC molecules and be subsequently displayed upon the surface of antigen presenting cells. If the peptide/MHC complexes are present upon the antigen presenting cell surface in sufficient numbers they may then activate T cells which bear the appropriate peptide/MHC-specific T cell receptors.

Due to the polymorphic nature of the MHC, individuals in an outbred population such as man will express different combinations of MHC molecules on their cell surfaces. Since different MHC molecules can bind different peptides from the same molecule based on the size, charge and shape of the peptide, different individuals will display a different repertoire of peptides bound to their MHC molecules. Identification of universal MHC-binding peptide epitopes in an outbred population such as man is more difficult than in inbred animals (such as certain strains of laboratory mice). On the basis of differential MHC expression between individuals and the inherent differences in peptide binding and presentation which this brings, it is unlikely that a single peptide can be identified which will be of use for desensitisation therapy in man.

The peptides of the invention, however, provide a broad coverage of efficacy over the human population by targeting multiple different MHC molecules. A vaccine formulated with a peptide of the invention would therefore have broad utility. Accordingly, the present invention provides a composition suitable for use in preventing or treating allergy to birch pollen by tolerisation comprising:

i) the polypeptideBir12B (AKYMVIQGEPGRVIRGK) or a variant thereof, and

ii) at least one of the polypeptideBir01I (FNYETETTSVIPAARK) or a variant thereof and the polypeptide Bir02J(PAARMFKAFILEGDKLVPK) or a variant thereof,

wherein said variant is:

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I) a longer polypeptide of up to 30 amino acids in length which comprises the sequence of the said polypeptide specified in (i) or (ii), or

II) a polypeptide of 9 to 30 amino acids in length which is capable of binding to the same MHC class II molecule as the said polypeptide specified in (i) or (ii) and which comprises a sequence that has at least 75% homology to the sequence of the said polypeptide specified in (i) or (ii); or

III) a polypeptide of 9 to 30 amino acids in length which is capable of binding to the same MHC class II molecule as the said polypeptide specified in (i) or (ii) and which comprises:

- at least 9 contiguous amino acids of the sequence of the said polypeptide specified in (i) or (ii), or
- a sequence that has at least 75% homology to at least 9 contiguous amino acids.

The present invention also provides an *in vitro* method of determining whether T cells recognize a composition as defined above comprising contacting said T cells with said composition and detecting whether said T cells are stimulated by said composition. The invention further provides use of the polypeptide i) or a variant thereof as defined above and at least one of the polypeptides ii) or a variant thereof as defined above for the manufacture of a medicament for preventing or treating allergy to birch pollen. The invention also provides a method of treating or preventing allergy to birch pollen in a subject in need of such treatment or prevention, which method comprises the administration to said subject of an effective amount of a composition of the invention.

Description of the sequences mentioned herein

SEQ ID NOS: 1 to 80 provide the polypeptide sequences of the invention as set out in Tables 1 to 8. SEQ ID NOS: 1 to 34 and 45 to 70 correspond to peptides derived from Bet v1. SEQ ID NOS: 71 to 76 correspond to peptides derived from Bet v2. SEQ ID NOS: 35, 36 and 77 correspond to peptides derived from Bet v3. SEQ ID NOS: 37 to 39, 78 and 79 correspond to peptides derived from Bet v4. SEQ ID NOS: 40 to 43 and 80 correspond to peptides derived from Bet v6. SEQ ID NO: 44

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corresponds to a peptide derived from Bet v7.

Detailed description of the invention

The invention concerns peptides which can be used in tolerisation. Such peptides may comprise, consist of, or consist essentially of the sequences shown in any of SEQ ID NOS: 1 to 80. Variants of these specific peptides may also be used. The variants may comprise, consist of, or consist essentially of sequences which are fragments of either any of SEQ ID NOS: 1 to 80 or homologues of any of SEQ ID NOS: 1 to 80.

The invention also provides compositions and formulations comprising the polypeptides of the invention. Such tolerisation will typically be to an epitope (for example a MHC class II-binding T cell epitope) present in any of SEQ ID NOS: 1 to 80.

Tree species

Species of tree from the family *Betulaceae*, commonly known as birch, are responsible for a high proportion of tree allergy worldwide, particularly allergies associated with tree pollen, such as hayfever. Other important tree species include alder, hazel, hornbeam and oak.

Birch trees, for example Silver Birch (*Betula pendula*), tolerate a wide range of habitats, with soil pH from approximately 3.5 to approximately 7. They are native to most of Europe and parts of Asia, but are common throughout the world, being found in the temperate, boreal, and arctic zones of the Northern Hemisphere, especially in Canada and other parts of North America. Birch trees typically flower between April and May (Northern Hemisphere).

Peptide fragments of birch pollen allergens

The present inventors have identified the regions in certain birch pollen allergen proteins which comprise MHC Class II-binding T cell epitopes. The present inventors have also shown that regions corresponding to MHC Class II-binding T cell epitopes within the major birch pollen allergens are highly conserved between different

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isoforms of said allergens. Based on this information, peptides derived from the relevant regions of each protein are suitable for preventing or treating birch allergy by tolerisation to all isoforms of that protein.

The peptides of the invention are derived directly or by homology from the protein allergens Bet v2 (SEQ ID NOS: 71 to 76), Bet v1 (SEQ ID NOS: 1 to 34 and 45 to 70), Bet v3 (SEQ ID NOS: 35, 36 and 77), Bet v4 (SEQ ID NOS: 37 to 39, 78 and 79), Bet v6 (SEQ ID NOS: 40 to 43 and 80) and Bet v7 (SEQ ID NO: 44). The terms "peptide" and "polypeptide" are used interchangeably herein. The above proteins are also referred to herein as "the allergens". Tables 1 to 7 set out the sequences of the peptides of the invention (SEQ ID NOS: 1 to 80), indicating the parent protein from which each peptide derives. The composition of the invention comprises the polypeptide of SEQ ID NO: 74 or a variant thereof as defined herein and at least one of the polypeptide of SEQ ID NO: 48 or a variant thereof as defined herein and the polypeptide of SEQ ID NO:53 or variant thereof as defined herein.

In other words, the invention provides a composition for use in the prevention or treatment of birch allergy comprising the polypeptide of SEQ ID NO: 74 or a variant thereof as defined herein and at least one of the polypeptide of SEQ ID NO: 48 or a variant thereof as defined herein and the polypeptide of SEQ ID NO:53 or variant thereof as defined herein.

The composition comprises polypeptides which derive from more than one allergen. The composition comprises one or more polypeptides or variants thereof derived from Bet v 2 and one or more polypeptides or variants thereof derived from Bet v 1. Additional polypeptides may optionally be included which derive from Bet v 3, Bet v 4, Bet v 6 and/or Bet v 7. Accordingly the composition comprises the polypeptide of SEQ ID NO: 74 (which is derived from Bet v 2) or a variant thereof as defined herein and at least one of the polypeptides of SEQ ID NO: 48 or SEQ ID NO: 53 (which are derived from Bet v 1), or a variant thereof as defined herein. The composition may further comprise:

i) at least one of the polypeptides of SEQ ID NO: 72, 71, 73, 75 and 76 (which are derived from Bet v 2), or a variant thereof as defined herein; and/or

ii) at least one of the polypeptides of SEQ ID NOS: 1 to 34, 45 to 47, 49 to 52 and 54 to 70 (which are derived from Bet v 1), or a variant thereof; and/or

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iii) at least one of the polypeptides of:

(a) SEQ ID NOs: 35, 36 and 77 (which are derived from Bet v 3), or a variant thereof as defined herein; and/or

(b) SEQ ID NOs: 37 to 39, 78 and 79 (which derived from Bet v 4), or a variant thereof as defined herein; and/or

(c) SEQ ID NO:s 40 to 43 and 80 (which are derived from Bet v 6), or a variant thereof as defined herein; and/or

(d) SEQ ID NO: 44 (which is derived from Bet v 7), or a variant thereof as defined herein.

Groups (i), (ii) and (iii) (a) to (d) correspond to peptides derived from different Bet allergens, as described above. Combining polypeptides derived from different Bet allergens may allow for broad coverage of birch pollen allergy observed in the general population by providing tolerising epitopes from more than one birch pollen allergen. The composition may further comprise at least one additional polypeptide of SEQ ID NO: 72 (BIR11; FPQFKPQEITGIMK), SEQ ID NO: 71 (BIR10; GSVWAQSSSFPQFK), SEQ ID NO: 73 (BIR12A; PTGMFVAGAKYMVIQGR), SEQ ID NO: 75 (BIR13; IKYMVIQGEAGAVIRGK), SEQ ID NO: 76 (BIR14; EAGAVIRGKKGSGGIT), SEQ ID NO: 54 (Bir04; PGTIKKISFPEGFPFKYV), SEQ ID NO: 67 (Bir09; ETLLRAVESYLLAHSDAY), SEQ ID NO: 60 (BIR07; SNEIKIVATPDGGSILK) and SEQ ID NO: 63 (Bir07C; SNEIKIVATPEGGSILK), or a variant thereof as defined herein. The composition may further comprise at least one additional polypeptide of SEQ ID NO: 77 (BIR15; SLNTLRLRRIFDLFDK) or SEQ ID NO: 78 (BIR16A; AERERIFKRFDANGEGK), or a variant thereof as defined herein. In a preferred embodiment, the composition comprises:

(a) the polypeptide Bir12B (AKYMVIQGEPGRVIRGK), or a variant thereof as defined herein;

(b) the polypeptide Bir02J (PAARMFKAFILEGDKLVPK), or a variant thereof as defined herein; and

(c) the polypeptide Bir01I (FNYETETTSVIPAARK) or a variant thereof as defined herein.

In a particularly preferred embodiment, the composition comprises the polypeptide Bir12B (AKYMVIQGEPGRVIRGK) or a variant thereof, the polypeptide

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Bir02J (PAARMFKAFILEGDKLVPK) or a variant thereof, the polypeptide Bir01I (FNYETETTSVIPAARK) or a variant thereof, the polypeptide Bir04 (PGTIKKISFPEGFPFKYV) or a variant thereof, the polypeptide Bir09 (ETLLRAVESYLLAHSDAY) or a variant thereof, the polypeptide Bir07C (SNEIKIVATPEGGSILK) or a variant thereof, and the polypeptide Bir16A (AERERIFKRFDANGEGK) or a variant thereof, and optionally no further polypeptides.

In a further particularly preferred embodiment, the composition comprises the polypeptide Bir12B (AKYMVIQGEPGRVIRGK) or a variant thereof, the polypeptide Bir02J (PAARMFKAFILEGDKLVPK) or a variant thereof, the polypeptide Bir01I (FNYETETTSVIPAARK) or a variant thereof, the polypeptide Bir04 (PGTIKKISFPEGFPFKYV) or a variant thereof, the polypeptide Bir07C (SNEIKIVATPEGGSILK) or a variant thereof, the polypeptide Bir16A (AERERIFKRFDANGEGK) or a variant thereof, and the polypeptide Bir09B (KEMGETLLRAVESYLLAHS) or a variant thereof, and optionally no further polypeptides.

In a further particularly preferred embodiment, the composition comprises the polypeptide Bir12B (AKYMVIQGEPGRVIRGK) or a variant thereof, the polypeptide Bir02J (PAARMFKAFILEGDKLVPK) or a variant thereof, the polypeptide Bir01I (FNYETETTSVIPAARK) or a variant thereof, the polypeptide Bir04 (PGTIKKISFPEGFPFKYV) or a variant thereof, the polypeptide Bir07C (SNEIKIVATPEGGSILK) or a variant thereof, and the polypeptide Bir16A (AERERIFKRFDANGEGK) or a variant thereof, and optionally no further polypeptides.

In a further particularly preferred embodiment, the composition comprises the polypeptide Bir12B (AKYMVIQGEPGRVIRGK) or a variant thereof, the polypeptide Bir02I (PAARMFKAFILD) or a variant thereof, the polypeptide Bir01I (FNYETETTSVIPAARK) or a variant thereof, the polypeptide Bir04 (PGTIKKISFPEGFPFKYV) or a variant thereof, the polypeptide Bir09 (ETLLRAVESYLLAHSDAY) or a variant thereof, the polypeptide Bir07C (SNEIKIVATPEGGSILK) or a variant thereof, and the polypeptide Bir16A

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(AERERIFKRFDANGEGK) or a variant thereof, and optionally no further polypeptides.

In a further particularly preferred embodiment, the composition comprises the polypeptide Bir12B (AKYMVIQGEPGRVIRGK) or a variant thereof, the polypeptide Bir02I (PAARMFKAFILD) or a variant thereof, the polypeptide Bir01I (FNYETETTSVIPAARK) or a variant thereof, the polypeptide Bir04 (PGTIKKISFPEGFPFKYV) or a variant thereof, the polypeptide Bir07C (SNEIKIVATPEGGSILK) or a variant thereof, the polypeptide Bir16A (AERERIFKRFDANGEGK) or a variant thereof, and the polypeptide Bir09B (KEMGETLLRAVESYLLAHS) or a variant thereof, and optionally no further polypeptides.

In a further particularly preferred embodiment, the composition comprises the polypeptide Bir12B (AKYMVIQGEPGRVIRGK) or a variant thereof, the polypeptide Bir02I (PAARMFKAFILD) or a variant thereof, the polypeptide Bir01I (FNYETETTSVIPAARK) or a variant thereof, the polypeptide Bir04 (PGTIKKISFPEGFPFKYV) or a variant thereof, the polypeptide Bir07C (SNEIKIVATPEGGSILK) or a variant thereof, and the polypeptide Bir16A (AERERIFKRFDANGEGK) or a variant thereof, and optionally no further polypeptides.Variants of the polypeptides of SEQ ID NOS: 1 to 80 are mentioned herein. A variant of any of SEQ ID NOS: 1 to 80 will typically be functional. By functional it is meant that the variant is one which:

(a) comprises or consists of a sequence which binds to the same MHC class II molecule as the corresponding polypeptide of SEQ ID NOS: 1 to 80; and/or

(b) comprises or consists of a sequence which is recognised by a T cell which recognises the corresponding polypeptide of SEQ ID NOS: 1 to 80; and/or

(c) is capable of inducing a late phase response in an individual with birch allergy; and/or

(d) is capable of tolerising an individual to the corresponding polypeptide.

Recognition by a T cell may be tested by measuring the ability of a peptide or variant to induce T cell proliferation in a sample of T cells. The induction of a late phase response may also be tested in this way when the sample of T cells is taken from an individual with birch allergy. Methods of testing the induction of T cell proliferation

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are well known in the art and one such method is exemplified in Example 8.

Variants of SEQ ID NOS: 1 to 80 may be fragments derived by truncation, e.g. by removal of one or more amino acids from the N and/or C-terminal ends of a polypeptide. Fragments may also be generated by one or more internal deletions, provided that the core 9 amino acids that makes up the T cell epitope is not substantially disrupted.

For example, a variant of SEQ ID NO: 1 may comprise a fragment of SEQ ID NO: 1, i.e. a shorter sequence. This may include a deletion of one, two, three or four amino acids from the N-terminal end of SEQ ID NO: 1 or from the C-terminal end of SEQ ID NO: 1. Such deletions may be made from both ends of SEQ ID NO: 1.

A variant of SEQ ID NO: 1 may include additional amino acids (for example from the sequence of the parent protein from which the peptide derives) extending beyond the end(s) of SEQ ID NO: 1. A variant of a polypeptide may typically be a longer polypeptide of up to 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 amino acids in length which comprises the sequence of the corresponding polypeptide of SEQ ID NOS: 1 to 80.

A variant may include a combination of the deletions and additions discussed above. For example, amino acids may be deleted from one end of SEQ ID NO: 1, but additional amino acids from the full length parent protein sequence may be added at the other end of SEQ ID NO: 1. The same discussion of variants above also applies to SEQ ID NOS: 2 to 80.

A variant may alternatively be a polypeptide of 9 to 30, 11 to 20 or 13 to 17 amino acids in length which comprises a sequence that has at least 75% sequence identity to the sequence of the corresponding polypeptide of SEQ ID NOS: 1 to 80. More preferably a suitable variant may comprise at least 80%, at least 85%, at least 90%, at least 95%, or at least 98% amino acid identity to the corresponding polypeptide of SEQ ID NOS: 1 to 80.

A variant may be a polypeptide of length 9 to 30, 11 to 20 or 13 to 17 amino acids which comprises a sequence of, or a sequence that has, at least 75% sequence identity to at least 9 (for example at least 10, 11, 12 or 13) or more contiguous amino acids of the sequence of the corresponding polypeptide of SEQ ID NOS: 1 to 80. These contiguous amino acids may typically comprise a MHC class II epitope, for example

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which binds to any of the MHC molecules mentioned herein.

A variant peptide may include one or more amino acid substitutions from the amino acid sequence of any of SEQ ID NOS: 1 to 80 or a fragment thereof. A variant peptide may comprise sequence having at least 75% sequence identity to at least 9 or more contiguous amino acids in any of SEQ ID NOS: 1 to 80. More preferably a suitable variant may comprise at least 80%, at least 85%, at least 90%, at least 95%, or at least 98% amino acid identity to at least 9 contiguous amino acids of any of SEQ ID NOS: 1 to 80. This level of amino acid identity may be seen at any section of the peptide, although it is preferably the core region. The level of amino acid identity is over at least 9 contiguous amino acids but it may be at least 10, 11, 12, 13, 14, 15 or at least 16 or 17 amino acids, depending on the size of the peptides of comparison. Accordingly, any of the above-specified levels of identity may be across the entire length of sequence.

In connection with amino acid sequences, "sequence identity" refers to sequences which have the stated value when assessed using ClustalW (Thompson et al., 1994, supra) with the following parameters:
Pairwise alignment parameters -Method: accurate, Matrix: PAM, Gap open penalty: 10.00, Gap extension penalty: 0.10; Multiple alignment parameters -Matrix: PAM, Gap open penalty: 10.00, % identity for delay: 30, Penalize end gaps: on, Gap separation distance: 0, Negative matrix: no, Gap extension penalty: 0.20, Residue-specific gap penalties: on, Hydrophilic gap penalties: on, Hydrophilic residues: GPSNDQEKR. Sequence identity at a particular residue is intended to include identical residues which have simply been derivatized.

A variant peptide may comprise 1, 2, 3, 4, 5 or more, or up to 10 amino acid substitutions from any of SEQ ID NOS: 1 to 80. Substitution variants preferably involve the replacement of one or more amino acids with the same number of amino acids and making conservative amino acid substitutions. For example, an amino acid may be substituted with an alternative amino acid having similar properties, for example, another basic amino acid, another acidic amino acid, another neutral amino acid, another charged amino acid, another hydrophilic amino acid, another hydrophobic amino acid, another polar amino acid, another aromatic amino acid or another aliphatic amino acid. Some properties of the 20 main amino acids which can be used to select

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suitable substituents are as follows:

Ala	aliphatic, hydrophobic, neutral	Met	hydrophobic, neutral
Cys	polar, hydrophobic, neutral	Asn	polar, hydrophilic, neutral
Asp	polar, hydrophilic, charged (-)	Pro	hydrophobic, neutral
Glu	polar, hydrophilic, charged (-)	Gln	polar, hydrophilic, neutral
Phe	aromatic, hydrophobic, neutral	Arg	polar, hydrophilic, charged (+)
Gly	aliphatic, neutral	Ser	polar, hydrophilic, neutral
His	aromatic, polar, hydrophilic, charged (+)	Thr	polar, hydrophilic, neutral
Ile	aliphatic, hydrophobic, neutral	Val	aliphatic, hydrophobic, neutral
Lys	polar, hydrophilic, charged(+)	Trp	aromatic, hydrophobic, neutral
Leu	aliphatic, hydrophobic, neutral	Tyr	aromatic, polar, hydrophobic

Further variants include those in which instead of the naturally occurring amino acid the amino acid which appears in the sequence is a structural analog thereof. Amino acids used in the sequences may also be modified, e.g. labelled, providing the function of the peptide is not significantly adversely affected.

Where the peptide has a sequence that varies from the sequence of any of SEQ ID NOS: 1 to 80 or a fragment thereof, the substitutions may occur across the full length of the sequence, within the sequence of any of SEQ ID NOS: 1 to 80 or outside the sequence of any of SEQ ID NOS: 1 to 80. For example, the variations described herein, such as additions, deletions, substitutions and modifications, may occur within the sequence of any of SEQ ID NOS: 1 to 80. A variant peptide may comprise or consist essentially of the amino acid sequence of any of SEQ ID NOS: 1 to 80 in which one, two, three, four or more amino acid substitutions have been made. A variant peptide may comprise a fragment of the parent protein that is larger than any of SEQ ID NOS: 1 to 80. In this embodiment, the variations described herein, such as substitutions and modifications, may occur within and/or outside the sequence of any of SEQ ID NOS: 1 to 80. For example, one or more positively charged residues may be added at the N and/or C terminus of the native sequence of the peptide of any of SEQ ID NOS: 1 to 80.

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The variant peptides of the invention are up to 30 or 9 to 30 amino acids in length

inclusive. Preferably, they may be from 9 to 20 or more preferably 13 to 17 amino acids in length. The peptides may be the same length as the peptide sequences in any one of SEQ ID NOS: 1 to 80.

The peptides may be chemically derived from the polypeptide allergen, for example by proteolytic cleavage or can be derived in an intellectual sense from the polypeptide allergen, for example by making use of the amino acid sequence of the polypeptide allergen and synthesising peptides based on the sequence. Peptides may be synthesised using methods well known in the art.

The term "peptide" includes not only molecules in which amino acid residues are joined by peptide (-CO-NH-) linkages but also molecules in which the peptide bond is reversed. Such retro-inverso peptidomimetics may be made using methods known in the art, for example such as those described in Meziere *et al* (1997) J. Immunol.159, 3230-3237. This approach involves making pseudopeptides containing changes involving the backbone, and not the orientation of side chains. Meziere *et al* (1997) show that, at least for MHC class II and T helper cell responses, these pseudopeptides are useful. Retro-inverse peptides, which contain NH-CO bonds instead of CO-NH peptide bonds, are much more resistant to proteolysis.

Similarly, the peptide bond may be dispensed with altogether provided that an appropriate linker moiety which retains the spacing between the carbon atoms of the amino acid residues is used; it is particularly preferred if the linker moiety has substantially the same charge distribution and substantially the same planarity as a peptide bond. It will also be appreciated that the peptide may conveniently be blocked at its N-or C-terminus so as to help reduce susceptibility to exoproteolytic digestion. For example, the N-terminal amino group of the peptides may be protected by reacting with a carboxylic acid and the C-terminal carboxyl group of the peptide may be protected by reacting with an amine. Other examples of modifications include glycosylation and phosphorylation. Another potential modification is that hydrogens on the side chain amines of R or K may be replaced with methylene groups ($-NH_2 \rightarrow -NH(Me)$ or $-N(Me)_2$).

Analogues of peptides according to the invention may also include peptide variants that increase or decrease the peptide's half-life *in vivo*. Examples of analogues capable of increasing the half-life of peptides used according to the invention include

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peptoid analogues of the peptides, D-amino acid derivatives of the peptides, and peptide-peptoid hybrids. A further embodiment of the variant polypeptides used according to the invention comprises D-amino acid forms of the polypeptide. The preparation of polypeptides using D-amino acids rather than L-amino acids greatly decreases any unwanted breakdown of such an agent by normal metabolic processes, decreasing the amounts of agent which needs to be administered, along with the frequency of its administration.

The peptides provided by the present invention may be derived from splice variants of the parent proteins encoded by mRNA generated by alternative splicing of the primary transcripts encoding the parent protein chains. The peptides may also be derived from amino acid mutants, glycosylation variants and other covalent derivatives of the parent proteins which retain at least an MHC-binding property of the allergens. Exemplary derivatives include molecules wherein the peptides of the invention are covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid. Further included are naturally occurring variants of the parent proteins found in different mites. Such a variant may be encoded by an allelic variant or represent an alternative splicing variant.

Variants as described above may be prepared during synthesis of the peptide or by post- production modification, or when the peptide is in recombinant form using the known techniques of site- directed mutagenesis, random mutagenesis, or enzymatic cleavage and/or ligation of nucleic acids.

In any of the embodiments of the invention, typical examples of variants as described herein may be as follows:

- a variant of Bir01I is Bir01F (FNYETEATSVIPAARK), Bir01G (FNYEIEATSVIPAARK) or Bir01H (FNYEIETTSVIPAARK); and/or
- a variant of Bir02J is Bir02E (PAARLFKAFILEGDTLIPK), Bir02G (PAARLFKAFILEGDNLIPK), Bir02I (PAARMFKAFILD) or Bir02D (PAARMFKAFILDGDKLVPK); and/or
- a variant of Bir09 is selected from Bir09A (GETLLRAVESYLLAHS), Bir09B (KEMGETLLRAVESYLLAHS) or Bir09C (KEKGETLLRAVESYLLAHS); and/or
- a variant of Bir16A is Bir16B (AERERIFKRFDAGGEGK).

It will be understood that SEQ ID NOS: 1 to 80 are polypeptide sequences

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which comprise a T cell epitope that consists of a core of typically 9 amino acids, which are the minimal essential sequence required for MHC class II binding. However, the polypeptides of SEQ ID NOS: 1 to 80 may also comprise additional residues flanking the core. The peptides may therefore comprise a region containing a T cell epitope, in which some residues may be modified without affecting the function of the epitope. Thus, for example, the sequences of any of SEQ ID NOS: 1 to 80 may be altered to improve their solubility, and accordingly a variant of any of SEQ ID NOS: 1 to 80 will preferably be more soluble than the corresponding polypeptide of SEQ ID NOS: 1 to 80 under equivalent conditions. Methods for evaluating the solubility of peptides are well known in the art and one such method is exemplified in Example 9.

Improved solubility is advantageous for the tolerisation of subjects to allergens from which the peptides of the invention derive, since administration of poorly soluble agents to subjects causes undesirable, non-tolerising inflammatory responses. The solubility of the peptides may be improved by altering the residues which flank the region containing a T cell epitope. A peptide of the invention may be engineered to be more soluble such that it comprises:

i) N terminal to the residues of the peptide which flank a T cell epitope: one to six contiguous amino acids corresponding to the one to six contiguous amino acids immediately N terminal to said residues in the sequence of the protein from which the peptide derives; and/or

ii) C terminal to the residues of the peptide which flank a T cell epitope: one to six contiguous amino acids corresponding to the one to six contiguous amino acids immediately C terminal to the said residues in the sequence of the protein from which the peptide derives; or

iii) both N and C terminal to the residues of the peptide which flank a T cell epitope, at least one amino acid selected from arginine, lysine, histidine, glutamate and aspartate,

wherein the polypeptide has a solubility of at least 3.5 mg/ml and the T cell epitope has a solubility of less than 3.5 mg/ml.

Optionally, the peptides may additionally be engineered to be more soluble such that:

i) any cysteine residues in the native sequence of the peptide are replaced with serine or 2-aminobutyric acid; and /or

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ii) any hydrophobic residues in the up to three amino acids at the N or C terminus of the native sequence of the peptide, which are not comprised in a T cell epitope, are deleted; and/or

iii) any two consecutive amino acids comprising the sequence Asp-Gly in the up to four amino acids at the N or C terminus of the native sequence of the peptide, which are not comprised in a T cell epitope, are deleted; and/or

iv) one or more positively charged residues are added at the N and/or C terminus of the native sequence of the peptide.

Preferably the peptides and variants of the invention are capable of causing T cell proliferation in at least 20 % of samples of T cells, wherein each sample is obtained from different birch allergic individuals in the population. The compositions of the invention are preferably capable of inducing T cell proliferation in 30 % or more samples of T cells obtained from of a panel of birch allergic individuals. More preferably, the compositions are capable of inducing T cell proliferation in 35% or more, 40 % or more, 45 %, 50 %, 55 %, 60 %, 65 %, 70 %, 75 %, 80 %, 85 %, or 90 % or more of samples obtained from sensitized individuals in a panel. The number of individuals in a panel of birch allergic individuals may be any number greater than one, for example at least 2, 3, 5, 10, 15, 20, 30, 50, 80, or at least 100 individuals.

It is preferred if the peptides, variants and compositions of the invention cause T cell proliferation, but do not lead to the release of histamine from enriched basophils or mast cell preparations from a sensitised individual. There may be some histamine release, but preferably the peptides, variants and compositions do not cause significant amounts of histamine to be released. Significant histamine release may be considered to be the release of 20% or more of the total available leukocyte histamine when a sample of leukocytes from an individual is stimulated with a composition *in vitro*. A peptide, variant or composition of the invention preferably causes the release of less than 5%, less than 4%, less than 3%, less than 2% or less than 1% of the total available leukocyte histamine when a sample of leukocytes from an individual is stimulated with a composition *in vitro*. A normal individual typically has an approximate leukocyte histamine content of $150ng/10^7$ cells.

Suitable peptides or variants capable of binding to TCRs may be derived

empirically or selected according to known criteria. Within a single peptide there are certain residues which contribute to binding within the MHC antigen binding groove and other residues which interact with hypervariable regions of the T cell receptor (Allen et al (1987) Nature 327: 713-5).

Within the residues contributing to T cell receptor interaction, a hierarchy has been demonstrated which pertains to dependency of T cell activation upon substitution of a given peptide residue. Using peptides which have had one or more T cell receptor contact residues substituted with a different amino acid, several groups have demonstrated profound effects upon the process of T cell activation. Evavold & Allen (1991) Nature 252: 1308-10) demonstrated the dissociation of T cell proliferation and cytokine production. In this *in vitro* model, a T cell clone specific for residues 64-76 of haemoglobin (in the context of I-E^k), was challenged with a peptide analogue in which a conservative substitution of aspartic acid for glutamic acid had been made. This substitution did not significantly interfere with the capacity of the analogue to bind to I-E^k.

Following *in vitro* challenge of a T cell clone with this analogue, no proliferation was detected although IL-4 secretion was maintained, as was the capacity of the clone to help B cell responses. In a subsequent study the same group demonstrated the separation of T cell-mediated cytolysis from cytokine production. In this instance, the former remained unaltered while the latter was impaired. The efficacy of altered peptide ligands *in vivo* was initially demonstrated in a murine model of EAE (experimental allergic encephalomyelitis) by McDevitt and colleagues (Smilek *et al* (1991) Proc Natl Acad Sci USA 88 : 9633-9637). In this model EAE is induced by immunisation with the encephalitogenic peptide Ac1-11 of MBP (myelin basic protein). Substitution at position four (lysine) with an alanine residue generated a peptide which bound well to its restricting element ($A\alpha^uA\beta^u$), but which was non-immunogenic in the susceptible PL/JxSJLF1 strain and which, furthermore prevented the onset of EAE when administered either before or after immunisation with the encephalitogenic peptide. Thus, residues can be identified in peptides which affect the ability of the peptides to induce various functions of T-cells.

Advantageously, peptides may be designed to favour T-cell proliferation and induction of desensitisation. Metzler and Wraith have demonstrated improved

tolerogenic capacity of peptides in which substitutions increasing peptide-MHC affinity have been made (Metzler & Wraith(1993) Int Immunol ~ : 1159-65). That an altered peptide ligand can cause long-term and profound anergy in cloned T cells was demonstrated by Sloan-Lancaster *et al* (1993) Nature 363: 156-9.

The compositions of the invention are capable of inducing a late phase response in an individual that is sensitised to the allergens. The term "late phase response" includes the meaning as set forth in Allergy and Allergic Diseases (1997) A. B. Kay (Ed.), Blackwell Science, pp 1113-1130. The late phase response may be any late phase response (LPR). Preferably, the peptides are capable of inducing a late asthmatic response (LAR) or a late rhinitic response, or a late phase skin response or a late phase ocular response. Whether or not a particular peptide can give rise to a LPR can be determined using methods well known in the art; a particularly preferred method is that described in Cromwell O, Durham SR, Shaw RJ, Mackay J and Kay AB. Provocation tests and measurements of mediators from mast cells and basophils in asthma and allergic rhinitis. In: Handbook of Experimental Immunology (4) Chapter 127, Editor: Weir DM, Blackwell Scientific Publications, 1986.

Thus, preferably, the individual peptides and variants of the invention are able to induce a LPR in an individual who has been sensitised to the allergens. Whether or not an individual has been sensitised to the allergens may be determined by well known procedures such as skin prick testing with solutions of allergen extracts, induction of cutaneous LPRs, clinical history, allergen challenge and radioallergosorbent test (RAST) for measurement of allergen specific IgE. Whether or not a particular individual is expected to benefit from treatment may be determined by the physician based, for example, on such tests.

Desensitising or tolerising an individual to the allergens means inhibition or dampening of allergic tissue reactions induced by the allergens in appropriately sensitised individuals. It has been shown that T cells can be selectively activated, and then rendered unresponsive. Moreover the anergising or elimination of these T-cells leads to desensitisation of the patient for a particular allergen. The desensitisation manifests itself as a reduction in response to an allergen or allergen-derived peptide, or preferably an elimination of such a response, on second and further administrations of the allergen or allergen-derived peptide. The second administration may be made after

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a suitable period of time has elapsed to allow desensitisation to occur; this is preferably any period between one day and several weeks. An interval of around two weeks is preferred.

Although the compositions of the invention are able to induce a LPR in a birch allergic individual, it should be appreciated that when a composition is used to treat a patient it is preferable that a sufficiently low concentration of the composition is used such that no observable LPR will occur but the response will be sufficient to partially desensitise the T cells such that the next (preferably higher) dose may be given, and so on. In this way the dose is built up to give full desensitisation but often without ever inducing a LPR in the patient. Although, the composition or peptide is able to do so at a higher concentration than is administered.

The compositions of the invention preferably are capable of inducing a late phase response in 50 % or more of a panel of birch allergic individuals from the population. More preferably, the compositions are capable of inducing a LPR in 55% or more, 60 % or more, 65 % or more, 70% or more, 75% or more, 80% or more, 85% or more, or 90 % or more of sensitized individuals in a panel. Whether or not the compositions are able to induce a LPR in a certain percentage of a panel of subjects can be determined by methods which are well known in the art.

The polypeptides of the invention may be present in a substantially isolated form. They may be mixed with carriers or diluents which will not interfere with their intended use and still be regarded as substantially isolated. They may also be in a substantially purified form, in which case they will generally comprise at least 90%, e.g. at least 95%, 98% or 99%, of the proteins or dry mass of the preparation.

Formulations and compositions

The peptides of the invention may be provided to an individual either singly or in combination. Each molecule of the invention may be provided to an individual in an isolated, substantially isolated, purified or substantially purified form. For example, a peptide of the invention may be provided to an individual substantially free from the other peptides. Alternatively, four or more peptides in the composition may be coupled chemically together, using standard peptide coupling reagents, to provide a single peptide containing the preferred epitopes. Such peptides would be screened for

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basophil histamine release to confirm lack of histamine release as per the individual peptides. In a further embodiment, four or more peptides in the composition may be provided as part of a single polypeptide chain i.e by recombinant means from an encoding polynucleotide. The four or more peptides may be fused contiguously, or may alternatively be separated by appropriate linkers.

Whilst it may be possible for the peptides or compositions according to the invention to be presented in raw form, it is preferable to present them as a pharmaceutical formulation. Thus, according to a further aspect of the invention, the present invention provides a pharmaceutical formulation for use in preventing or treating allergy to birch by tolerisation comprising a composition according to the invention together with one or more pharmaceutically acceptable carriers or diluents and optionally one or more other therapeutic ingredients. The carrier (s) must be 'acceptable' in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. Typically, carriers for injection, and the final formulation, are sterile and pyrogen free. Preferably, the carrier or diluent is thioglycerol or thioanisole.

Formulation of a composition comprising the peptide of the invention can be carried out using standard pharmaceutical formulation chemistries and methodologies all of which are readily available to the reasonably skilled artisan.

For example, compositions containing one or more molecules of the invention can be combined with one or more pharmaceutically acceptable excipients or vehicles. Auxiliary substances, such as wetting or emulsifying agents, pH buffering substances and the like, may be present in the excipient or vehicle. These excipients, vehicles and auxiliary substances are generally pharmaceutical agents that do not induce an immune response in the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, polyethyleneglycol, hyaluronic acid, glycerol, thioglycerol and ethanol. Pharmaceutically acceptable salts can also be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients, vehicles and auxiliary substances is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

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Such compositions may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable compositions may be prepared, packaged, or sold in unit dosage form, such as in ampoules or in multi-dose containers containing a preservative. Compositions include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such compositions may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a composition for parenteral administration, the active ingredient is provided in dry (for e.g., a powder or granules) form for reconstitution with a suitable vehicle (e. g., sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition. The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a a suitable period of time has elapsed to allow desensitisation to occur; this is preferably any period between one day and several weeks. An interval of around two weeks is preferred.

Although the compositions of the invention are able to induce a LPR in a birch allergic individual, it should be appreciated that when a composition is used to treat a patient it is preferable that a sufficiently low concentration of the composition is used such that no observable LPR will occur but the response will be sufficient to partially desensitise the T cells such that the next (preferably higher) dose may be given, and so on. In this way the dose is built up to give full desensitisation but often without ever inducing a LPR in the patient. Although, the composition or peptide is able to do so at a higher concentration than is administered.

The compositions of the invention preferably are capable of inducing a late phase response in 50 % or more of a panel of birch allergic individuals from the population. More preferably, the compositions are capable of inducing a LPR in 55% or more, 60 % or more, 65 % or more, 70% or more, 75% or more, 80% or more, 85% or more, or 90 % or more of sensitized individuals in a panel. Whether or not the compositions are able to induce a LPR in a certain percentage of a panel of subjects

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can be determined by methods which are well known in the art.

The polypeptides of the invention may be present in a substantially isolated form. They may be mixed with carriers or diluents which will not interfere with their intended use and still be regarded as substantially isolated. They may also be in a substantially purified form, in which case they will generally comprise at least 90%, e.g. at least 95%, 98% or 99%, of the proteins or dry mass of the preparation.

Formulations and compositions

The peptides of the invention may be provided to an individual either singly or in combination. Each molecule of the invention may be provided to an individual in an isolated, substantially isolated, purified or substantially purified form. For example, a peptide of the invention may be provided to an individual substantially free from the other peptides. Alternatively, four or more peptides in the composition may be coupled chemically together, using standard peptide coupling reagents, to provide a single peptide containing the preferred epitopes. Such peptides would be screened for basophil histamine release to confirm lack of histamine release as per the individual peptides. In a further embodiment, four or more peptides in the composition may be provided as part of a single polypeptide chain i.e by recombinant means from an encoding polynucleotide. The four or more peptides may be fused contiguously, or may alternatively be separated by appropriate linkers.

Whilst it may be possible for the peptides or compositions according to the invention to be presented in raw form, it is preferable to present them as a pharmaceutical formulation. Thus, according to a further aspect of the invention, the present invention provides a pharmaceutical formulation for use in preventing or treating allergy to birch by tolerisation comprising a composition according to the invention together with one or more pharmaceutically acceptable carriers or diluents and optionally one or more other therapeutic ingredients. The carrier (s) must be 'acceptable' in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. Typically, carriers for injection, and the final formulation, are sterile and pyrogen free. Preferably, the carrier or diluent is thioglycerol or thioanisole.

Formulation of a composition comprising the peptide of the invention can be

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carried out using standard pharmaceutical formulation chemistries and methodologies all of which are readily available to the reasonably skilled artisan.

For example, compositions containing one or more molecules of the invention can be combined with one or more pharmaceutically acceptable excipients or vehicles. Auxiliary substances, such as wetting or emulsifying agents, pH buffering substances and the like, may be present in the excipient or vehicle. These excipients, vehicles and auxiliary substances are generally pharmaceutical agents that do not induce an immune response in the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, polyethyleneglycol, hyaluronic acid, glycerol, thioglycerol and ethanol. Pharmaceutically acceptable salts can also be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients, vehicles and auxiliary substances is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

Such compositions may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable compositions may be prepared, packaged, or sold in unit dosage form, such as in ampoules or in multi-dose containers containing a preservative. Compositions include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such compositions may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a composition for parenteral administration, the active ingredient is provided in dry (for e.g., a powder or granules) form for reconstitution with a suitable vehicle (e. g., sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition. The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol,

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for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono-or di-glycerides.

Other parentally-administrable compositions which are useful include those which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

Alternatively, the peptides of the present invention may be encapsulated, adsorbed to, or associated with, particulate carriers. Suitable particulate carriers include those derived from polymethyl methacrylate polymers, as well as PLG microparticles derived from poly(lactides) and poly(lactide-co-glycolides). See, e.g., Jeffery et al. (1993) Pharm. Res. 10:362-368. Other particulate systems and polymers can also be used, for example, polymers such as polylysine, polyarginine, polyornithine, spermine, spermidine, as well as conjugates of these molecules.

The formulation of any of the peptides mentioned herein will depend upon factors such as the nature of the substance and the method of delivery. Any such substance may be administered in a variety of dosage forms. It may be administered orally (e.g. as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules), topically, parenterally, subcutaneously, by inhalation, intravenously, intramuscularly, intrasternally, transdermally, intradermally, sublingually, intranasally, buccally or by infusion techniques. The substance may also be administered as suppositories. A physician will be able to determine the required route of administration for each particular individual.

The compositions of formulations of the invention will comprise a suitable concentration of each peptide to be effective without causing adverse reaction. Typically, the concentration of each peptide in the composition will be in the range of 0.03 to 200 nmol/ml. More preferably in the range of 0.3 to 200 nmol/ml, 3 to 180 nmol/ml, 10 to 150 nmol/ml, 50 to 200nmol/ml or 30 to 120 nmol/ml. The composition or formulations should have a purity of greater than 95% or 98% or a purity of at least 99%.

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In one aspect of the invention an adjuvant may be used in combination with the polypeptides of the invention. The adjuvant is preferably administered in an amount which is sufficient to augment the effect of the polypeptides of the invention or vice versa. The adjuvant or other therapeutic agent may be an agent that potentiates the effects of the molecule of the invention. For example, the other agent may be an immunomodulatory molecule or an adjuvant which enhances the response to the peptide or cell of the invention.

In one embodiment, therefore, the peptides or compositions of the invention are used for therapy in combination with one or more other therapeutic agents. The agents may be administered separately, simultaneously or sequentially. They may be administered in the same or different compositions. Accordingly, in a method of the invention, the subject may also be treated with a further therapeutic agent.

A composition may therefore be formulated which comprises a molecule of the invention and also one or more other therapeutic molecules. A composition of the invention may alternatively be used simultaneously, sequentially or separately with one or more other therapeutic compositions as part of a combined treatment.

Non-limiting examples of adjuvants include vitamin D, rapamycin and glucocorticoid steroids such as dexamethasone, fluticasone, budesonide, mometasone, beclomethasone, hydrocortisone, cortisone acetate, prednisone, prednisolone, methylprednisolone, betamethasone and triamcinolone. A preferred glucocorticoid is dexamethasone.

Therapeutic methods and individual to be treated

The present invention relates to peptides that are capable of desensitising or tolerising human individuals to the allergens described above and are therefore useful in the prevention or treatment of birch allergy. The invention provides compositions and formulations for use in preventing or treating allergy to birch by tolerisation. Also disclosed is a method of tolerising or desensitizing a birch allergic individual comprising administering, either singly or in combination the polypeptides of the invention as described above.

The individual to be treated or provided with the composition or formulation of the invention is preferably human. It will be appreciated that the individual to be treated may be known to be sensitised to the allergens, at risk of being sensitised or

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suspected of being sensitised. The individual can be tested for sensitisation using techniques well known in the art and as described herein. Alternatively, the individual may have a family history of allergy to birch. It may not be necessary to test an individual for sensitisation to birch because the individual may display symptoms of allergy when exposed to birch. By exposure is meant proximity to, for example, a birch plant, or a substance or product derived from a birch plant, or a substance or product containing or comprising either of the above. The substance or product derived from a birch plant is typically birch pollen. By proximity is meant 10 metres or less, 5 metres or less, 2 metres or less, 1 metre or less, or 0 metres from the items described above. Symptoms of allergy can include itchy eyes, runny nose, breathing difficulties, red itchy skin or rash.

The individual to be treated may be of any age. However, preferably, the individual may be in the age group of 1 to 90, 5 to 60, 10 to 40, or more preferably 18 to 35.

Preferably, the individual to be treated is from a population that has MHC allele frequencies within the range of frequencies that are representative of the Caucasian population. Reference population allele frequencies for 11 common DRB1 allele families are shown in Table 1 (Data from HLA Facts Book, Parham and Barber).

Table 1

DRB1	1	3	4	7	8	11	12	13	14	15	16
%	6.4	14.7	15.7	8.8	3.4	8.3	3.9	14.7	2.9	17.6	2.5
Reference population %	9.4	11.1	12.8	13.2	3.7	13.4	2.3	10.2	3.2	10.7	3.6

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Reference frequencies were obtained by analysis of multiple studies reporting frequencies and the figures shown are mean values. Preferably therefore, the individual to be treated is from a population that has equivalent MHC allele frequencies as the reference population for the alleles referred to Table 1 (such as for at least 1, 2, 3, 4, 5 or all of the alleles), for example within the ranges of those figures plus or minus 1, 2, 3, 5, 10, 15 or 20%.

Preferably the individual is from a population where the allele frequencies of the following DRB1 alleles is:

4 – at least 9%

7 – at least 10%

11 – at least 8%.

The individual may have had allergy to birch for at least 2 weeks, 1 month, 6 months, 1 year or 5 years. The individual may suffer from a rash, nasal congestion, nasal discharge and/or coughing caused by the allergy. The individual may or may not have been administered with other compositions/compounds which treat birch allergy. The individual may live in a geographical region which has:

- a temperate, boreal or arctic climate, and/or:
- a typical soil pH in the range of about 3.5, to about 7.5.

The individual typically suffers from allergy to birch pollen in a particular season. The season typically corresponds to the flowering season of birch, which is typically spring, preferably early spring (for example from April to May in the Northern hemisphere). The allergic individual is typically allergic to birch pollen from any tree in the subgenus *Betula*, for example *Betula pendula* or *Betula pubescens*.

Combination immunotherapy

Since many individuals are allergic, or may require desensitizing to several polypeptide antigens, the current invention also provides means of desensitizing individuals that are allergic to multiple antigens. "Tolerance" induced in an individual to a first polypeptide antigen or allergen can create in the individual a "tolergeneic environment" wherein inappropriate immune responses to other antigens can be downregulated in order to provide tolerance to other antigens.

This finding means that individuals allergic to multiple allergens can be treated

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in a greatly reduced time period, and that individuals seriously allergic to some allergens (e.g., peanuts) but more mildly allergic to other allergens (e.g., cat dander) can benefit from a therapy wherein tolerance to the milder allergen is established and then this tolergeneic environment is used to provide tolerance to the other, more extreme allergen. In addition, individuals suffering from an autoimmune disorder who are additionally sensitised (or otherwise immune) to an unrelated antigen or allergen can benefit from a treatment regime wherein tolerance to the unrelated antigen or allergen is first established and then this tolergeneic environment is used to provide tolerance to the autoantigen associated with the autoimmune disorder.

A method is therefore provided for desensitising a birch allergic individual to birch allergen as described above and one or more further different polypeptide antigens. The method entails, in a first step, administering to the individual a composition/formulation (primary composition) according to the invention as described herein and wherein the administration is carried out in a manner sufficient to generate a hyporesponsive state against birch allergen. Once a hyporesponsive state has been established toward birch allergen, or at least a shift toward desensitisation has occurred, the method entails administration of a secondary composition comprising a second, different polypeptide antigen to which the individual is to be sensitised. Administration of the secondary composition is carried out in such a way as to take advantage of the tolerogenic environment established by use of the primary composition, where it is now possible to establish tolerance to the second, different polypeptide antigen. The secondary composition is coadministered with either the first primary composition or a larger fragment of the birch allergen. By "coadministered" it is meant either the simultaneous or concurrent administration, e.g., when the two are present in the same composition or administered in separate compositions at nearly the same time but at different sites, as well as the delivery of polypeptide antigens in separate compositions at different times. For example, the secondary composition may be delivered prior to or subsequent to delivery of the first composition at the same or a different site. The timing between deliveries can range from about several seconds apart to about several minutes apart, several hours apart, or even several days apart. Furthermore, different delivery methods can be employed.

The second polypeptide antigen is preferably an allergen different to the birch allergen. Suitable allergens for use in the methods of the invention can of course be

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obtained and/or produced using known methods. Classes of suitable allergens include, but are not limited to, dust mite allergens, pollens, animal dander (especially cat dander), grass allergens, molds, dusts, antibiotics, stinging insect venoms, and a variety of environmental (including chemicals and metals), drug and food allergens. Common tree allergens include pollens from cottonwood, poplar, ash, birch, maple, oak, elm, hickory, and pecan trees; common plant allergens include those from mugwort, ragweed, English plantain, sorrel-dock and pigweed; plant contact allergens include those from poison oak, poison ivy and nettles; common grass allergens include rye grass, Timothy, Johnson, Bermuda, fescue and bluegrass allergens; common allergens can also be obtained from molds or fungi such as Alternaria, Fusarium, Hormodendrum, Aspergillus, Micropolyspora, Mucor and thermophilic actinomycetes; epidermal allergens can be obtained from house or organic dusts (typically fungal in origin), or from animal sources such as feathers, and dog dander; common food allergens include milk and cheese (dairy), egg, wheat, nut (e.g., peanut), seafood (e.g., shellfish), pea, bean and gluten allergens; common environmental allergens include metals (nickel and gold), chemicals (formaldehyde, trinitrophenol and turpentine), Latex, rubber, fiber (cotton or wool), burlap, hair dye, cosmetic, detergent and perfume allergens; common drug allergens include local anesthetic and salicylate allergens; antibiotic allergens include penicillin, tetracycline and sulfonamide allergens; and common insect allergens include bee, wasp and ant venom, and cockroach calyx allergens. Particularly well characterized allergens include, but are not limited to, the major cat allergen Fel d1, bee venom phospholipase A2 (PLA) (Akdis et al. (1996) *J. Clin. Invest.* 98:1676-1683) and the multi-epitopic recombinant grass allergen rKBG8.3 (Cao et al. (1997) *Immunology* 90:46-51). These and other suitable allergens are commercially available and/or can be readily prepared as extracts following known techniques.

Preferably, the second polypeptide allergen is a whole tree pollen allergen or allergen fragment selected from the list of allergen sequences and database accession numbers (NCBI Entrez accession numbers) below. NCBI is the National Center for Biotechnology information and is a division of the US National Institutes of Health. The NCBI web site, from which access to the database may be sought, is

www.ncbi.nlm.nih.gov/. Allergen sequences and database accession numbers (NCBI Entrez accession numbers):

Olive tree

Olive sequences

416610 Ole e 1
EDIPQPPVSQFHIQGQVYCDTCRAGFITELSEFIPGASLRLQCKDKENGDVTFTE
VGYTRAEGLYSMLVERDHKNEFCEITLISSGRKDCNEIPTEGWAKPSLKFKLNT
VNGTTRTVNPLGFFKKEALPKCAQVYNKLGMYPPNM

Tree allergen sequences (mainly birch) sequences:

130975 Bet v 2
MSWQTYVDEHLMCDIDGQASNSLASAIVGHDGSVWAQSSSFPQFKPQEITGIM
KDFEEPGHLAPTGLHLGGIKYMVIQGEAGAVIRGKKGSGGITIKKTGQALVFGI
YEEPVTPGQCNMVVERLGDYLIDQGL

1942360 Bet v 2
MSWQTYVDEHLMCDIDGQGEELAASAIVGHDGSVWAQSSSFPQFKPQEITGIM
KDFEEPGHLAPTGLHLGGIKYMVIQGEAGAVIRGKKGSGGITIKKTGQALVFGI
YEEPVTPGQCNMVVERLGDYLIDQGL

166953 Bet v 2
MSWQTYVDEHLMCDIDGQASNSLASAIVGHDGSVWAQSSSFPQFKPQEITGIM
KDFEEPGHLAPTGLHLGGIKYMVIQGEAGAVIRGKKGSGGITIKKTGQALVFGI
YEEPVTPGQCNMVVERLGDYLIDQGL

541814 Bet v 2
MSWQTYVDEHLMCDIDGQASNSLASAIVGHDGSVWAQSSSFPQFKPQEITGIM
KDFEEPGHLAPTGLHLGGIKYMVIQGEAGAVIRGKKGSGGITIKKTGQALVFGI

YEEPVTPGQCNMVVERLGDYLIDQGL

2488678 Bet v 2

MSWQTYVDEHLMCDIDGQASNSLASAIVGHDGSVWAQSSSFPQFKPQEITGIM
KDFEEPGHLAPTGLHLGGIKYMVIQGEAGAVIRGKKGSGGITIKKTGQALVFGI
YEEPVTPGQCNMVVERLGDYLIDQGL

1829894 Bet v 2

MSWQTYVDEHLMCDIDGQASNSLASAIVGHDGSVWAQSSSFPQFKPQEITGIM
KDFEEPGHLAPTGLHLGGIKYMVIQGEAGAVIRGKKGSGGITIKKTGQALVFGI
YEEPVTPGQCNMVVERLGDYLIDQGL

1168696 Bet v 3

MPCSTEAMEKAGHGHASTPRKRSLSNSSFRLRSESLNTLRLRRIFDLFDKNSDG
IITVDELSRALNLLGLETDLSELESTVKSFTREGNIGLQFEDFISLHQSLNDSYFA
YGGEDEDDNEEDMRKSILSQEEADSFGGFKVFDEDGDGYISARELQMVLGKL
GFSEGSEIDRVEKMIVSVDSNRDGRVDFFEFKDMMRSVLVRSS

809536 Bet v 4

MADDHPQDKAERERIFKRFDANGDGKISAAELGEALKTLGSITPDEVKHMMA
EIDTDGDGFISFQEFTDFGRANRGLLKDVAKIF

543675 Que a I - Quercus alba=oak trees (fragment)

GVFTXESQETSVIAPAXLFKALFL

543509 Car b I - Carpinus betulus=hornbeam trees (fragment)

GVFNYEAETPSVIPAARLFKSYVLDGDKLIPKVAPQAIXK

543491 Aln g I - Alnus glutinosa=alder trees (fragment)

GVFNYEAETPSVIPAARLFKAFILDGDKLLPKVAPEAVSSVENI

1204056 Rubisco

VQCMQVWPPLGLKKFETLSYLPPLSSEQLAKEVDYLLRKNLIPCLEFELEHGFV YREHNRSPGYYDGRYWTMWKLPMFGCNDSSQVLKELEECKKAYPSAFIRIIGF DDK

Additional tree allergen sequences (NCBI entrez accession number):

131919; 128193; 585564; 1942360; 2554672; 2392209; 2414158; 1321728; 1321726; 1321724; 1321722; 1321720; 1321718; 1321716; 1321714; 1321712; 3015520; 2935416; 464576; 1705843; 1168701; 1168710; 1168709; 1168708; 1168707; 1168706; 1168705; 1168704; 1168703; 1168702; 1842188; 2564228; 2564226; 2564224; 2564222; 2564220; 2051993; 1813891; 1536889; 534910; 534900; 534898; 1340000; 1339998; 2149808; 66207; 2129477; 1076249; 1076247; 629480; 481805; 81443; 1361968; 1361967; 1361966; 1361965; 1361964; 1361963; 1361962; 1361961; 1361960; 1361959; 320546; 629483 ; 629482; 629481; 541804; 320545; 81444; 541814:; 629484; 474911; 452742; 1834387; 298737; 298736; 1584322; 1584321; 584320; 1542873; 1542871; 1542869; 1542867; 1542865; 1542863; 1542861; 1542859; 1542857; 1483232; 1483230; 1483228; 558561; 551640; 488605; 452746; 452744; 452740; 452738; 452736; 452734; 452732; 452730; 452728; 450885; 17938; 17927; 17925; 17921; 297538; 510951; 289331; 289329; 166953 .

Cedar sequences

493634 Cry j IB precursor

MDSPCLVALLVFSFVIGSCFSDNPIDSCWRGDSNWAQNRMKLADCAVGFGSST MGGKGGDLYTVTNSDDDPVNPPGTLRYGATRDRPLWIIFSGNMNIKLKMPMY IAGYKTFDGRGAQVYIGNGGPCVFIKRVSNVIIHGLYLYGCSTSVLGNVLINESF GVEPVHPQDGDALTLRTATNIWIDHNSFSNSSDGLVDVTLTSTGVTISNNLFFN HHKVMSLGHDDAYSDDKSMKVTVAFNQFGPNCGQRMPRARYGLVHVANNN YDPWTIYAIGGSSNPTILSEGNSFTAPNESYKKQVTIRIGCKTSSSCSNWVWQST QDVFYNGAYFVSSGKYEGGNIYTKKEAFNVENGNATPHLTQNAGVLTCSLSK RC

493632 Cry j IA precursor

MDSPCLVALLVLSFVIGSCFSDNPIDSCWRGDSNWAQNRMKLADCAVGFGSS
TMGGKGGDLYTVTNSDDDPVNPAPGTLRYGATRDRPLWIIFSGNMNIKLKMP
MYIAGYKTFDGRGAQVYIGNGGPCVFIKRVSNVIIHGLHLYGCSTSVLGNVLIN
ESFGVEPVHPQDGDALTLRTATNIWIDHNSFSNSSDGLVDVTLSSTGVTISNNLF
FNHHKVMLLGHDDAYSDDKSMKVTVAFNQFGPNCGQRMPRARYGLVHVAN
NNYDPWTIYAIGGSSNPTILSEGNSFTAPNESYKKQVTIRIGCKTSSSCSNWVW
QSTQDVFYNGAYFVSSGKYEGGNIYTKKEAFNVENGNATPQLTKNAGVLTCS
LSKRC

1076242 Cry j II precursor - Japanese cedar

MAMKLIAPMAFLAMQLIIMAAAEDQSAQIMLDSVVEKYLRSNRSLRKVEHSR
HDAINIFNVEKYGAVGDGKHDCTEAFSTAWQAACKNPSAMLLVPGSKKFVVN
NLFFNGPCQPHFTFKVDGIIAAYQNPASWKNNRIWLQFAKLTGFTLMGKGVID
GQGKQWWAGQCKWVNGREICNDRDRPTAIKFDFSTGLIIQGLKLMNSPEFHL
VFGNCEGVKIIGISITAPRDSPNTDGIDIFASKNFHLQKNTIGTGDDCVAIGTGSS
NIVIEDLICGPGHGISIGSLGRENSRAEVSYVHVNGAKFIDTQNGLRIKTWQGGS
GMASHIIYENVEMINSENPILINQFYCTSASACQNQRSAVQIQDVTYKNIRGTSA
TAAAIQLKCSDSMPCKDIKLSDISLKLTSGKIASCLNDNANGYFSGHVIPACKNL
SPSAKRKESKSHKHPKTVMVENMRAYDKGNRTRILLGSRPPNCTNKCHGCSP
CKAKLVIVHRIMPQEYYPQRWICSCHGKIYHP

1076241 Cry j II protein - Japanese cedar

MAMKFIAPMAFVAMQLIIMAAAEDQSAQIMLDSDIEQYLRSNRSLRKVEHSRH
DAINIFNVEKYGAVGDGKHDCTEAFSTAWQAACKKPSAMLLVPGNKKFVVN
NLFFNGPCQPHFTFKVDGIIAAYQNPASWKNNRIWLQFAKLTGFTLMGKGVID
GQGKQWWAGQCKWVNGREICNDRDRPTAIKFDFSTGLIIQGLKLMNSPEFHL
VFGNCEGVKIIGISITAPRDSPNTDGIDIFASKNFHLQKNTIGTGDDCVAIGTGSS
NIVIEDLICGPGHGISIGSLGRENSRAEVSYVHVNGAKFIDTQNGLRIKTWQGGS
GMASHIIYENVEMINSENPILINQFYCTSASACQNQRSAVQIQDVTYKNIRGTSA
TAAAIQLKCSDSMPCKDIKLSDISLKLTSGKIASCLNDNANGYFSGHVIPACKNL

SPSAKRKESKSHKHPKTVMVKNMGAYDKGNRTRILLGSRPPNCTNKCHGCSP
CKAKLVIVHRIMPQEYYPQRWMCSRHGKIYHP

541803 Cry j I precursor - Japanese cedar

MDSPCLVALLVLSFVIGSCFSDNPIDSCWRGDSNWAQNRMKLADCAVGFGSS
TMGGKGGDLYTVTNSDDDPVNPPGTLRYGATRDRPLWIIFSGNMNIKLKMPM
YIAGYKTFDGRGAQVYIGNGGPCVFIKRVSNVIIHGLHLYGCSTSVLGNVLINES
FGVEPVHPQDGDALTLRTATNIWIDHNSFSNSSDGLVDVTLSSTGVTISNNLFF
NHHKVMLLGHDDAYSDDKSMKVTVAFNQFGPNCGQRMPRARYGLVHVANN
NYDPWTIYAIGGSSNPTILSEGNSFTAPNESYKKQVTIRIGCKTSSSCSNWVWQS
TQDVFYNGAYFVSSGKYEGGNIYTKKEAFNVENGNATPQLTKNAGVLTCSLS
KRC

541802 Cry j I precursor- Japanese cedar

MDSPCLVALLVFSFVIGSCFSDNPIDSCWRGDSNWAQNRMKLADCAVGFGSST
MGGKGGDLYTVTNSDDDPVNPAPGTLRYGATRDRPLWIIFSGNMNIKLKMPM
YIAGYKTFDGRGAQVYIGNGGPCVFIKRVSNVIIHGLYLYGCSTSVLGNVLINES
FGVEPVHPQDGDALTLRTATNIWIDHNSFSNSSDGLVDVTLTSTGVTISNNLFF
NHHKVMSLGHDDAYSDDKSMKVTVAFNQFGPNCGQRMPRARYGLVHVANN
NYDPWTIYAIGGSSNPTILSEGNSFTAPNESYKKQVTIRIGCKTSSSCSNWVWQS
TQDVFYNGAYFVSSGKYEGGNIYTKKEAFNVENGNATPHLTQNAGVLTCSLS
KRC

Delivery methods

Once formulated the compositions of the invention can be delivered to a subject *in vivo* using a variety of known routes and techniques. For example, a composition can be provided as an injectable solution, suspension or emulsion and administered via parenteral, subcutaneous, epidermal, intradermal, intramuscular, intraarterial, intraperitoneal, intravenous injection using a conventional needle and syringe, or using a liquid jet injection system, or using a patch. Compositions can also be administered topically to skin or mucosal tissue, such as nasally, intratracheally, intestinal, rectally or vaginally, or provided as a finely divided spray suitable for respiratory or pulmonary

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administration. Other modes of administration include oral administration, suppositories, sublingual administration, and active or passive transdermal delivery techniques.

Where a peptide of the invention is to be administered, it is preferred to administer the peptide to a site in the body where it will have the ability to contact suitable antigen presenting cells, and where it, or they, will have the opportunity to contact T cells of the individual.

Delivery regimes

Administration of the peptides (such as the composition containing a plurality of peptides) may be by any suitable method as described above. Suitable amounts of the peptide may be determined empirically, but typically are in the range given below. A single administration of each peptide may be sufficient to have a beneficial effect for the patient, but it will be appreciated that it may be beneficial if the peptide is administered more than once, in which case typical administration regimes may be, for example, once or twice a week for 2-4 weeks every 6 months, or once a day for a week every four to six months. As will be appreciated, each peptide or combination of peptides may be administered to a patient singly or in combination.

Dosages for administration will depend upon a number of factors including the nature of the composition, the route of administration and the schedule and timing of the administration regime. Suitable doses of a molecule of the invention may be in the order of up to 15μg, up to 20μg, up to 25μg, up to 30μg, up to 50μg, up to 100μg, up to 500 μg or more per administration. Suitable doses may be less than 15μg, but at least 1ng, or at least 2ng, or at least 5ng, or at least 50ng, or least 100ng, or at least 500ng, or at least 1μg, or at least 10μg. For some molecules of the invention, the dose used may be higher, for example, up to 1 mg, up to 2 mg, up to 3 mg, up to 4 mg, up to 5 mg or higher. Such doses may be provided in a liquid formulation, at a concentration suitable to allow an appropriate volume for administration by the selected route.

Kits

The present disclosure also relates to a combination of components described herein suitable for use in a treatment of the invention which are packaged in the form of a kit in a container. Such kits may comprise a series of components to allow for a

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treatment of the invention. For example, a kit may comprise one or more different peptides of the invention and one or more additional therapeutic agents suitable for simultaneous administration, or for sequential or separate administration. The kit may optionally contain other suitable reagent(s) or instructions and the like.

The invention is illustrated by the following Examples:

Example 1

MHC Class II binding search

The aim of this study is to identify a distinct panel of peptides with strong affinities for the eight most common human MHC Class II HLA-DRB1* allotypes. In order to identify binding peptides in the major birch allergens Bet v1, Bet v2, Bet v3, Bet v4, Bet v6, an *in silico* approach was carried out using the commercially available EpiMatrix algorithm (EpiVax Inc.) This is a bioinformatic analysis of peptides from a sequence for the potential to be accommodated within the binding groove of MHC class II HLA-DR molecules.

EpiMatrix is a matrix-based algorithm that ranks 9 amino acid residue sequences, overlapping by 8 amino acids, from any polypeptide sequence by estimated probability of binding to each of the selected MHC molecules. (De Groot et al., AIDS Research and Human Retroviruses 13:539-41 (1997). The procedure for developing matrix motifs was published by Schafer et al, Vaccine 16:1880-4 (1998). In this Example, binding potential for HLA DR1, DR3, DR4, DR7, DR8, DR11, DR13 and DR15 is assessed. Putative MHC ligands are selected by scoring each 9-mer frame in a protein sequence. This score is derived by comparing the sequence of the 9-mer to the matrix of amino acid sequences known to bind to each MHC allele. Retrospective studies have demonstrated that EpiMatrix accurately predicts published MHC ligands (Jesdale et al., in Vaccines '97 (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.,

1997)). Successful Prediction of peptides which bind to multiple MHC molecules has also been confirmed.

The EpiMatrix data for each allergen is shown either:

As overlapping 9 mer peptide data with the binding (Z) score for each allele and the number of 'hits' for the eight alleles (Z scores of equal to or greater than the top 5% of predicted binders): or

As a cluster report where the data from analysing multiple sequences from the database is 'clustered' to give an overview of binding for all variants of the protein. The "EpiMatrix hits' refers to the number of high predicted Z binding scores for the eight alleles within that sequence whilst the "EpiMatrix Cluster Score" is derived from the number of hits normalized for the length of the cluster. Cluster Score is thus the excess or shortfall in predicted aggregate MHC binding properties relative to a random peptide standard. A cluster score above 10 is considered to indicate broad MHC binding properties.

EpiMatrix analyses were performed on the entire sequences of known isoforms of Bet v1, listed below with their corresponding NCBI accession numbers:

Bet v1 L	P43185;	Bet v1 E	P43178;
Bet v1 M/N	P43186;	Bet v1 D/H	P43177;
Betv1 K	P43184;	Bet v1 C	P43176;
Bet v1 J	P43183;	Bet v1 B	P45431;
Bet v1 G	P43180;	Bet v1 A	P15494;
Bet v1 F/I	P43179;		

Epimatrix analyses were also performed on additional known Bet v1 sequences indexed by accession number in Table 2.

These analyses identified core peptides (and their flanking sequences) derived from the above sequences which are predicted to have good MHC class-II binding. These sequences are shown below in Tables 1 and 2. As shown, many of the peptides identified are highly conserved between different Bet v1 isoforms.

In Tables 1 and 2:

"Residues in main sequence" gives the location of the peptide within the sequences that were analysed. The core peptide (underscored middle amino acids in bold) defines the actual binding sequence that was identified during the analysis. The stabilizing flanks (N-terminal and C-terminal, not bold) were included for use with the core sequence and are typically required to aid manufacture of the peptides. "Number of

hits" refers to the number of high predicted binding affinities for all MHC types tested within the sequence. The "EpiMatrix Cluster Score" is derived from the number of hits normalized for the length of the cluster. Cluster Score is thus the excess or shortfall in predicted aggregate MHC binding properties relative to a random peptide standard. A score above 10 is considered to indicate broad MHC binding properties.

Table 1 - Bet v1

INPUT SEQUENCE	RESIDUES IN SEQUENCE (Incl. FLANKS)	SEQUENCE	Hydrophobicity	EpiMatrix HITS (Excl FLANKS)	EpiMatrix CLUSTER SCORE (Excl FLANKS)	Peptide ID NO	SEQ ID NO:
P43185	13 - 28	VIPAARMFKAFILDGD	0.78	6	11.3	P1	1
P15494	100 - 114	SNEIKIVATPDGGSI	0.03	7	15.57	P2	2
P43176	100 - 114	CNEIKIVATPDGGSI	0.25	7	15.57	P3	3
P43177	100 - 114	SNEIKIVATPDGGCV	0.23	7	15.57	P4	4
P43178	100 - 116	SNEIKIVATPNGGSILK	0.02	10	20.13	P5	5
P43179	100 - 116	SNEIKIVATPNGGSILK	0.02	10	20.13	P6	
P43180	100 - 114	SNEIKIVATPDGGCV	0.23	7	15.57	P7	
P43183	100 - 116	SNEIKIVATPNGGSILK	0.02	10	20.13	P8	
P43184	100 - 114	CNEIKIVATPDGGSI	0.25	7	15.57	P9	
P43185	100 - 114	SNEIKIVATPDGGCV	0.23	7	15.57	P10	
P43186	100 - 114	CNEIKIVATPDGGSI	0.25	7	15.57	P11	
P45431	100 - 114	CNEIKIVATPDGGSI	0.25	7	15.57	P12	
P43178	112 - 126	GSILKINNKYHTKGD	-1.08	6	12.34	P13	
P43179	112 - 126	GSILKINNKYHTKGD	-1.08	6	12.34	P14	
P43183	112 - 126	GSILKINNKYHTKGD	-1.08	6	12.34	P15	
P15494	142 - 160	ETLLRAVESYLLAHSDAYN	-0.09	8	12.06	P16	6
P43176	142 - 160	EALLRAVESYLLAHSDAYN	0.04	8	12.06	P17	7
P43177	142 - 160	ETLLRAVESYLLAHSDAYN	-0.09	8	12.06	P18	
P43178	142 - 160	ETLLRAVESYLLAHSDAYN	-0.09	8	12.06	P19	
P43179	142 - 160	ETLLRAVESYLLAHSDAYN	-0.09	8	12.06	P20	
P43180	142 - 160	ETLLRAVESYLLAHSDAYN	-0.09	8	12.06	P21	
P43183	142 - 160	ETLLRAVESYLLAHSDAYN	-0.09	8	12.06	P22	
P43184	142 - 160	EALLRAVESYLLAHSDAYN	0.04	8	12.06	P23	
P43185	142 - 160	ETLLRAVESYLLAHSDAYN	-0.09	8	12.06	P24	
P43186	142 - 160	EALLRAVESYLLAHSDAYN	0.04	8	12.06	P25	
P45431	142 - 160	EALLRAVESYLLAHSDAYN	0.04	8	12.06	P26	

Table 1A

EpiMatrix analysis of Bet v1 Sequence: P15494: predicted multiple HLA DR allele binding region: FNYETETTSVIPAARLFKAFILDGDNLF (4-31).

Frame Start	AA Sequence	Frame Stop	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits
4	FNYETETTS	12	.96	1.29	1.58	-.42	.10	.93	-.21	.27	0

5	NYETETTSV	13	.32	-.86	.59	.81	-1.32	-.80	-.75	.22	0
6	YETETTSVI	14	2.15	1.37	2.25	1.72	1.09	1.15	-.03	.70	3
7	ETETTSVIP	15	-.11	-1.34	.48	.58	-.89	-.60	-.84	-.03	0
8	TETTSVIPA	16	.16	-1.10	.97	.51	-.86	-.69	-.48	.04	0
9	ETTSVIPAA	17	-.04	.16	.64	-.18	-.89	.43	-.39	-.17	0
10	TTSVIPAAR	18	.64	.66	-.12	-.56	.14	.47	.50	-.43	0
11	TSVIPAARL	19	1.52	-.44	.23	.47	-.63	.28	-.96	-.33	0
12	SVIPAARLF	20	.69	-.06	-.07	1.07	-.44	.25	1.05	-.20	0
13	VIPAARLFK	21	.67	2.00	.65	.50	1.21	1.32	1.98	1.21	2
14	IPAARLFKA	22	.55	1.17	.00	-.28	.32	.16	1.27	1.60	0
15	PAARLFKAF	23	-1.29	-1.65	-1.57	.28	-.29	-.88	-.32	-.58	0
16	AARLFKAFI	24	1.13	1.39	-.25	-.68	1.25	1.50	1.32	-.06	0
17	ARLFKAFIL	25	2.12	.89	.25	1.84	1.11	.18	1.54		3
18	RLFKAFILD	26	-1.54	-.90	-1.35	-.86	1.38	-1.29	-.38	-.67	0
19	LFKAFILDG	27	.86	1.13	1.48	.68	.94	.39	1.14	1.37	0
20	FKAFILDGD	28	-.17	.58	.09	-.43	1.90	.24	.30	.96	1
21	KAFILDGDN	29	-.49	-1.35	-.67	-.69	-.59	-.02	-2.05	.29	0
22	AFILDGDNL	30	1.32	-.28	-.21	.67	-.85	-.69	-.80	.30	0
23	FILDGDNLF	31	-.02	2.27	1.73	1.07	.47	-.09	-.22	.74	2

Table 2 – Bet v 1

INPUT SEQUENCE	RESIDUES IN SEQUENCE (Incl. FLANKS)	SEQUENCE	Hydro-phobicity	EpiMatrix HITS (Excl. FLANKS)	EpiMatrix CLUSTER SCORE (Excl. FLANKS)	Peptide ID NO	SEQ ID NO:
CAA04829	1 - 16	GV**FNYEIGATSVI**PAA	0.84	7	11.23	P27	8
2122374C	14 - 34	IAP**ARLFKSFVLDADNLI**PKV	0.62	9	13.67	P28	9
ABC41588	97 - 111	CNE**IKLVATPDG**GST	-0.15	7	15.45	P29	10
ABC41605	97 - 111	SKE**IKIAAAPDG**GSI	0.01	6	13.05	P30	11
ABC41615	97 - 111	SNE**IKIVATPDG**GCI	0.25	7	15.57	P31	12
ABC41617	97 - 111	CNE**IKLVATPDG**GSI	0.20	7	15.45	P32	13
ABC41596	97 - 113	CNE**IKIVAAPGGGS**ILK	0.54	9	17.42	P33	14
ABC41602	97 - 113	CNE**IKIVPAPGGGS**ILK	0.34	9	16.98	P34	15
ABC41609	97 - 113	SYE**IKIVAAPGGGS**ILK	0.48	9	17.42	P35	16
1QMR_A	99 - 113	SNE**IKIVATGDG**GSI	0.11	5	10.32	P36	17
CAA96546	100 - 114	CNE**IKIVAAPDG**GSI	0.41	7	14.59	P37	18
CAA96547	100 - 114	SNE**IKIVATPDG**RSI	-0.25	7	15.57	P38	19
CAA07324	100 - 114	SNE**IKLVATPDG**GSI	-0.02	7	15.45	P39	20
CAA07327	100 - 114	CNE**IKIVATPDG**GCV	0.45	7	15.57	P40	21
CAA07318	100 - 114	SNE**IKIVTTPDG**GCV	0.06	7	15.57	P41	22
AAD26561	100 - 114	SNE**IKIVATPDG**GPI	-0.03	7	15.57	P42	23
ABC41589	109 - 125	GSI**LKIRNKYHTKG**DHE	-1.41	9	15.34	P43	24
ABC41609	139 - 150	AGL**FKAVENYLV**	0.82	5	10.95	P44	25
ABC41583	139 - 150	ETL**LRAVESYLL**	0.58	6	13.44	P45	26
ABC41589	139 - 150	EAL**LRAVESYLL**	0.78	6	13.44	P46	27
ABC41602	139 - 150	EAL**FRAVESYLL**	0.70	7	16.84	P47	28
CAA96544	139 - 156	EKA**VGLLKAVESYLL**AHS	0.43	7	12.67	P48	29
CAA07319	141 - 160	GET**LLRAVEGYLLAHSDAY**N	-0.09	8	11.13	P49	30
AAD26561	142 - 156	ETL**LRAVESYPLA**HS	-0.05	6	13.44	P50	31
2122374C	142 - 160	AGL**FKAVENYLVAHPNAY**N	0.02	10	15.5	P51	32

CAA96539	142 - 160	ETLLRAVERYLLAHSDAYN	-0.29	10	18.69	P52	33
2122374A	142 - 160	EALFRAVESYLLAHSDAYN	-0.02	9	15.46	P53	34

Example 2

EpiMatrix analyses as above were performed on the entire sequence of a known isoform of Bet v 3 (NCBI accession no: P43187). These analyses identified a core peptide (and its flanking sequence) derived from the above sequence which is predicted to have good MHC class-II binding. The sequence is shown below in Table 3. Headings and notes for Table 3 are as with Table 1 above.

Table 3 - Bet v 3

INPUT SEQUENCE	RESIDUES IN SEQUENCE (Incl FLANKS)	SEQUENCE	Hydro-phobicity	EpiMatrix HITS (Excl FLANKS)	EpiMatrix CLUSTER SCORE (Excl FLANKS)	Peptide ID NO	SEQ ID NO:
P43187	188 - 205	VDFFEFKDMMRSVLVRSS	0.16	10	14.02	P54	35

A sequence at residues 80 to 94 of P43187, TVKSFTREGNIGLQF (Peptide ID NO. P55, SEQ ID NO: 36), was also predicted to have good MHC-Class II binding. Additional *in silico* analysis of other birch allergen sequences from Bet v 3 is shown here:

Table 3A

EpiMatrix analysis of Bet v3 Sequence: GI1168696_SPP43187: predicted multiple HLA DR allele binding region SLNTLRLRRIFDLFDK (35-50).

Frame start	sequence	end	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits
35	SLNTLRLRR	43	.28	.97	-.38	.38	.64	.53	1.73	.68	1
36	LNTLRLRRI	44	1.30	.87	-.15	.46	.82	1.35	1.41	.17	0
37	NTLRLRRIF	45	-.24	.12	-1.52	.43	1.21	1.13	1.42	-.47	0
38	TLRLRRIFD	46	.47	.40	-.46	.42		.66	.49	-.34	1
39	LRLRRIFDL	47	1.09	.83	.08		1.68	.31	1.94		4
40	RLRRIFDLF	48	-1.83	-.86	-1.40	-.38	-.94	-1.34	-.63	-.87	0
41	LRRIFDLFD	49	.84	.60	1.39	.46	1.41	.88	.39	1.55	0
42	RRIFDLFDK	50	-.31	.38	-.31	-.58	.85	.33	1.05	1.61	0

Example 3

EpiMatrix analyses as above were performed on the entire sequence of known isoforms of Bet v 4 (NCBI accession nos: Q39419, CAA73147). These analyses identified core peptides (and their flanking sequences) derived from the above sequences which are predicted to have good MHC class-II binding. These sequences

are shown below in Table 4. Headings and notes for Table 4 are as with Table 1 above.

Table 4 - Bet v 4

INPUT SEQUENCE	RESIDUES IN SEQUENCE (Incl. FLANKS)	SEQUENCE	Hydro-phobicity	EpiMatrix HITS (Excl FLANKS)	EpiMatrix CLUSTER SCORE (Excl FLANKS)	Peptide ID NO.	SEQ ID NO:
Q39419	10 - 27	AER**ERIFKRFDANGD**GKI	-1.19	8	13.83	P56	37
Q39419	67 - 81	FTD**FGRANRGLL**KDV	-0.38	7	13.49	P57	38
CAA73147	67 - 81	FTD**FARANRGLL**KDV	-0.23	7	13.53	P58	39

Example 4

EpiMatrix analyses as above were performed on the entire sequence of a known isoform of Bet v 6 (NCBI accession no: O65002). These analyses identified core peptides (and their flanking sequences) derived from the above sequences which are predicted to have good MHC class-II binding. These sequences are shown below in Table 5. Headings and notes for Table 5 are as with Table 1 above.

Table 5 – Bet v 6

INPUT SEQUENCE	RESIDUES IN SEQUENCE (Incl. FLANKS)	SEQUENCE	Hydro-phobicity	EpiMatrix HITS (Excl FLANKS)	EpiMatrix CLUSTER SCORE (Excl FLANKS)	Peptide ID NO	SEQ ID NO:
O65002	43 - 63	PVK**GKLVERFKGLGVTLL**HGD	0.04	9	14.09	P59	40
O65002	67 - 90	HES**LVKAFKQVDVVISTVGHL**QLA	0.52	11	15.54	P60	41
O65002	149 - 170	YVS**SNFFAGYFLPTLAQPG**LTS	0.46	10	12.92	P61	42
O65002	258 - 274	PIN**VILAINHSVFV**KGD	0.83	7	10.54	P62	43

Example 5

EpiMatrix analyses as above were performed on the entire sequence of a known isoform of Bet v 7 (NCBI accession no: CAC84116). These analyses identified a core peptide (and its flanking sequence) derived from the above sequence which is predicted to have good MHC class-II binding. This sequence is shown below in Table 6. Headings and notes for Table 6 are as with Table 1 above.

Table 6 – Bet v 7

INPUT SEQUENCE	RESIDUES IN SEQUENCE (Incl. FLANKS)	SEQUENCE	Hydro-phobicity	EpiMatrix HITS (Excl FLANKS)	EpiMatrix CLUSTER SCORE (Excl FLANKS)	Peptide ID NO.	SEQ ID NO:
CAC84116	34 - 48	AEN**FRALCTGEK**GNG	-0.77	5	10.46	P63	44

Example 5A

Additional *in silico* analysis of other birch allergen sequences from Bet v 2 is shown here:

Table 6A

EpiMatrix analysis of Bet v2 Sequence: GI1942360_PDB1CQA: predicted multiple HLA DR allele binding region: SVWAQSSSFPQFKPQEITGIMK (33-54).

Frame start	sequence	end	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits
33	SVWAQSSSF	41	.75	.40	1.04	1.19	-.29	-.22	-.17	.71	0
34	VWAQSSSFP	42	.90	.82	1.31	.46	-.02	.21	.64	1.01	0
35	WAQSSSFPQ	43	1.70	.36	2.24		1.23	1.44	.12	1.53	3
36	AQSSSFPQF	44	-.65	.38	-.28	-.01	-1.26	-.18	-.18	-.56	0
37	QSSSFPQFK	45	-.31	.01	.24	.04	-1.01	.00	-1.52	-.51	0
38	SSSFPQFKP	46	-.45	-.73	-1.50	-1.02	-.04	-.76	-.02	.21	0
39	SSFPQFKPQ	47	-1.10	-2.37	-.57	.24	-.52	-.64	-1.22	-.98	0
40	SFPQFKPQE	48	-.23	1.11	-.75	-1.69	1.34	.47	1.32	-.32	0
41	FPQFKPQEI	49	1.55	.73	.67	.99	.88	.49	.09	2.02	1
42	PQFKPQEIT	50	-.26	.27	-.85	.12	1.00	.28	.59	-.60	0
43	QFKPQEITG	51	-1.28	-.42	-.84	-.60	-.27	-1.13	.23	.00	0
44	FKPQEITGI	52	1.94	1.68	1.69	1.76	.07	.45	1.37	1.43	4
45	KPQEITGIM	53	.15	.15	.32	.12	-.39	.10	-1.15	-.26	0
46	PQEITGIMK	54	-.06	-1.11	-.06	-.24	-1.55	-.85	-1.48	-.15	0

Table 6B

EpiMatrix analysis of Bet v2 Sequence: GI1942360_PDB1CQA: predicted multiple HLA DR allele binding region: IKYMVIQGEAGAVIRGKKGSGG (72-93).

Frame start	sequence	end	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits
72	IKYMVIQGE	80	1.21	1.60	1.25	.49	1.87	1.30	-.52	.97	1
73	KYMVIQGEA	81	.45	.63	-.35	.37	.56	1.73	.28	.53	1
74	YMVIQGEAG	82	1.68	.22	1.47	.98	.61	1.06	-.37	.26	1
75	MVIQGEAGA	83	1.49	2.23	1.51	-.42	1.07	1.87	2.27	.85	3
76	VIQGEAGAV	84	1.12	1.46	-.18	1.12	-.11	.16	-.07	2.02	1
77	IQGEAGAVI	85		1.64	1.28	.54	.46	.67	.50	.86	1
78	QGEAGAVIR	86	.56	.61	-.43	-.01	-.68	-1.08	.29	.12	0
79	GEAGAVIRG	87	.02	.36	.41	-.45	-.52	-1.38	-.79	.28	0
80	EAGAVIRGK	88	-.12	-.55	.17	-.08	-.44	.08	-.23	-1.21	0
81	AGAVIRGKK	89	-.65	.95	-1.14	.00	.75	1.71	.58	.53	1
82	GAVIRGKKG	90	.82	-1.39	-.20	-.20	-.70	-.26	-1.31	.08	0
83	AVIRGKKGS	91	-.08	.16	-.55	-.21	1.97	1.49	1.43	1.07	1
84	VIRGKKGSG	92	.10		-.64	-.90	1.35	1.18	.92	1.79	2
85	IRGKKGSGG	93	1.18	1.05	.64	.02	2.09	.95	1.11	1.83	2

Example 6

Based on the analyses performed in Examples 1 to 5A, the following peptides shown in Table 7 were designed for screening in subsequent assays. The design process involved modification of native sequences to enhance solubility and other physicochemical characteristics. For example, for Bir12A, residues in parent 62-77R indicates that the peptide sequence of Bir12A corresponds to residues 62 to 77 of the parent sequence, with an additional R residue added to the C terminus to improve solubility. Similarly, for Bir01F, G, H and I, residues in parent 4-18K indicates that these peptide sequences correspond to residues 4 to 18 of the parent sequence, with an additional K residue added to the C terminus to improve solubility.

Table 7

Peptide	Sequence	Residues	SEQ. ID. NO
BIR01F	FNYETEATSVIPAARK	4-18K (P43185) Bet v1	45
BIR01G	FNYEIEATSVIPAARK	4-18K (P43179) Bet v1	46
BIR01H	FNYEIETTSVIPAARK	4-18K (P43177) Bet v1	47
BIR01I	FNYETETTSVIPAARK	4-18K (P15494) Bet v1	48
BIR02D	PAARMFKAFILDGDKLVPK	15-33(P43185) Bet v1	49
BIR02E	PAARLFKAFILEGDTLIPK	15-33 (P43184) Bet v1	50
BIR02G	PAARLFKAFILEGDNLIPK	15-33 (P41380) Bet v1	51
BIR02I	PAARMFKAFILD	15-26 (P41385) Bet v1	52
BIR02J	PAARMFKAFILEGDKLVPK	D to E variant of BIR02D	53
BIR04	PGTIKKISFPEGFPFKYV	51-68 (P43185) Bet v1	54
BIR05	SPFKYVKERVDEVDHA	63-78 (P43186) Bet v1	55
BIR05A	FPFKYVKDRVDEVDHT	63-78 (P43185) Bet v1	56
BIR06	ANFKYSYSMIEGGALGD	78-94(P43186) Bet v1	57
BIR06B	TNFKYSYSVIEGGPVGD	78-94 (P43183) Bet v1	58
BIR06D	TNFKYNYSVIEGGPIG	78-93 (P) Bet v1	59
BIR07	SNEIKIVATPDGGSILK	100-116 Bet v1	60
BIR07A	SNEIKIVATPNGGSILK	100-116 Bet v1	61
BIR07B	SNEIKIVATPQGGSILK	100-116 Bet v1	62
BIR07C	SNEIKIVATPEGGSILK	100-116 Bet v1	63
BIR07D	SNEIKIVATPGGGSILK	100-116 Bet v1	64
BIR08	GSILKINNKYHTKGD	112-126 Bet v1	65
BIR08A	SILKISNKYHTKGD	113-125 (P43186) Bet v1	66
BIR09	ETLLRAVESYLLAHSDAY	142-159 Bet v1	67
BIR09A	GETLLRAVESYLLAHS	141-156 Bet v1	68
BIR09B	KEMGETLLRAVESYLLAHS	138-156 Bet v1	69
BIR09C	KEKGETLLRAVESYLLAHS	M to K variant of above	70
BIR10	GSVWAQSSSFPQFK	33-45 (P25816) Bet v 2	71
BIR11	FPQFKPQEITGIMK	41-54 (AAB44348) Bet v2	72
BIR12A	PTGMFVAGAKYMVIQGR	62-77R (P35079) Phl p12	73
BIR12B	AKYMVIQGEPGRVIRGK	70-86 (P35079) Phl p12	74

BIR13	GIKYMVIQGEAGAVIRGK	71-88 (AAB44348) Bet v2	75
BIR14	EAGAVIRGKKGSGGIT	80-95 (P25816) Bet v2	76
BIR15	SLNTLRLRRIFDLFDK	35-50 Bet v3	77
BIR16A	AERERIFKRFDANGEGK	10-26 D to E variant Bet v4	78
BIR16B	AERERIFKRFDAGGEGK	N to G variant of above	79
BIR17	VKGKLVEKFKGLGVTLLHG	44-62 Bet v6	80

Example 7

In vitro binding analysis

The peptides identified as being potential MHC Class II-binding are pre-screened for solubility in an aqueous, acidic milieu and the peptides are tested in an in vitro MHC Class II binding assay.

Methods

The assay employed is a competitive MHC class II binding assay, wherein each peptide is analysed for its ability to displace a known control binder from each of the human MHC class II allotypes investigated. The allotypes and control peptides used in this study are typically those shown below:

Control peptides used in the in vitro binding assays

Allotype	Control Peptide	Sequence
DRB1*0301	Myco. tuberculosis/leprae hsp 65 2-16	AKTIAYDEEARRGLE
DRB1*1101	Influenza haemagglutinin 307-319	PKYVKQNTLKLAT
DRB1*1501	Human myelin basic protein 85-99	ENPVVHFFKNIVTPR

Each of the peptides from Tables 1 to 7 are analysed in the competition assay and screened for relative binding compared to the control peptides. Due to the nature of the competitive assay the data for each peptide is determined as a ratio of its own IC50 to that of the control peptide. Thus, a peptide that has an IC50 value that is parity to the control peptide has an identical binding affinity, while peptides with a ratio less than one have a higher affinity and those with a ratio greater than one have a lower affinity.

Solubility in aqueous solution is an essential criterion for a peptide to be an effective therapeutic agent. Therefore, as a consequence of the solubility screen very hydrophobic peptides with a high frequency of large hydrophobic amino acid residues in multiple binding registers will be eliminated. This is a characteristic of promiscuous HLA-DRB1* binders. Peptides which bind to one or more of the MHC Class II

allotypes are identified. It would be expected that such peptides would have the ability to bind similar allotypes that have not been tested through the homology of MHC structures.

Example 8

The following methods are applied to the same peptides as in Example 7.

Cell proliferation assay

The cell proliferation assay is performed on PBMC's ($140x10^6$ cells required for all parameters to be tested). Proliferation is measured by the incorporation of the radiolabelled compound 3H-thymidine. In more detail, 100µl of the appropriate antigen or peptide concentration is distributed into the appropriate wells of 96 well plates. The plates are then placed into a humidified 5% CO2 incubator set at 37°C for a maximum of 4 hours. PBMC's isolated as described above are prepared to a concentration of $2x10^6$ cells/ml in complete medium at room temperature. 100µl of cell solution is then distributed into each of the wells of the 96 well plates containing antigen/peptide. The plates are then incubated for 6 to 8 days. The cultures are pulsed with tritiated thymidine solution by adding 10µl of tritiated thymidine stock solution (1.85MBq/ml in serum-free RPMI medium) to each well. The plates are then returned to the incubator for between 8 and 16 hours. Cultures are then harvested using a Canberra Packard FilterMate 196 cell harvester. Dried filter mats are counted using an appropriate beta scintillation counter.

Counts from wells containing peptide are compared statistically to wells containing media alone (12 wells per group). The non-parametric Mann-Whitney test is used. The same statistical test is used for all subjects. A statistically significant difference between media only wells and peptide-stimulated wells is considered a positive stimulation of PBMC's by the peptide.

Cytokine release assay

The 36 peptides were manufactured at small scale (approximately 10mg batch size, non-GMP). The purity of each peptide was at least 95% by HPLC. 96 well culture plates containing peptides and controls (the negative control was culture medium and the positive controls were staphylococcal enterotoxin B (SEB) 25ng/ml and whole birch pollen allergen extract 100 µg/ml) were prepared in advance and stored at -20°C

prior to the day of assay. Peptides were added to wells in a volume of 100 µl containing peptides at a concentration of 200 µg/ml, such that subsequent addition of 100 µl of cells would create a final assay concentration of 100 µg/ml.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood by Ficoll density gradient centrifugation. A 100 µl aliquot of a $5x10^6$ cell/ml PBMC suspension was then added to each well and the plates placed in a humidified 5% CO2 incubator at 37°C for 5 days. Following stimulation, culture supernatants (100 µl) were harvested for testing by multiplex bead assay.

Multiplex cytokine bead assays (IL-10, IL-13, Interferon gamma (IFN-g)) were performed on thawed culture supernatants according to the manufacturer's instructions. Single measurements were performed for each culture supernatant sample. After completion of the multiplex assay, individual cytokine levels were determined by interpolation from the standard curve generated in the assay. A positive result was taken as being greater than 100 pg/ml for the IL-13 and IFN-g assays or >4 times the background for the IL-10 assays. The number of responders out of 47 birch allergic subjects tested was calculated for each peptide for the three cytokines. Results for IL-13 or IFN-g are summarized in Table 9.

Table 8

% responders indicates the proportion of subjects in which each peptide induced IL-13 or IFN-g above a threshold level of 100pg/ml

Peptide	% responders	Peptide	% responders
Bir02J	48	Bir15	27
Bir01I	42	Bir16B	27
Bir01F	38	Bir01H	25
Bir12B	38	Bir06D	25
Bir01G	33	Bir07B	25
Bir04	33	Bir07D	25
Bir09	33	Bir10	25
Bir02E	31	Bir14	25
Bir02G	31	Bir17	25
Bir02I	31	Bir05A	23
Bir07	31	Bir06	23
Bir07C	31	Bir07A	23
Bir09A	31	Bir13	23
Bir09B	31	Bir06B	19
Bir11	31	Bir08A	19
Bir16A	31	Bir05	17
Bir02D	29	Bir08	17
Bir09C	27	Bir12A	17

Peptides which induce positive response in a high proportion of subjects are desirable for inclusion in a vaccine. As shown, the top performing peptides were

Bir02J (top of the 02 series), Bir 01I (top of the 01 series) and Bir12B. The core of any vaccine should ideally contain these peptides. The second best performing peptides were Bir04 and Bir09 (top of the 09 series) which may be added to the core mixture of Bir02J, Bir01I and Bir12B. The third best performing peptides were Bir07, Bir07C, Bir11 and Bir16A. Additional peptides from this group may be added to the vaccine mixture to further increase coverage. Bir15 was the fourth best performing peptide and may also be added to the vaccine mixture. In terms of other peptides in the various series, Bir01F, 01G or 01H, in that order of preference, are useful variants of Bir01I; Bir02E, 02G, 02I or lastly 02D are useful variants of Bir02J; Bir09A, 09B or lastly 09C are useful variants of Bir09; and Bir16B is a useful variant of Bir16A. A possible preferred mixture would therefore include Bir02J, Bir01I, Bir12B, Bir04, Bir09, Bir07C and Bir16A. Bir11 and/or Bir15 may also be included, or alternatively substituted for Bir07C and/or Bir16A.

In terms of IL-10 release, Bir01I, listed above as one of the top 3 peptides for IL-13 or IFN-g production, induced IL-10 responses in 49% of individuals. Bir02I also induced IL-10 production in a high proportion of individuals (43%). Inclusion of a strong IL-10 inducing peptide may assist in the induction of tolerance following vaccination.

Example 9 – Solubility screening

A) Introduction

Table 9-1: Birch peptides to be included in solubility testing

Peptide	Sequence	Mw (Da)	Length (a.a.)	Theoretical Isoelectric point (pI)
BIR01I	FNYETETTSVIPAARK	1825.92	16	6.14
BIR02I	PAARMFKAFILD	1378.74	12	9.18
BIR02J	PAARMFKAFILEGDKLVPK	2130.26	19	9.72
BIR04	PGTIKKISFPEGFPFKYV	2054.12	18	9.56
BIR07C	SNEIKIVATPEGGSILK	1754.98	17	5.86
BIR09	ETLLRAVESYLLAHSDAY	2050.04	18	4.65
BIR09B	KEMGETLLRAVESYLLAHS	2146.11	19	5.50
BIR12B	AKYMVIQGEPGRVIRGK	1901.07	17	10.28
BIR16A	AERERIFKRFDANGEGK	2022.04	17	8.63

B)

Solubility Testing

A series of matrices containing 260mM trehalose and spanning a pH range of

3.0 to 7.0 plus a solution modified with 2mM HCl were prepared as indicated in Appendix 2. The solubility of each of the nine peptides was evaluated in each of the matrices in accordance with Appendix 1. Where solubility was achieved initially, but the peptide precipitated out of solution subsequently then an additional quantity of the relevant matrix was added to try and achieve solubility of the peptide at ca. 200µM.

Details of the Birch reference peptides used are indicated in Table 9-2. All peptides were manufactured by Bachem AG, Bubendorf, Switzerland.

Table 9-2. Details of Birch peptides

Peptide	Batch no.	MW (Da)	Peptide purity (%)	Peptide content (%)
BIR1I	1028882	1827.03	99.2	87.1
BIR02I	1028883	1379.69	99.1	86.2
BIR02J	1028884	2131.61	97.4	84.1
BIR04	1028885	2055.45	98.1	88.3
BIR07C	1029310	1756.03	97.9	89.8
BIR09	1028886	2051.28	97.5	86.9
BIR09B	1029311	2147.48	97.3	89.4
BIR12B	1028887	1902.30	97.3	85.9
BIR16A	1028888	2023.24	98.4	88.1

C) Results

The results of the solubility screening are displayed in tables 9-3 to 9-8 below:

Table 3: 2mM HCl and 260mM trehalose dihydrate, pH 2.65

2mM hydrochloric Acid			Weight of peptide 'as is' (mg)	Volume required (µL)	Solubility [mg per ml 'as is']	Solubility [mg per ml]	Solubility [µmol per ml]
Peptide	Comments	Solubility after 24 Hours					
BIR1I	Completely dissolved	Completely dissolved	0.975	50	19.50	16.849	9.222
BIR02I	Completely dissolved	Completely dissolved	1.175	50	23.50	20.075	14.550
BIR02J	Completely dissolved	Completely dissolved	1.180	50	23.60	19.332	9.069
BIR04	Completely dissolved	Completely dissolved	1.025	50	20.50	17.758	8.639
BIR07C	Completely dissolved	Completely dissolved	1.148	100	11.48	10.093	5.747
BIR09	Completely dissolved - filter debris	Completely dissolved - filter debris	1.147	1250	0.92	0.777	0.379
BIR09B	Completely dissolved	Completely dissolved	1.220	100	12.20	10.612	4.942
BIR12B	Completely dissolved	Completely dissolved	1.022	100	10.22	8.542	4.490
BIR16A	Completely dissolved	Completely dissolved	1.243	50	24.86	21.551	10.652

Table 4[a] 10mM sodium citrate and 260mM trehalose dihydrate, pH 3.01

Citrate buffer pH 3.0			Weight of peptide 'as is' (mg)	Volume required (µL)	Solubility [mg per ml 'as is']	Solubility [mg per ml]	Solubility [µmol per ml]
Peptide	Comments	Solubility after 24 Hours					
BIR1I	Completely dissolved	Completely dissolved	1.071	650	1.65	1.424	0.779
BIR02I	Completely dissolved	Completely dissolved	1.112	1100	1.01	0.864	0.626
BIR02J	Completely dissolved in 100uL, redissolved in 3mL after 24hrs	Precipitated out, redissolved	1.150	3000	0.38	0.314	0.147
BIR04	Completely dissolved	Completely dissolved	1.129	50	22.58	19.559	9.516
BIR07C	Completely dissolved	Completely dissolved	1.108	850	1.30	1.146	0.653
BIR09	Undissolved in 1.5mL- diluted to 3mL still undissolved	Undissolved	1.111	3000	0.37	0.314	0.153
BIR09B	Completely dissolved	Completely dissolved	1.162	100	11.62	10.108	4.707
BIR12B	Completely dissolved	Completely dissolved	1.094	200	5.47	4.572	2.403
BIR16A	Completely dissolved	Completely dissolved	1.076	150	7.17	6.219	3.074

Table 4[b] 10mM sodium citrate and 260mM trehalose dihydrate, pH 3.99

Citrate buffer pH 4.0			Weight of peptide 'as is' (mg)	Volume required (µL)	Solubility [mg per ml 'as is']	Solubility [mg per ml]	Solubility [µmol per ml]
Peptide	Comments	Solubility after 24 Hours					
BIR1I	Completely dissolved	Completely dissolved	1.026	450	2.28	1.970	1.078
BIR02I	Cloudy solution with suspended material. Frothing on vortex	Undissolved	1.051	3000	0.35	0.299	0.217
BIR02J	Completely dissolved in 50uL, redissolved in 3mL after 24hrs	Precipitated out, redissolved	1.155	3000	0.39	0.335	0.148
BIR04	Completely dissolved	Completely dissolved	1.018	50	20.36	17.636	8.580
BIR07C	Clear solution with suspended material. Frothing on vortex	Clear solution with suspended material	1.126	3000	0.38	0.330	0.188
BIR09	Cloudy solution with undissolved material	Cloudy solution with undissolved material	1.074	3000	0.36	0.303	0.148
BIR09B	Completely dissolved	Completely dissolved	1.140	100	11.40	9.916	4.618
BIR12B	Completely dissolved	Completely dissolved	1.032	100	10.32	8.626	4.534
BIR16A	Completely dissolved	Completely dissolved	1.123	150	7.49	6.490	3.208

Table 5: 10mM sodium citrate and 260mM trehalose dihydrate, pH 5.02

Citrate buffer pH 5.0			Weight of peptide 'as is' (mg)	Volume required (µL)	Solubility [mg per ml 'as is']	Solubility [mg per ml]	Solubility [µmol per ml]
Peptide	Comments	Solubility after 24 Hours					
BIR1I	Completely dissolved	Completely dissolved	1.036	450	2.30	1.989	1.089
BIR02I	Cloudy solution with suspended material	Completely dissolved	1.085	3000	0.36	0.309	0.224
BIR02J	Completely dissolved in 150uL, redissolved in 3mL after 24hrs	Precipitated out, redissolved	1.029	3000	0.34	0.281	0.132
BIR04	Completely dissolved	Completely dissolved	1.076	50	21.52	18.641	9.069
BIR07C	Completely dissolved	Completely dissolved	1.167	1350	0.86	0.760	0.433
BIR09	Clear solution with suspended material	Clear solution with suspended material	1.115	3000	0.37	0.315	0.154
BIR09B	Completely dissolved	Completely dissolved	1.275	100	12.75	11.091	5.165
BIR12B	Completely dissolved	Completely dissolved	1.167	200	5.84	4.877	2.564
BIR16A	Completely dissolved	Completely dissolved	1.158	50	23.16	20.077	9.923

Table 6: 10mM sodium citrate and 260mM trehalose dihydrate, pH 6.01

Citrate buffer pH 6.0			Weight of peptide 'as is' (mg)	Volume required (µL)	Solubility [mg per ml 'as is']	Solubility [mg per ml]	Solubility [µmol per ml]
Peptide	Comments	Solubility after 24 Hours					
BIR1I	Completely dissolved	Completely dissolved	1.117	150	7.45	6.434	3.522
BIR02I	Cloudy solution with suspended material	Cloudy solution with suspended material	1.143	3000	0.38	0.325	0.236
BIR02J	Completely dissolved in 100uL, redissolved in 3mL after 24hrs	Precipitated out, redissolved	1.172	3000	0.39	0.320	0.150
BIR04	Completely dissolved. Evidence of filter debris	Completely dissolved. Filter debris	1.011	100	10.11	8.758	4.261
BIR07C	Completely dissolved in 350uL, redissolved in 3mL after 24hrs	Precipitated out, redissolved	1.045	3000	0.35	0.306	0.174
BIR09	Completely dissolved	completely dissolved	1.084	1050	1.03	0.875	0.426
BIR09B	Completely dissolved	Completely dissolved	1.230	250	4.92	4.280	1.993
BIR12B	Completely dissolved. Evidence of filter debris	Completely dissolved. Filter debris	1.026	450	2.28	1.906	1.002
BIR16A	Completely dissolved	Completely dissolved	1.293	50	25.86	22.418	11.080

Table 7: 10mM potassium dihydrogen phosphate and 260mM trehalose dihydrate, pH 6.03

Phosphate buffer pH 6.0			Weight of peptide 'as is' (mg)	Volume required (µL)	Solubility [mg per ml 'as is']	Solubility [mg per ml]	Solubility [µmol per ml]
Peptide	Comments	Solubility after 24 Hours					
BIR1I	Completely dissolved	Completely dissolved	1.107	50	22.14	19.130	10.470
BIR02I	Completely dissolved	Completely dissolved	1.145	100	11.45	9.781	7.089
BIR02J	Completely dissolved	Completely dissolved	1.112	100	11.12	9.109	4.273
BIR04	Completely dissolved	Completely dissolved	0.986	100	9.86	8.541	4.155
BIR07C	Completely dissolved in 250uL, redissolved in 3mL after 24hrs	Precipitated out, redissolved	1.245	3000	0.42	0.365	0.208
BIR09	Clear solution with suspended material	Clear solution with suspended material	1.037	3000	0.35	0.293	0.143
BIR09B	Completely dissolved in 100uL, redissolved in 3mL after 24hrs	Precipitated out, redissolved	1.086	3000	0.36	0.315	0.147
BIR12B	Completely dissolved	Completely dissolved	1.192	50	23.84	19.926	10.474
BIR16A	Completely dissolved	Completely dissolved	1.077	50	21.54	18.673	9.229

Table 8: 10mM potassium dihydrogen phosphate and 260mM trehalose dihydrate, pH 7.03

Phosphate Buffer pH7.0			Weight of peptide 'as is' (mg)	Volume required (µL)	Solubility [mg per ml 'as is']	Solubility [mg per ml]	Solubility [µmol per ml]
Peptide	Comments	Solubility after 24 Hours					
BIR1I	Completely dissolved	Completely dissolved	1.039	50	20.78	17.955	9.827
BIR02I	Completely dissolved	Completely dissolved	1.190	100	11.90	10.165	7.368
BIR02J	Completely dissolved	Completely dissolved	1.231	100	12.31	10.084	4.730
BIR04	Completely dissolved	Completely dissolved	1.075	50	21.50	18.624	9.061
BIR07C	Completely dissolved	Completely dissolved	1.005	150	6.70	5.890	3.354
BIR09	Completely dissolved	Completely dissolved	1.138	750	1.52	1.286	0.627
BIR09B	Completely dissolved	Completely dissolved	1.085	50	21.70	18.876	8.790
BIR12B	Completely dissolved	Completely dissolved	1.031	50	20.62	17.234	9.060
BIR16A	Completely dissolved	Completely dissolved	1.099	50	21.98	19.055	9.418

Example 9 - Annex 1
PEPTIDE SOLUBILITY STUDIES

Solubility Methodology

The formulation vehicles were prepared and measurement of pH taken.

- Weighing of peptides.
  - Approximately 1mg was required for each evaluation.
  - Materials were dispensed into containers suitable for subsequent solubility evaluation , i.e. clear glass HPLC vials (with screw cap) .
- Evaluation of solubility (for each matrix).
  - Aliquots of matrix (50 to 100~L) were added as required .
  - The peptide solubility was interpreted by visual inspection.
  - The description of the sample characteristics following addition of each aliquot of the solvent was recorded.
  - Repeat visual assessment of solubility after 24 hours.
- Where a peptide precipitated out of solution after 24 hours, additional buffer was added to produce a final concentration of ca. 0.2mM (200nmol per mL should equate to roughly 0.35mg/mL).
- Calculation of peptide solubilities (initial evaluation).
  - Based on absolute amount of powder weighed.
  - Determination of molar concentration at which solubility was achieved using peptide molecular masses and peptide content and purity values.

Calculalions

$$\text{Solubility mg/ml 'as is'} = \frac{\text{weight(mg)} \times 1000}{\text{dilution}(\mu l)}$$

$$\text{Solubility mg/ml} = \frac{\text{weight(mg)} \times 1000 \times \%\text{Content} \times \%\text{Purity}}{\text{dilution}(\mu l)}$$

$$\text{Solubility } \mu\text{mol/ml} = \frac{\text{weight(mg)} \times 1000 \times \%\text{Content} \times \%\text{Purity} \times \frac{1}{\text{MolWt}} \times 1000}{\text{dilution}(\mu l)}$$

Example 9 - Annex 2

BUFFERS FOR INITIAL SOLUBILITY AND STABILITY SCREENING

Each matrix was prepared at a concentration of 10mM of the buffering agent. Each buffer contained 260mM Trehalose dihydrate (FW 378.3).

Preparation of matrix

The procedure indicated is for the preparation of 100mL of each buffer, but alternative volumes can be prepared by adjusting the quantities.

- 0.1M stock solutions of sodium citrate and potassium dihydrogen phosphate were prepared.
- Weight of trehalose dihydrate equivalent to 260mM was transferred to an appropriate mixing vessel containing 70-80mL of distilled deionised water and allowed to dissolve.
- 10mLof the appropriate stock 0.1M buffer solution was added to the mixing vessel and stirred .
- The pH of the matrix was adjusted to the desired value by adding 2mM hydrochloric acid or 0.1M sodium hydroxide as required.
- The solutions were finally diluted to 100g weight and the pH re-assessed.

Buffers for initial solubility and stability screening, shown as Buffer salt or pH modifier / pH:

2mM HCl and 260mM trehalose dihydrate/ pH 2.65

10mM sodium citrate and 260mM trehalose dihydrate/ pH 3.01

10mM sodium citrate and 260mM trehalose dihydrate/ pH 3.99

10mM sodium citrate and 260mM trehalose dihydrate/ pH 5.02

10mM sodium citrate and 260mM trehalose dihydrate/ pH 6.01

10mM potassium dihydrogen phosphate and 260mM trehalose dihydrate/ pH 6.03

10mM potassium dihydrogen phosphate and 260mM trehalose dihydrate/ pH 7.03

Example 10 - Histamine release assay

The purpose of this assay was to identify compositions that are capable of activating blood basophils (as a surrogate for tissue mast cells) resulting in histamine release that may result in allergic reactions during therapy. A composition comprising

a mixture of peptides that induce histamine release frequently may be considered unsuitable for use as a vaccine.

Histamine release requires the crosslinking of adjacent specific IgE molecules on the surface of the basophil. The peptides being evaluated were small (11 to 18 amino acids in length) and should not, therefore, possess significant tertiary structure that would enable them to retain the conformation of an IgE-binding epitope of the whole molecule. Furthermore, peptide monomers in solution, even if they are bound by IgE, should not be able to crosslink adjacent IgE molecules.

Histamine release from fresh peripheral whole blood from birch allergic subjects was evaluated. Peripheral blood basophils were used as a surrogate for tissue mast cells which were not practical to assay. Blood was incubated in vitro with mixtures of peptides identified as suitable based on the results of Examples 1 to 9 above. Specifically, the following mixtures were assayed:

Mix 1 – BIR01I, BIR02J, BIR04, BIR12B, BIR16A, BIR07C

Mix 2 – BIR01I, BIR02J, BIR04, BIR12B, BIR16A, BIR07C, BIR09

Mix 3 – BIR01I, BIR02J, BIR04, BIR12B, BIR16A, BIR07C, BIR09B

Mix 4 - BIR01I, BIR02I, BIR04, BIR12B, BIR16A, BIR07C

Mix 5 - BIR01I, BIR02I, BIR04, BIR12B, BIR16A, BIR07C, BIR09

Mix 6 - BIR01I, BIR02I, BIR04, BIR12B, BIR16A, BIR07C, BIR09B

Histamine release in response to whole birch allergen extract was measured in each subject to confirm basophil sensitisation. A positive control, representing total histamine release, generated by freeze/thawing the cells twice, was included in each assay. A negative control for spontaneous histamine release was generated by incubating cells in buffer only.

The assay was performed using the Immunotech Histamine Release Immunoassay kit according to the manufacturer's instructions. Following the *in vitro* challenge of blood basophils with peptide mixtures, whole allergen or buffer in microtitre plate wells, supernatants were removed and the histamine in the samples converted to acyl histamine. Acylated samples were tested by a competitive acyl histamine ELISA.

Peptide mixtures were assayed for their ability to induce histamine release over a 5 log10 range (1 to 10,000 ng/ml). The concentration range assayed was selected

based on theoretical *in vivo* doses of peptide that may be achieved during therapy. For example, a 31μg dose (approximately 3 nmol/peptide equivalent) of each peptide entering a blood volume of 5 litres, would result in a blood concentration of 6ng/ml, at the lower end of the histamine release assay dose range. The whole birch allergen extract was used over the same concentration range.

Single measurements were performed for each dilution. After completion of the ELISA, individual histamine levels were determined by interpolation from the standard curve generated in the ELISA assay. Results from samples were adjusted to allow for dilution. Where two or more consecutive dilutions of a peptide/allergen preparation elicited >15% of the total histamine release seen in the freeze thawed positive control (>15% of positive control), or where a single value of >15% of positive control was achieved at the highest concentration tested (10 μg/mL for peptides), this was considered a "*positive histamine release*".

A total of 40 histamine release assays were completed during the study. Of these 5 assays were rejected because of failure to meet appropriate QC controls, e.g. due to unacceptably high levels (>15% of positive control) of spontaneous release in the medium plus buffer negative control wells.

The mixtures tested all showed good histamine release properties. The study findings are summarised as follows: (WA = whole allergen)

Mix	Peptide conc:μg/ml	Average % histamine release	Mix	Peptide conc:μg/ml	Average % histamine release
1	10	1%	5	10	2%
1	1	2%	5	1	2%
1	0.1	3%	5	0.1	2%
1	0.01	3%	5	0.01	2%
1	0.001	2%	5	0.001	2%
2	10	2%	6	10	3%
2	1	2%	6	1	2%
2	0.1	3%	6	0.1	1%
2	0.01	3%	6	0.01	1%
2	0.001	2%	6	0.001	2%
3	10	4%	WA	10	65%
3	1	2%	WA	1	38%
3	0.1	3%	WA	0.1	38%
3	0.01	2%	WA	0.01	42%
3	0.001	2%	WA	0.001	43%
4	10	2%			
4	1	2%			
4	0.1	2%			
4	0.01	1%			
4	0.001	1%			

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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.

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.

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CLAIMS

1. A composition suitable for use in preventing or treating allergy to birch pollen comprising:

i) the polypeptide set forth in SEQ ID NO: 74 (Bir12B; AKYMVIQGEPGRVIRGK) or a variant thereof, and

ii) at least one of the polypeptides set forth in SEQ ID NO: 48 (Bir01I; FNYETETTSVIPAARK) or a variant thereof and SEQ ID NO: 53 (Bir02J; PAARMFKAFILEGDKLVPK) or a variant thereof,

wherein said variant is:

I) a longer polypeptide of up to 30 amino acids in length which comprises the sequence of the said polypeptide specified in (i) or (ii), or

II) a polypeptide of 9 to 30 amino acids in length which is capable of binding to the same MHC class II molecule as the said polypeptide specified in (i) or (ii) and which comprises a sequence that has at least 75% homology to the sequence of the said polypeptide specified in (i) or (ii); or

III) a polypeptide of 9 to 30 amino acids in length which is capable of binding to the same MHC class II molecule as the said polypeptide specified in (i) or (ii) and which comprises:

- at least 9 contiguous amino acids of the sequence of the said polypeptide specified in (i) or (ii), or
- a sequence that has at least 75% homology to said at least 9 contiguous amino acids.

2. A composition according to claim 1, comprising:

(a) the polypeptide set forth in SEQ ID NO: 74 (Bir12B; AKYMVIQGEPGRVIRGK), or a said variant thereof;

(b) the polypeptide set forth in SEQ ID NO: 53 (Bir02J; PAARMFKAFILEGDKLVPK), or a said variant thereof; and

(c) the polypeptide set forth in SEQ ID NO: 48 (Bir01I; FNYETETTSVIPAARK) or a said variant thereof.

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3. A composition according to claim 1 or 2, wherein:

- said variant of the polypeptide set forth in SEQ ID NO: 48 (Bir01I) is the polypeptide set forth in SEQ ID NO: 45 (Bir01F; FNYETEATSVIPAARK), the polypeptide set forth in SEQ ID NO: 46 (Bir01G; FNYEIEATSVIPAARK) or the polypeptide set forth in SEQ ID NO: 47 (Bir01H; FNYEIETTSVIPAARK); and/or
- said variant of the polypeptide set forth in SEQ ID NO: 53 (Bir02J) is the polypeptide set forth in SEQ ID NO: 50 (Bir02E; PAARLFKAFILEGDTLIPK), the polypeptide set forth in SEQ ID NO: 51 (Bir02G; PAARLFKAFILEGDNLIPK), the polypeptide set forth in SEQ ID NO: 52 (Bir02I; PAARMFKAFILD) or the polypeptide SEQ ID NO: 49 (Bir02D; PAARMFKAFILDGDKLVPK.

4. A composition according to any one of the preceding claims, comprising at least one additional polypeptide selected from the polypeptide set forth in SEQ ID NO: 72 (Bir11; FPQFKPQEITGIMK), the polypeptide set forth in SEQ ID NO: 71 (Bir10; GSVWAQSSSFPQFK), the polypeptide set forth in SEQ ID NO: 73 (Bir12A; PTGMFVAGAKYMVIQGR), the polypeptide set forth in SEQ ID NO: 75 (Bir13; IKYMVIQGEAGAVIRGK), the polypeptide set forth in SEQ ID NO: 76 (Bir14; EAGAVIRGKKGSGGIT), the polypeptide set forth in SEQ ID NO: 54 (Bir04; PGTIKKISFPEGFPFKYV), the polypeptide set forth in SEQ ID NO: 67 (Bir09; ETLLRAVESYLLAHSDAY), the polypeptide set forth in SEQ ID NO: 60 (Bir07; SNEIKIVATPDGGSILK) and the polypeptide set forth in SEQ ID NO: 63 (Bir07C; SNEIKIVATPEGGSILK), or a said variant thereof.

5. A composition according to claim 4, wherein said variant of the polypeptide set forth in SEQ ID NO: 67 (Bir09) is the polypeptide set forth in SEQ ID NO: 68 (Bir09A; GETLLRAVESYLLAHS), the polypeptide set forth in SEQ ID NO: 69 (Bir09B; KEMGETLLRAVESYLLAHS) or the polypeptide set forth in SEQ ID NO: 70 (Bir09C; KEKGETLLRAVESYLLAHS).

6. A composition according to any one of the preceding claims, comprising the additional polypeptide of SEQ ID NO: 77 (Bir15; SLNTLRLRRIFDLFDK) or SEQ ID NO: 78 (Bir16A; AERERIFKRFDANGEGK), or a said variant thereof.

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7. A composition according to claim 6, wherein said variant of the polypeptide set forth in SEQ ID NO: 78 (Bir16A) is the polypeptide set forth in SEQ ID NO: 79 (Bir16B; AERERIFKRFDAGGEGK).

8. A composition according to claim 1, comprising:

(a) the polypeptide set forth in SEQ ID NO: 74 (Bir12B; AKYMVIQGEPGRVIRGK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 53 (Bir02J; PAARMFKAFILEGDKLVPK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 48 (Bir01I; FNYETETTSVIPAARK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 54 (Bir04; PGTIKKISFPEGFPFKYV) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 67 (Bir09; ETLLRAVESYLLAHSDAY) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 63 (Bir07C; SNEIKIVATPEGGSILK) or a said variant thereof, and the polypeptide set forth in SEQ ID NO: 78 (Bir16A; AERERIFKRFDANGEGK) or a said variant thereof; or

(b) the polypeptide set forth in SEQ ID NO: 74 (Bir12B; AKYMVIQGEPGRVIRGK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 53 (Bir02J; PAARMFKAFILEGDKLVPK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 48 (Bir01I; FNYETETTSVIPAARK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 54 (Bir04; PGTIKKISFPEGFPFKYV) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 63 (Bir07C; SNEIKIVATPEGGSILK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 78 (Bir16A; AERERIFKRFDANGEGK) or a said variant thereof, and the polypeptide set forth in SEQ ID NO: 69 (Bir09B; KEMGETLLRAVESYLLAHS) or a said variant thereof; or

(c) the polypeptide set forth in SEQ ID NO: 74 (Bir12B; AKYMVIQGEPGRVIRGK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 53 (Bir02J; PAARMFKAFILEGDKLVPK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 48 (Bir01I; FNYETETTSVIPAARK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 54 (Bir04; PGTIKKISFPEGFPFKYV) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 63 (Bir07C;

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SNEIKIVATPEGGSILK) or a said variant thereof, and the polypeptide set forth in SEQ ID NO: 78 (Bir16A; AERERIFKRFDANGEGK) or a said variant thereof; or (d) the polypeptide set forth in SEQ ID NO: 74 (Bir12B; AKYMVIQGEPGRVIRGK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 52 (Bir02I; PAARMFKAFILD) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 48 (Bir01I; FNYETETTSVIPAARK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 54 (Bir04; PGTIKKISFPEGFPFKYV) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 67 (Bir09; ETLLRAVESYLLAHSDAY) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 63 (Bir07C; SNEIKIVATPEGGSILK) or a said variant thereof, and the polypeptide set forth in SEQ ID NO: 78 (Bir16A; AERERIFKRFDANGEGK) or a said variant thereof; or (e) the polypeptide set forth in SEQ ID NO: 74 (Bir12B; AKYMVIQGEPGRVIRGK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 52 (Bir02I; PAARMFKAFILD) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 48 (Bir01I; FNYETETTSVIPAARK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 54 (Bir04; PGTIKKISFPEGFPFKYV) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 63 (Bir07C; SNEIKIVATPEGGSILK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 78 (Bir16A; AERERIFKRFDANGEGK) or a said variant thereof, and the polypeptide set forth in SEQ ID NO: 69 (Bir09B; KEMGETLLRAVESYLLAHS) or a said variant thereof; or (f) the polypeptide set forth in SEQ ID NO: 74 (Bir12B; AKYMVIQGEPGRVIRGK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 52 (Bir02I; PAARMFKAFILD) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 48 (Bir01I; FNYETETTSVIPAARK) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 54 (Bir04; PGTIKKISFPEGFPFKYV) or a said variant thereof, the polypeptide set forth in SEQ ID NO: 63 (Bir07C; SNEIKIVATPEGGSILK) or a said variant thereof, and the polypeptide set forth in SEQ ID NO: 78 (Bir16A; AERERIFKRFDANGEGK) or a said variant thereof.

9. A composition according to any one of the preceding claims, wherein the composition:

- is capable of tolerising at least 60% of a panel of birch pollen allergic individuals in

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the population and/or

- comprises at least one further polypeptide up to a total of thirteen unique/different polypeptides, wherein the further polypeptides:

(a) comprise a sequence having at least 75% sequence identity to at least 9 or more contiguous amino acids in any of SEQ ID NOs: 1 to 80 not selected above; and

(b) are 9 to 30 amino acids in length.

10. A composition according to any one of the preceding claims, comprising at least one said variant polypeptide which is 9 to 20 amino acids in length

11. A composition according to claim 10, wherein:

(a) the at least one said variant polypeptide is 13 to 17 amino acids in length; and/or

(b) the at least one said variant polypeptide has at least 75% sequence identity to any of SEQ ID NOS: 1 to 80.

12. A composition according to any one of the preceding claims, wherein:

(a) one or more of the polypeptides have one or more modifications selected from the following:

(i) N terminal acetylation;

(ii) C terminal amidation;

(iii) one or more hydrogens on the side chain amines of Arginine and/or Lysine replaced with a methylene group;

(iv) glycosylation; and

(v) phosphorylation; and/or

(b) at least one of the peptides has been engineered to be soluble such that it comprises:

i) N terminal to the residues of the peptide which flank a T cell epitope: one to six contiguous amino acids corresponding to the one to six contiguous amino acids immediately N terminal to said residues in the sequence of the protein from which the peptide derives; and/or

ii) C terminal to the residues of the peptide which flank a T cell epitope: one to six contiguous amino acids corresponding to the one to six contiguous

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amino acids immediately C terminal to the said residues in the sequence of the protein from which the peptide derives; or

iii) both N and C terminal to the residues of the peptide which flank a T cell epitope, at least one amino acid selected from arginine, lysine, histidine, glutamate and aspartate,

wherein the polypeptide has a solubility of at least 3.5mg/ml and the T cell epitope has a solubility of less than 3.5mg/ml; and/or

(c) at least one of the peptides has been engineered to be soluble such that additionally:

i) any cysteine residues in the native sequence of the peptide are replaced with serine or 2-aminobutyric acid; and /or

ii) any hydrophobic residues in the up to three amino acids at the N or C terminus of the native sequence of the peptide, which are not comprised in a T cell epitope, are deleted; and/or

iii) any two consecutive amino acids comprising the sequence Asp-Gly in the up to four amino acids at the N or C terminus of the native sequence of the peptide, which are not comprised in a T cell epitope, are deleted; and/or

iv) one or more positively charged residues are added at the N and/or C terminus of the native sequence of the peptide.

13. A composition according to any one of the preceding claims, which is a solution wherein each polypeptide has a concentration in the range of 0.03 to 200 nmol/ml or 0.3 to 200 nmol/ml.

14. A composition according to any one of the preceding claims, comprising no further peptides.

15. A composition according to any one of the preceding claims, which is a pharmaceutical formulation further comprising a pharmaceutically acceptable carrier or diluent.

16. The composition according to claim 15, formulated for oral administration,

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nasal administration, topical administration, subcutaneous administration, sublingual administration, intradermal administration, buccal administration, epidermal administration, or for administration by inhalation, by injection, or by a patch.

17. A composition according to any one of the preceding claims, additionally comprising a further polypeptide allergen for use in tolerising an individual to the further polypeptide allergen.

18. An *in vitro* method of determining whether T cells recognize a composition as defined in claim 1 comprising contacting said T cells with said composition and detecting whether said T cells are stimulated by said composition.

19. An *in vitro* method according to claim 18 which is carried out to determine whether an individual has, or is at risk of having, an allergy to birch pollen.

20. Use of the polypeptide i) or a said variant thereof as defined in claim 1 and at least one of the polypeptides ii) or a said variant thereof as defined in claim 1 for the manufacture of a medicament for preventing or treating allergy to birch pollen.

21. A method of treating or preventing allergy to birch pollen in a subject in need of such treatment or prevention, which method comprises the administration to the said subject of an effective amount of a composition as defined in any one of claims 1 to 17.

22. A composition according to any one of claims 1 to 17, an *in vitro* method according to claim 18 or 19, a use according to claim 20 or a method according to claim 21, substantially as herein described, with reference to the Examples and/or Figures.

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SEQUENCE LISTING

<110> Circassia Ltd.

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<130> N110180A

<140> PCT/GB11/000206
<141> 2011-02-15

<150> GB 1002559.1
<151> 2010-02-15

<160> 102

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35 40 45

Gly Asp Val Thr Phe Thr Glu Val Gly Tyr Thr Arg Ala Glu Gly Leu

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50 55 60

Tyr Ser Met Leu Val Glu Arg Asp His Lys Asn Glu Phe Cys Glu Ile
65 70 75 80

Thr Leu Ile Ser Ser Gly Arg Lys Asp Cys Asn Glu Ile Pro Thr Glu
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Gly Trp Ala Lys Pro Ser Leu Lys Phe Lys Leu Asn Thr Val Asn Gly
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35 40 45

Ile Thr Gly Ile Met Lys Asp Phe Glu Glu Pro Gly His Leu Ala Pro
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Thr Gly Leu His Leu Gly Gly Ile Lys Tyr Met Val Ile Gln Gly Glu
65 70 75 80

Ala Gly Ala Val Ile Arg Gly Lys Lys Gly Ser Gly Gly Ile Thr Ile
85 90 95

Lys Lys Thr Gly Gln Ala Leu Val Phe Gly Ile Tyr Glu Glu Pro Val
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35 40 45

Ile Thr Gly Ile Met Lys Asp Phe Glu Glu Pro Gly His Leu Ala Pro
50 55 60

Thr Gly Leu His Leu Gly Gly Ile Lys Tyr Met Val Ile Gln Gly Glu
65 70 75 80

Ala Gly Ala Val Ile Arg Gly Lys Lys Gly Ser Gly Gly Ile Thr Ile
85 90 95

Lys Lys Thr Gly Gln Ala Leu Val Phe Gly Ile Tyr Glu Glu Pro Val
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35 40 45

Ile Thr Gly Ile Met Lys Asp Phe Glu Glu Pro Gly His Leu Ala Pro
50 55 60

Thr Gly Leu His Leu Gly Gly Ile Lys Tyr Met Val Ile Gln Gly Glu
65 70 75 80

Ala Gly Ala Val Ile Arg Gly Lys Lys Gly Ser Gly Gly Ile Thr Ile
85 90 95

Lys Lys Thr Gly Gln Ala Leu Val Phe Gly Ile Tyr Glu Glu Pro Val
100 105 110

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20 25 30

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35 40 45

Ile Thr Gly Ile Met Lys Asp Phe Glu Glu Pro Gly His Leu Ala Pro
50 55 60

Thr Gly Leu His Leu Gly Gly Ile Lys Tyr Met Val Ile Gln Gly Glu
65 70 75 80

Ala Gly Ala Val Ile Arg Gly Lys Lys Gly Ser Gly Gly Ile Thr Ile
85 90 95

Lys Lys Thr Gly Gln Ala Leu Val Phe Gly Ile Tyr Glu Glu Pro Val
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20 25 30

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35 40 45

Ile Thr Gly Ile Met Lys Asp Phe Glu Glu Pro Gly His Leu Ala Pro
50 55 60

Thr Gly Leu His Leu Gly Gly Ile Lys Tyr Met Val Ile Gln Gly Glu
65 70 75 80

Ala Gly Ala Val Ile Arg Gly Lys Lys Gly Ser Gly Gly Ile Thr Ile
85 90 95

Lys Lys Thr Gly Gln Ala Leu Val Phe Gly Ile Tyr Glu Glu Pro Val
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115 120 125

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20 25 30

Ser Val Trp Ala Gln Ser Ser Ser Phe Pro Gln Phe Lys Pro Gln Glu
35 40 45

Ile Thr Gly Ile Met Lys Asp Phe Glu Glu Pro Gly His Leu Ala Pro
50 55 60

Thr Gly Leu His Leu Gly Gly Ile Lys Tyr Met Val Ile Gln Gly Glu
65 70 75 80

Ala Gly Ala Val Ile Arg Gly Lys Lys Gly Ser Gly Gly Ile Thr Ile
85 90 95

Lys Lys Thr Gly Gln Ala Leu Val Phe Gly Ile Tyr Glu Glu Pro Val
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115 120 125

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20 25 30

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35 40 45

Asp Lys Asn Ser Asp Gly Ile Ile Thr Val Asp Glu Leu Ser Arg Ala
50 55 60

Leu Asn Leu Leu Gly Leu Glu Thr Asp Leu Ser Glu Leu Glu Ser Thr
65 70 75 80

Val Lys Ser Phe Thr Arg Glu Gly Asn Ile Gly Leu Gln Phe Glu Asp
85 90 95

Phe Ile Ser Leu His Gln Ser Leu Asn Asp Ser Tyr Phe Ala Tyr Gly
100 105 110

Gly Glu Asp Glu Asp Asp Asn Glu Glu Asp Met Arg Lys Ser Ile Leu
115 120 125

Ser Gln Glu Glu Ala Asp Ser Phe Gly Gly Phe Lys Val Phe Asp Glu
130 135 140

Asp Gly Asp Gly Tyr Ile Ser Ala Arg Glu Leu Gln Met Val Leu Gly
145 150 155 160

Lys Leu Gly Phe Ser Glu Gly Ser Glu Ile Asp Arg Val Glu Lys Met
165 170 175

Ile Val Ser Val Asp Ser Asn Arg Asp Gly Arg Val Asp Phe Phe Glu
180 185 190

Phe Lys Asp Met Met Arg Ser Val Leu Val Arg Ser Ser
195 200 205

<210> 92
<211> 85
<212> PRT
<213> Betulaceae

<400> 92

Met Ala Asp Asp His Pro Gln Asp Lys Ala Glu Arg Glu Arg Ile Phe
1 5 10 15

Lys Arg Phe Asp Ala Asn Gly Asp Gly Lys Ile Ser Ala Ala Glu Leu
20 25 30

Gly Glu Ala Leu Lys Thr Leu Gly Ser Ile Thr Pro Asp Glu Val Lys
35 40 45

His Met Met Ala Glu Ile Asp Thr Asp Gly Asp Gly Phe Ile Ser Phe

50 55 60

Gln Glu Phe Thr Asp Phe Gly Arg Ala Asn Arg Gly Leu Leu Lys Asp
65 70 75 80

Val Ala Lys Ile Phe
85

<210> 93
<211> 24
<212> PRT
<213> Quercus alba

<220>
<221> misc_feature
<222> (5)..(5)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (17)..(17)
<223> Xaa can be any naturally occurring amino acid

<400> 93

Gly Val Phe Thr Xaa Glu Ser Gln Glu Thr Ser Val Ile Ala Pro Ala
1 5 10 15

Xaa Leu Phe Lys Ala Leu Phe Leu
20

<210> 94
<211> 40
<212> PRT
<213> Carpinus betulus

<220>
<221> misc_feature
<222> (39)..(39)
<223> Xaa can be any naturally occurring amino acid

<400> 94

Gly Val Phe Asn Tyr Glu Ala Glu Thr Pro Ser Val Ile Pro Ala Ala
1 5 10 15

Arg Leu Phe Lys Ser Tyr Val Leu Asp Gly Asp Lys Leu Ile Pro Lys
20 25 30

Val Ala Pro Gln Ala Ile Xaa Lys
35 40

<210> 95
<211> 44

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<212> PRT
<213> Alnus glutinosa

<400> 95

Gly Val Phe Asn Tyr Glu Ala Glu Thr Pro Ser Val Ile Pro Ala Ala
1 5 10 15

Arg Leu Phe Lys Ala Phe Ile Leu Asp Gly Asp Lys Leu Leu Pro Lys
20 25 30

Val Ala Pro Glu Ala Val Ser Ser Val Glu Asn Ile
35 40

<210> 96
<211> 110
<212> PRT
<213> Betula pendula

<220>
<223> Rubisco

<400> 96

Val Gln Cys Met Gln Val Trp Pro Pro Leu Gly Leu Lys Lys Phe Glu
1 5 10 15

Thr Leu Ser Tyr Leu Pro Pro Leu Ser Ser Glu Gln Leu Ala Lys Glu
20 25 30

Val Asp Tyr Leu Leu Arg Lys Asn Leu Ile Pro Cys Leu Glu Phe Glu
35 40 45

Leu Glu His Gly Phe Val Tyr Arg Glu His Asn Arg Ser Pro Gly Tyr
50 55 60

Tyr Asp Gly Arg Tyr Trp Thr Met Trp Lys Leu Pro Met Phe Gly Cys
65 70 75 80

Asn Asp Ser Ser Gln Val Leu Lys Glu Leu Glu Glu Cys Lys Lys Ala
85 90 95

Tyr Pro Ser Ala Phe Ile Arg Ile Ile Gly Phe Asp Asp Lys
100 105 110

<210> 97
<211> 373
<212> PRT
<213> Pinaceae

<400> 97

Met Asp Ser Pro Cys Leu Val Ala Leu Leu Val Phe Ser Phe Val Ile
1 5 10 15

Gly Ser Cys Phe Ser Asp Asn Pro Ile Asp Ser Cys Trp Arg Gly Asp
20 25 30

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Ser Asn Trp Ala Gln Asn Arg Met Lys Leu Ala Asp Cys Ala Val Gly
35 40 45

Phe Gly Ser Ser Thr Met Gly Gly Lys Gly Gly Asp Leu Tyr Thr Val
50 55 60

Thr Asn Ser Asp Asp Asp Pro Val Asn Pro Pro Gly Thr Leu Arg Tyr
65 70 75 80

Gly Ala Thr Arg Asp Arg Pro Leu Trp Ile Ile Phe Ser Gly Asn Met
85 90 95

Asn Ile Lys Leu Lys Met Pro Met Tyr Ile Ala Gly Tyr Lys Thr Phe
100 105 110

Asp Gly Arg Gly Ala Gln Val Tyr Ile Gly Asn Gly Gly Pro Cys Val
115 120 125

Phe Ile Lys Arg Val Ser Asn Val Ile Ile His Gly Leu Tyr Leu Tyr
130 135 140

Gly Cys Ser Thr Ser Val Leu Gly Asn Val Leu Ile Asn Glu Ser Phe
145 150 155 160

Gly Val Glu Pro Val His Pro Gln Asp Gly Asp Ala Leu Thr Leu Arg
165 170 175

Thr Ala Thr Asn Ile Trp Ile Asp His Asn Ser Phe Ser Asn Ser Ser
180 185 190

Asp Gly Leu Val Asp Val Thr Leu Thr Ser Thr Gly Val Thr Ile Ser
195 200 205

Asn Asn Leu Phe Phe Asn His His Lys Val Met Ser Leu Gly His Asp
210 215 220

Asp Ala Tyr Ser Asp Asp Lys Ser Met Lys Val Thr Val Ala Phe Asn
225 230 235 240

Gln Phe Gly Pro Asn Cys Gly Gln Arg Met Pro Arg Ala Arg Tyr Gly
245 250 255

Leu Val His Val Ala Asn Asn Asn Tyr Asp Pro Trp Thr Ile Tyr Ala
260 265 270

Ile Gly Gly Ser Ser Asn Pro Thr Ile Leu Ser Glu Gly Asn Ser Phe
275 280 285

Thr Ala Pro Asn Glu Ser Tyr Lys Lys Gln Val Thr Ile Arg Ile Gly
290 295 300

Cys Lys Thr Ser Ser Ser Cys Ser Asn Trp Val Trp Gln Ser Thr Gln
305 310 315 320

Asp Val Phe Tyr Asn Gly Ala Tyr Phe Val Ser Ser Gly Lys Tyr Glu
325 330 335

Gly Gly Asn Ile Tyr Thr Lys Lys Glu Ala Phe Asn Val Glu Asn Gly

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340 345 350

Asn Ala Thr Pro His Leu Thr Gln Asn Ala Gly Val Leu Thr Cys Ser
355 360 365

Leu Ser Lys Arg Cys
370

<210> 98
<211> 374
<212> PRT
<213> Pinaceae

<400> 98

Met Asp Ser Pro Cys Leu Val Ala Leu Leu Val Leu Ser Phe Val Ile
1 5 10 15

Gly Ser Cys Phe Ser Asp Asn Pro Ile Asp Ser Cys Trp Arg Gly Asp
20 25 30

Ser Asn Trp Ala Gln Asn Arg Met Lys Leu Ala Asp Cys Ala Val Gly
35 40 45

Phe Gly Ser Ser Thr Met Gly Gly Lys Gly Gly Asp Leu Tyr Thr Val
50 55 60

Thr Asn Ser Asp Asp Asp Pro Val Asn Pro Ala Pro Gly Thr Leu Arg
65 70 75 80

Tyr Gly Ala Thr Arg Asp Arg Pro Leu Trp Ile Ile Phe Ser Gly Asn
85 90 95

Met Asn Ile Lys Leu Lys Met Pro Met Tyr Ile Ala Gly Tyr Lys Thr
100 105 110

Phe Asp Gly Arg Gly Ala Gln Val Tyr Ile Gly Asn Gly Gly Pro Cys
115 120 125

Val Phe Ile Lys Arg Val Ser Asn Val Ile Ile His Gly Leu His Leu
130 135 140

Tyr Gly Cys Ser Thr Ser Val Leu Gly Asn Val Leu Ile Asn Glu Ser
145 150 155 160

Phe Gly Val Glu Pro Val His Pro Gln Asp Gly Asp Ala Leu Thr Leu
165 170 175

Arg Thr Ala Thr Asn Ile Trp Ile Asp His Asn Ser Phe Ser Asn Ser
180 185 190

Ser Asp Gly Leu Val Asp Val Thr Leu Ser Ser Thr Gly Val Thr Ile
195 200 205

Ser Asn Asn Leu Phe Phe Asn His His Lys Val Met Leu Leu Gly His
210 215 220

Asp Asp Ala Tyr Ser Asp Asp Lys Ser Met Lys Val Thr Val Ala Phe

225 230 235 240

Asn Gln Phe Gly Pro Asn Cys Gly Gln Arg Met Pro Arg Ala Arg Tyr
245 250 255

Gly Leu Val His Val Ala Asn Asn Asn Tyr Asp Pro Trp Thr Ile Tyr
260 265 270

Ala Ile Gly Gly Ser Ser Asn Pro Thr Ile Leu Ser Glu Gly Asn Ser
275 280 285

Phe Thr Ala Pro Asn Glu Ser Tyr Lys Lys Gln Val Thr Ile Arg Ile
290 295 300

Gly Cys Lys Thr Ser Ser Ser Cys Ser Asn Trp Val Trp Gln Ser Thr
305 310 315 320

Gln Asp Val Phe Tyr Asn Gly Ala Tyr Phe Val Ser Ser Gly Lys Tyr
325 330 335

Glu Gly Gly Asn Ile Tyr Thr Lys Lys Glu Ala Phe Asn Val Glu Asn
340 345 350

Gly Asn Ala Thr Pro Gln Leu Thr Lys Asn Ala Gly Val Leu Thr Cys
355 360 365

Ser Leu Ser Lys Arg Cys
370

<210> 99
<211> 514
<212> PRT
<213> Cryptomeria japonica

<400> 99

Met Ala Met Lys Leu Ile Ala Pro Met Ala Phe Leu Ala Met Gln Leu
1 5 10 15

Ile Ile Met Ala Ala Ala Glu Asp Gln Ser Ala Gln Ile Met Leu Asp
20 25 30

Ser Val Val Glu Lys Tyr Leu Arg Ser Asn Arg Ser Leu Arg Lys Val
35 40 45

Glu His Ser Arg His Asp Ala Ile Asn Ile Phe Asn Val Glu Lys Tyr
50 55 60

Gly Ala Val Gly Asp Gly Lys His Asp Cys Thr Glu Ala Phe Ser Thr
65 70 75 80

Ala Trp Gln Ala Ala Cys Lys Asn Pro Ser Ala Met Leu Leu Val Pro
85 90 95

Gly Ser Lys Lys Phe Val Val Asn Asn Leu Phe Phe Asn Gly Pro Cys
100 105 110

Gln Pro His Phe Thr Phe Lys Val Asp Gly Ile Ile Ala Ala Tyr Gln

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        115                 120                 125

Asn Pro Ala Ser Trp Lys Asn Asn Arg Ile Trp Leu Gln Phe Ala Lys
    130                 135                 140

Leu Thr Gly Phe Thr Leu Met Gly Lys Gly Val Ile Asp Gly Gln Gly
145                 150                 155                 160

Lys Gln Trp Trp Ala Gly Gln Cys Lys Trp Val Asn Gly Arg Glu Ile
                165                 170                 175

Cys Asn Asp Arg Asp Arg Pro Thr Ala Ile Lys Phe Asp Phe Ser Thr
            180                 185                 190

Gly Leu Ile Ile Gln Gly Leu Lys Leu Met Asn Ser Pro Glu Phe His
        195                 200                 205

Leu Val Phe Gly Asn Cys Glu Gly Val Lys Ile Ile Gly Ile Ser Ile
    210                 215                 220

Thr Ala Pro Arg Asp Ser Pro Asn Thr Asp Gly Ile Asp Ile Phe Ala
225                 230                 235                 240

Ser Lys Asn Phe His Leu Gln Lys Asn Thr Ile Gly Thr Gly Asp Asp
                245                 250                 255

Cys Val Ala Ile Gly Thr Gly Ser Ser Asn Ile Val Ile Glu Asp Leu
            260                 265                 270

Ile Cys Gly Pro Gly His Gly Ile Ser Ile Gly Ser Leu Gly Arg Glu
        275                 280                 285

Asn Ser Arg Ala Glu Val Ser Tyr Val His Val Asn Gly Ala Lys Phe
    290                 295                 300

Ile Asp Thr Gln Asn Gly Leu Arg Ile Lys Thr Trp Gln Gly Gly Ser
305                 310                 315                 320

Gly Met Ala Ser His Ile Ile Tyr Glu Asn Val Glu Met Ile Asn Ser
                325                 330                 335

Glu Asn Pro Ile Leu Ile Asn Gln Phe Tyr Cys Thr Ser Ala Ser Ala
            340                 345                 350

Cys Gln Asn Gln Arg Ser Ala Val Gln Ile Gln Asp Val Thr Tyr Lys
        355                 360                 365

Asn Ile Arg Gly Thr Ser Ala Thr Ala Ala Ala Ile Gln Leu Lys Cys
    370                 375                 380

Ser Asp Ser Met Pro Cys Lys Asp Ile Lys Leu Ser Asp Ile Ser Leu
385                 390                 395                 400

Lys Leu Thr Ser Gly Lys Ile Ala Ser Cys Leu Asn Asp Asn Ala Asn
                405                 410                 415

Gly Tyr Phe Ser Gly His Val Ile Pro Ala Cys Lys Asn Leu Ser Pro
            420                 425                 430
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Ser Ala Lys Arg Lys Glu Ser Lys Ser His Lys His Pro Lys Thr Val
435 440 445

Met Val Glu Asn Met Arg Ala Tyr Asp Lys Gly Asn Arg Thr Arg Ile
450 455 460

Leu Leu Gly Ser Arg Pro Pro Asn Cys Thr Asn Lys Cys His Gly Cys
465 470 475 480

Ser Pro Cys Lys Ala Lys Leu Val Ile Val His Arg Ile Met Pro Gln
485 490 495

Glu Tyr Tyr Pro Gln Arg Trp Ile Cys Ser Cys His Gly Lys Ile Tyr
500 505 510

His Pro

<210> 100
<211> 514
<212> PRT
<213> Cryptomeria japonica

<400> 100

Met Ala Met Lys Phe Ile Ala Pro Met Ala Phe Val Ala Met Gln Leu
1 5 10 15

Ile Ile Met Ala Ala Ala Glu Asp Gln Ser Ala Gln Ile Met Leu Asp
20 25 30

Ser Asp Ile Glu Gln Tyr Leu Arg Ser Asn Arg Ser Leu Arg Lys Val
35 40 45

Glu His Ser Arg His Asp Ala Ile Asn Ile Phe Asn Val Glu Lys Tyr
50 55 60

Gly Ala Val Gly Asp Gly Lys His Asp Cys Thr Glu Ala Phe Ser Thr
65 70 75 80

Ala Trp Gln Ala Ala Cys Lys Lys Pro Ser Ala Met Leu Leu Val Pro
85 90 95

Gly Asn Lys Lys Phe Val Val Asn Asn Leu Phe Phe Asn Gly Pro Cys
100 105 110

Gln Pro His Phe Thr Phe Lys Val Asp Gly Ile Ile Ala Ala Tyr Gln
115 120 125

Asn Pro Ala Ser Trp Lys Asn Asn Arg Ile Trp Leu Gln Phe Ala Lys
130 135 140

Leu Thr Gly Phe Thr Leu Met Gly Lys Gly Val Ile Asp Gly Gln Gly
145 150 155 160

Lys Gln Trp Trp Ala Gly Gln Cys Lys Trp Val Asn Gly Arg Glu Ile
165 170 175

Cys Asn Asp Arg Asp Arg Pro Thr Ala Ile Lys Phe Asp Phe Ser Thr
180 185 190

Gly Leu Ile Ile Gln Gly Leu Lys Leu Met Asn Ser Pro Glu Phe His
195 200 205

Leu Val Phe Gly Asn Cys Glu Gly Val Lys Ile Ile Gly Ile Ser Ile
210 215 220

Thr Ala Pro Arg Asp Ser Pro Asn Thr Asp Gly Ile Asp Ile Phe Ala
225 230 235 240

Ser Lys Asn Phe His Leu Gln Lys Asn Thr Ile Gly Thr Gly Asp Asp
245 250 255

Cys Val Ala Ile Gly Thr Gly Ser Ser Asn Ile Val Ile Glu Asp Leu
260 265 270

Ile Cys Gly Pro Gly His Gly Ile Ser Ile Gly Ser Leu Gly Arg Glu
275 280 285

Asn Ser Arg Ala Glu Val Ser Tyr Val His Val Asn Gly Ala Lys Phe
290 295 300

Ile Asp Thr Gln Asn Gly Leu Arg Ile Lys Thr Trp Gln Gly Gly Ser
305 310 315 320

Gly Met Ala Ser His Ile Ile Tyr Glu Asn Val Glu Met Ile Asn Ser
325 330 335

Glu Asn Pro Ile Leu Ile Asn Gln Phe Tyr Cys Thr Ser Ala Ser Ala
340 345 350

Cys Gln Asn Gln Arg Ser Ala Val Gln Ile Gln Asp Val Thr Tyr Lys
355 360 365

Asn Ile Arg Gly Thr Ser Ala Thr Ala Ala Ala Ile Gln Leu Lys Cys
370 375 380

Ser Asp Ser Met Pro Cys Lys Asp Ile Lys Leu Ser Asp Ile Ser Leu
385 390 395 400

Lys Leu Thr Ser Gly Lys Ile Ala Ser Cys Leu Asn Asp Asn Ala Asn
405 410 415

Gly Tyr Phe Ser Gly His Val Ile Pro Ala Cys Lys Asn Leu Ser Pro
420 425 430

Ser Ala Lys Arg Lys Glu Ser Lys Ser His Lys His Pro Lys Thr Val
435 440 445

Met Val Lys Asn Met Gly Ala Tyr Asp Lys Gly Asn Arg Thr Arg Ile
450 455 460

Leu Leu Gly Ser Arg Pro Pro Asn Cys Thr Asn Lys Cys His Gly Cys
465 470 475 480

Ser Pro Cys Lys Ala Lys Leu Val Ile Val His Arg Ile Met Pro Gln
485 490 495

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Glu Tyr Tyr Pro Gln Arg Trp Met Cys Ser Arg His Gly Lys Ile Tyr
500 505 510

His Pro

<210> 101
<211> 373
<212> PRT
<213> Cryptomeria japonica

<400> 101

Met Asp Ser Pro Cys Leu Val Ala Leu Leu Val Leu Ser Phe Val Ile
1 5 10 15

Gly Ser Cys Phe Ser Asp Asn Pro Ile Asp Ser Cys Trp Arg Gly Asp
20 25 30

Ser Asn Trp Ala Gln Asn Arg Met Lys Leu Ala Asp Cys Ala Val Gly
35 40 45

Phe Gly Ser Ser Thr Met Gly Gly Lys Gly Gly Asp Leu Tyr Thr Val
50 55 60

Thr Asn Ser Asp Asp Asp Pro Val Asn Pro Pro Gly Thr Leu Arg Tyr
65 70 75 80

Gly Ala Thr Arg Asp Arg Pro Leu Trp Ile Ile Phe Ser Gly Asn Met
85 90 95

Asn Ile Lys Leu Lys Met Pro Met Tyr Ile Ala Gly Tyr Lys Thr Phe
100 105 110

Asp Gly Arg Gly Ala Gln Val Tyr Ile Gly Asn Gly Gly Pro Cys Val
115 120 125

Phe Ile Lys Arg Val Ser Asn Val Ile Ile His Gly Leu His Leu Tyr
130 135 140

Gly Cys Ser Thr Ser Val Leu Gly Asn Val Leu Ile Asn Glu Ser Phe
145 150 155 160

Gly Val Glu Pro Val His Pro Gln Asp Gly Asp Ala Leu Thr Leu Arg
165 170 175

Thr Ala Thr Asn Ile Trp Ile Asp His Asn Ser Phe Ser Asn Ser Ser
180 185 190

Asp Gly Leu Val Asp Val Thr Leu Ser Ser Thr Gly Val Thr Ile Ser
195 200 205

Asn Asn Leu Phe Phe Asn His His Lys Val Met Leu Leu Gly His Asp
210 215 220

Asp Ala Tyr Ser Asp Asp Lys Ser Met Lys Val Thr Val Ala Phe Asn
225 230 235 240

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Gln Phe Gly Pro Asn Cys Gly Gln Arg Met Pro Arg Ala Arg Tyr Gly
                245                 250                 255

Leu Val His Val Ala Asn Asn Asn Tyr Asp Pro Trp Thr Ile Tyr Ala
            260                 265                 270

Ile Gly Gly Ser Ser Asn Pro Thr Ile Leu Ser Glu Gly Asn Ser Phe
        275                 280                 285

Thr Ala Pro Asn Glu Ser Tyr Lys Lys Gln Val Thr Ile Arg Ile Gly
    290                 295                 300

Cys Lys Thr Ser Ser Ser Cys Ser Asn Trp Val Trp Gln Ser Thr Gln
305                 310                 315                 320

Asp Val Phe Tyr Asn Gly Ala Tyr Phe Val Ser Ser Gly Lys Tyr Glu
                325                 330                 335

Gly Gly Asn Ile Tyr Thr Lys Lys Glu Ala Phe Asn Val Glu Asn Gly
            340                 345                 350

Asn Ala Thr Pro Gln Leu Thr Lys Asn Ala Gly Val Leu Thr Cys Ser
        355                 360                 365

Leu Ser Lys Arg Cys
    370
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<210> 102
<211> 374
<212> PRT
<213> Cryptomeria japonica

<400> 102

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Met Asp Ser Pro Cys Leu Val Ala Leu Leu Val Phe Ser Phe Val Ile
1               5                   10                  15

Gly Ser Cys Phe Ser Asp Asn Pro Ile Asp Ser Cys Trp Arg Gly Asp
            20                  25                  30

Ser Asn Trp Ala Gln Asn Arg Met Lys Leu Ala Asp Cys Ala Val Gly
        35                  40                  45

Phe Gly Ser Ser Thr Met Gly Gly Lys Gly Gly Asp Leu Tyr Thr Val
    50                  55                  60

Thr Asn Ser Asp Asp Asp Pro Val Asn Pro Ala Pro Gly Thr Leu Arg
65                  70                  75                  80

Tyr Gly Ala Thr Arg Asp Arg Pro Leu Trp Ile Ile Phe Ser Gly Asn
                85                  90                  95

Met Asn Ile Lys Leu Lys Met Pro Met Tyr Ile Ala Gly Tyr Lys Thr
            100                 105                 110

Phe Asp Gly Arg Gly Ala Gln Val Tyr Ile Gly Asn Gly Gly Pro Cys
        115                 120                 125
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Val Phe Ile Lys Arg Val Ser Asn Val Ile Ile His Gly Leu Tyr Leu
130 135 140

Tyr Gly Cys Ser Thr Ser Val Leu Gly Asn Val Leu Ile Asn Glu Ser
145 150 155 160

Phe Gly Val Glu Pro Val His Pro Gln Asp Gly Asp Ala Leu Thr Leu
165 170 175

Arg Thr Ala Thr Asn Ile Trp Ile Asp His Asn Ser Phe Ser Asn Ser
180 185 190

Ser Asp Gly Leu Val Asp Val Thr Leu Thr Ser Thr Gly Val Thr Ile
195 200 205

Ser Asn Asn Leu Phe Phe Asn His His Lys Val Met Ser Leu Gly His
210 215 220

Asp Asp Ala Tyr Ser Asp Asp Lys Ser Met Lys Val Thr Val Ala Phe
225 230 235 240

Asn Gln Phe Gly Pro Asn Cys Gly Gln Arg Met Pro Arg Ala Arg Tyr
245 250 255

Gly Leu Val His Val Ala Asn Asn Asn Tyr Asp Pro Trp Thr Ile Tyr
260 265 270

Ala Ile Gly Gly Ser Ser Asn Pro Thr Ile Leu Ser Glu Gly Asn Ser
275 280 285

Phe Thr Ala Pro Asn Glu Ser Tyr Lys Lys Gln Val Thr Ile Arg Ile
290 295 300

Gly Cys Lys Thr Ser Ser Ser Cys Ser Asn Trp Val Trp Gln Ser Thr
305 310 315 320

Gln Asp Val Phe Tyr Asn Gly Ala Tyr Phe Val Ser Ser Gly Lys Tyr
325 330 335

Glu Gly Gly Asn Ile Tyr Thr Lys Lys Glu Ala Phe Asn Val Glu Asn
340 345 350

Gly Asn Ala Thr Pro His Leu Thr Gln Asn Ala Gly Val Leu Thr Cys
355 360 365

Ser Leu Ser Lys Arg Cys
370