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(71) Applicant (for all designated States except US): **Phadia AB** [SE/SE]; Box 6460, S-751 37 Uppsala (SE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **Mattsson, Lars** [SE/SE]; Dragonvägen 16, S-757 56 Uppsala (SE). **Lidholm, Jonas** [SE/SE]; Björknäsvägen 9, S-741 42 Knivsta (SE). **Lundgren, Thomas** [SE/SE]; Stenhagsvägen 102, S-752 60 Uppsala (SE).

(74) Agent: **BRANN AB**; PO Box 12246, S-102 26 Stockholm (SE).

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(54) Title: NOVEL ALLERGEN

(57) Abstract: An isolated horse allergen is disclosed which is a secretoglobin having a molecular weight of 15 kDa under non-reducing conditions and comprising a first peptide chain having a molecular weight of about 5 kDa and a second peptide chain having a molecular weight of about 10 kDa linked together, and variants and fragments thereof sharing epitopes for antibodies therewith. The use of the allergen in diagnosis and therapy is also disclosed as well as a diagnostic kit and a pharmaceutical composition containing the allergen.

**NOVEL ALLERGEN****Field of the invention**

The present invention relates to the field of allergy. More specifically, the invention relates to the identification of novel allergens from mammals and to diagnosis and treatment of allergy towards mammals.

**Background**

Approximately 20% of the populations of the industrialized world become hypersensitive (allergic) upon exposure to antigens from a variety of environmental sources. Those antigens that induce immediate and/or delayed types of hypersensitivity are known as allergens (Breiteneder et al. 1997). These include products of grasses, trees, weeds, animal dander, insects, food, drugs and chemicals. The antibodies involved in atopic allergy belong primarily to the immunoglobulin E isotype (IgE). IgE binds to basophils mast cells and dendritic cells via a specific high affinity receptor Fc $\epsilon$ RI. Upon exposure to an allergen, allergen-specific IgE antibodies on the cell surface become cross linked leading to the release of inflammatory mediators such as histamine and leukotrienes resulting in physiological manifestations of allergy (Akdis 2006).

Diagnostic tests for allergy involve the detection of IgE antibodies from patients with a specificity to proteins from an allergen source. Typically, an aqueous extract from the allergen source, containing a mixture of proteins, is used in these tests. For most allergen sources, the allergenic proteins present in crude extract have only partly been identified and characterised. Diagnostic test procedures for detection of specific IgE antibodies in patients can either utilize an *in vitro* immunoassay using serum from the patient, or be a skin prick test (SPT), performed by topical application of the specific extract on the skin of the patient (Wainstein et al. 2007).

In recent years, many important allergenic proteins in the allergenic extracts have been identified and characterized. This has enabled the quantitation of specific IgE antibodies to each of these individual allergenic components, often referred to as component resolved diagnostics (CRD) (Valenta et al. 1999)(Hiller et al. 2002) which in many cases can lead to an improved diagnosis of hypersensitivity (Stumvoll et al. 2003). The use of CRD has also been suggested as an aid in the selection of optimal

immunotherapy treatment (Valenta et al. 2007) . Further, individual allergens can in some cases be used to enhance the diagnostic sensitivity of an extract by spiking the extract with a component. In conclusion, it is thus of great importance to identify and characterise all important allergenic proteins in each allergen source.

Apart from reducing symptoms of allergy by e. g. anti-histamines, more long-term and curative treatment of allergy can be performed with specific immunotherapy. Application of the disease causing allergenic extract, most commonly either subcutaneously or sublingually, that causes a specific activation of a protective immune response to the allergenic proteins. Although the exact mechanisms are not fully known, such a specific activation of the immune system alleviates the symptoms of allergy upon subsequent environmental exposure of the same allergen (Akdis et al. 2007). A further development of regular immunotherapy has been to use one or several purified allergenic proteins instead of a crude natural extract. Such immunotherapy has been successfully performed for grass pollen allergic patients (Jutel et al. 2005) and it has also been suggested for treating allergy against animal dander (Gronlund et al. 2009).

Horse dander is an increasingly common cause of respiratory allergy (Liccaldi et al. 2011), with symptoms including rhinitis, conjunctivitis, bronchial inflammation and asthma. Occupational exposure to horse allergens is a significant risk factor for allergic sensitisation (Tutluoglu et al. 2002) but considerable concentrations of allergens can be detected also in other places such as schools (Kim et al. 2005). IgE sensitisation to horse dander was in one study shown to be associated with a high risk of developing asthma (Ronmark et al. 2003).

Extracts of horse hair and dander contain a complexity of allergenic proteins and four horse allergens have so far been identified: Equ c 1, Equ c 2, Equ c 3 and Equ c 4/5. The first two are both members of the lipocalin protein family and have been purified from their natural source (Dandeu et al. 1993; Goubran Botros et al. 1998) while only Equ c 1 has been expressed as a recombinant protein (Gregoire et al. 1996). The amino acid sequence of Equ c 1 is 67% similar to that of the cat allergen Fel d 4 (Smith et al. 2004). Equ c 3, horse serum albumin, is a relatively conserved protein showing extensive cross-reactivity to other mammalian albumins (Goubran Botros et al. 1996). Equ c 4/5, was first purified and reported as an IgE binding

protein in horse dander (Goubran Botros et al. 1998; Goubran Botros et al. 2001) and only later identified as horse sweat latherin (McDonald et al. 2009).

Equ c 1 is claimed to be the most important one of the known horse allergens (Dandeu et al. 1993) and IgE antibody recognition of the recombinant protein was present in 76% of a population of horse allergic subjects studied (Saarelainen et al. 2008). In another study using purified native allergens, only 33% of horse allergic patients were sensitized to Equ c 2 and 23% to Equ c 4/5 (Goubran Botros et al. 1998). The frequency of IgE binding to horse serum albumin has been addressed in several studies demonstrating reactivity in up to 40% of horse allergic subjects (Spitzauer et al. 1993; Cabañas et al. 2000). However, as sensitization to serum albumins is often accompanied by higher concentrations of IgE antibodies to other allergen components, its specific clinical relevance is uncertain.

Although the horse dander allergens Equ c 1, Equ c 2, Equ c 3 and Equ c 4/5 have been known for a long time, no quantitative estimation of each component's contribution to the total IgE response to horse dander has been made.

### **Summary of the invention**

As stated above, a well designed laboratory immunoassay for specific IgE antibodies can detect most cases of sensitization to horse using natural horse dander extract. However, in a miniaturized or non-laboratory immunoassay, such as an allergen microarray or a doctor's office test, the combination of less favourable assay conditions, lower capacity for antibody-binding allergen reagent and natural allergen extract of limited potency, may cause insufficient diagnostic sensitivity. A similar situation may exist also for immunoassays for specific IgE to other animal epithelia. Thus, there is a need in some cases to use pure allergenic proteins to achieve sufficient sensitivity in diagnostic tests for specific IgE antibodies to animal epithelia.

Such allergens may be useful not only as reagents for increased sensitivity in routine diagnostic tests, but also in different types of component-resolved diagnostic applications (Valenta et al. 1999)(Hiller et al. 2002). Pure allergenic proteins, or fragments and variants thereof with improved non-anaphylactic properties, may also be used as novel reagents in immunotherapy (Valenta et al. 1999)(Cromwell et al. 2006) (Saarne et al. 2005); (Jutel et al. 2005); (Cromwell et al. 2006).

The purification and analysis of all the known horse allergen components resulted in the identification of some patients' sera having a significantly higher IgE response to horse dander extract than could be collectively accounted for by the sum of the individual horse allergen components. These sera were found to have IgE binding reactivity to a previously unknown horse allergen component.

With the aid of the sera described above, a new major allergen could be purified from horse dander and identified as a member of the secretoglobin protein family. The novel horse protein, herein referred to as Equ c 15k, consists of one 5 kDa amino acid chain and one 10 kDa amino acid chain joined together by disulfide bridges. Considering the fact that the two polypeptide chains are encoded by separate genes, this study demonstrates the presence of a heterodimeric protein that has not previously been anticipated by bioinformatic studies of the horse genome. It is in all aspects distinct from previously known horse allergens. This allergen represents an important addition to the panel of known horse allergens and will be useful in the diagnosis of horse allergy.

In one aspect the present invention relates to an isolated horse allergen, Equ c 15k belonging to the secretoglobin family, showing an electrophoretic mobility (apparent molecular weight) corresponding to approximately 15 kDa under non-reducing conditions, and comprising a first peptide chain having a molecular weight in the order of 5 kDa and a second peptide chain having a molecular weight in the order of 10 kDa, linked together by one or more disulfide bonds. This aspect of the invention also comprises variants and fragments of Equ c 15k, sharing epitopes for antibodies therewith, such that the variants and fragments cross-react with such antibodies to at least about 50%. Such variants and fragments include, for example, related allergens from the same species. Also in the other aspects of the invention described below, the term "Equ c 15k" is, for simplicity, used to also include such variants and fragments thereof.

In another aspect, the invention relates to an isolated nucleic acid encoding the allergen according to the first-mentioned aspect, as well as to a vector containing the nucleic acid molecule, and to a host cell containing the vector. Recombinant proteins or peptides produced by such a vector-containing host cell may be glycosylated or not depending on the host cell used.

In a further aspect, the invention relates to the use of Equ c 15k for the manufacture of a composition for diagnosis of Type I allergy.

In a further aspect, the invention relates to an allergen composition “spiked” with Equ c 15k. Such an allergen composition may be an allergen extract or a mixture of purified or recombinant allergen components having no or a low Equ c 15k content, wherein Equ c 15k is added in order to bind IgE from patients whose IgE would not bind, or bind poorly, to the other allergen components in the composition. This aspect of the invention also relates to a method for producing such a composition, which method comprises the step of adding Equ c 15k to an allergen composition, such as an allergen extract (optionally spiked with other components) or a mixture of purified native or recombinant allergen components.

In yet a further aspect, the invention relates to an *in vitro* diagnostic method for diagnosing a Type I allergy in a patient, wherein a body fluid sample, such as a blood or serum sample from the patient, is brought into contact with Equ c 15k or a composition according to the previous aspect, whereby it can be determined whether or not the patient sample contains IgE antibodies that bind specifically to the Equ c 15k. Such a diagnostic method may be carried out in any manner known in the art. The Equ c 15k may e.g. be immobilized on a solid support, such as in a conventional laboratory immunoassay, in a microarray or in a lateral flow assay, or used as a fluid-phase reagent.

In another aspect, the invention relates to a diagnostic kit for performing the method according to the previous aspect.

In the above mentioned aspects, the wildtype Equ c 15k molecule may, as mentioned above, be replaced with fragments or variants of Equ c 15k, natural or man-made, sharing epitopes for antibodies with the wildtype protein, as defined below.

The invention further relates to a method of treatment of Type I allergy comprising administering to a patient in need of such treatment Equ c 15k or a modified Equ c 15k, as explained below. This aspect of the invention also relates to the use of the Equ c 15k in such immunotherapy, including e.g. component-resolved

immunotherapy (Valenta et al. 2007). In one embodiment of this aspect, the Equ c 15k may be used in its natural form or in a recombinant form displaying biochemical and immunological properties similar to those of the natural molecule. In another embodiment, the Equ c 15k may be used in a modified form, generated chemically or genetically, in order to abrogate or attenuate its IgE antibody binding capacity, while preferably being capable of eliciting an IgG response in a treated individual. Examples of modifications include, but are not limited to, fragmentation, truncation, tandemization or aggregation of the molecule, deletion of internal segment(s), substitution of amino acid residue(s), domain rearrangement, or disruption at least in part of the tertiary structure by disruption of disulfide bridges or its binding to another macromolecular structure, or other low molecular weight compounds. In yet another embodiment of this aspect, the individual 10 kDa and/or 5 kDa subunits of Equ c 15k, which display reduced IgE binding activity as compared to the intact molecule, are used as modified Equ c 15k.

In all of the above mentioned aspects of the invention, the Equ c 15k protein may be purified from its natural source, such as from urine, saliva or other body fluids, or from tissue, such as hair or dander, from horse. It may also, as mentioned above, be produced by recombinant DNA technology or chemically synthesized by methods known to a person skilled in the art.

The invention also relates to the Equ c 15k for use in prophylactic or therapeutic treatment of Type 1 allergy, as well as in diagnosis.

### **Definitions**

The allergenic horse protein described here, Equ c 15k, belongs to the secretoglobin protein family, specifically one subfamily which comprises tetrameric proteins formed by two heterodimeric subunits. The heterodimer consists of two chains derived from different genes linked together by disulfide bridges (Klug et al. 2000). The horse secretoglobin described here is a 15 kDa heterodimer, herein referred to as Equ c 15k, consisting of a 5±2 kDa and a 10 ±2 kDa subunit, respectively, which for the purposes of this invention are referred to as the 5 and 10 kDa subunits, respectively. The molecular weight assignments are according to their apparent molecular weight as observed in SDS-PAGE, as described in Example 4 below. It is understood that the apparent molecular weights will vary depending on the separation conditions, including electrophoretic separation medium and

concentration thereof, linear or gradient buffer used, etc. Also, the 10 kDa subunit contains an N-glycosylation site, the occupation of which by a glycan structure may affect the apparent molecular weight.

The amino acid sequence of the 5 kDa chain has the predicted amino acid sequence  
ATCPAVATDIASFFLLPDSLFKLQLIKYQAPPEAKDATMQVKQCINEIS  
AGDRYIITETLGKIVLQCGA (SEQ ID NO: 4) and a theoretical molecular weight of 7.5 kDa.

The amino acid sequence of the 10 kDa chain has the predicted amino acid sequence

GSGCQLLEDVVEKTITAELSPAELYVEAVQEFIPDEATEKAAIQLKQCYLKQSNETLNDFR  
TMMNSMYNSAYCALF (SEQ ID NO: 5) and a theoretical molecular weight of 8.4 kDa.

It is to be noted that structurally related proteins have been described in a wide range of mammalian species but only one protein has been defined as an allergen, i.e. the major cat allergen Fel d 1 (Acc no P30438 and P30440).

Variants and fragments of Equ c 15k should be construed as meaning proteins or peptides with a length of at least 10 amino acids, more preferably at least 40, even more preferably at least 50 or 60 amino acid residues of each chain in the heterodimer, and a sequence identity to said Equ c 15k of at least 50 %, preferably over 60 %, 70 %, 80 %, 90 % or 95 %.

A modified Equ c 15k should in the context of the present invention be construed as meaning an Equ c 15k variant that has been chemically or genetically modified to change its immunological properties, e.g. as exemplified above in relation to the immunotherapy aspect of the invention.

Variants and fragments of Equ c 15k sharing epitopes for antibodies with Equ c 15k should be construed as being those fragments and variants whose binding of antibodies, such as IgE or IgG antibodies, from a serum sample from a representative Equ c 15k sensitized patient can be significantly inhibited by Equ c 15k. Such an inhibition assay may e.g. be performed according to the methods

described by (Mattsson et al. 2009) (the disclosure of which is incorporated by reference herein).

A hypoallergenic modified Equ c 15k or variant or fragment of Equ c 15k should be construed as being a modified Equ c 15k or variant or fragment of Equ c 15k that is not capable of binding Equ c 15k reactive IgE antibodies from a serum sample of a representative Equ c 15k sensitized patient, as determined e.g. by the protocol according to Example 7 below or which displays no or significantly reduced biological allergen activity, as determined by a cellular activation assay such as the basophil histamine release assay (Demoly et al. 2003; Ebo et al. 2004).

#### **Brief description of drawings**

Figure 1A shows the fractionation of horse dander proteins by size exclusion chromatography (SEC). Peaks A and B that were used for subsequent purification steps are indicated by arrows.

Figure 1B shows the purification of nEqu c 1 by hydrophobic interaction chromatography. Peaks C and D were used for subsequent purification steps.

Figure 1C shows the purification of nEqu c 2 and Equ c 4/5 by hydrophobic interaction chromatography. Peaks E, F and G were used for subsequent purification steps.

Figure 1D shows the purification of nEqu c 2 by anion exchange chromatography. Peaks H and I were used for subsequent analysis.

Figure 1E shows SDS-PAGE analysis of the purified proteins Equ c 1 forms A and B, Equ c 2 and Equ c 4/5 form 14 kDa and 19 kDa. Lane M contains molecular weight marker proteins with the molecular weight indicated to the left.

Figure 2A compares the IgE binding of the two forms, A and B, of nEqu c 1 (from peak C and peak D respectively) using 35 horse dander reactive sera. Dotted lines indicate the 0.35 kU<sub>A</sub>/L cut-off level.

Figure 2B compares the IgE binding of the 19 kDa and the 14 kDa forms of nEqu c 4/5 using 38 horse dander reactive sera. Dotted lines indicate the 0.35 kU<sub>A</sub>/L cut-off level.

Figure 3 shows the purification of fractions used to search for novel IgE binding proteins. A: Purification of fraction A by anion exchange chromatography. B: Purification of fractions B and C by hydrophobic interaction chromatography.

Figure 4 shows the purification of a 15 kDa horse dander protein. A: Fractionation of horse dander extract by size exclusion chromatography. Peak A was used for subsequent purification steps. B: Fractionation of peak A by hydrophobic interaction chromatography. Peak J, pooled as indicated in the figure, was used for subsequent purification steps. C: Fractionation of peak J by anion exchange chromatography. Peaks K and L were used for subsequent analysis and/or further purification steps. D: shows SDS-PAGE analysis reduced (Red) and non-reduced (Ox) samples of the purified 15 kDa horse dander protein. Lane M contains molecular weight marker proteins with the molecular weight indicated to the left. E: Refining purification of peak K by reversed-phase chromatography. Peak M was used for subsequent immunological analysis.

Figure 5 shows the predicted sequence of the 5 kDa and 10 kDa amino acid chains of nEqu c 15k. Amino acids identified by N-terminal sequencing are underlined and amino acids identified by MS/MS analysis are shown in bold.

Figure 6 shows the IgE reactivity to nEqu c 15k in sera of two of the horse allergic patients (Nos. 3 and 12), as detected by immunoblotting. The first two strips show total protein stain and positions of the 5 and 10 kDa subunits and the 15 kDa protein, respectively, are indicated by arrows. The four strips to the right shows the IgE binding to reduced (Red) and non-reduced (Ox) samples of Equ c 15k.

Figure 7 shows the correlation between the IgE reactivity of native and recombinant Equ c 15k. The 0.35 kU<sub>A</sub>/L and 0.1 kU<sub>A</sub>/L levels are indicated by dotted lines.

Figure 8 shows levels of IgE antibodies to horse dander extract (HDE), Equ c 1, nEqu c 2, nEqu c 3, nEqu c 4/5 and rEqu c 15k in a cohort of 25 horse dander allergic subjects. The number of observations below 0.1 kU<sub>A</sub>/L is indicated in

brackets for each component. Dotted line indicates the 0.35 kU<sub>A</sub>/L level and solid line indicates the 0.1 kU<sub>A</sub>/L level. Horizontal bars indicate median levels of IgE.

Figure 9 compares IgE antibody binding to nEqu c 15k and rFel d 1. The 0.35 kU<sub>A</sub>/L and 0.1 kU<sub>A</sub>/L levels are indicated by dotted lines.

Figure 10 shows the ability of soluble Equ c 15k and Fel d 1 to self- and cross-inhibit IgE binding to immobilized Equ c 1 and Fel d 1. Sera from horse dander allergic patients (labelled according to table 3) or horse dander sensitised subjects (labelled A to E) were used.

#### **Detailed description of the invention**

The examples below illustrate the present invention with the isolation and use of secretoglobin from horse. The examples are only illustrative and should not be considered as limiting the invention, which is defined by the scope of the appended claims.

#### **EXAMPLE 1: Purification and characterisation of known allergens from horse dander and serum**

Horse dander was used as a starting material for purification of Equ c 1, Equ c 2 and Equ c 4/5 while Equ c 3 was purified from horse serum.

Horse dander (Allergon, Välinge, Sweden) was extracted in 20 mM MOPS, pH 7.6, 0.5 M NaCl (MBS = MOPS-buffered saline), clarified by centrifugation and filtered through a 0.45 µm mixed cellulose ester filter (Millipore, Billerica, MA, USA). As a first purification step for all three horse dander allergens, the clarified extract was applied to a Superdex™ 75 column (XK26/100, V<sub>t</sub>= 505 mL, GE Healthcare Life Sciences, Uppsala, Sweden) for size exclusion chromatography (SEC) and elution was performed with MBS at a flow rate of 2 mL/min.

#### **Equ c 1**

In order to purify Equ c 1, peak A in Fig. 1A was adjusted to 2 M NH<sub>4</sub>SO<sub>4</sub> and applied to a Phenyl Sepharose™ HP column (HR10/10, V<sub>t</sub>= 9.0 mL, GE Healthcare Life Sciences) equilibrated with 2 M NH<sub>4</sub>SO<sub>4</sub> in 20 mM tris pH 8.0. Elution was performed in a linear NH<sub>4</sub>SO<sub>4</sub> gradient from 2 M to 0 M NH<sub>4</sub>SO<sub>4</sub>. Two peaks containing Equ c 1 were eluted in the middle of the gradient, peaks C and D in Fig.

1B. After desalting each peak on a Sephadex™ G25 fine column (XK16/20,  $V_t$ = 34 mL, GE Healthcare Life Sciences) equilibrated with 20 mM MOPS pH 7.6 0.5 M NaCl, each preparation of of nEqu c 1 was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the NuPAGE MES buffer system (10% NuPAGE gel, Invitrogen, Carlsbad, CA, USA) of a reduced sample prepared by mixing the sample 1:3 with NuPAGE LDS sample buffer (Invitrogen) containing 100 mM  $\beta$ -mercaptoethanol. As an indication of apparent molecular weight the Mark 12™ standard (Invitrogen) was used. Both nEqu c 1 preparations were pure as judged by SDS-PAGE (Fig. 1E).

The protein preparations was unambiguously identified as Equ c 1 by peptide mass fingerprinting (PMF) performed in a Bruker Daltonics Autoflex 2 instrument (Bruker Daltonics, Bremen, Germany) as described in (Mattsson et al. 2009).

Both forms of the protein were immobilized to ImmunoCAP™ solid phase as described (Marknell DeWitt et al. 2002).

#### Equ c 2

In order to purify Equ c 2, the second peak from SEC, peak B in Fig. 1A, was adjusted to 1 M NH<sub>4</sub>SO<sub>4</sub> and subjected to hydrophobic interaction chromatography (HIC) on a phenyl Sepharose™ HP column (HR10/10,  $V_t$ = 9.0 mL, GE Healthcare Life Sciences) equilibrated with 1 M NH<sub>4</sub>SO<sub>4</sub> in 20 mM Tris pH 8.0. Elution was performed in a linear NH<sub>4</sub>SO<sub>4</sub> gradient from 1 M to 0 M NH<sub>4</sub>SO<sub>4</sub> in the same buffer. Equ c 2 was contained in the flow through fraction (peak E in Fig. 1C) that was pooled and desalting on a Sephadex™ G25 fine column (XK26/20,  $V_t$ = 90 mL, GE Healthcare Life Sciences) equilibrated with 20 mM Bis-Tris propane, pH 8.5. The desalting Equ c 2 pool was finally applied to an anion exchange column Source™ 15Q (HR16/10,  $V_t$ = 9 mL, GE Healthcare Life Sciences) equilibrated with 20 mM Bis-Tris propane, pH 8.5. Upon elution in a linear 0-0.40 M NaCl gradient in the same buffer, the protein was resolved into three peaks that all displayed pure 17 kDa band upon SDS-PAGE analysis. The two largest peaks were analysed by N-terminal sequencing (Procise™ LC452, Applied Biosystems, Foster city CA, USA) and both had the sequence DQDPQSEDTY, identifying them as Equ c 2.0201 (Fig. 1D peak H and I). For the purpose of evaluating IgE binding reactivity, the peaks were pooled and immobilised to ImmunoCAP™ solid phase as described (Marknell DeWitt et al. 2002).

#### Equ c 4/5

Purification of Equ c 4/5 was performed by using the second peak from SEC, peak B in Fig. 1A. This pool was adjusted to 1 M NH<sub>4</sub>SO<sub>4</sub> and subjected to hydrophobic interaction chromatography (HIC) on a phenyl Sepharose™ HP column (HR10/10, V<sub>t</sub> = 9.0 mL, GE Healthcare Life Sciences) equilibrated with 1 M NH<sub>4</sub>SO<sub>4</sub> in 20 mM Tris pH 8.0. Elution was performed in a linear NH<sub>4</sub>SO<sub>4</sub> gradient from 1 M to 0 M NH<sub>4</sub>SO<sub>4</sub> in the same buffer. Equ c 4/5 protein eluted in two distinct peaks in the middle of the gradient, (peak F and G in Fig. 1C). SDS-PAGE analysis of the first peak revealed a protein migrating as a 14 kDa band while the second peak contained a 19 kDa band. After desalting on a Sephadex™ G25 fine column (XK26/20, V<sub>t</sub> = 90 mL, GE Healthcare Life Sciences) equilibrated with 20 mM Bis-Tris propane, pH 8.5. Both proteins were pure as judged by SDS-PAGE (Fig. 1E). Both of the two preparations displayed the N-terminal sequence VGPLLGPSDA, identifying them as horse latherin or Equ c 4/5.

The two forms of nEqu c 4/5 were immobilized separately to ImmunoCAP™ solid phase as described (Marknell DeWitt et al. 2002).

### Equ c 3

Native Equ c 3 was purified from horse serum by affinity chromatography using Blue Sepharose FF, (GE Healthcare Life Sciences), anion exchange chromatography (AIEC) and SEC essentially as described (van Eijk et al. 1999).

### **EXAMPLE 2: Assessment of IgE binding levels to individual horse dander allergen components in a panel of sera from horse sensitized individuals**

The IgE binding activity of the two forms of Equ c 1, named form A and B, was evaluated using a set of horse dander sensitized sera (obtained from an in-house serum collection. The two forms of Equ c 1 displayed equivalent IgE binding activity, as shown in Fig. 2A. Therefore only the values obtained with nEqu c 1 A were used in the analysis below. Using a similar set of horse dander reactive sera, IgE antibody binding to the two forms of Equ c 4/5 were compared and found to be very similar, as shown in Fig. 2B.

IgE antibody binding to horse dander extract and the purified horse allergens was examined using regular and experimental ImmunoCAP™ tests (Phadia, Uppsala, Sweden). Experimental ImmunoCAP™ tests were prepared as described above. A panel of 29 sera from horse dander sensitised individuals were used.

Determinations of IgE responses to horse dander extract, nEqu c 1, nEqu c 2, nEqu

c 4/5 and were performed. The results are presented in Table 1 where the IgE antibody concentrations in sera of patients A1 to A29 to HDE and the components and sum of the three components are displayed as kU<sub>A</sub>/L. The component coverage is the ratio of component sum and horse dander extract, expressed as percentage. A number of sera were identified as having a significantly higher level of IgE binding to horse dander extract than could be accounted for by the individual components, e.g. sera no A1, A21 and A22. Apart from possible Equ c 3 reactivity, which was not evaluated at this stage, the identified sera could aid in the search for novel IgE binding proteins from horse dander.

**EXAMPLE 3: Identification of a fraction from horse dander having a novel IgE binding reactivity**

During the process of purifying the previously characterised horse dander allergens, several fractions were identified which contained proteins other than the previously known horse allergens. Three fractions of particular interest were selected for analysis of IgE binding activity using the sera identified in Example 2 above. Fraction A contained a 10 kDa band (reducing SDS-PAGE) obtained from an anion exchange purification step of Equ c 2 indicated by an arrow (Fig. 3A). Fractions B and C, containing a 13 kDa and a 10 kDa band (reducing SDS-PAGE), respectively, were obtained from a HIC purification step of Equ c 1 and are indicated by arrows in Fig. 3B. Experimental ImmunoCAP™ (Phadia) tests were prepared as described (Marknell DeWitt et al. 2002) and used for serum analysis.

The results are summarised in Table 2, which also includes the previous determinations of horse dander extract and the sum of nEqu c 1, nEqu c 2 and nEqu c 4/5, all displayed as kU<sub>A</sub>/L. The highest IgE binding levels were found in fraction C. Notably, in serum A1, the level of IgE binding to fraction C was much higher than the sum of IgE binding to nEqu c 1, nEqu c 2 and nEqu c 4/5. The fact that this serum had an albumin IgE reactivity of only 1.5 kUA/L (not shown) suggested that fraction C contained a novel horse dander allergen.

**EXAMPLE 4: Purification and identification of the dominant protein constituent of fraction C**

**Purification of a horse dander protein from fraction C**

To purify the 10 kDa protein present in fraction C in a more targeted way, horse dander extract was subjected to SEC as described in Example 1. The Equ c 1 containing peak was pooled according to SDS-PAGE analysis as indicated in the figure (peak A in Fig. 4A). Only the right-hand part of peak A contained a 10 kDa band and was included in the pool. The pool was adjusted to 2 M NH<sub>4</sub>SO<sub>4</sub> and subjected to HIC (Fig. 4B) as described for Equ c 1 in Example 1. Peak J in Fig. 4B was diluted 1:3 in 20 mM Tris, pH 8.0, and applied to a Source<sup>TM</sup> 15Q column (PE 4.6/100, V<sub>t</sub>= 1.7 mL; GE Healthcare Life Sciences) equilibrated in the same buffer. Elution was performed with a linear gradient of 0-0.4 M NaCl, yielding a dominant peak in the middle of the gradient, followed by a smaller peak (peaks K and L, Fig. 4C). SDS-PAGE analysis was performed using the NuPAGE MES buffer system as described in Example 1 where samples were prepared by diluting the sample 1:3 in NuPAGE LDS buffer with or without 4% β-mercaptoethanol for reducing and non-reducing conditions, respectively. SDS-PAGE analysis of both peaks (Fig. 4D) revealed a band of approximately 15 kDa under non-reducing conditions. Upon reduction of the samples, the 15 kDa band disappeared while two bands of approximately 5 and 10 kDa appeared, suggesting that the unreduced 15 kDa band was made up of the polypeptides forming the 5 kDa and 10 kDa bands, linked to one another by one or more disulfide bridges. Although both peaks appeared to contain the same protein, only the large peak (K) was subjected to further biochemical analysis. For the purpose of IgE binding studies, a refining RPC purification step was included by applying the sample to a Source<sup>TM</sup> 5 RPC column (ST 2.1/150, V<sub>t</sub>= 0.52 mL; GE Healthcare Life Sciences) equilibrated with 0.065% TFA in water. Elution was performed in a linear 0-70% gradient of buffer B, consisting of 0.05% TFA in 90% acetonitrile. The protein eluted in a single peak near the end of the gradient (Peak M, Fig. 4E).

**Identification of the 15 kDa horse dander protein as a secretoglobin**

The reduced 5 kDa and 10 kDa protein bands, excised and extracted from a SDS-PAGE gel, were analysed by N-terminal sequencing. Analysis of the 5 kDa band revealed the amino acid sequence ATxPAVATDIASFFLLPDSL (x: unresolved

residue), matching residues 22-41 of the predicted *Equus caballus* sequence denoted “similar to LppAB” (Genbank Acc no XP\_001502544) (SEQ ID NO: 1). Analysis of the 10 kDa band revealed the sequence GSGxQLLEDVVEKTITAELS (x: unresolved residue), matching residues 19-38 of a predicted sequence denoted “similar to Lipophilin CL2” from *Equus caballus* (GenBank Acc no XP\_001494564) (SEQ ID NO: 2).

Peptide mass fingerprinting (PMF) analysis of the purified 15 kDa protein by MALDI-TOF MS of an in-solution trypsin digest did not result in any significant match ( $p<0.05$ ) to known database entries. However, MS-MS analysis of peptides  $m/z=2281$  and  $m/z=1262.5$  identified the sequence QCINEISAGDRYIITETLGK (SEQ ID NO: 3) from the predicted sequence “similar to LppAB (*Equus caballus*) (GenBank Acc no XP\_001502544.

Peptide mass fingerprinting (PMF) analysis by MALDI-TOF MS of in-gel trypsin digested 5 kDa fragment did not result in any significant match ( $p<0.05$ ) to known database entries. However, the five dominant peptides detected did all correspond to anticipated trypsin fragments from the SEQ ID NO: 4 where  $m/z=903.47$  (corresponding to residue 28-35),  $m/z=1037.6$  (residues 43-53),  $m/z=1262.6$  (residue 43-53),  $m/z=2281.1$  (residues 43-62) and  $m/z=2384.2$  (residues 1-22), which in total cover 50 (72%) out of the predicted amino acid residues of SEQ ID NO: 4.

Peptide mass fingerprinting (PMF) analysis by MALDI-TOF MS of in-gel trypsin digested 10 kDa band did not result in any significant match ( $p<0.05$ ) to known database entries. However, the two dominant peptides detected were  $m/z=1433.6$  and  $m/z=2880.4$  which is consistent with the mass of the peptides GSGCQLLEDVVEK and TITAELSPAELYVEAVQEFIPDEATEK, respectively, corresponding to residues 1-13 and 14-39 of SEQ ID NO: 5.

The amino acid sequences of both of the identified database entries, XP\_001502544 (SEQ ID NO: 6) AND XP\_001494564 (SEQ ID NO: 7) , contained features characteristic of the secretoglobin protein family. Thus, taken together, the results identified the 15 kDa horse dander protein as a secretoglobin. This protein is hereinafter referred to as Equ c 15k. The predicted full length sequences precursor sequences of the two chains of Equ c 15k are shown in Fig. 5 (5 kDa fragment -

SEQ ID NO: 6; 10 kDa fragment - SEQ ID NO 7) where the amino acids identified by N-terminal sequencing are underlined and those identified by MS-MS analysis are shown in bold. The precursor sequence for the 5 kDa fragment includes an N-terminal signal peptide of 21 amino acids, and the precursor sequence for the 10 kDa fragment includes an N-terminal signal peptide of 18 amino acids. It is to be noted that signal peptide prediction of the precursor sequence using SignalP ([www.cbs.dtu.dk/services/SignalP](http://www.cbs.dtu.dk/services/SignalP)) results in the same mature sequences as those obtained experimentally for both the 5 kDa and 10 kDa chains.

The SDS-PAGE analysis in Fig. 4D provides evidence that the 5 and 10 kDa amino acid chains are held together by one or more disulfide bridges under non-reducing conditions, thereby forming a heterodimeric protein. Thus, the analysis links together a gene encoding a the sequence SEQ ID No 4 with a different gene encoding SEQ ID No 5 that together make up a previously unknown heterodimeric secretoglobin protein.

#### **EXAMPLE 5: Assessment of IgE binding to Equ c 15k using immunoblot analysis**

With the aim of determining to which subunit of Equ c 15k the IgE reactivity against the protein was directed, an immunoblot analysis was performed employing both reducing and non-reducing conditions.

Immunoblot analysis was performed on reduced and non-reduced samples of purified Equ c 15k separated by SDS-PAGE using a 4-20% NuPAGE gel (Invitrogen) and electroblotted onto a Hybond ECL nitrocellulose membrane (GE Healthcare Life Sciences). Protein blots were blocked for 1 h at room temperature using blocking buffer (50 mM phosphate pH 7.4, 0.1 % (v/v) Tween<sup>TM</sup> 20, 0.9% (w/v) NaCl, 0.3% (w/v) Dextran T10) and then incubated overnight with serum from patient 3 and 12, diluted 1: 4.8 and 1:13.5, respectively, in blocking buffer. After washing with 0.15 M NaCl containing 0.5 % (v/v) Tween-20, the membrane was incubated 3 hours with a HRP-labelled anti-human IgE antibody in blocking buffer and, after washing, bound IgE was fluorometrically detected using an ECL Advance Western Blotting Detection Kit (GE Healthcare Life Sciences) and a LAS 4000 mini CCD camera (Fujifilm, Tokyo, Japan).

The two sera used in the analysis (patients No. 3 and 12) both had a dominant reactivity to Equ c 15k according to the ImmunoCAP™ analysis (see example 7 below). Both sera reacted only weakly with the subunits of Equ c 15k, dissociated under reducing conditions, visible as faint bands corresponding to the reduced 5 kDa and 10 kDa subunits (Fig. 6). Under non-reducing conditions, a much stronger reactivity was observed with a band coinciding with the non-reduced 15 kDa band of Equ c 15k. No significant reactivity to other bands was observed in this analysis. This immunoblot analysis demonstrates that the IgE binding reactivity is indeed directed to the major protein bands in the Equ c 15k preparation.

**EXAMPLE 6: Production and immunological characterization recombinant Equ c 15k**

*Cloning and purification of recombinant Equ c 15k*

A synthetic Equ c 15k single chain gene was designed by combining nucleotide sequences encoding the amino acid sequences of the 5 kDa and the 10 kDa subunits with a sequence encoding a linker peptide comprising 3x (Gly-Gly-Gly-Gly-Ser). The full-length synthetic gene was cloned into the NdeI and XhoI sites of vector pET23a(+) (Novagen, Madison, WI, USA), adding a C-terminal hexahistidine tag to enable protein purification by immobilised metal ion affinity chromatography (IMAC).

The amino acid sequence for the whole recombinant protein is shown in SEQ ID NO: 8. The nucleotide sequence was designed for optimal codon usage in *E. coli* (DNA2.0, Menlo Park, CA, USA). The nucleic acid sequence encoding the whole recombinant protein is shown in SEQ ID NO: 9.

The plasmid DNA construct was transformed into *E. coli* strain BL21-AI (Invitrogen) and the recombinant Equ c 15k single chain protein was produced using a 3-litre bioreactor (Belach Bioteknik, Solna, Sweden).

For purification of recombinant Equ c 15k, harvested cells was resuspended in 20 mM Tris-HCl pH 8.0 and lysed by passing the suspension through an Emulsiflex C5 homogenizor (Avestin, Ottawa, Ontario, Canada) at 10 000-15 000 kPa. After centrifugation of the suspension, the pelleted inclusion bodies were dissolved in 6 M Guanidine-HCl, 20 mM Tris pH 8.0, 0.5 M NaCl, 5 mM imidazol and filtered

through 0.45 µm mixed cellulose filters (Millipore). The filtered supernatant was applied to a Chelating Sepharose FF column (GE Healthcare Life Sciences), charged with NiSO<sub>4</sub>. Column washing was performed with 6 M urea in 20 mM Tris-HCl pH 8.0, 0.15 M NaCl, 20 mM imidazole followed by renaturation *in situ* by a linear 6 M to 2 M gradient of urea in the same buffer. Following renaturation, the recombinant protein was eluted in a linear 20-500 mM gradient of imidazole in the same buffer. Further purification of the recombinant protein was performed by AIEC in 20 mM Tris-HCl pH 8.0 using a Q Sepharose™ FF column (GE Healthcare Life Sciences). The protein was eluted using a linear 0-0.5 M NaCl gradient and fractions were pooled according to SDS-PAGE results. The protein concentration of the final preparation was determined from absorbance at 280 nm, using a calculated extinction coefficient of 0.44 per mg/mL

#### Assessment of IgE binding to recombinant Equ c 15k

Recombinant Equ c 15k was immobilised to experimental ImmunoCAP™ and the IgE reactivity to sera from 36 horse dander sensitized subjects was determined as described (Marknell DeWitt et al. 2002).

There was a good agreement ( $r=0.98$ ) between IgE binding to purified native Equ c 15k and recombinant Equ c 15k (Fig. 7), demonstrating that the recombinant protein was immunologically active and structurally similar to the native protein. These data provide strong evidence that the amino acid sequence of the 5 kDa (SEQ ID NO: 4) and 10 kDa (SEQ ID NO: 5) fragments of Equ c 15k, as predicted from the genomic sequence information identified, are correct and represents the amino acid sequence of the purified horse dander allergen Equ c 15k.

#### **EXAMPLE 7: Assessment of IgE binding activity of nEqu c 1, nEqu c 2, nEqu c 3, nEqu c 4/5 and Equ c 15k in a cohort of horse allergic patients**

Sera from 25 horse allergic subjects from Spain (n=20) and Sweden (n=5) were used in the study. All patients had a doctors' diagnosis of horse allergy with symptoms such as asthma, rhinoconjunctivitis and urticaria, and a positive skin prick test to horse dander extract. All samples and clinical data were collected under the approval of the local ethics committee at each center contributing to the biobank in which the samples and data had been deposited.

The levels of specific IgE antibodies to horse dander extract, nEqu c 1, nEqu c 2, nEqu c 3 and nEqu c 4/5 and rEqu c 15k among the 25 horse allergic subjects

were determined using ImmunoCAP™ (Fig. 8, Table 3). In Table 3, all ImmunoCAP™ levels are displayed as kU<sub>A</sub>/L and the origin of each patient is indicated by ES (Spain) or SE (Sweden). Recorded allergic symptoms on exposure to horse are rhinitis (rhin), asthma (astm), urticaria (urt) or anaphylaxis (anaph).

Of the 25 sera tested, 12 (48%) showed an IgE response  $\geq 0.35$  kU<sub>A</sub>/L to rEqu c 15k, 16 (64%) to nEqu c 2 and 19 (76%) to nEqu c 1. Both nEqu c 3 and nEqu c 4/5 appeared as minor allergens among the subjects studied, binding IgE ab from only 5 (20%) and 7 (28%) of the tested sera, respectively. Four of the 25 sera (16%) reacted exclusively to Equ c 15k. On average among all Equ c 15k-reactive sera, the concentration of IgE antibody to Equ c 15k amounted to 37% of that to horse dander. The corresponding relative concentration of IgE antibody to nEqu c 1 was 52%, whereas for nEqu c 2, nEqu c 3 and nEqu c 4/5 the relative concentrations were 35%, 69% and 9%, respectively, among sera specifically reactive to those allergens. Twenty-four of the 25 sera showed IgE antibody binding to horse dander extract. All of those sera showed binding to at least one of the five individual horse allergens tested. The sum of the IgE binding levels to the individual component matched or exceeded that to horse dander extract.

**EXAMPLE 8: Independent sensitisation to Equ c 15k and secretoglobin from cat, the major cat allergen Fel d 1**

Since Equ c 15k belongs to the secretoglobin protein family, the immunological relationship to the major cat allergen, Fel d 1, which belongs to the same protein family, was investigated. The levels of IgE binding to Fel d 1 was evaluated in sera of 36 horse dander sensitized subjects, including those 25 horse allergic patients described in Example 7. No significant correlation ( $r=0.36$ ) between the IgE levels to recombinant Equ c 15 and rFel d 1 could be detected (Fig. 9), suggesting that the IgE antibody response to Equ c 15k was predominantly not a result of cross-reactivity between Equ c 15k and Fel d 1, and vice versa.

In order to further investigate potential cross reactivity between Equ c 15k and Fel d 1, eight sera displaying significant IgE antibody binding reactivity to both Fel d 1 and Equ c 15k were tested for cross-inhibition, using both rEqu c 15k and rFel d 1 on solid phase as well as nEqu c 15k and rFel d 1 as inhibitors at a final concentration of 100 µg/ml (Fig. 10). As an inhibition control, IgE diluent (Phadia) was used. Means of duplicate determinations of each inhibition were calculated and the fraction of inhibition was calculated as the fraction of the binding using diluent

inhibitor that could be quenched with each inhibitor. In these selected sera, inhibition by Fel d 1 could only be achieved when binding to Fel d 1 on the solid phase. Likewise, inhibition using Equ c 15k was only possible on Equ c 15k on the solid phase indicating that in these subjects sensitisation to these two molecules occurred independently of one another and was not a result of cross reactivity. However, the presence of weak cross reactivity between the two proteins cannot be ruled out completely.

Table 1

No	horse dander	nEqu c 1 A	nEqu c 4/5	nEqu c 2	Component sum	component coverage (%)
A1	44,25	4,39	0,18	0,63	5,21	12%
A2	17,08	7,96	0,83	1,40	10,20	60%
A3	136,97	58,75	14,99	30,51	104,24	76%
A4	11,57	1,82	0,14	0,13	2,09	18%
A5	10,43	6,47	0,20	0,54	7,21	69%
A6	12,86	2,34	0,12	4,96	7,42	58%
A7	9,19	5,60	1,15	1,12	7,87	86%
A8	7,26	3,37	0,17	2,07	5,60	77%
A9	11,34	4,69	1,11	1,90	7,70	68%
A10	33,75	3,65	0,26	20,01	23,92	71%
A11	7,39	1,09	5,34	0,85	7,28	99%
A12	42,79	26,87	1,06	6,34	34,27	80%
A13	0,45	0,06	0,07	0,26	0,39	87%
A14	36,70	13,32	4,25	9,41	26,98	74%
A15	16,94	3,73	0,11	2,78	6,63	39%
A16	1,01	3,05	0,07	0,10	3,22	319%
A17	4,97	1,36	0,42	2,03	3,81	77%
A18	13,40	6,10	0,15	0,91	7,16	53%
A19	7,14	2,36	0,08	0,10	2,54	36%
A20	5,20	0,24	0,50	0,20	0,94	18%
A21	32,75	16,16	0,24	0,87	17,27	53%
A22	13,46	3,49	0,15	0,74	4,38	33%
A23	15,28	5,09	0,16	4,30	9,55	62%
A24	198,77	130,15	94,19	87,00	311,33	157%
A25	42,70	11,66	7,05	13,30	32,01	75%
A26	6,55	3,23	0,36	1,77	5,36	82%
A27	27,61	8,55	0,24	5,86	14,65	53%
A28	8,43	4,85	0,14	0,13	5,11	61%
A29	27,12	12,81	0,67	3,29	16,77	62%

Table 2

No	Fraction A	Fraction B	Fraction C	Horse dander	Component sum
A1	0,16	3,54	17,82	<b>44,25</b>	5,21
A4	0,10	1,28	1,09	<b>11,57</b>	2,09
A6	0,00	4,94	6,80	<b>12,86</b>	7,42
A15	0,25	2,64	3,73	<b>16,94</b>	6,63
A18	0,06	4,01	6,06	<b>13,40</b>	7,16
A19	0,05	1,08	1,97	<b>7,14</b>	2,54
A20	0,00	1,93	1,67	<b>5,20</b>	0,94
A21	0,09	21,23	25,99	<b>32,75</b>	17,27
A22	0,05	8,45	11,75	<b>13,46</b>	4,38
A27	0,50	14,11	15,39	<b>27,61</b>	14,65

Table 3

Patient no	Symptoms	Country	e3	nEqu c 1	nEqu c 2	nEqu c 3	nEqu c 4/5	nEqu c 4/5	rEqu c 15k
1	Rhin	SE	1,55	0,06	0,21	0,12	0,30	0,30	1,31
2	Rhin, astm	SE	1,28	1,24	0,56	0,00	0,16	0,04	0,03
3	Rhin	ES	4,79	1,42	0,13	0,00	0,04	0,04	1,89
4	Rhin, astm	ES	5,87	4,96	2,32	0,07	0,53	0,53	0,16
5	Rhin, astm	ES	1,79	1,28	0,26	0,01	0,15	0,02	0,04
6	Rhin, astm	ES	8,74	5,41	5,56	0,00	0,34	0,02	0,02
7	Rhin, astm	ES	0,21	0,00	0,02	0,00	0,02	0,02	0,20
8	Rhin, astm	ES	4,55	1,41	2,02	0,00	0,53	0,53	0,86
9	Rhin	ES	0,55	0,00	0,01	0,00	0,02	0,02	0,63
10	Rhin, astm, urt, anaph	ES	17,31	6,20	2,67	11,90	3,11	3,11	6,07
11	Rhin	ES	16,62	1,30	15,15	5,04	0,86	0,86	0,10
12	Rhin, urt	ES	13,49	2,91	1,19	0,03	0,30	0,30	12,96
13	Rhin, astm, urt	SE	26,19	11,04	7,48	0,05	2,94	2,94	5,68
14	Rhin	SE	6,58	3,42	1,08	0,01	0,48	0,48	1,16
15	Rhin, astm	SE	7,01	0,03	0,04	0,04	0,20	0,20	7,45
16	Rhin	ES	6,78	5,77	0,95	9,43	0,28	0,28	0,02
17	Rhin	ES	28,73	21,92	5,89	33,75	1,19	1,19	0,24
18	Rhin, urt	ES	13,81	5,44	14,10	0,05	0,13	0,13	0,07
19	Rhin, astm	ES	5,18	0,06	0,08	0,06	0,18	0,18	5,81
20	Rhin, astm	ES	0,78	0,76	0,09	0,01	0,02	0,02	0,02
21	Rhin, astm	ES	1,96	1,63	0,58	0,01	0,16	0,16	0,04
22	Rhin, urt	ES	1,28	0,24	1,75	0,01	0,02	0,02	0,06
23	Rhin, astm	ES	6,94	2,49	0,30	0,00	0,10	0,10	1,36
24	Rhin, astm	ES	3,18	1,46	1,31	0,00	0,13	0,13	0,60
25	Rhin	ES	7,78	5,46	1,32	4,61	0,08	0,08	0,03

SEQUENCE LISTINGSEQ ID NO: 1

5 ATCPAVATDIASFFLLPDSL

SEQ ID NO: 2

GSGCQLLEDVVEKTITAELS

10 SEQ ID NO: 3

QCINEISAGDRYIITETL GK

SEQ ID NO: 415 ATCPAVATDIASFFLLPDSLFKLQLI KYQAPPEAKDATMQVKQCINEIS  
AGDRYIITETL GKIVLQCGASEQ ID NO: 520 GSGCQLLEDVVEKTITAELSPA EYVEAVQEFIPDEATEKAAIQLKQCYLKQSNETLND FRTM  
MNSMYNSAYCALF

20

SEQ ID NO: 6MRLFLPVLLVTLALCCETNAATCPAVATDIASFFLLPDSLFKLQLI KYQAPPEAKDATMQV  
KQCINEISAGDRYIITETL GKIVLQCGA25 SEQ ID NO: 7MKLTVLMLVAFPLYCYAGSGCQLLEDVVEKTITAELSPA EYVEAVQEFIPDEATEKAAIQL  
KQCYLKQSNETLND FRTMMNSMYNSAYCALFSEQ ID NO: 830 MATCPAVATD IASFFLLPDS LFKLQLI KYQ APPEAKDATM QVKQCINEIS 50  
AGDRYIITET LGKIVLQCGA GGGGSGGGGS GGGGSGSGCQ LLEDVVEKTI 100  
TAEELSPA EYV EAVQEFIPDE ATEKAAIQLK QCYLKQSNET LND FRTMMNS 150  
MYNSAYCALF LEHHHHHH 16835 SEQ ID NO: 9**ATG**GCCACGTGCCCTGCAGTCGCTACGGACATCGCATCGTCTTCTTGCTGCC  
GGACAGCCTGTTAAGCTGCAACTGATCAAATATCAGGCTCCGCCGGAGGCCAAGACGC



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20 Valenta, R. and V. Niederberger (2007). "Recombinant allergens for immunotherapy." *J Allergy Clin Immunol* **119**(4): 826-830.

Valenta, R., T. Twaroch, et al. (2007). "Component-resolved diagnosis to optimize allergen-specific immunotherapy in the Mediterranean area." *J Investig Allergol Clin Immunol* **17 Suppl 1**: 36-40.

25 van Eijk, H. M., D. R. Rooyakkers, et al. (1999). "Automated isolation of high-purity plasma albumin for isotope ratio measurements." *Journal of Chromatography. B, Biomedical Sciences and Applications* **731**(2): 199-205.

**Claims**

1. An isolated horse allergen which is a secretoglobin having a molecular weight of 15 kDa under non-reducing conditions and comprising a first peptide chain having a molecular weight of 5 kD and a second peptide chain having a molecular weight of 10 kDa linked together, and variants and fragments thereof sharing epitopes for antibodies therewith.  
5
2. The horse allergen according to claim 1 which is purified from horse or recombinantly produced.  
10
3. The horse allergen according to claim 1 or 2, wherein the first peptide chain comprises an N-terminal amino acid sequence ATCPAVATDIASFLLPDSL (SEQ ID NO: 1), and variants and fragments thereof sharing epitopes for antibodies therewith.  
15
4. The horse allergen according to claim 1, 2 or 3, wherein the second peptide chain comprises an N-terminal amino acid sequence GSGCQLLEDVVEKTITAELS (SEQ ID NO: 2), and variants and fragments thereof sharing epitopes for antibodies therewith.  
20
5. The horse allergen according to any one of claims 1 to 4, wherein the first peptide chain comprises a segment having the amino acid sequence QCINEISAGDRYIITETL GK (SEQ ID NO: 3), and variants and fragments thereof sharing epitopes for antibodies therewith.  
25
6. The horse allergen according to any one of claims 1 to 5, wherein the first peptide segment comprises the amino acid sequence ATCPAVATDIASFLLPDSL FK LQLIKYQAPPEAKDATMQVKQCINEIS AGDRYIITETL GKIVLQCGA (SEQ ID NO: 4), and variants and fragments thereof sharing epitopes for antibodies therewith.  
30
7. The horse allergen according to any one of claims 1 to 5, wherein the second peptide segment comprises the amino acid sequence

GSGCQLLEDVVEKTITAELSPAELYVEAVQEFIPDEATEKAAIQLKQCYLKQSNET  
LNDFRTMMNSMYNSAYCALF (SEQ ID NO: 5), and variants and fragments  
thereof sharing epitopes for antibodies therewith.

5 8. An isolated nucleic acid sequence encoding the horse allergen according to  
any one of claims 1 to 7.

9. A vector comprising the nucleic acid molecule according to claim 8.

10 10. A host cell comprising the vector according to claim 9.

11. A horse allergen according to any one of claims 1 to 7 for use in *in vitro*  
diagnosis of Type 1 allergy.

15 12. Use of a horse allergen according to any one of claims 1 to 7 for the  
manufacture of a diagnostic composition for the *in vitro* diagnosis of Type 1  
allergy.

20 13. A method for producing an allergen composition, comprising the step of  
adding a horse allergen according to any one of claims 1 to 7 to a  
composition comprising an allergen extract and/or at least one purified  
allergen component.

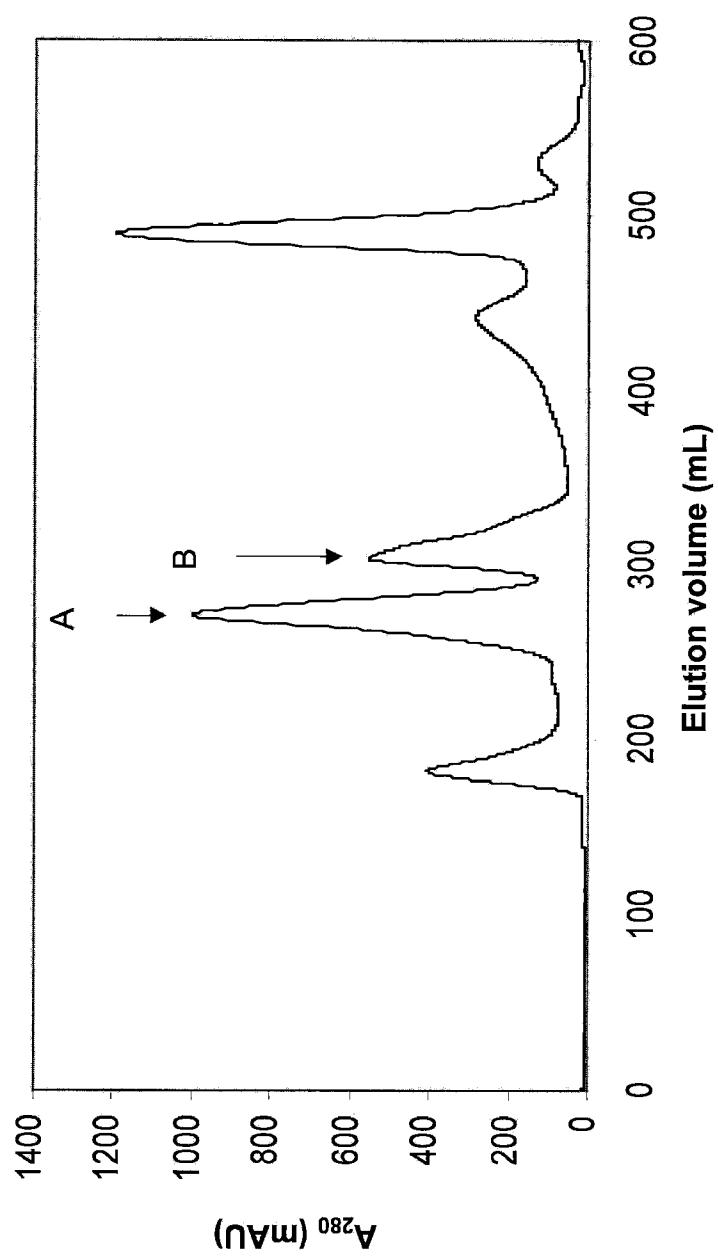
25 14. An allergen composition obtainable by the method according to claim 13.

15. A method for *in vitro* diagnosis of type 1 allergy comprising the steps of  
contacting an immunoglobulin-containing body fluid sample from a patient  
suspected of having Type 1 allergy with a horse allergen according to any  
one of claims 1 to 7, or with an allergen composition according to claim 14,  
30 and  
detecting the presence, in the sample, of IgE antibodies specifically binding  
to the horse allergen,  
wherein the presence of such IgE antibodies specifically binding to the  
horse allergen is indicative of Type 1 allergy.

16. A diagnostic kit for performing the method according to claim 15, comprising a horse allergen according to any one of claims 1 to 7 or a composition according to claim 14.
- 5 17. A horse allergen according to any one of claims 1 to 7 for use in prophylactic or therapeutic treatment of Type 1 allergy.
- 10 18. Use of a horse allergen according to any one of claims 1 to 7 for the manufacture of a therapeutic composition for prophylactic or therapeutic treatment of Type 1 allergy.
- 15 19. A method for treatment of Type 1 allergy, comprising administering, to an individual susceptible to such treatment, a horse allergen according to any one of claims 1 to 7, or a form of the horse allergen that is modified to abrogate or attenuate its IgE binding response.
20. 20. A pharmaceutical composition comprising a horse allergen according to any one of claims 1 to 7, or a form of the horse allergen that is modified to abrogate or attenuate its IgE binding response, and optionally at least one of pharmaceutically acceptable carriers, excipients, buffers and diluents.

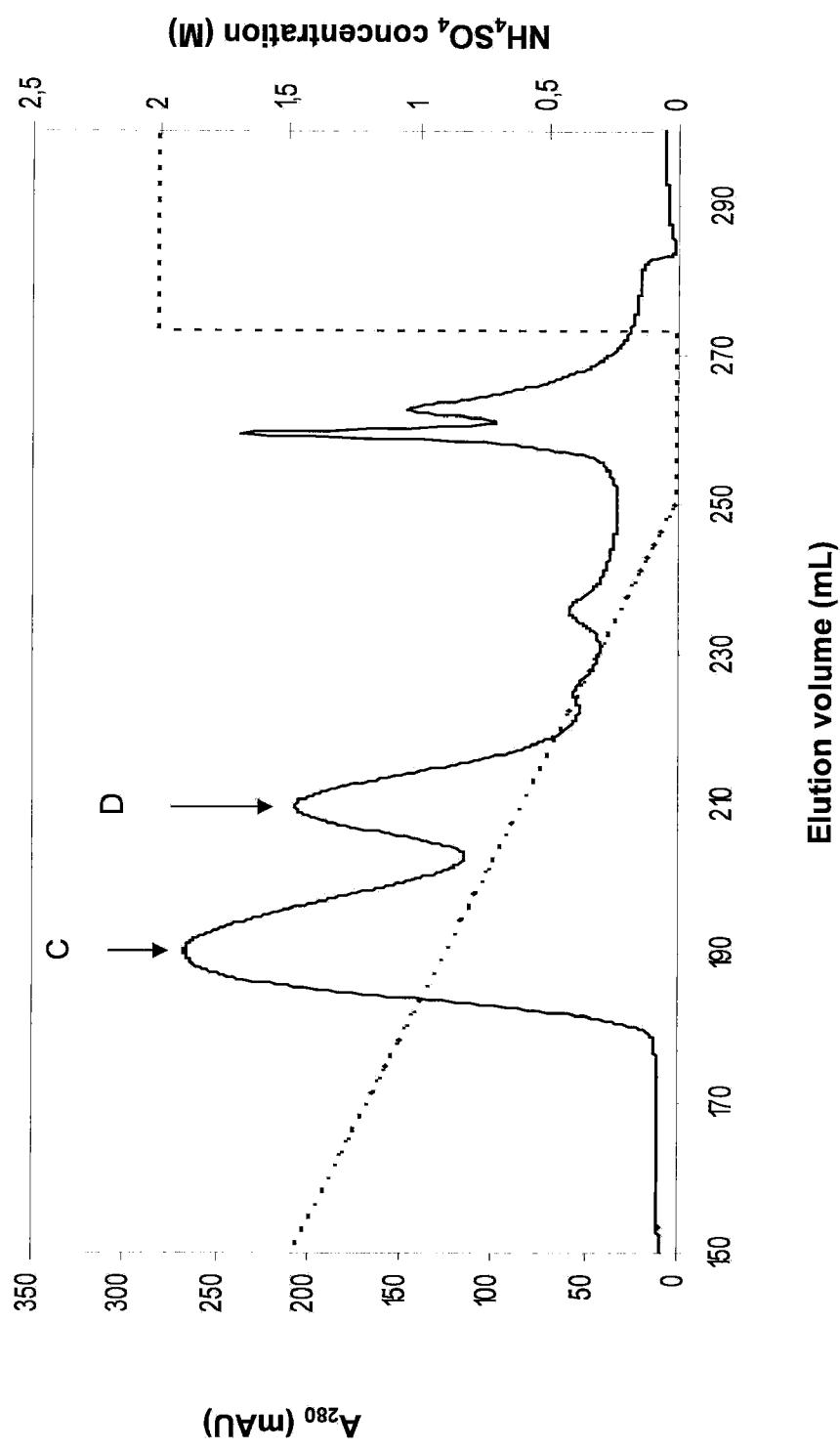
1/20

Fig 1A



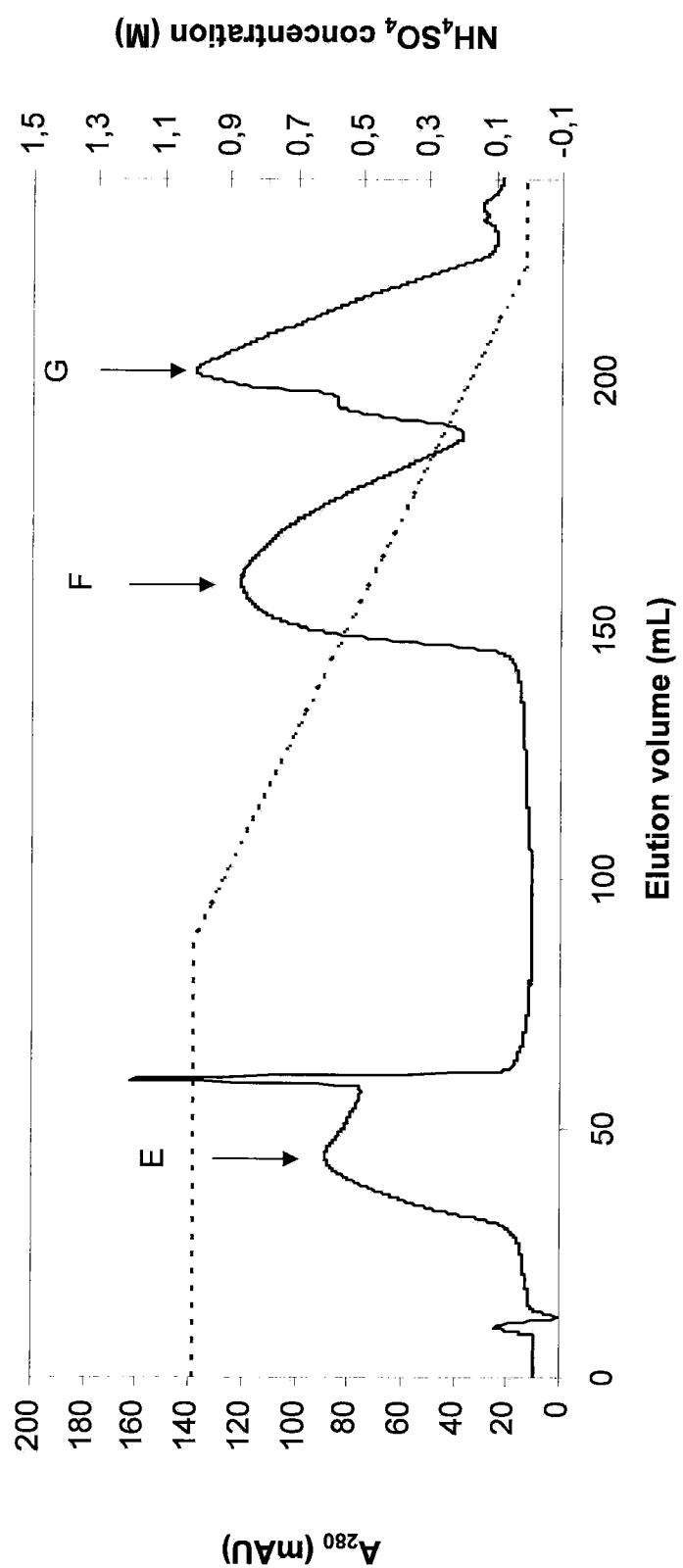
2/20

Fig 1B



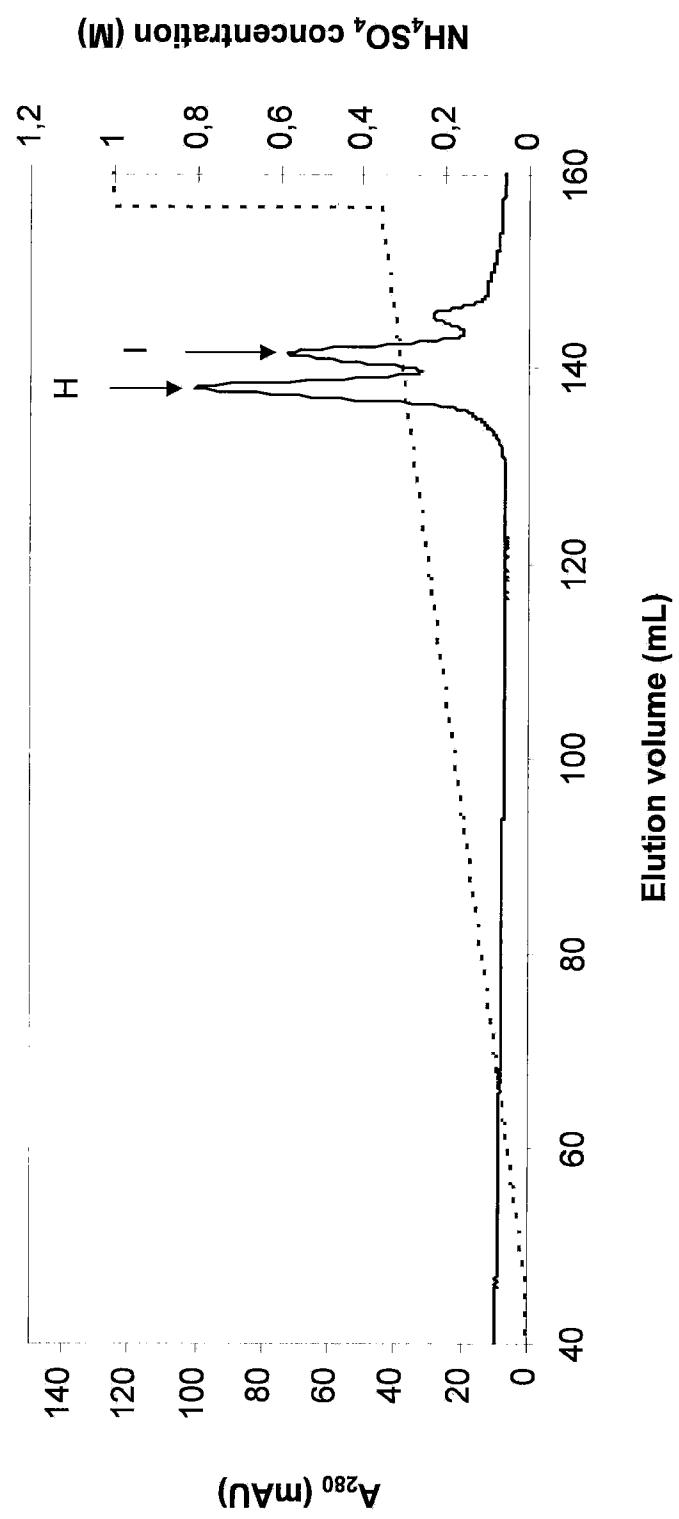
3/20

Fig 1C

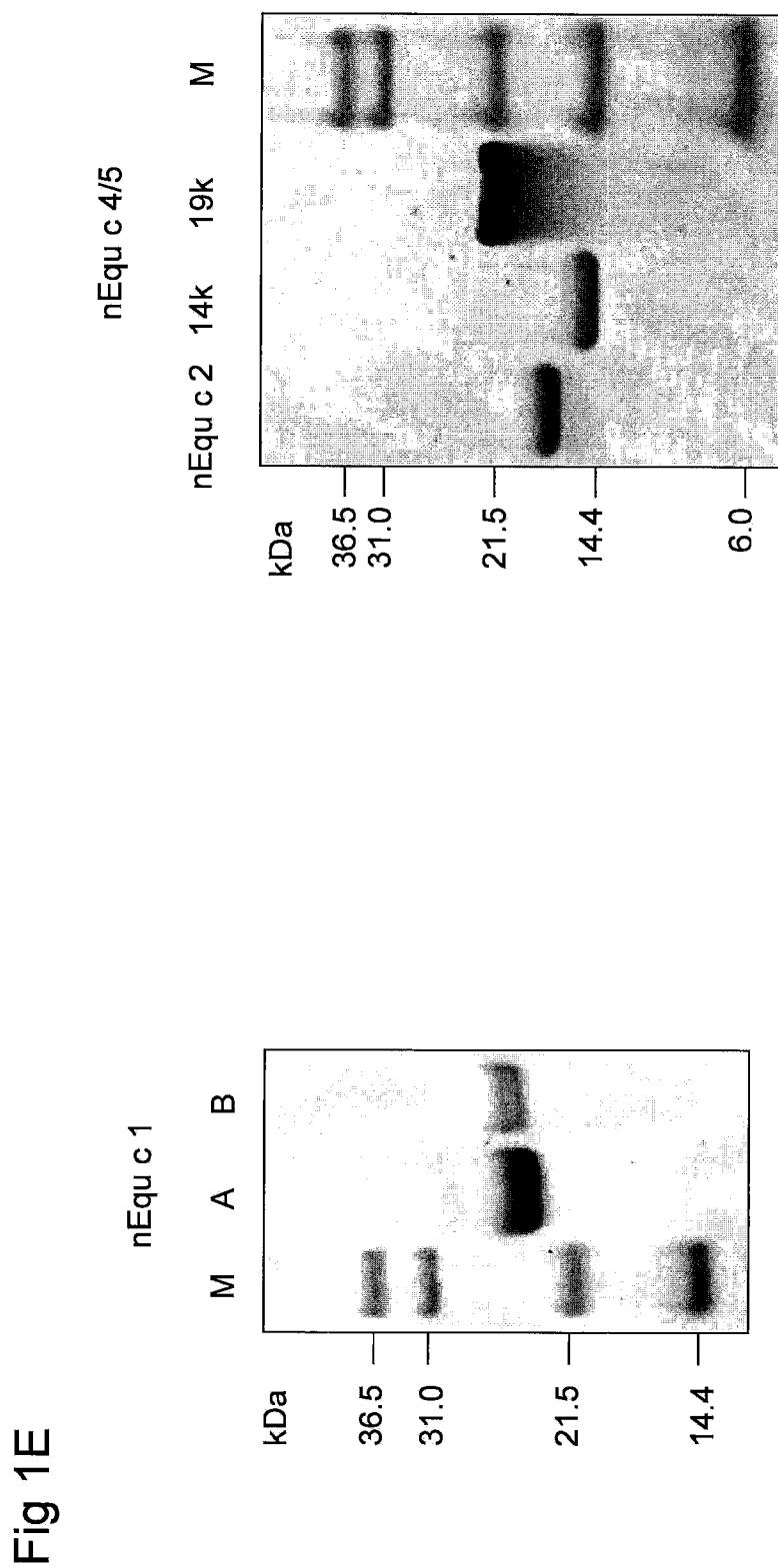


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Fig 1D



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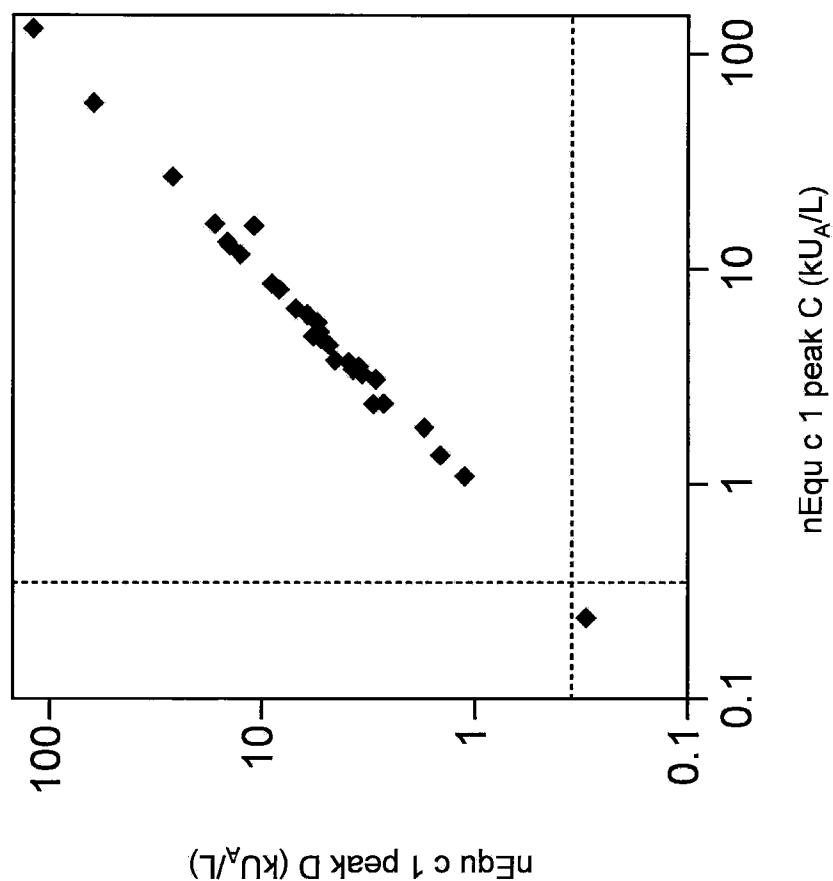


Fig 2A

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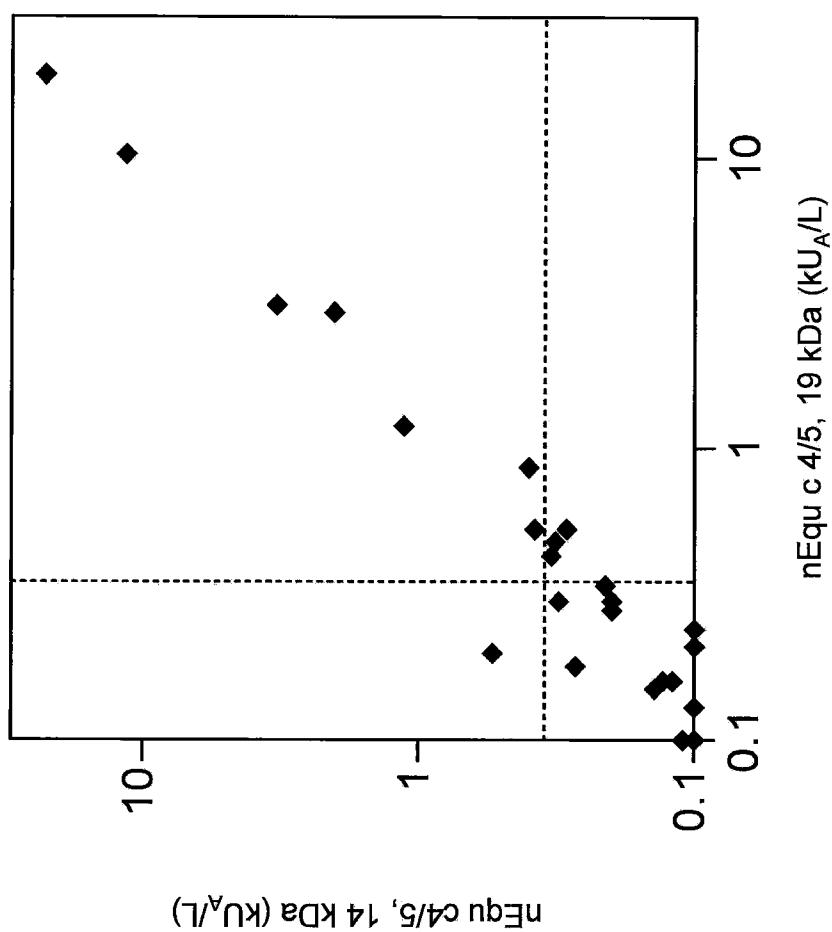
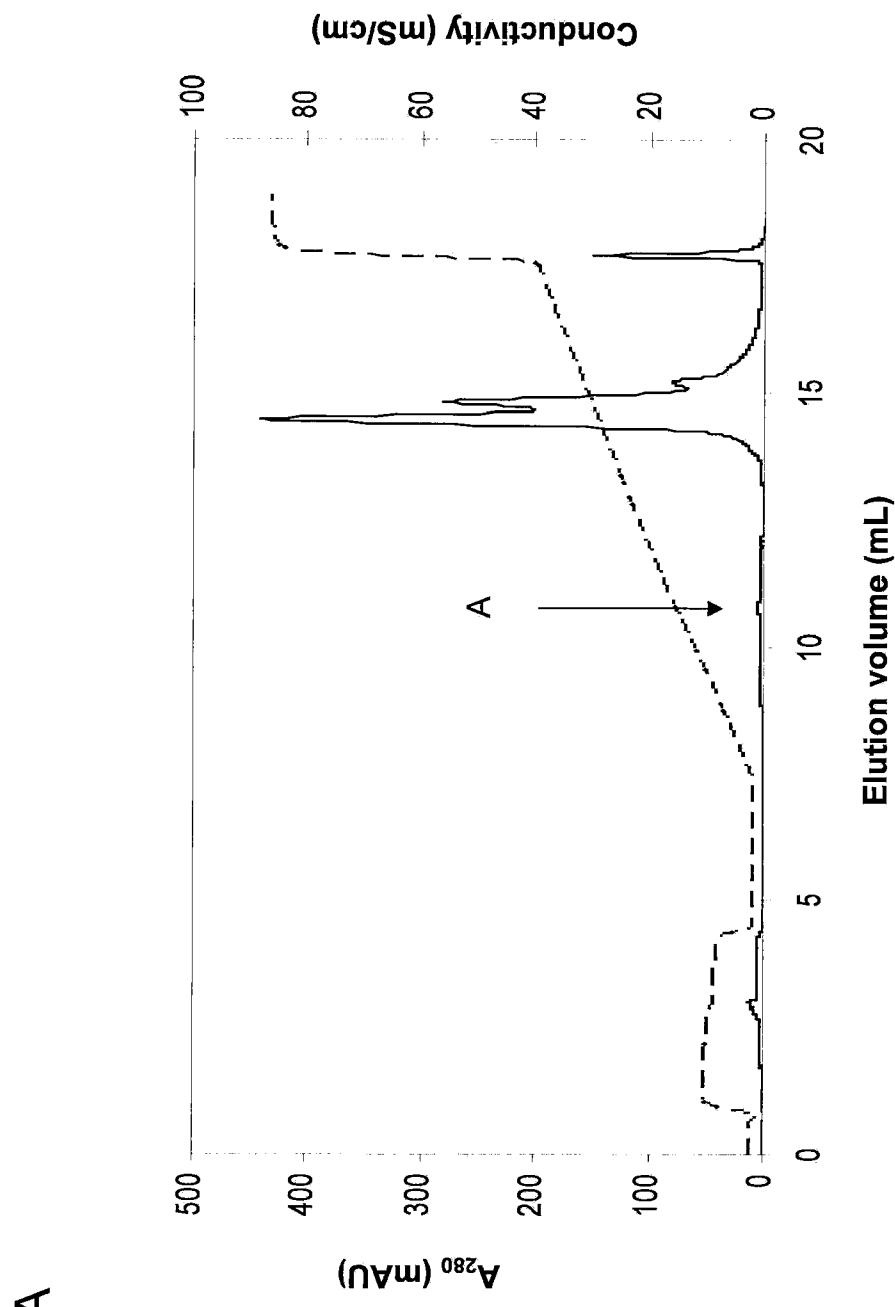


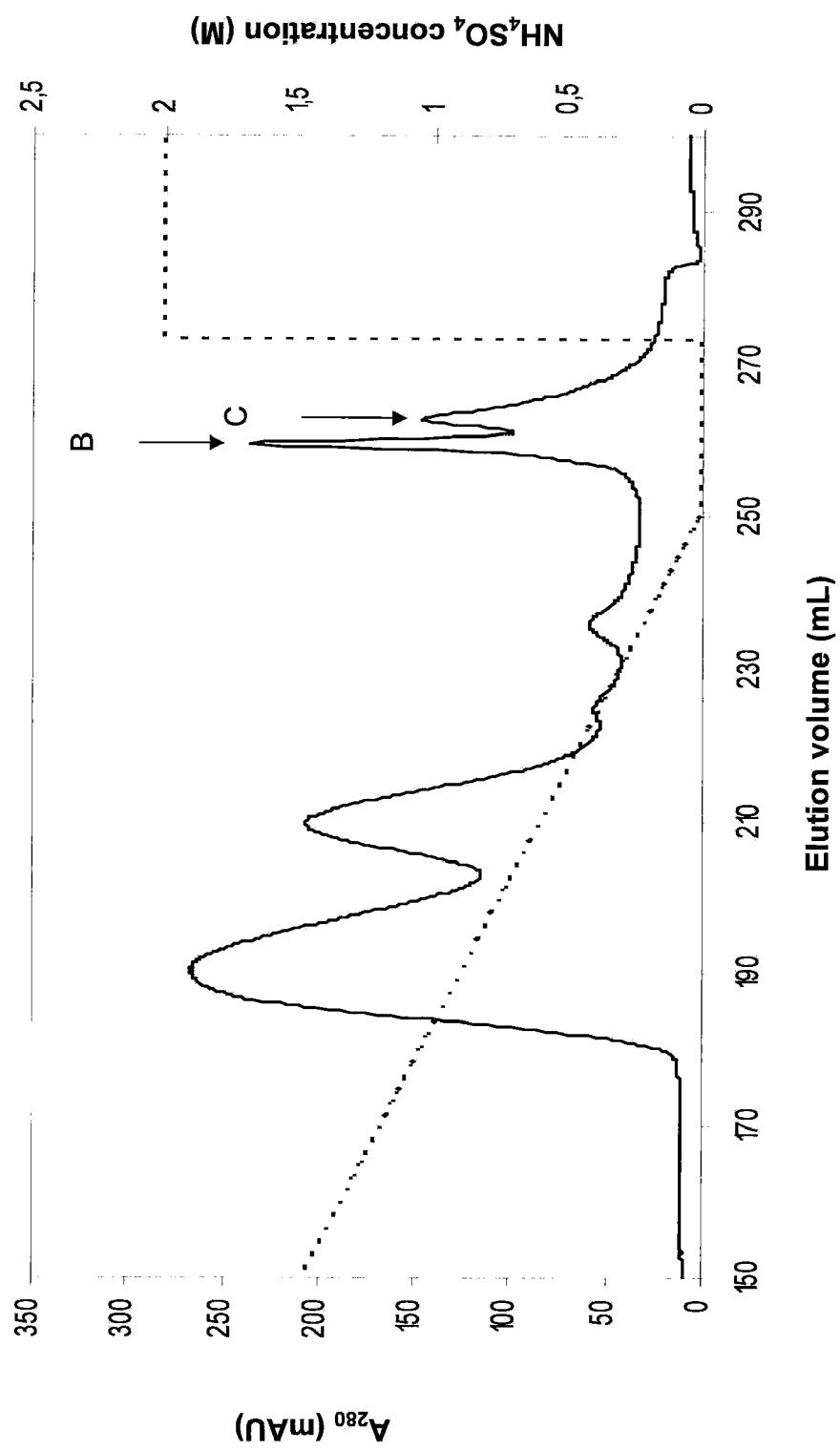
Fig 2B

8/20



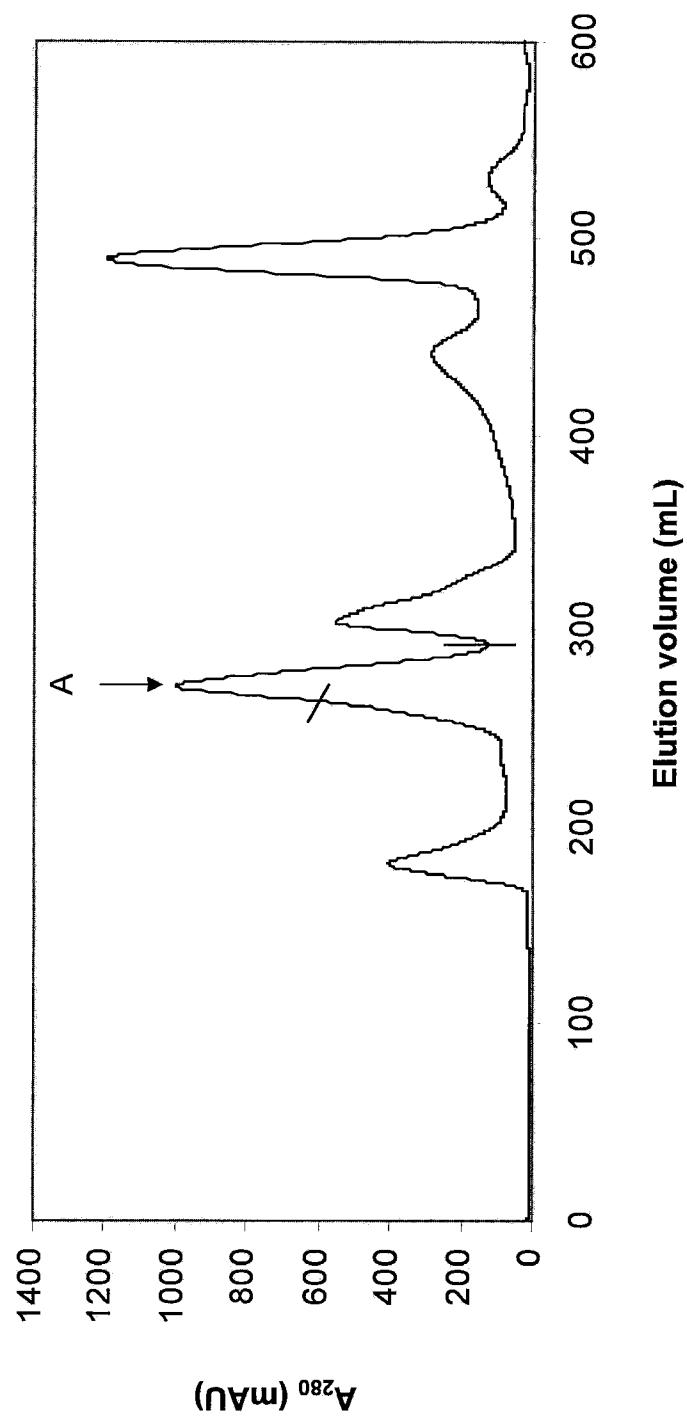
9/20

Fig 3B



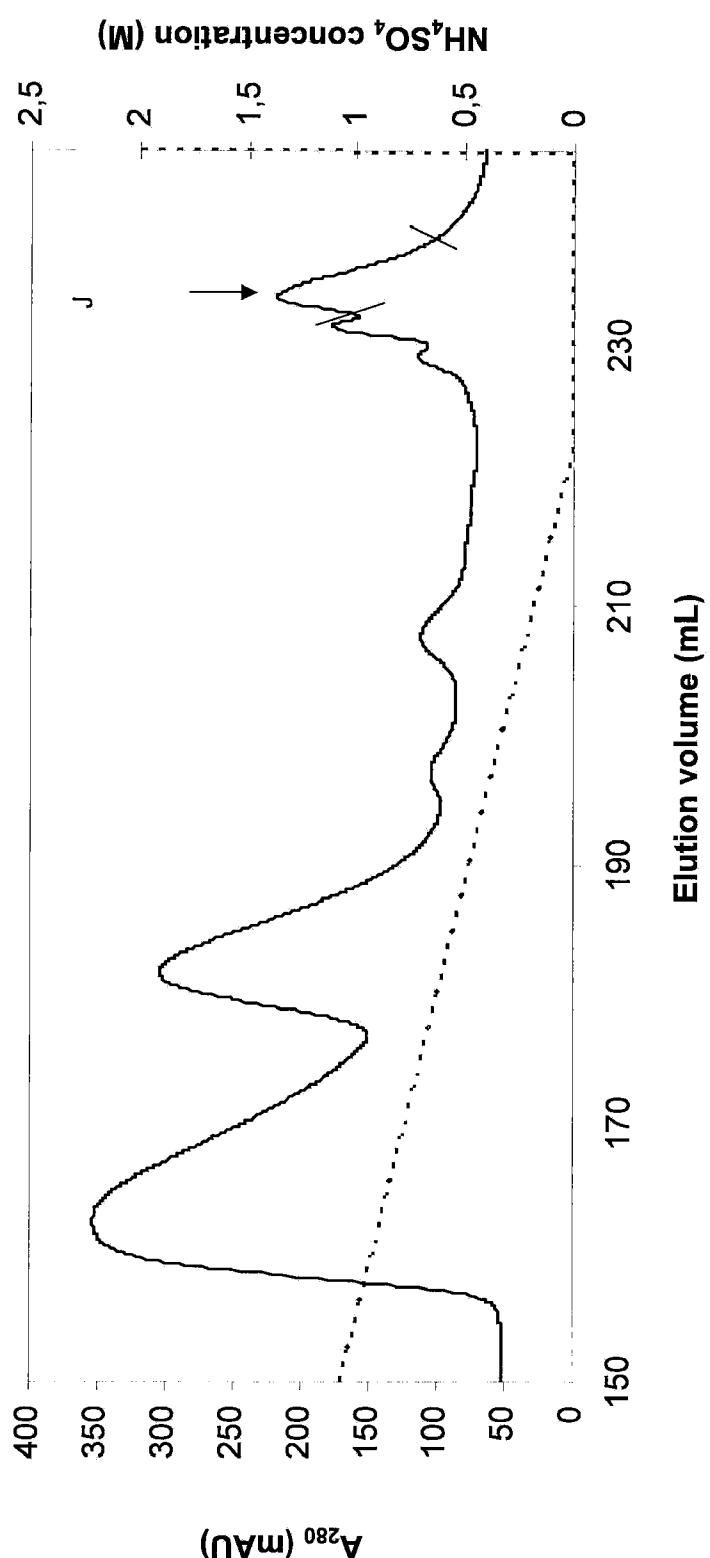
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Fig 4A



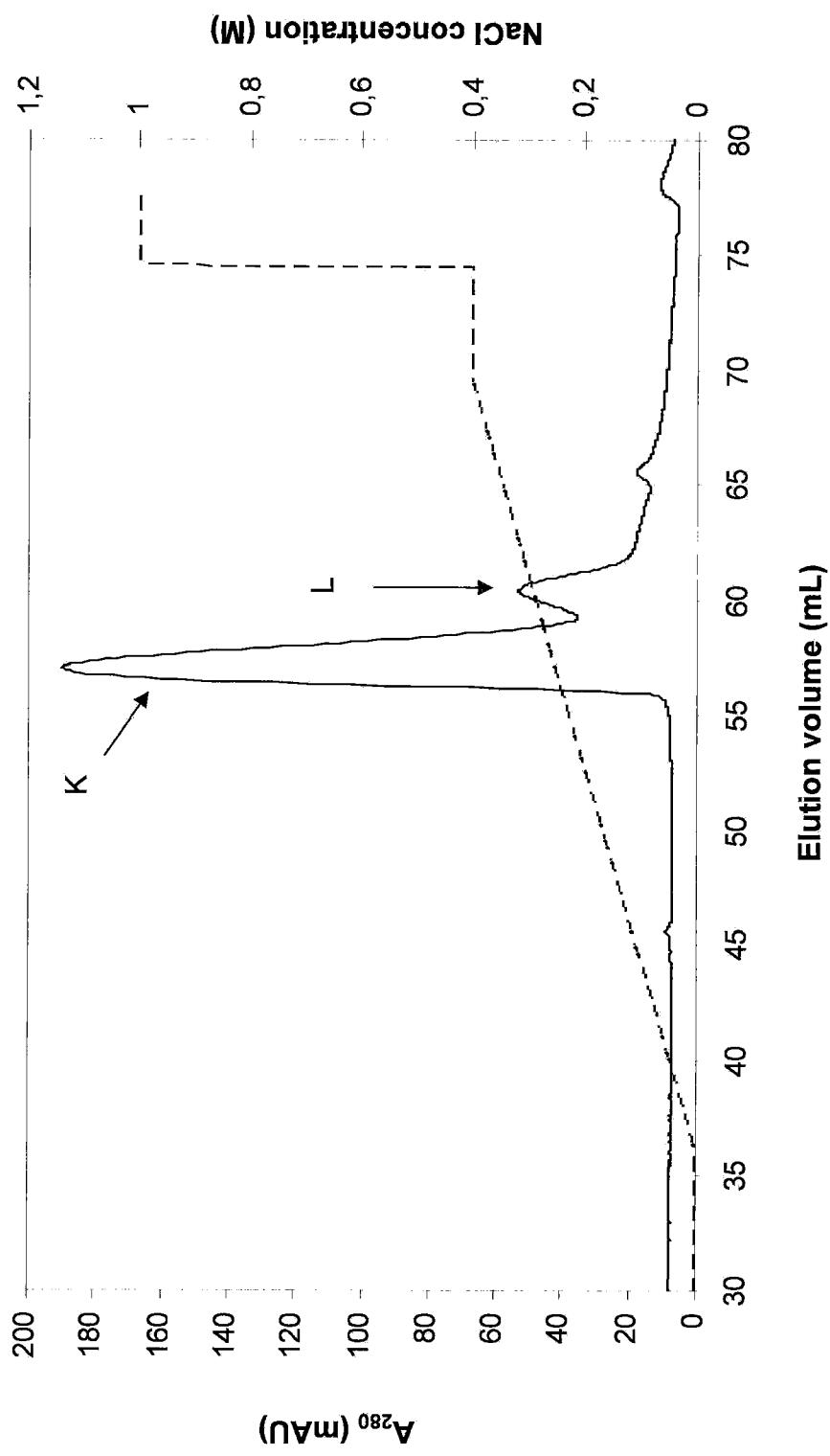
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Fig 4B



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Fig 4C



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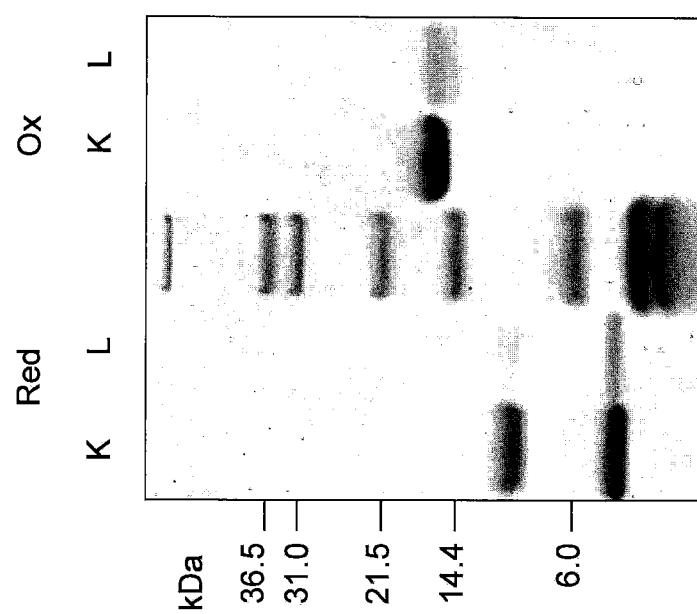
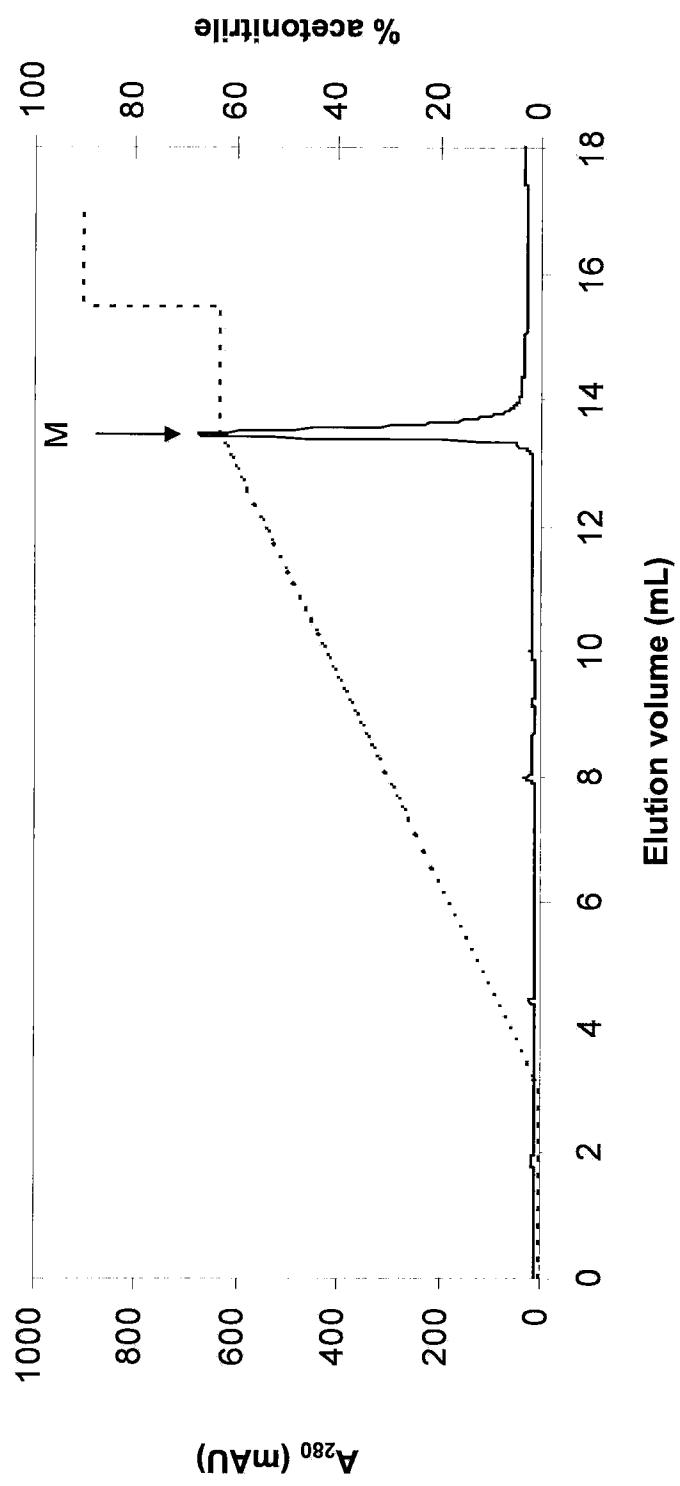


Fig 4D

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Fig 4E



**Fig 5****Equ c 15k - 5 kDa fragment**

Predicted full length precursor sequence:

MRLFLPVILLVTLLALCCCTNAATCPAVATDIASFFLLPDSLFKLQLI**KYQAPPEAKDA**  
TMQVKQCINEISAGDRYIITETL**GKJVLQCGA**

**Equ c 15k - 10 kDa fragment**

Predicted full length precursor sequence:

MKLVTVLMILVAFFPLYCYAGSGCQLEDVVEKTITAELSPAEYVEAVQEFIPDEATEK  
AAIQLKQCYKLQSNETLNDFRMMNSM**YNSAYCALF**

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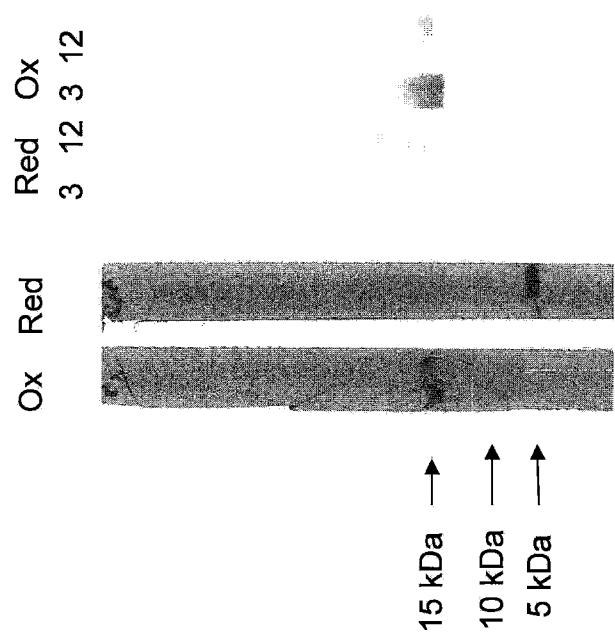


Fig 6

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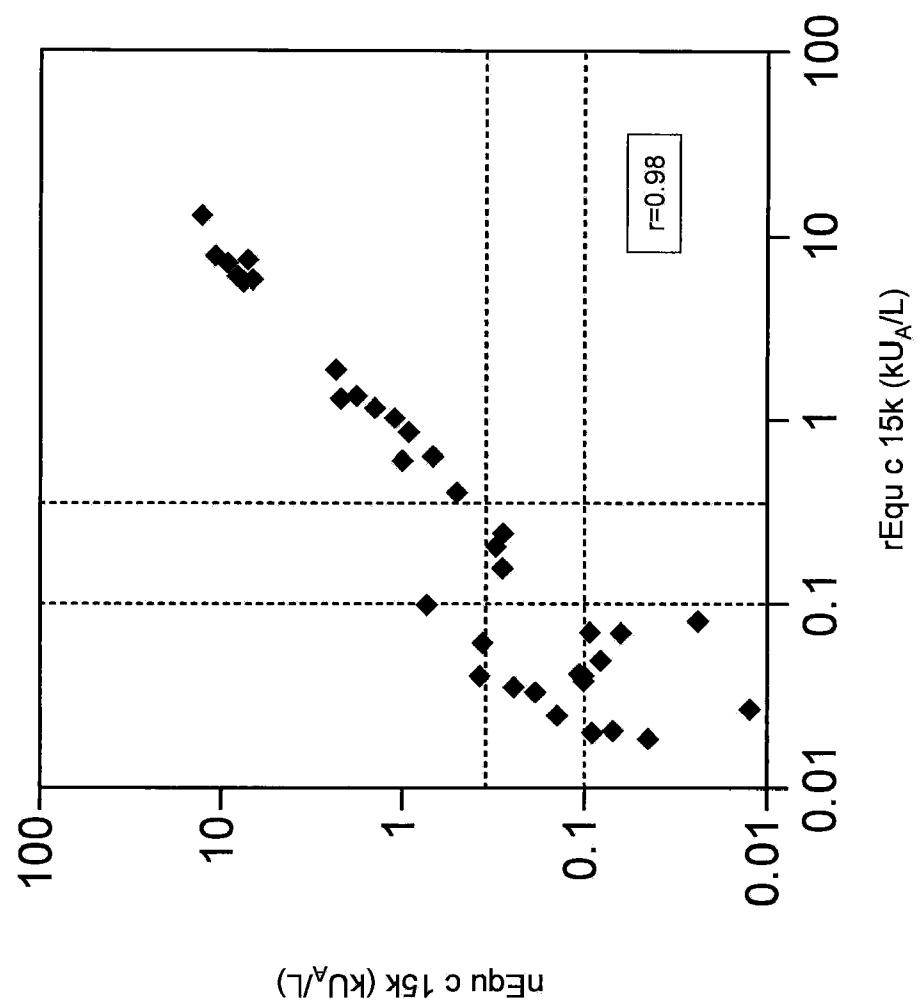
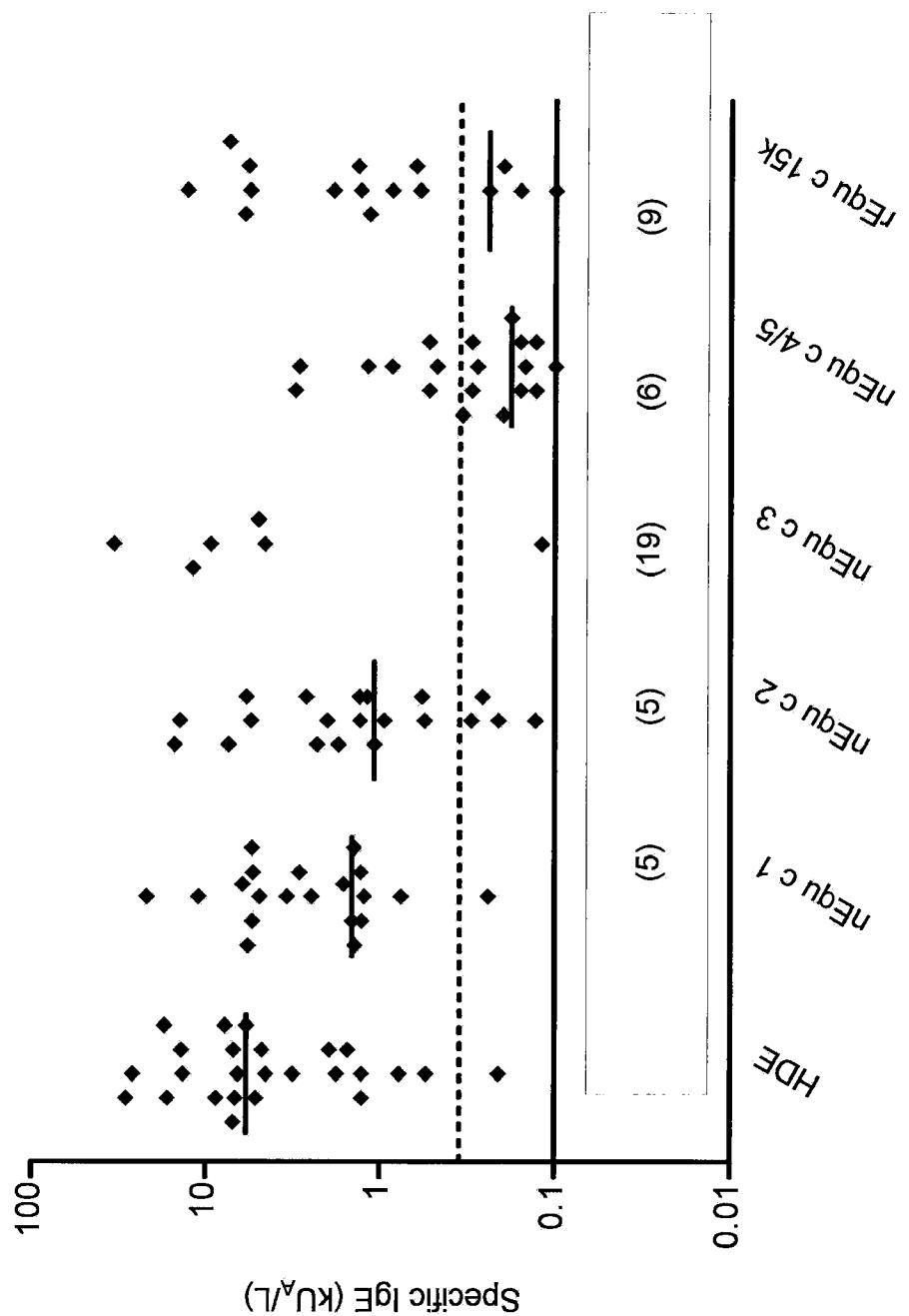


Fig 7

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8  
Fig

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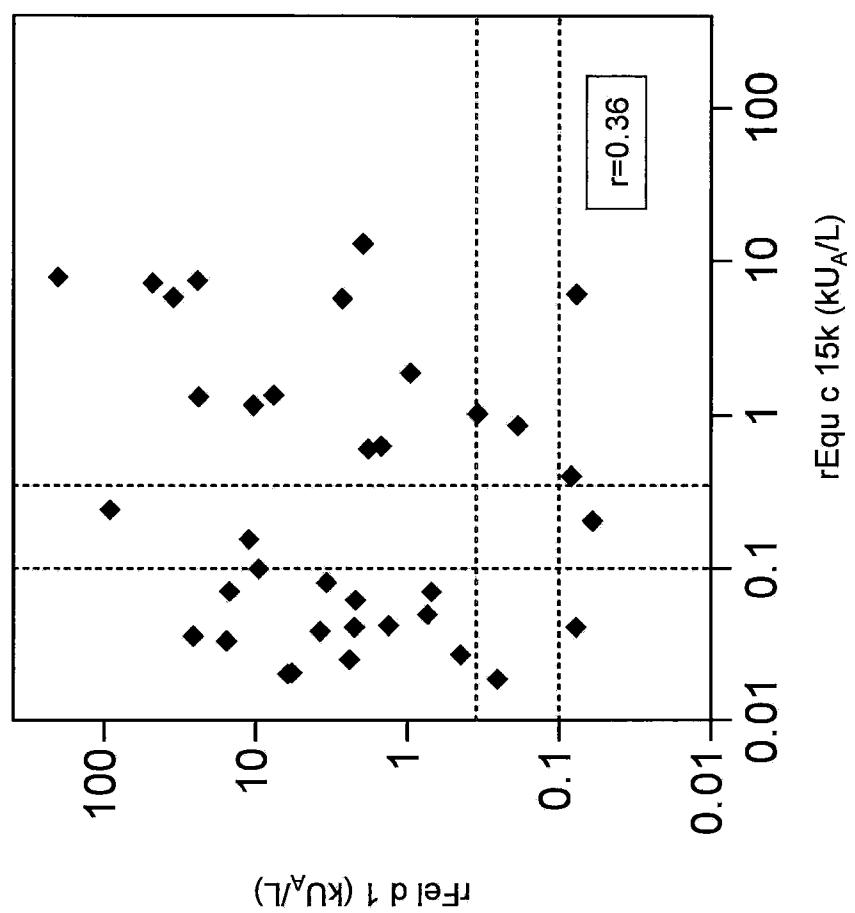
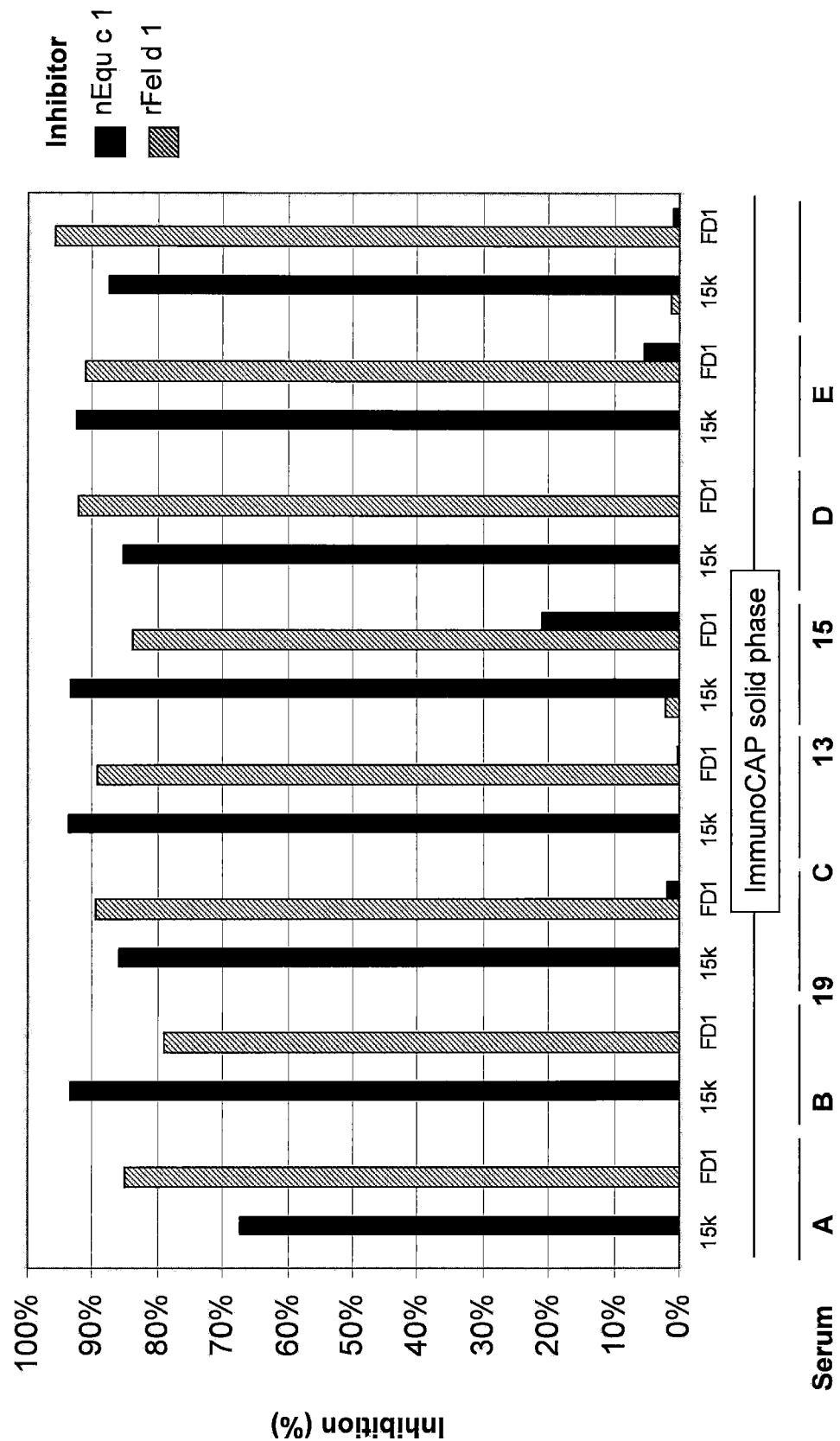


Fig 9

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**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/SE2011/050503

**A. CLASSIFICATION OF SUBJECT MATTER**

**IPC: see extra sheet**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

**IPC: A61K, A61P, C07K, G01N**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

**SE, DK, FI, NO classes as above**

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**EPO-Internal, PAJ, WPI data, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE, EBI (sekvenssökn)**

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Kumar, F. 'Allergens of horse dander:Comparison among breeds and individual animals by immunoblotting' In:J Allergy Clin Immunol, 1996, Vol. 98, pp. 169-71; abstract; see page 169, left column, third paragraph; page 169, right column, last paragraph; page 170, right column, second paragraph	1-20
Y	--	15, 17-20
X	Fjeldsgaard B.E. 'Comparison of IgE-binding antigens in horse dander and a mixture of horse hair and skin scrapings' In:Allergy, 1993, Vol. 48, pp. 535-541; abstract; see page 537, right column, paragraphs 2-3; table 2 and the abstract	1-20
Y	--	15, 17-20



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance

“E” earlier application or patent but published on or after the international filing date

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

02-07-2011

Date of mailing of the international search report

19-07-2011

Name and mailing address of the ISA/SE

Patent- och registreringsverket

Box 5055

S-102 42 STOCKHOLM

Facsimile No. + 46 8 666 02 86

Authorized officer

**Carolina Palmcrantz**

Telephone No. + 46 8 782 25 00

**INTERNATIONAL SEARCH REPORT**International application No.  
PCT/SE2011/050503**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: **19**  
because they relate to subject matter not required to be searched by this Authority, namely:  

Claim 19 relates to a method for treatment of the human or animal body by therapy, see PCT rule 39.1(iv). Nevertheless, a search has been made for this claim. The search has been directed to the technical content of the claim.
2.  Claims Nos.: **1-20 (partly)**  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  

Present claims 1-20 relate, in respect of the wording "variants thereof sharing ...../....
3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

**Continuation of:** Box No. II

epitopes for antibodies therewith", to variants defined by reference to a desirable characteristic or property. The claims cover all variants having this characteristic or property, whereas the application provides no support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for any such variants. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the variants by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed. These are the characteristics of the allergen which has been isolated in the application, i.e. the molecular weight, that it is a secretoglobin and the sequences given in claims 3-7. The search has also been directed to the isolation/identification of horse allergens in general, but no specific allergens, except for the one which has been isolated in the application, have been searched.

Thence it follows that a reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability only can be established for those parts which have been searched.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/SE2011/050503

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Valenta R. 'The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT)' In: Clinical and Experimental Allergy, 1999, Vol. 29, pp. 896-904; abstract; see page 897, left column, first paragraph and the abstract --	15, 17-20
X	Bulone V. 'Separation of horse dander allergen proteins by two-dimensional electrophoresis Molecular characterisation and identification of Equ c 2.0101 and Equ c 2.0102 as lipocalin proteins' In: Eur J Biochem, 1998, Vol. 253, pp. 202-211; abstract; see page 208 "Discussion-page 209, first paragraph --	1-20
X	Bulone V. et al., "Characterisation of horse dander allergen glycoproteins using amino acid and glycan structure analyses", Int Arch Allergy Immunol., 2000, Vol. 123, pp. 220-227; see page 221, left column, last paragraph and page 226, left column, second paragraph --	1-20
A	Gregoire C. 'cDNA Cloning and Sequencing Reveal the Major Horse Allergen Equ c1 to Be a Glycoprotein Member of the Lipocalin Superfamily' In: The Journal of Biological Chemistry, 1996, Vol. 271, No. 51, pp. 32951-32959; abstract; see the abstract --	1-20
A	Lidholm J. 'The importance of molecular allergens for in vitro testing: a critical evaluation of component-resolved diagnostics', 2009 (Abstract), Retrieved from: NLM database, PMID 20799456, In: Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frank A M., 2009, Vol. 96, pp. 157-161, discussion 161-163.; abstract --	1-20
A	Virtanen T. et al., "Important animal allergens are lipocalin proteins: why are they allergenic?", Int Arch Allergy Immunol, 1999, Vol. 120, pp. 247-258; see the whole document -----	1-20

**Continuation of:** second sheet

**International Patent Classification (IPC)**

***C07K 14/47*** (2006.01)

***A61K 39/35*** (2006.01)

***A61P 37/08*** (2006.01)

***G01N 33/68*** (2006.01)