

Biochemical characterization and surfactant properties of horse allergens

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A new allergen from horse dander, Equ c 5 has been purified. Its biochemical and biophysical properties have been characterized and compared with those of Equ c 1, Equ c 2 and Equ c 4. Their molecular masses, determined by mass spectrometry, were 22 kDa for Equ c 1, 16 kDa for Equ c 2, 18.7 kDa for Equ c 4 and 16.7 kDa for Equ c 5. Their pI values were between 3.8 and 5.25. Equ c 2 and Equ c 5 are not glycosylated, while Equ c 4 contains a tri-antennary tri-sialylated N-linked glycan. Linkages of terminal *N*-acetylneuraminic acid to galactose were: α -(2→6) in Equ c 4, and both α -(2→3) and α -(2→6) in Equ c 1. Oligosaccharide portions of Equ c 1 or Equ c 4 were barely involved in IgE-immunoreactivity. Partial

N-terminal sequence of Equ c 4 shares a significant sequence homology with the rat submandibular gland protein A. No matching was found for two internal peptides of Equ c 5. Surfactant properties of horse allergens as well as other proteins were investigated. In contrast to Equ c 2 and Equ c 3, solutions of Equ c 1, Equ c 4 and Equ c 5 significantly lowered the surface tension. Relationship between a property such as this, involving oriented hydrophobic patches of a molecule and allergenicity, is addressed.

Keywords: horse allergen; surfactant protein; hydrophobicity; surface tension.

Allergies are among the most common diseases all over the world and consequently diagnostic and therapeutic procedures have been widely developed. These are based on identification and physicochemical characterization of individual allergens. Allergens are grouped in families having significant homology with proteins of known function or a given biological activity, as well as proteins involved in transport or regulatory proteins [1,2].

Horses are considered to be one of the most important and significant sources of mammalian allergens, provoking a hypersensitivity response in genetically predisposed individuals. Inhalation of horse allergens in sensitized individuals leads to the production of inflammatory mediators and type I allergic symptoms. Exposure to horse allergens may occur directly through horse-riding or indirectly

through transport of antigens by means of riding clothes [3–6]. The importance of horse dander as a source of allergens has long been recognized. Comparisons of the composition of allergen extracts from different sources (horse dander, horse hair and skin scraping) and from horses of different breeds revealed the presence of several allergenic proteins, glycosylated or not, mainly acidic proteins (pI ranging between 3.5 and 4.6), with an apparent molecular mass (M_r) range between 10 kDa and 75 kDa [7–19].

The most important horse allergen, from a clinical point of view, is Equ c 1, a glycoprotein with a M_r of 22 kDa, and a pI of 3.9 [20]. Sequence analysis and crystallographic studies of the recombinant form revealed that Equ c 1 is dimeric in solution and is a member of the lipocalin family [21–23], a large group of predominantly extracellular proteins involved in the transport of small hydrophobic molecules [24]. Two other allergens were then described [25], designated as Equ c 2 and Equ c 4 in this report. Moreover, global studies of allergens from horse dander extracts, performed by two-dimensional PAGE identified 16 spots, among which 13 belong to the lipocaline family including two nonglycosylated variants of Equ c 2 and 7 are likely isoforms of Equ c 1 [26,27]. Global studies by two-dimensional gel analysis, although informative, do not allow a precise physico-chemical characterization of the allergen. Structural protein analysis especially requires unlimited amount of allergen in solution. Indeed, the structure of an allergen has been addressed in several reports. Thus, besides genetic predisposition, dose and mode of entry of the allergen, the structure/function of the allergen itself may contribute to its allergenicity. Such structural basis may play a role in skewing the immune

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Abbreviations: DIG, digoxigenin; HR index, hydrophobic retention index; MAA, *maackia amurensis* lectin; PLA2, phospholipase A2; SMG-A, sub mandibular gland protein-A; SNA, *Sambucus nigra* lectin; ST index, surface tension index; RAST, radio allerge sorbent test; RPC, reverse-phase chromatography; SEC, size-exclusion chromatography; TIC, thiophilic interaction chromatography; ECL, enhanced chemiluminescence; IEF, isoelectric focusing.

Note: the partial sequences reported in this paper have been submitted to the Swiss-Prot data bank with accession numbers P82615 (Equ c 4) and P82616 (Equ c 5).

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response in favor of a Th2 dominance or in maintaining a persistent inflammatory status by IgE-independent mechanisms [2,28–31].

In this study, four purified horse dander allergens, Equ c 1, Equ c 2, Equ c 4 and a newly described, Equ c 5 were biochemically and immunochemically compared. Partial amino-acid sequences are also reported for Equ c 4 and Equ c 5. Furthermore, surface activity of various allergens was studied using an original approach by measuring their capacity to lower the surface tension at the liquid–air interface using Wilhelmy balance. The results are discussed in relation to the putative interactions that allergens might have with hydrophobic surfaces such as pulmonary surfactant or membrane phospholipids because of their surfactant-like properties and how such a characteristic could be a contributing factor to their allergenicity in an IgE-independent manner.

EXPERIMENTAL PROCEDURES

Chemicals

HPLC gradient grade acetonitrile, trifluoroacetic acid and Coomassie Brilliant Blue R250, were obtained from Merck (Darmstadt, Germany). Biotinylated goat anti-(human IgE) Ig (ϵ chain specific) was purchased from Vector Laboratories Inc. (Burlingame, CA, USA). Nitrocellulose membrane (0.2 μ m) was obtained from Millipore (MA, USA). Western blotting detection reagent kit (ECL) and hyperfilm MP from Amersham Pharmacia Biotech UK Limited (Buckinghamshire, England). Digoxigenin-labeled lectins from *Sambucus nigra* (SNA) and from *Maackia amurensis* (MAA), as well as DIG glycan detection kits, and *N*-glycosidase F deglycosylation kits were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). *O*-Glycosidase was procured from Roche Diagnostics GmbH (Mannheim, Germany). Phospholipase A₂ (from bee venom), surfactin (from *Bacillus subtilis*), lysozyme, β -lactoglobulin, ananain, glucose oxydase, horse serum albumin, bovine serum albumin, ovalbumin, bovine γ -globulin, pepsin, α -chymotrypsin, gelatin and streptavidin peroxidase were purchased from Sigma (St Louis, MO, USA). Extracts from animal origin were prepared in our laboratory; Fel d 1 as described previously in [32], horse sweat in [25] and horse dander (this report). Cat saliva extract was collected by a veterinary from anaesthetized cats and was centrifuged and filtered before use.

Horse dander extract

The raw material, which is a commercial horse dander extract (batch 204695001) obtained from Allergon AB (Ängelholm, Sweden), was extracted with 20 mM Tris/HCl, pH 8.6 at 4 °C for 20 h. Insoluble material was removed by centrifugation (13 000 g, 10 min) and the supernatant was filtered, dialyzed and lyophilized.

Patients' allergic sera

Twenty-two patients (with radio allerge sorbent test, RAST, between 1.35 to >100), were selected by the Allergy clinic, Institut Pasteur (Paris, France) on the basis of clinical symptoms such as rhinitis, conjunctivitis, asthma,

Quincke oedema or urticaria upon contact with horse allergens. Individual sera were used for ELISA and for Western-blot experiments. Sera from individuals either nonallergic or sensitive to allergens other than equine were used as negative controls.

Chromatographic procedures

Purification of Equ c 5 from horse dander extract was performed as described previously for the purification of Equ c 2 and Equ c 4 [25], by a combination of (a) size-exclusion chromatography (SEC) on Superose 12 column (HR 16/50, Pharmacia (Uppsala, Sweden); (b) thiophilic interaction chromatography (TIC) a 10 $\times$ 1 cm (ID) column (Tentacle type gel; Merck, Darmstadt, Germany); and (c) reversed-phase chromatography (RPC) using a 10 R1 (10 cm $\times$ 4.6 mm ID) column Poros perfusion chromatography media, PerSeptive Biosystems (MA, USA).

Electrophoresis and immuno-blot analysis

SDS/PAGE. Protein samples were analyzed using homogeneous 18% acrylamide/bisacrylamide (29 : 1) gel [33] under reducing and nonreducing conditions in a Novex (San Diego, CA, USA) electrophoresis unit. Proteins were visualized with Coomassie Blue.

Immunoblot analysis. After completion of the SDS/PAGE, the separated proteins were electrophoretically transferred [34] onto nitrocellulose membrane by the Novex blotter according to the manufacturer's instructions. After blocking the unoccupied sites by 1 h incubation with 1% BSA in NaCl/P_i, strips of the membrane were incubated overnight at 4 °C with different sera (diluted 1 : 3) from horse sensitive patients or sera from subjects allergic to allergens other than horse (cat and mites) as negative control. After washing, binding of human IgE to the allergens was detected with biotinylated anti-(human IgE) Ig. The biotinyl residues were visualized by streptavidin-peroxidase followed by using the enhanced chemiluminescence (ECL) assay [35,36] according to the manufacturer's instructions. Briefly, the nitrocellulose strips were incubated for 1 min in luminescence reagent and the exposed film was subsequently developed immediately.

Isoelectric focusing (IEF). Analysis was performed in the pH range 3–7, in polyacrylamide gel, according to the recommendations of the manufacturer (Novex, CA, USA). The focused proteins were stained with Coomassie Brilliant Blue R250. Isoelectric point markers, pI 2.4–6.1 were purchased from BDH Laboratory Supplies (Poole, Dorset, England).

Mass spectrometry

Purified horse allergens were dissolved in water:methanol:formic acid (50 : 50 : 5). Samples were introduced to an API 365 triple-quadrupole mass spectrometer (PE-Sciex, Thornhill, Canada) at 5 μ L·min⁻¹ with a syringe pump (Harvard Apparatus, South Natick, MA, USA). The device was equipped with an atmospheric pressure ion source used to sample positive ions produced from a pneumatically assisted electrospray interface. Data were collected on a

Power Macintosh 8600/200 and processed through the BIOTOOLBOX 2.3 software from AB/MDS-SCIEX (Thornhill, Canada). Mass spectrometry analysis of horse allergens was performed at the department of structural chemistry of macromolecules, Institut Pasteur.

Detection of glycoproteins

The DIG glycan detection kit was used to detect sugars in glycoproteins, briefly: glycans of glycoproteins were oxidized to produce aldehyde groups allowing the covalent attachment of the steroid hapten digoxigenin (DIG). The latter is subsequently detected using specific antidigoxigenin labeled antibodies. Purified horse allergens were first labeled with digoxigenin before separation on SDS/PAGE and transferred to nitrocellulose membrane. The presence of the transferred proteins on the membrane was checked by staining with a Ponceau S solution. The incorporated digoxigenin was then detected on the membrane using polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase and visualized by soaking the membrane in 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate. Transferrin was used as positive control-glycoprotein and creatinase as a nonglycosylated control protein.

Monosaccharide analysis

Monosaccharides from glycoproteins were analysed as their pertrimethylsilyl methyl glycosides by gas/liquid chromatography [37]. Briefly, sugars were released by methanolysis (HCl 1 M in dry methanol, 80 °C, 24 h). Osamine residues were N-reacetylated with acetic anhydride in presence of silver carbonate powder. After derivatization with *N,O*-bis(trimethylsilyl)trifluoroacetamide/pyridine mixture, monosaccharides were separated and quantified by gas liquid chromatography-flame ionization detection. The gas chromatograph was equipped with a DB1 column (SGE, Villeneuve Saint-Georges, France, 25 m × 0.32 mm ID) using the following conditions: gas vector, helium (0.7 bar), temperature gradient 120–180 °C at 2 °C·min⁻¹; 180–240 °C at 4 °C·min⁻¹; 240 °C for 10 min

Analysis of carbohydrates on glycosylated allergens by digoxigenin-labeled lectins

The glycosylated purified allergens were subjected to SDS/PAGE followed by blotting onto nitrocellulose membranes. After blocking of nonspecific binding sites, the membranes were incubated for 1 h in the digoxigenin-labeled lectin solutions: lectin from MAA and lectin from SNA. Bound lectins were detected by alkaline phosphatase-labeled anti-digoxigenin Ig and visualized by immersing the membranes in a substrate solution (4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate) according to the manufacturer's instructions. Control glycoproteins (transferrin for SNA and fetuin for MAA) were used in order to check the specificity of the lectins.

Enzymic deglycosylation of glycoproteins

N-linked carbohydrate chains of glycoproteins were split with recombinant *N*-glycosidase F, whereas O-glycans

were released with O-glycosidase from *Diplococcus pneumoniae* according to the manufacturer's instructions. Control glycoproteins (transferrin, α_1 -acid glycoprotein and ribonuclease B) were used to check the efficiency of the enzymic treatment.

N-terminal sequencing

Allergens were sequenced at the Protein Sequencing Laboratory (Institut Pasteur) using an Applied Biosystems model 477A protein sequencer. The partial sequences reported in this paper have been submitted to the SWISS-PROT data bank with accession numbers P82615(Equ c 4) and P82616(Equ c 5). Sequence data were analyzed and compared with the protein database using BLAST program [38].

Amino-acid analysis

Purified Equ c 5 containing 0.2% (w/v) phenol and norleucine as internal standard was hydrolysed under vacuum in 6 M HCl and its amino-acid composition was determined using Bechman 6300 amino-acid analyser at the Organic Chemistry Department (Institut Pasteur).

Direct and inhibition ELISA

Microtiter plate was coated with the native allergen and unoccupied sites in the wells were saturated by human serum albumin in NaCl/P_i. A pool of sera from horse-sensitized patients (1 : 10 dilution, giving 80% of the maximum binding activity) was previously mixed with an equal volume of different concentrations of the inhibitor and incubated for 1 h at 37 °C. These samples were then added to the ELISA plate and incubated at 37 °C for 1 h. Binding of human IgE was detected by biotinylated-conjugated goat anti-(human IgE) Ig followed by the streptavidin-peroxidase and the substrate. Absorbance (A) values were measured at 490 nm. Negative control was performed using nonallergic sera or from patients allergic to other allergens such as pollen, Fel d 1 or dust mites, where the A obtained was subtracted from all other values. The percentage of inhibition was calculated according to the following formula:

$$[1 - (A \text{ in presence of inhibitor} / A \text{ in absence of inhibitor})] \times 100.$$

The sera of 22 horse-sensitized patients were examined by direct ELISA for the presence of IgE antibodies to highly purified Equ c 5.

Surface tension measurements by Wilhelmy balance

Surface tension measurements of the purified horse allergens, as well as several other protein solutions, were performed using Wilhelmy balance [39]. It is composed of filter paper (10 mm × 17 mm) suspended vertically, dipped into a glass Petri dish (diameter 5 cm) containing 10 mL of NaCl/P_i, pH 7.4, and connected to an KSV 5000 electronic balance. The shift in baseline was corrected by setting the signal to zero at the beginning of each measurement. All experiments were performed under a hood at ≈ 24 °C. Solutions of sample proteins were injected

into the subphase without disturbing the surface and the surface tension was recorded 2 min later. All tested proteins were dissolved in NaCl/P_i, pH 7.4 while the experiment with surfactin was performed at two pH values: pH 7.4 and pH 9.0 in 5 mM Tris.

RESULTS

Nomenclature of horse allergens

A certain number of horse allergens from horse dander extract have previously been identified and characterized. For clarity, an official designation has been proposed to the International Committee for Allergen Nomenclature [40] as follows: Equ c 1 [20,21], Equ c 2 [25,26], Equ c 3 (horse serum albumin) [20], Equ c 4 [25] and finally, in the course of purification of Equ c 4, we have identified and characterized in this report a new horse allergen that we propose to name as Equ c 5 (Table 1).

Comparative physico-chemical characteristics of horse allergens

Purification of Equ c 5 from horse dander extract was performed by three successive chromatographic steps. Following fractionation of horse dander extract by SEC, we obtained different fractions. The Equ c 5-enriched fraction was then further fractionated on TIC (as reported in [20]) and final purification was achieved by RPC, where around 60% acetonitrile was required to separate Equ c 5 from contaminating Equ c 4 (Fig. 1).

Under nonreducing conditions, comparative SDS/PAGE analysis (Fig. 2) showed that Equ c 5 migrated with an apparent M_r of 17.2 kDa while Equ c 1, Equ c 2 and Equ c 4 had a M_r of 22, 16 and 17 kDa, respectively [20,25]. Under reducing conditions the M_r of Equ c 5 slightly increased to 20.5 kDa due to decreased accessibility of SDS which induced a reduced electrophoretic mobility. A single band of 4.5 kDa was observed for reduced Equ c 4 suggesting a tertiary structure of four chains as previously reported [25]. The faint band observed at ≈ 9 kDa, could be attributed to an incomplete reduction of the molecule.

Additional determination of the exact molecular masses of horse allergens were performed by mass spectrometry. They were 22 kDa for Equ c 1, 16 kDa for Equ c 2, 18.7 kDa for Equ c 4 and 16.7 kDa for Equ c 5.

Table 1. Nomenclature and biochemical characteristics of horse allergens. M_r was determined by mass spectrometry. – Indicates no former name (new allergen).

Name	Former name	M_r (kDa)	Reference
Equ c 1	Equ c 1	22	[20]
Equ c 2	Equ c 3	16	[25]
	Equ c 2		[26]
Equ c 3 ^a	Antigen 3	65	[20]
Equ c 4	Equ c 2	18.7	[25]
Equ c 5	–	16.7	This report

^a Horse serum albumin.

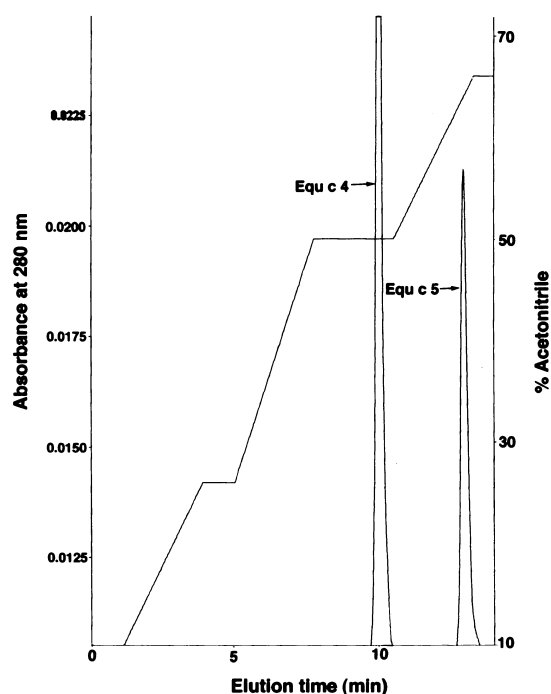


Fig. 1. Purification of Equ c 5 by reverse-phase chromatography. Following size-exclusion and thiophilic-interaction chromatographies, enriched fraction of Equ c 5 was passed through a 10 R1 (10 cm $\times$ 4.6 mm ID) column (Poros perfusion chromatography media) and elution was performed using a linear gradient from 10 to 90% acetonitrile/trifluoroacetic acid at a flow rate of 3 mL $\cdot$ min⁻¹. The eluate was monitored at 280 nm.

In isoelectric focusing (pH-gradient between 3 and 7), Equ c 5 exhibited a pI of 5.25 (Fig. 3). As compared to Equ c 1, pI:3.9 [20], Equ c 2 and Equ c 4, pI: 3.8 [25], horse dander allergens comprise a family of rather acidic proteins.

Purified Equ c 5 was shown to be an allergen as a specific binding of IgE from nine individual sera of horse-sensitive patients was observed by immunoblotting (Fig. 4). Moreover, the allergenicity of Equ c 5 was further analyzed by direct ELISA using 22 sera from horse-sensitized patients. Seventeen sera exhibited IgE binding capacity to Equ c 5 (77%), while control sera from patients allergic to other allergens (pollen, cat, dust mites etc.) were negative (Table 2).

Oligosaccharide portions of Equ c 1 and Equ c 4 do not play a major role in IgE immunoreactivity

The glycoprotein detection assay carried out on blotted purified horse allergens demonstrated that in contrast to Equ c 1 and Equ c 4, Equ c 2 and Equ c 5 were not glycosylated (Fig. 5). Gas chromatographic analysis showed that Equ c 4 was glycosylated and contained approximately 14.4% (w/w) of carbohydrates, corresponding to a molecular mass of about 2715 Da. Carbohydrate analysis revealed the presence of the following molar ratios of monosaccharides: 3 mannose, 3 galactose, 4.6 *N*-acetylglucosamine and 3.1 *N*-acetyl neuraminic acid, suggesting the presence of one tri-antennary tri-sialylated *N*-linked glycan molecule. The

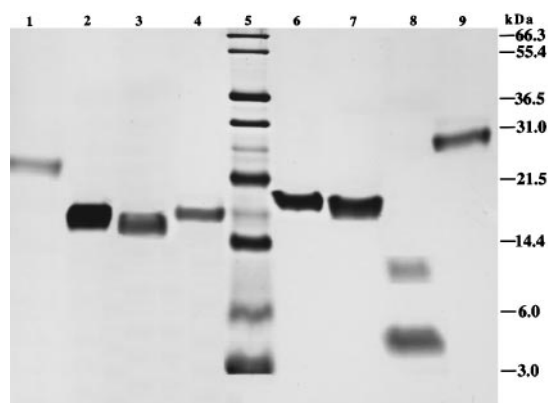


Fig. 2. SDS/PAGE of purified horse allergens. Electrophoresis was performed on a 18% homogenous gel containing SDS. Lanes 1–4, Equ c 1, Equ c 4, Equ c 2 and Equ c 5, respectively, under nonreducing conditions; lane 5, molecular mass protein markers; lanes 6–9, Equ c 5, Equ c 2, Equ c 4 and Equ c 1, respectively, under reducing conditions.

type of glycan linkage was also investigated using the specific binding of SNA and MAA lectins on blotted Equ c 1 and Equ c 4. SNA recognized both allergens (Fig. 6A), whereas MAA reacted weakly with Equ c 1 (Fig. 6B). These results indicated that terminal *N*-acetylneuraminic acids of Equ c 1 were mainly α -(2→6) and also α -(2→3) glycosidically linked to galactose, whereas in Equ c 4 only α -(2→6) linkages were encountered.

We then evaluated the involvement of glycosylation in IgE immunoreactivity against Equ c 1 and Equ c 4 by using inhibition ELISA. Removal of carbohydrate chains was enzymically completed after treatment with *N*- (for Equ c 4); *N*- plus *O*-glycosidase (for Equ c 1) as assessed by the glycoprotein detection assay and SDS/PAGE analysis (data not shown). As depicted in Fig. 7, the binding of IgE from a pool of sera from horse sensitive patients to the native allergen was inhibited to the same extent by deglycosylated and native Equ c 1 or Equ c 4. Fifty percent inhibition was achieved with 3.4 nM of the

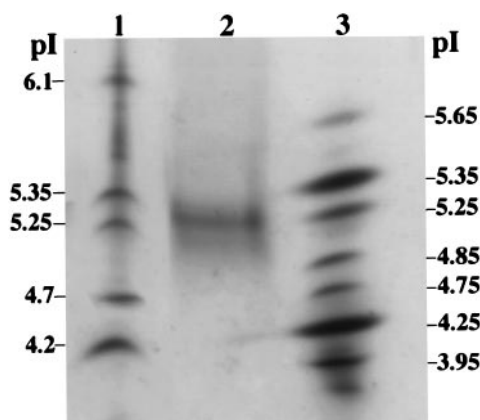


Fig. 3. Isoelectric-focusing. IEF was performed in the pH range 3–7. Lane 1, isoelectric point markers; lane 2, Equ c 5; lane 3, isoelectric point markers. The focused proteins were stained with Coomassie Brilliant Blue R250.

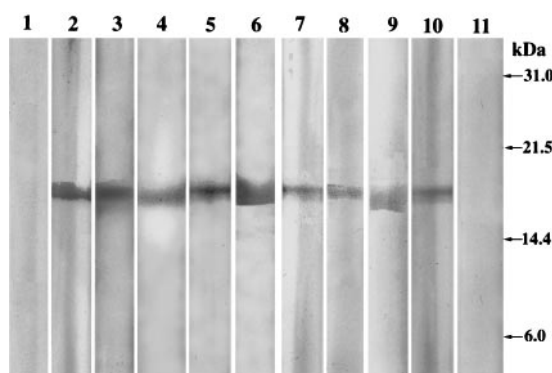


Fig. 4. Immunoblot analysis of Equ c 5. The purified protein was electrophoresed on a 18% homogeneous polyacrylamide gel containing SDS and was transferred electrophoretically to nitrocellulose membrane. The blotted protein was probed with: lane 1, a pool of sera from individuals sensitized to allergens other than horse; lanes 2–10, sera from horse-sensitized patients; lane 11, negative control incubated only with second antibody without prior incubation with patient's serum. IgE binding was then revealed with biotinylated anti-(human IgE) Ig. The biotinyl residues were visualized by streptavidin-peroxidase-based detection system by enhanced chemiluminescence assay.

native Equ c 1 and with 4.7 nM of the deglycosylated protein, while 2.7 nM of the native Equ c 4 and 3.3 nM of the deglycosylated allergen were required. Our results suggest that carbohydrate moieties of Equ c 1 or Equ c 4 are not significantly involved in IgE-binding epitopes.

Partial sequences of Equ c 4 and Equ c 5

Partial N-terminal amino-acid sequence of Equ c 2 was found to be identical (not shown) to that described by Bulone *et al.* [26]. Fifty-four amino acids of the N-terminal portion of Equ c 4 were sequenced and comparison with other sequences in the protein database revealed a 35% identity and 64% homology with the rat submandibular gland protein A (SMG-A) [41]. SMG-A is a leucine rich protein of M_r 23.5 kDa and pI of 4.5; it is secreted by the submandibular gland of the rat suggesting that Equ c 4 may originate from the salivary gland as does Equ c 1. Our attempt to identify the N-terminal sequence of Equ c 5

Table 2. Distribution of IgE reactivity against Equ c 5 determined by ELISA in sera of 22 horse-allergic patients. *, Cut-off corresponding to the mean $A + 3$ SD obtained from 11 sera from subjects sensitized to allergens other than horse.

	Numbers of patient	Percentages
Negative	5	22.73
$A < 0.037^*$		
+	8	36.36
$0.037 < A < 0.1$		
++	5	22.73
$0.1 < A < 0.5$		
+++	4	18.18
$0.5 < A$		

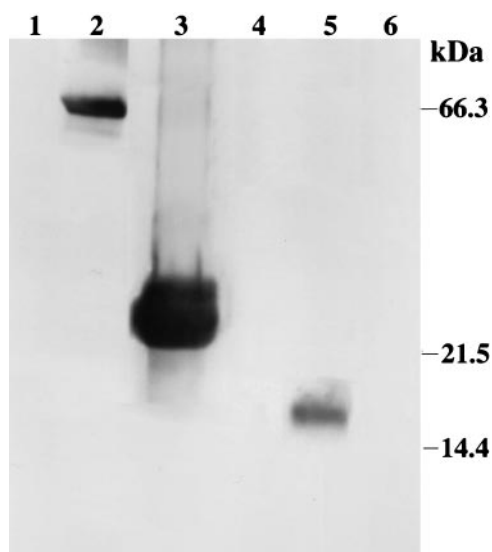


Fig. 5. Detection of glycoproteins. After electrophoresis and blotting of the purified allergens, the glycoproteins were detected using the DIG glycan detection kit. Lane 1, creatinase (a nonglycosylated control protein); lane 2, transferrin (control-glycoprotein); lanes 3–6, Equ c 1, Equ c 2, Equ c 4 and Equ c 5, respectively.

failed, probably due to a blocked N-terminus. Consequently, we digested the protein with trypsin and two internal peptides P1 and P2 (11 and 12 amino acids) were sequenced. A search for homologous sequences in the protein data base showed no homology with any known protein sequence. Sequence data are illustrated in Fig. 8.

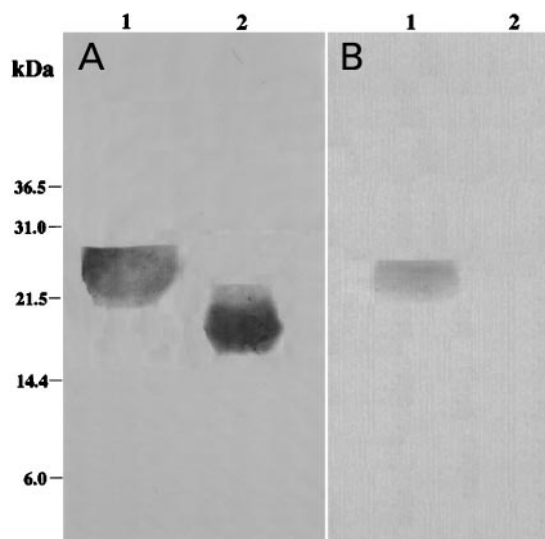


Fig. 6. Glycoproteins probed with *Sambucus nigra* (SNA) and *Maackia amurensis* (MAA) lectins. Equ c 1 and Equ c 4 were subjected to SDS/PAGE followed by blotting onto nitrocellulose membranes. The membranes were then incubated in the digoxigenin-labeled lectin solutions: lectin from MAA (A) and lectin from SNA (B). Lane 1, Equ c 1; lane 2, Equ c 4. Bound lectins were detected by alkaline phosphatase-labeled anti-digoxigenin Ig.

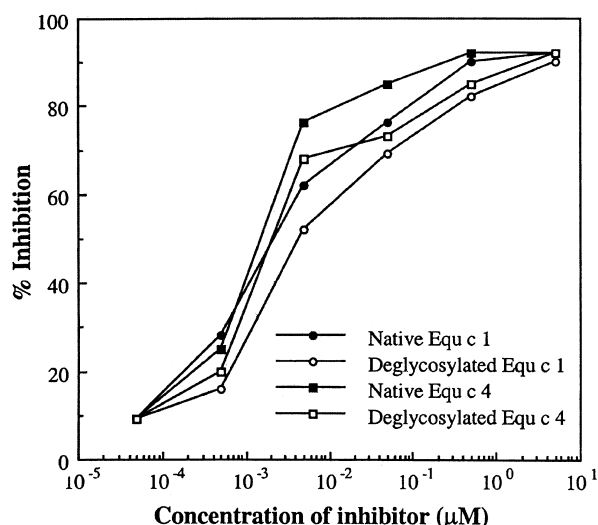


Fig. 7. Inhibition of IgE binding. Influence of deglycosylation treatment on binding of human IgE from a pool of sera of horse sensitive patients to Equ c 1-coated wells by (●) native, and (○) deglycosylated Equ c 1; and to Equ c 4-coated wells by (■) native, and (□) deglycosylated Equ c 4. Inhibitors were added after coincubation with the human serum, and binding was determined after addition of biotinylated-labeled goat anti-(human IgE) Ig.

Equ c 1, Equ c 4 and Equ c 5 exhibit surfactant-like properties

The global amino-acid composition carried out on Equ c 5 (Table 3) as well as the analysis of the 54 N-terminal amino acids of Equ c 4 (Fig. 8) revealed a high incidence of the hydrophobic amino-acid leucine; 21.3% and 24%, respectively. This is consistent with their delayed elution profile as observed in reversed phase chromatography and prompted us to study the hydrophobic properties of horse allergens as well as various proteins by measuring their ability to reduce the surface tension of water using Wilhelmy balance [39].

All 18 proteins tested had the ability to lower the surface tension of water. However, differences in the profile of the curves were observed between proteins with regard to, first, the value of the plateau reached and second, the slope of the curve at inflexion point. These two parameters reflect the propensity of the protein to adsorb at the air/liquid interface. In these studies, when the surfactant power of the tested molecule was high, the slope was high and the plateau was low. According to Laughlin [42] 'A useful measure of the efficiency with which a particular compound lowers surface tension is the concentration at which this plateau is attained'. As this condition could not be fulfilled because of the high amounts of proteins required, we considered the slope of the curves as a relative index of surface tension (ST index) in order to facilitate the comparison among the proteins. Figure 9 shows the measurements of surface tension of varying concentrations of proteins. A starting value of $72.5 \text{ mN}\cdot\text{m}^{-1}$ was considered for the surface tension of NaCl/P₁ alone [43].

As a positive control, surfactin (Fig. 9A), a bacterial lipopeptide with powerful surfactant properties [44], showed the highest ST index (51 at pH 7.2 and 26.8 at pH 9.5). Three phases in the profile of the curve may be distinguished

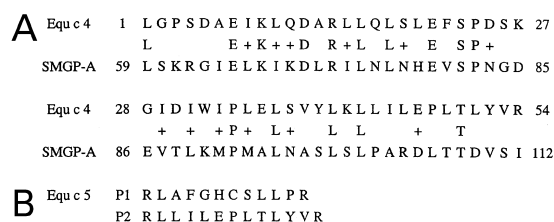


Fig. 8. Partial amino-acid sequences. (A), sequence alignment of a partial N-terminal sequence of Equ c 4 with the rat submandibular gland protein-A. (B) Two internal tryptic fragments of Equ c 5 (P1 and P2). (+), amino-acid similarities.

which correspond to the progressive increase of the amount of adsorbed molecules onto the surface (Gibbs monolayer) [45]. At low protein concentrations the surface tension is not affected. Increasing the concentration of the molecule results in its accumulation at the interface and thus in the abrupt lowering of the measured surface tension. Finally, a

plateau is reached when Gibbs monolayer is formed of a close-packed layer of protein. Thus, with a typical surface active molecule, the dose-dependent curve was sigmoidal by using a semilog representation.

Although the maximal decrease of the surface tension with proteins was always inferior to that observed with the lipopeptide surfactin, we could distinguish three families of tested proteins. In Fig. 9B,C,D representative results are depicted (including horse allergens and heterogeneous fluids) for two of these three families of proteins; (a) those which followed the surfactin profile (rather sigmoidal) and were, thus, considered as surfactant-like proteins such as bee venom PLA₂ (Api m 1), Equ c 1, Equ c 4, and Equ c 5, with a high ST index of more than 10 (16.5, 15.2, 14.5 and 13.5, respectively, Fig. 9B,C); (b) those exhibiting a weak and regular concentration-dependent decrease in surface tension (weak ST index < 7) such as β -lactoglobulin (Bos d 5, Fig. 9C), ananain, horse serum albumin (Equ c 3, Fig. 9B), ovalbumin (Gal d 2), pepsin, Fel d 1, gelatin, BSA (Fig. 9C),

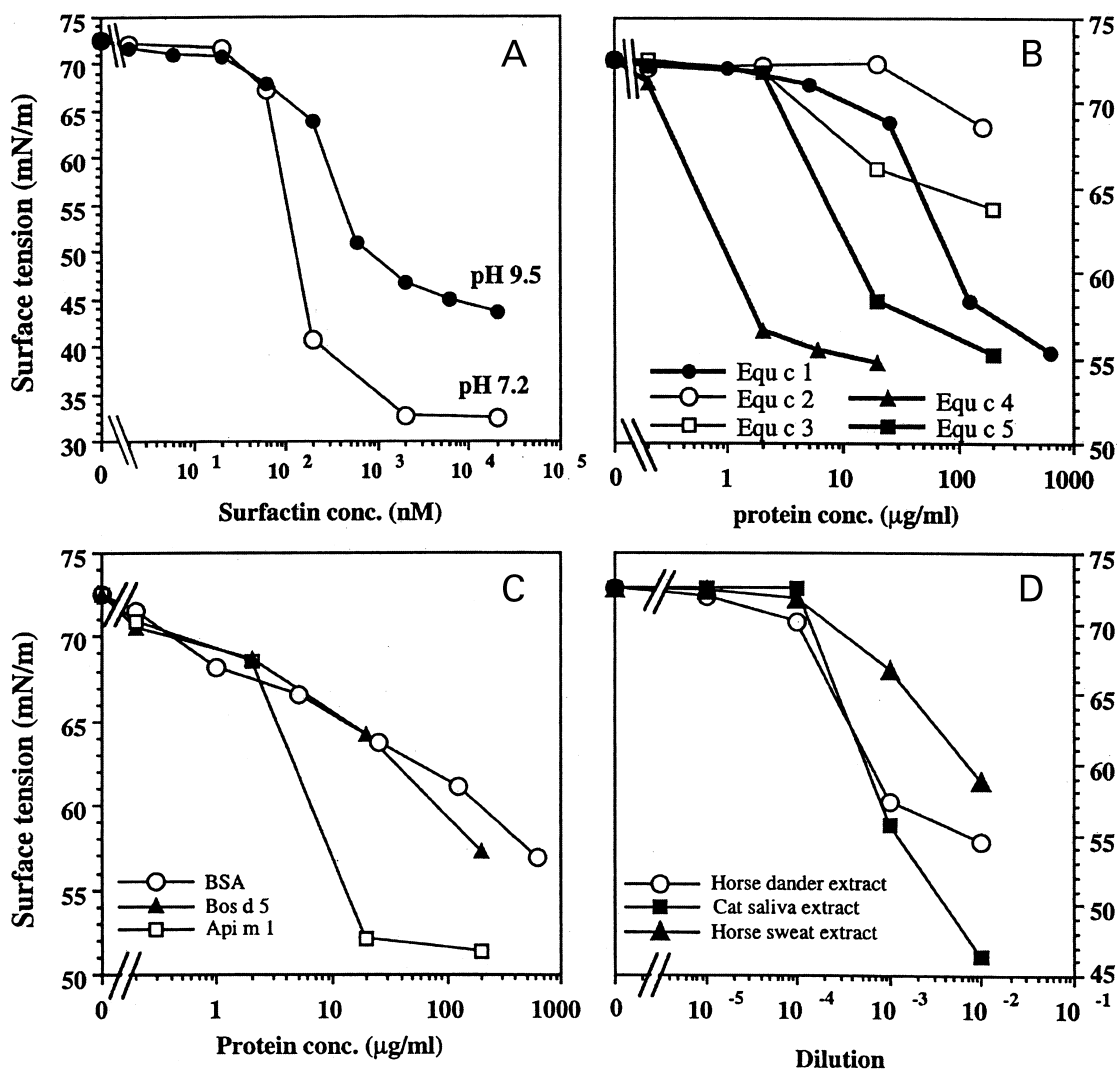


Fig. 9. Surface tension vs protein concentrations. The surface tensions of different protein concentrations were measured by Wilhelmy balance. (A) surfactin solution at pH 9.5 (●) and pH 7.2 (○). (B) Different concentrations of Equ c 1 (●); Equ c 2 (○), Equ c 3 (□), Equ c 4 (▲) and Equ c 5 (■). (C) BSA (○), Bos d 5 (▲), Api m 1 (□). (D) Different allergen extracts from horse dander (○), cat saliva (■) and horse sweat (▲).

Table 3. Amino-acid composition of Equ c 5. ND, not determined.

Amino acid	Equ c 5 (molar %)
Asx	13.2
Thr	4.1
Ser	7.5
Glx	11.4
Pro	5.2
Gly	6.9
Ala	5.6
Val	5.6
Met	0.2
Ile	6.8
Leu	21.3
Tyr	2.1
Phe	2.4
His	1.3
Lys	3.0
Arg	3.3
Cys	0.2
Trp	ND

Equ c 2 (Fig. 9B), bovine γ -globulin (Bos d 7) and α -chymotrypsin; and (c) those showing intermediate profile with ST index between 7 and 10, i.e. lysozyme and glucose oxidase (profile not shown). The ST index is listed in Table 3. Interestingly, the three heterogeneous extracts, horse dander, sweat extract and cat saliva extract, exhibited surfactant-like properties (Fig. 9D).

As surfactant properties reflect the hydrophobicity of the proteins, we compared ST index with the percentage of hydrophobic residues (% LVIFM, collected in the IRPSEC-SAPS data bank <http://www.isrcc.isb-sib.ch/software>) and also with the elution time on RP-HPLC (HR index) performed on the same protein. The values for the tested proteins were listed in Table 4. With regard to horse allergens Equ c 1, Equ c 4 and Equ c 5, their degrees of surfactant-like properties were correlated with high hydrophobic retention indices. However, when surfactin was excluded (because of biased class effect), no global correlations among the 16 tested proteins were found between the ST and HR indices ($r = 0.116$) or ST index and the percentage of hydrophobic amino acids (% LVIFM, $r = 0.213$). Also, HR indices of the 18 proteins tested were not significantly correlated with the percentages of hydrophobic amino acid ($r = 0.335$).

DISCUSSION

Physicochemical characterization of allergens, besides fundamental protein knowledge, is an important step towards the development of efficient diagnosis and, ultimately, the treatment of allergic disorders. Allergy to horse dander, as well as to mammalian proteins, has long been reported and efforts have been made to biochemically and immunologically characterize the various allergen containing extracts. Until now, although several spots (which may include isoforms of one protein) were detected by 2-dimensional gel analysis [26], only four horse allergens have been purified to homogeneity and characterized

Table 4. Comparison between the surface tension index (ST index, see Fig. 8), the percent of hydrophobic amino-acids and the RP-HPLC retention time of 18 homogeneous proteins and three heterogeneous fluids. HR, hydrophobic retention index. LVIFM values are taken from the IRPSEC-SAPS database. ND, not determined.

Proteins	Designation	ST index	% LVIFM	HR index
Surfactin (pH 7.2)	Surfactin	50.93	71.4	10.025
Surfactin (pH 9.5)	Surfactin	26.79	71.4	
Phospholipase A2	Api m 1	16.44	21.6	7.062
Horse dander allergen	Equ c 1	15.22	34.2	9.549
Horse dander allergen	Equ c 4	14.54	40.74 ^a	9.492
Horse dander allergen	Equ c 5	13.5	36.3	9.793
Lysozyme	Gal d 4	8.83	24.5	7.293
Glucose oxidase	GOx	7.82	27.9	9.542
β -Lactoglobulin	Bos d 5	6.85	30.9	8.306
Ananain	Ananain	6.06	22.7	7.896
Horse serum albumin	Equ c 3	5.71	24.7	7.863
Ovalbumin	Gal d 2	5.48	32.2	7.79
Pepsin	Pepsin	4.97	29.5	5.383
Major cat allergen	Fel d 1	4.96	35.2	9.259
Gelatin	Gelatin	4.56	10.3	5.2
Bovine serum albumin	BSA	4.18	25.2	7.802
Horse dander allergen	Equ c 2	4.04	13.2	8.3
Bovine gamma globulin	Bos d 7	3.72	22	9.08
α -Chymotrypsin	Chymo	3.12	24.6	ND
Cat saliva extract ^b		16.74	ND	ND
Horse dander extract ^b		12.88	ND	ND
Horse sweat extract ^b		7.77	ND	ND

^a Calculated within 54 amino-acid residues; ^b heterogeneous mixture.

[20,25]. According to previous published articles, they were designated Equ c 1, Equ c 2, Equ c 3 and Equ c 4. We report herein the purification of a new horse allergen, Equ c 5, that we biochemically and biophysically investigated together with Equ c 1, Equ c 2 and Equ c 4.

The native Equ c 5, purified from hair dander extract was able to bind IgE-antibodies from the sera of horse-sensitized individuals. Its apparent molecular mass, determined by SDS/PAGE was higher under reducing conditions than in the absence of reducing agent (20.5 vs. 17 kDa). This phenomenon, also observed for Equ c 1 [20], and Equ c 2 [25], suggested the presence of intramolecular disulfide bond(s). Mass spectrometry experiments confirmed the molecular weight of all four allergens determined by SDS/PAGE. Equ c 5 was less acidic in nature than Equ c 1 [20], Equ c 2 and Equ c 4 [25], which fell within the pI range 2.5–5.0 reported [26]. Moreover, allergens of mammalian origin usually present in hair dandruff such as Fel d 1, Can f 1, Can f 2, Bos d 1, Mus m 1, Rat n 1 and Rat n 2 are also rather acidic [1].

Equ c 2 and Equ c 5 were not glycosylated whereas Equ c 4 was shown to be N- and Equ c 1 both N- and O-glycosylated [21]. In a previous report, only N-glycosylations in horse allergens were shown [19,26]. Specificities of linkage revealed by the binding of lectins could be used for the final steps of purification of these allergens, using lactose as an eluent.

Carbohydrate moieties of allergen can be part of epitopes recognized by IgE. Several studies showed that various allergens lost their IgE-binding activity after removal of their carbohydrate portions [19,26,46–48], while other investigations demonstrated that the binding sites for human IgE are mainly located in the protein moiety of the molecule [49–51]. In inhibition ELISA, we noticed that the enzymically deglycosylated Equ c 1 and Equ c 4 maintained their specific IgE inhibition capabilities, in comparison to that of the native allergens, suggesting that the oligosaccharide portions are barely involved in IgE epitope(s). These results are in agreement with those deduced from immunoblot experiments [21] which revealed that unglycosylated recombinant Equ c 1 was also recognized by IgE from sera of horse-sensitized patients. Other studies indicated that the carbohydrate moiety of allergens from horse dander is likely to interact at least partially with IgE from patient sera [26]. It was suggested that this interaction is most probably not based on the glycan moiety alone, but on the overall structure of the glycoprotein [27].

Equ c 1 is the major horse allergen from a clinical point of view. Its sequence and crystallographic three-dimensional structure revealed that it is a member of the lipocalin superfamily, as are significant numbers of mammalian allergens [2]. Using N-terminal sequencing we confirmed that Equ c 2 also belong to the lipocalin family as previously reported [26]. The comparison of the partial N-terminal amino-acid sequence of Equ c 4 with other sequences in the protein data base revealed a significant homology with the rat SMG-A [41], which is not a member of the lipocalin family. This result suggests that Equ c 4 may originate from the salivary gland as does Equ c 1 [21]. SMG-A is a leucine-rich protein (16.0% leucine) similar to other proteins from this family such as the rat parotid secretory protein (20.9%) and the mouse parotid secretory protein (23.0%). The proportion of leucine in the

N-terminal amino acid of Equ c 4 as well as in the amino-acid composition of Equ c 5 was also particularly high. In another study [14] it was also observed that an allergen identified in horse hair and dandruff and denoted antigen no. 9 had a high leucine content which constitutes about a quarter of the total number of amino acids. Leucine, as an hydrophobic amino-acid, contributes to the global hydrophobicity of the protein, a property which was also exhibited by Equ c 4 and Equ c 5 in RP-HPLC.

The ability of a protein to lower the surface tension of a solution is also a measure of hydrophobicity as it reflects the propensity of the protein to escape water and to preferentially adsorb at the air/liquid interface. Using the Wilhelmy balance, among the tested proteins, Equ c 1, Equ c 4, Equ c 5 and bee venom PLA₂ were found to possess the highest capacity to lower the surface tension of water although different degrees of surfactant property were observed for the different proteins tested. The surfactant property of PLA₂ is consistent with its enzymatic activity which requires the interaction with phospholipids [31] and thus PLA₂ might be considered as a positive control. The absence of correlation between surfactant-like properties and the composition in hydrophobic amino acids could be explained by the fact that a certain proportion of hydrophobic residues may be buried in the protein. Similarly, ST and HR indices, both reflecting the hydrophobic character of the protein, did not correlate. As the microenvironment surrounding the protein is different, namely, organic solvent, a highly hydrophobic matrix for RP-HPLC and a liquid/air interface in surface tension measurement, molecular requirements for hydrophobic interactions should be different, especially with regard to the distribution of the exposed hydrophobic patches of amino-acids which participates in the orientation of the molecule. Additional three-dimensional analysis of the protein should clarify these points.

Nevertheless, the observed surfactant-like property is neither restricted to allergens nor to lipocalins as, for instance, Equ c 3 (horse serum albumin) or the allergenic lipocalin β -lactoglobulin (Bos d 5) had no ability to significantly lower the surface tension of water. Such a surface-active property could, more likely, be an attribution of proteins associated with physiological fluids such as sweat, saliva, tears, or pulmonary or intestinal surfactant, where they may play a role in promoting the adsorption and spreading of liquids at the air–liquid interface [52–54]. Whether such a property attributed to an allergen, especially an aeroallergen, may have an implication on the primary sensitization of atopic individuals or on IgE-independent response in sensitized allergic patients remains to be further investigated. Different reports indicated a possible link between the structure of an allergen and its ability to trigger inflammatory or immunological responses in an IgE-independent manner. The allergenicity of lipocalins was proposed to be a consequence of molecular mimicry between endogenous lipocalins and exogenous lipocalin allergens at the T cell level [28]. In addition, enzymatic activity of some allergens was shown to directly contribute to specific IgE response through IgE-independent pathways [29,31,55,56].

Hydrophobic interactions may take place when proteins come in contact with membrane phospholipids [57] or hydrophobic surfaces such as pulmonary surfactant. Interaction of an inhaled aeroallergen possessing surface-active

properties with the pulmonary surfactant might result in the impairment of the physiological role of the latter and might favour allergen penetration in tissue. Furthermore, as we have shown in this report that three heterogeneous extracts exhibited surfactant-like properties due to several, but not all, of their components, this global property might exert an adjuvant effect even on nonsurfactant proteins included in the mixture.

In conclusion, our study provides an insight into the biochemical and immunochemical characterization of highly purified horse allergens including the new member, Equ c 5. A special emphasis is laid on a biophysical characteristic, namely, the surfactant-like property of Equ c 1, Equ c 4 and Equ c 5 probably relying on a nonequal distribution of exposed hydrophobic patches of amino acids. Besides biochemical activities of allergens, the spatial arrangement of amino acids should lead to important clues for the understanding of the structure/property relationships of allergens.

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