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Thiophilic adsorption chromatography: purification of Equ c2 and Equ c3, two horse allergens from horse sweat

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Abstract

Purification of two allergens from horse (*Equus caballus*) sweat, Equ c2 and Equ c3, by means of salt-promoted chromatography on a ''thiophilic'' (T-gel) adsorbent is described. Immobilization of these proteins was found to be dependent on the presence of water-structure-forming salts where the ammonium sulphate concentration in the equilibration buffer was 2 *M*. Equ c2 showed higher affinity towards the thiophilic matrix than Equ c3. Their molecular mass (M_r) values established by SDS–polyacrylamide gel electrophoresis were for Equ c2 ≈17 000 and for Equ c3 ≈16 000, and both proteins showed a low isoelectric point of ≈3.8. Their allergenic properties were also investigated using sera from horse-sensitized patients, where it was demonstrated that these proteins exhibited an IgE antibody binding capacity. In this report we show the broad potential applications of thiophilic adsorption chromatography for the efficient purification of allergens. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Thiophilic adsorption chromatography; Horse allergens

1. Introduction

Animals produce proteins which could be potentially allergenic to persons exposed to these molecules. These allergens provoke a hypersensitivity response mediated by immunoglobulin (Ig) E in genetically predisposed individuals, giving rise to asthma, rhinitis and atopic dermatitis. Allergen extracts are composed of a large number of antigenic molecules. Some of them are defined as major allergens when they elicit an IgE-mediated type I (immediate) allergic reaction in more than 50% of a selected group of patients while others, which only sensitize a few patients, are defined as minor allergens [1].

In a recent study [2], 30% of the patients who consulted for allergic reactions to animals, such as cats and dogs, also displayed clinical allergic symptoms to horses. Different horse dander allergen extracts have been studied in which several molecules, among which horse serum albumin, have been shown to be allergenic [3–7]. Several reports also showed that horse dandruff extract contains allergenic proteins with a molecular mass (M_r) range between 10 000 and 75 000, and isoelectric points (p*I*) that range between 3.5 and 4.5 [8–16].

In a previous study [6] we identified four allergens in horse dander extract which were also present in horse sweat, as shown by immunoelectrophoresis. The major horse allergen, Equ c1, was purified [6], and the gene encoding it was cloned [17]. Another allergen was horse serum albumin, whose antigenic

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structure and allergenic properties have been studied [18].

The aim of the present study is to purify and characterize the two other allergens, Equ c2 and Equ c3, from horse sweat. The latter source was used because it is rich in protein content and contains high concentrations of the allergens studied. We purified these two horse proteins by using an original type of protein–ligand recognition process introduced by Porath et al. in 1985 [19], and known as thiophilic adsorption chromatography. This chromatographic method is based on a salt promoted adsorption of proteins (salting-out adsorption) to a divinylsulfone-activated agarose to which the ligand (mercaptoethanol) is coupled (''thiophilic'' adsorbent or ''T-gel'') [19].

Thiophilic adsorption has been utilized for the purification of immunoglobulins from human serum [19,20]. This selective binding of immunoglobulins in the presence of structure-forming salts was also used for the purification of several classes of antibodies from different species [21–27]. However, we show that thiophilic adsorption chromatography can also be efficient for the purification of allergens, thus revealing their thiophilic properties. Finally, the allergenic properties of the purified proteins were also investigated.

2. Experimental

2.1. Chemicals

HPLC gradient grade acetonitrile, trifluoroacetic acid (TFA) and Coomassie brilliant blue R250, were obtained from Merck (Darmstadt, Germany). Agarose A, gel bond film and ^{125}I-labelled rabbit anti-human IgE antibody were from Pharmacia.

2.2. Horse sweat

Horse sweat was collected from thoroughbred horses after intensive training. Samples were centrifuged for 20 min at 4000 *g* to remove particulate material, dialysed and lyophilised.

2.3. Patients' allergic sera

Human sera were provided from volunteers at the Pasteur Hospital, Allergy consultation. They were presenting clinical symptoms of type-I (immediate) allergy to horse allergens. The sera selected had positive skin prick test [28] with horse allergens and a history of allergy to horses. A pool of sera prepared from non-allergic individuals was the control.

2.4. Protein assays

Sample concentration was evaluated using the bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL, USA) and used with 96-well microtiter plates [29].

2.5. Size-exclusion chromatography

For partial purification of the proteins of interest, horse sweat samples were analyzed on Superose 12 column (HR 16/50, Pharmacia, Uppsala, Sweden). The eluent was 20 m*M* phosphate buffer, pH 7.0 containing 0.25 *M* NaCl. Elution of proteins was performed at a flow-rate of 30 cm h^{-1} and the absorbance was monitored at 280 nm. Chromatographic experiments were carried out using an FPLC system (Pharmacia).

2.6. Thiophilic adsorption chromatography

The ''thiophilic'' adsorbent gel used for these investigations was a generous gift from Professor Jerker Porath. This ''T-gel'' consisted of agarose activated with divinylsulfone and coupled with mercaptoethanol. The structure of the immobilized ligand is: agarose–O-CH_2–CH_2–SO_2–CH_2–CH_2–S–CH_2–CH_2–OH [19]. The equilibration of the column was performed with 20 m*M* phosphate buffer containing 2 *M* $(NH_4)_2SO_4$ at a pH of 7.0. Samples were reconstituted in this equilibration buffer and then the solution was clarified by centrifugation. The clear supernatant was filtered through a 0.2-μm filter and was applied to a 50×5 mm column (bed volume=0.98 ml). The flow-rate was 304 cm h^{-1}.

The elution was achieved by applying a linear gradient for 50 min using 20 m*M* phosphate buffer, pH 7.0, without ammonium sulphate. The effluent was monitored at 280 nm. Using the BCA protein assay we found that nearly total protein recovery was obtained. Chromatographic experiments were carried out using an FPLC system (Pharmacia).

2.7. Reversed-phase chromatography

Minor contamination by the major horse allergen Equ c1 detected in the purified Equ c2 fraction was removed by a further step using reversed-phase on a Poros perfusion chromatography media (10 R1, 10 cm×4.6 mm I.D.) (PerSeptive Biosystems, MA, USA). Elution was performed using a linear gradient for 20 min from 10 to 90% acetonitrile–TFA at a flow-rate of 2 ml min^{-1}; the eluate was monitored at 280 nm. Chromatographic experiments were carried out using Biocad Sprint system (PerSeptive Biosystems).

2.8. Immunoelectrophoretic methods

To follow the degree of purification of the antigens/allergens, crossed line rocket immunoelectrophoresis (CLRIE) was used [30]. Briefly, CRLIE was performed in 1% agarose A in Tris–veronal buffer pH 8.6, on a gel bond film. A first-dimension electrophoretic separation, in agarose gel, of the extract containing horse sweat proteins was performed. Then immediately the gel strip with these separated antigens was cut out from the agarose gel and placed in a slit of corresponding size in the second-dimension plate. These separated proteins were then electrophoresed perpendicular to the first one into agarose gel containing rabbit serum raised against horse sweat extract. The antibody containing agarose gel was poured in two parts; the lower part contained 1 μl cm^{-2} and the upper part 4 μl cm^{-2}. By interposing an intermediate gel, containing horse sweat proteins, between the first-dimension and the second-dimension gel, it was possible to compare the content of the purified sample with that present in the horse sweat extract.

2.8.1. SDS–PAGE and rocket immunoelectrophoresis (RLIE)

After sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), we cut out the desired band separately from the gel, and the extracted protein is electrophoresed into agarose gel containing rabbit serum against horse sweat extract.

2.8.2. SDS–PAGE and Western blot analysis

Samples were analyzed on a homogeneous 16% acrylamide–bisacrylamide (29:1) gel electrophoresis in the presence and absence of SDS as well as under reducing and non-reducing conditions [31] in a Novex (San Diego, CA, USA) electrophoresis unit. Proteins were visualized with Coomassie Blue and were additionally stained with silver ions [32]. After SDS–PAGE, the separated proteins were subjected to electrophoretic transfer [33] to a nitrocellulose membrane, 0.2 μm, by the Novex blotter according to the manufacturer's instructions. Unoccupied sites on the membrane were blocked by incubation with human serum albumin for 45 min. Between each incubation step the membrane was washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20. The membrane was then incubated with patient serum (diluted 1:10) for 1 h and IgE-binding antigens were detected with ^{125}I-labelled rabbit anti-human IgE antibody, followed by autoradiography. A pool of sera from non-allergic healthy donors was used as a negative control.

2.8.3. Isoelectric focusing

Isoelectric focusing (IEF) was performed in the pH range 3–10. The focused proteins were stained with Coomassie brilliant blue R250 [34].

3. Results

3.1. Sensitivity pattern of horse-sensitive patients

Using IDALI (immunodetection of individual allergens) [6], we tested 21 horse-sensitive patients sera with respect to Equ c2 and Equ c3. We found that 33.3% of the tested sera reacted with Equ c2 and 23.8% reacted with Equ c3. No reaction was observed with control sera from non-allergic persons.

3.2. Chromatographic procedures

3.2.1. Size-exclusion chromatography

Horse sweat (50 mg) was fractionated by size-exclusion chromatography (SEC) on a Superose 12 HR 16/50 column. The first peak (S1) was eluted in the void volume of the column and was essentially composed of high molecular mass components. The second peak was divided into two parts (S2a) and (S2b) (Fig. 1A). The RIE analysis revealed that peak S1 contained non-antigenic material, the fraction (S2a) contained essentially the horse major allergen Equ c1, whereas the antigens/allergens of interest were present in the fraction (S2b). After several runs, the fraction (S2b) was pooled, dialysed, lyophilised and then submitted for a second fractionation by SEC (Fig. 1B). This second run enabled us to obtain a rich fraction containing both allergens of interest.

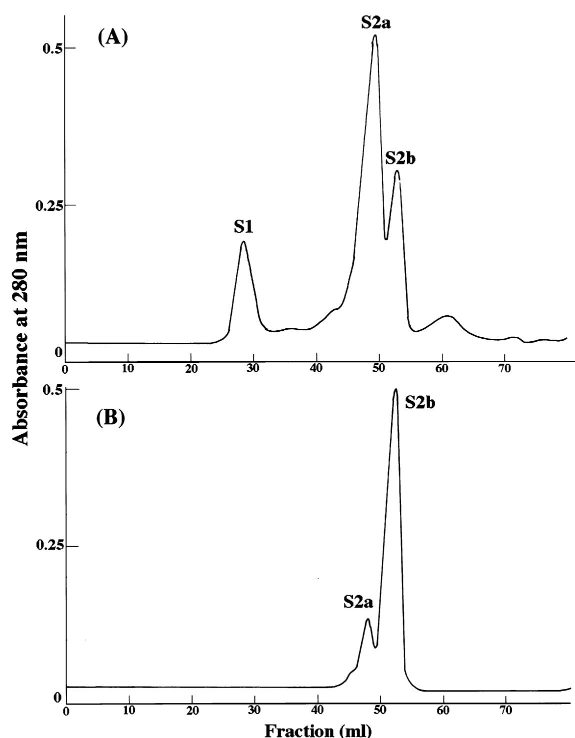

Fig. 1. Size-exclusion chromatography. Horse sweat (50 mg) was applied to a Superose 12 column (HR16/50, Pharmacia). The eluent was 20 m*M* phosphate buffer, pH 7.0 containing 0.25 *M* NaCl. Elution of proteins was performed at a flow-rate of 30 cm h^{-1} and the absorbance was monitored at 280 nm. (A) The fraction, S1 contained high-molecular-mass components; S2a, Equ c1; S2b, Equ c2 and Equ c3. (B) Only fraction S2b was submitted for a second fractionation.

3.2.2. Thiophilic adsorption chromatography

To further purify both proteins, the lyophilised fraction (S2b) was reconstituted in the equilibration buffer composed of 20 m*M* phosphate buffer containing 2 *M* $(NH_4)_2SO_4$ at a pH of 7.0. The solution was clarified by centrifugation, filtered through a 0.2-μm filter and then loaded onto the column. After an isocratic run, elution of proteins was performed by a gradient of decreasing ammonium sulphate concentration. The first peak (I) contained non-antigenic material, while the second peak (II) contained the Equ c3 fraction and the third peak (III) contained the Equ c2 fraction. The fraction eluted between both peaks (II and III) contained a small amount of the horse major allergen Equ c1. Equ c3 fraction was eluted at (40% of buffer B (corresponding to ≈1.2 *M* $(NH_4)_2SO_4$, whereas Equ c2 fraction was eluted in much smaller amount of $(NH_4)_2SO_4$ (90% buffer B corresponding to ≈0.2 *M* $(NH_4)_2SO_4$. Fig. 2 shows the "T-gel" elution of adsorbed horse sweat proteins as a function of ammonium sulphate concentration. Nearly total proteins were recovered in high yields.

3.2.3. Reversed-phase chromatography

Minor quantities of Equ c1 which contaminated the Equ c2 fraction were removed by a further step

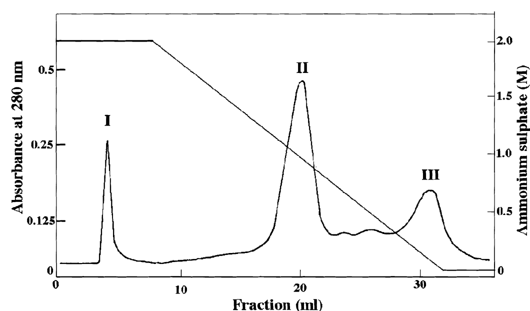

Fig. 2. Thiophilic adsorption chromatography. Chromatography of the fraction (S2b) was performed using a column (50×5 mm I.D.) packed with "T-gel". Flow-rate 304 cm h^{-1}. The equilibration buffer consisted of 20 m*M* phosphate containing 2 *M* $(NH_4)_2SO_4$ at a pH of 7.0. After washing the column with the same buffer, adsorbed proteins were eluted by applying a linear gradient for 50 min using 20 m*M* phosphate buffer, pH 7.0, without ammonium sulphate. The effluent was monitored at 280 nm. Peak I contained non-antigenic material; peak II, Equ c3 and peak III, Equ c2.

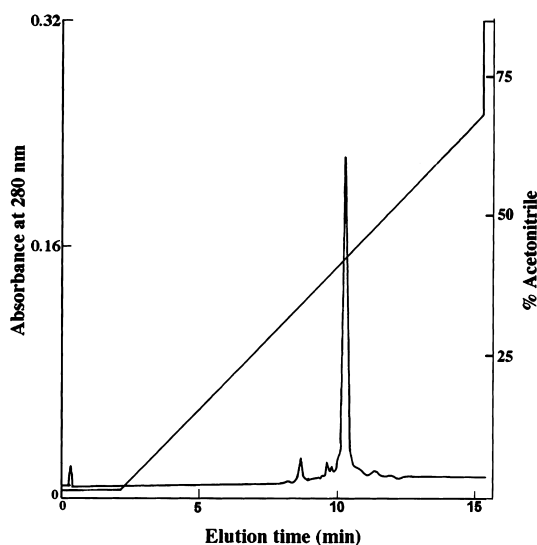

Fig. 3. Reversed-phase chromatography. Rechromatography of Equ c2 (fraction III of Fig. 2), was performed using Poros perfusion chromatography media (10 R1, 10 cm×4.6 mm I.D.) (PerSeptive Biosystems). Elution was performed using a linear gradient for 20 min from 10 to 90% acetonitrile–trifluoroacetic acid at a flow-rate of 2 ml min^{-1}; the eluate was monitored at 280 nm.

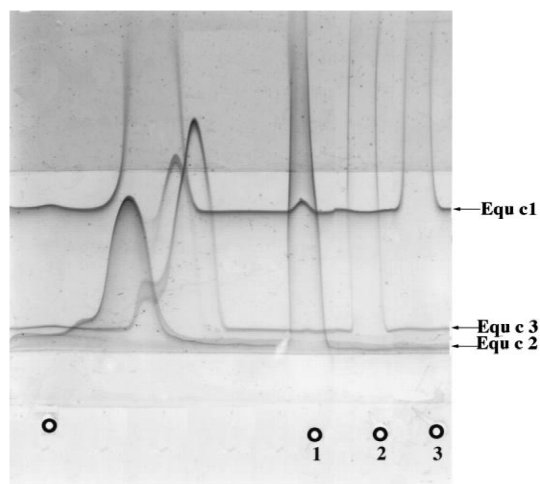

Fig. 4. Crossed line rocket immunoelectrophoresis. A first-dimension electrophoretic separation of the extract containing horse sweat proteins was performed. The separated proteins were then electrophoresed perpendicular to the first one into agarose gel containing rabbit serum raised against horse sweat extract. The antibody containing agarose gel was poured in two parts; the lower part contained 1 μl cm^{-2} and the upper part 4 μl cm^{-2}. An intermediate gel containing horse sweat proteins was interposed between the first-dimension and the second-dimension gel. Wells: (1) Equ c2; (2) Equ c3 and (3) Equ c1.

on reversed-phase column (Poros 10 R1, PerSeptive Biosystems). The pure Equ c2 was eluted from the column at around 42% acetonitrile (Fig. 3).

3.3. Analytical procedures

3.3.1. CLRIE

Fractions obtained after the chromatographic procedures were analysed by CLRIE (Fig. 4). Well No. 1 contained the Equ c2 fraction, No. 2 contained the Equ c3 fraction and No. 3 a sample of Equ c1. We can notice that each fraction gave rise to one rocket-like immunoprecipitate and that these peaks were not connected to any other peak, indicating a high degree of purity and a non-related antigenicity.

3.3.2. SDS and non-denaturing PAGE analysis

Fig. 5 illustrate the electrophoretic pattern of the purified proteins following the chromatographic procedures. In presence of SDS and under non-reducing conditions the M_r of Equ c2 was ≈17 000 (mean M_r of the double bands 17 400 and 16 800) and Equ c3 was ≈16 000. Under reducing conditions the 17 000 had a lower apparent M_r≈4500, whereas Equ c3 had a higher apparent M_r≈20 000. In absence of SDS (Fig. 6), Equ c2 appeared as only one band and Equ c3 showed to be the most negatively charged protein followed by Equ c2 and finally Equ c1.

3.3.3. Isoelectric focusing

Both purified horse sweat proteins had a p*I*≈3.8 showing their acidic nature (Fig. 7).

3.3.4. Immunoblotting

The immunoblot analysis showed that Equ c2 and Equ c3 were able to bind IgE antibodies from a pool of sera of horse-sensitive patients, and can be considered as allergens (Fig. 8).

3.3.5. SDS–PAGE and RLIE

Each of the double bands noticed in the electrophoretic pattern of Equ c2 (Fig. 5; ≈17 400 and ≈16 800), produced a peak and both of them were connected together by the same precipitated line (Fig. 9).

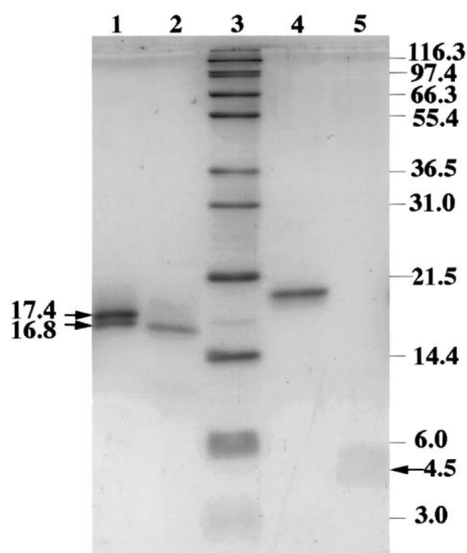

Fig. 5. Electrophoretic pattern of Equ c2 and Equ c3. Electrophoresis was performed on a 16% homogeneous gel containing SDS. Lanes 1 and 2, Equ c2 and Equ c3, respectively under non-reducing conditions; lanes 4 and 5 Equ c3 and Equ c2, respectively under reducing conditions; lane 3, proteins standards. Proteins were stained with silver ions.

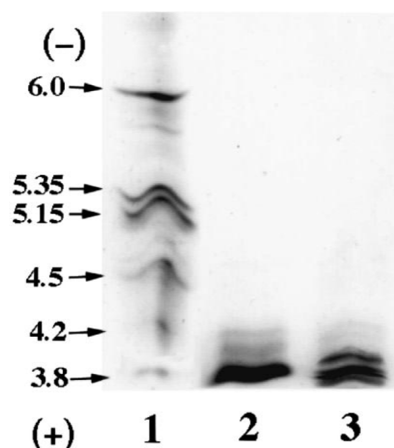

Fig. 7. Isoelectric focusing. IEF was performed in the pH range 3–10. Lanes: (1) Isoelectric point markers; (2) Equ c3; (3) Equ c2. The focused proteins were stained with Coomassie brilliant blue R250.

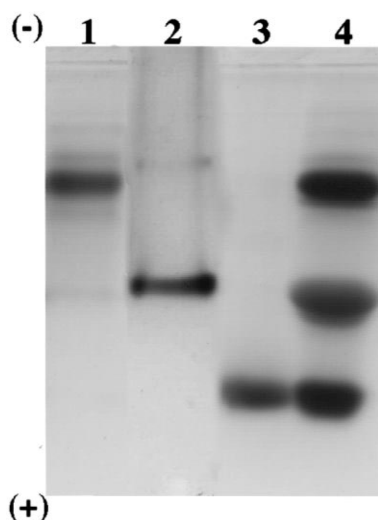

Fig. 6. Non-denaturing gel electrophoresis. Electrophoresis was performed on a 16% homogeneous polyacrylamide gel in absence of SDS. Lanes: (1) Equ c1; (2) Equ c2, (3) Equ c3 and (4) fraction S2b of Fig. 1.

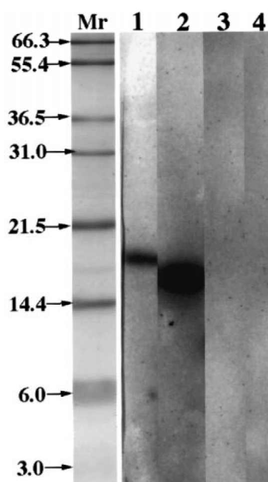

Fig. 8. Immunoblot analysis. Equ c2 and Equ c3 were electrophoresed on a 16% homogeneous polyacrylamide gel containing SDS. Proteins were transferred electrophoretically to a nitrocellulose membrane. The latter was then incubated with sera from horse-sensitized patients and IgE-binding antigens were detected with ^{125}I-labelled anti-human IgE antibody, followed by autoradiography. Lanes: (1) Equ c2; (2) Equ c3; (3) and (4) both allergens incubated with a pool of sera from non-allergic persons as control. Molecular mass markers are indicated.

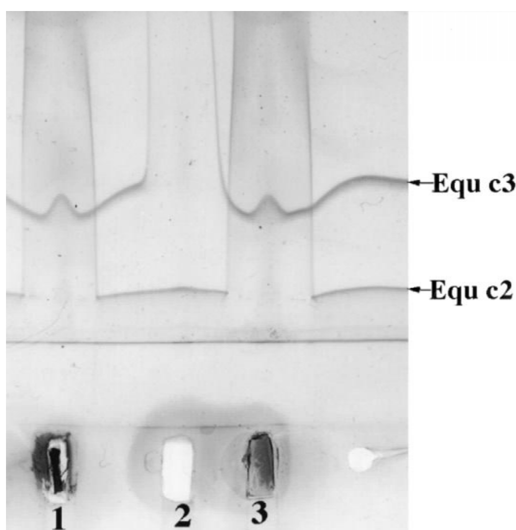

Fig. 9. Rocket line immunoelectrophoresis (RLIE). The double bands observed in the electrophoretic pattern of Equ c2 (≈17 400 and (16 800; lane 1 of Fig. 5) were cut out separately from the gel, and the extracted proteins were electrophoresed into agarose gel containing rabbit serum against horse sweat extract. An intermediate gel containing purified Equ c2 and Equ c3 was interposed between the agarose gel and the gel containing the rabbit serum. Wells: (1) The band ≈17 400; (2) Equ c3; and (3) the band ≈16 800.

4. Discussion

Animal allergen extracts, such as horse allergenic extract, are prepared from raw materials (e.g., hair, dandruff, epithelia and saliva). Several studies have been carried out to investigate the allergenic composition of horse hair/dander extracts [3–16]. In a previous study [6], we purified the major horse allergen Equ c1, whereas in the present study we purified the two allergens Equ c2 and Equ c3. The latter are not considered as major allergens since they elicited an IgE-mediated type-I allergic reaction in less than 50% of a selected group of patients. Since 33.3% of the tested sera reacted with the 17 000 allergen and 23.8% with the 16 000 allergen, both allergens were designated Equ c2 and Equ c3 respectively. Instead of using horse dander as the source of purification, as was done in the case of Equ c1, we preferred to use horse sweat to purify Equ c2 and Equ c3 because it contains not only higher concentrations of the proteins of interest, but also less non-proteinic material. We verified whether these two allergens were also present in horse sweat by means of rocket immunoelectrophoresis. In this technique, rabbit IgG antibodies raised against horse dander extract are also able to recognize the same proteins in horse sweat (results not shown).

Two SEC runs enabled us to obtain a rich fraction containing both proteins, Equ c2 and Equ c3. We further separated and purified both proteins by means of thiophilic adsorption chromatography. In this procedure, thiophilic matrices are shown to have a preferential affinity for immunoglobulins [19]. Immobilization of the horse sweat proteins was found to be dependent on the presence of water-structure-forming salts, where the breakthrough fraction was completely depleted of proteins, indicating selective binding to the thiophilic gel. The salt concentration we chose in the equilibration buffer was 2 *M* because in preliminary experiments, in which we used up to 1.5 *M* ammonium sulphate, we noticed that Equ c3 as well as the contaminating Equ c1 were eluted together within the same peak. Raising the salt concentration up to 2 *M* resulted in a higher retention of Equ c1 on the column leading hence to pure Equ c3 fraction. On the other hand, a small contaminating amount of Equ c1 was found in the peak containing Equ c2 fraction. This was removed by a further step on a reversed-phase column. These results showed that the ‘‘T-gel’’ was an efficient adsorbent not only for the purification of Equ c2 and Equ c3, but also that it was well suited for purifying the major horse allergen Equ c1. It is important to note that the purification procedures used did not alter the allergenic/antigenic activity of these proteins as demonstrated by the immunoblot experiment where IgE antibodies present in horse-sensitized patients exhibited a high reactivity towards Equ c2 and Equ c3. These results demonstrate that both proteins are allergens and that horse sweat is a source of allergens.

As analysed by SDS–PAGE, the apparent M_r of Equ c2 and Equ c3 are ≈17 000 and ≈16 000, respectively. Under reducing conditions, Equ c2 had a lower apparent M_r of ≈4500, suggesting it contains disulphide bonds formed between smaller polypeptide chains, maybe four. The double bands noticed in the electrophoretic pattern of Equ c2 (≈17 400 and ≈16 800) could represent an isomer. Different studies described the presence of many isoallergenic

forms such as tree pollen allergens and mite allergens [35]. We analyzed these two bands by means of rocket immunoelectrophoresis where each band produced one peak and both peaks were connected together by the same precipitated line, indicating that rabbit IgG antibodies recognized both bands as being antigenically related. In addition, non-denaturing gel electrophoresis showed that Equ c2 appeared as only one band and that Equ c3 showed to be more negatively charged than Equ c2. In the presence of reducing agent, Equ c3 had a higher apparent M_r of ≈20 000 suggesting that the tertiary structure of this protein could be maintained by intramolecular disulphide bond(s). Instead of ensuring complete unfolding and optimal interaction with SDS, the cleavage of these disulphide bonds within the polypeptide chain lead the protein to take a tertiary conformation where it could not be saturated with the detergent, as in non-reduced conditions. Consequently, the lower SDS binding resulted in a decreased charge-to-mass ratio leading to a decrease in mobility and hence yielding artifactually high molecular mass estimates. The acidic nature of both proteins was shown in IEF where their p*I* was ≈3.8.

Proteins interacting with the ‘‘T-gel’’ have been termed ‘‘thiophilic’’ in recognition of their affinity, through as yet unknown mechanisms, for the sulfone group in close proximity to a thioether in the ligand [19,20,36]. Like in hydrophobic interaction chromatography (HIC), a sufficient concentration of structure-forming salts (e.g., ammonium-, potassium-, sodium-sulphate) is required for the binding of solutes to the ‘‘T-gel’’. This is tentatively ascribed to conformational changes in the protein and/or ligand moiety creating the conditions necessary for their interaction. According to Porath et al. [19], the principle underlying adsorption to the thiophilic gel is different from that of HIC although high concentrations of structure-forming salts are necessary for efficient adsorption, but both methods belong to the group of salt-promoted chromatography [37,38]. The mechanism involved in the interaction of specific proteins with the ‘‘T-gel’’ as well as the thiophilic ligand acceptor site(s) is not known at this time [20,36]. However, aromatic amino acids on the surface of certain proteins are strongly implicated in such interactions [19,36,37] and the extent to which dipeptides of aromatic amino acids were bound to the ‘‘T-gel’’ was in the order $[Trp]_2 > [Phe]_2 > [Tyr]_2$ [37]. Consequently, we may postulate that the ‘‘T-gel’’ might interact primarily with aromatic side chains present in both allergens and that the high strength of binding of Equ c2 could be due to the high number of exposed aromatic amino acids. We may also speculate that Equ c2 is more ‘‘hydrophobic’’ than the other two proteins, Equ c1 and Equ c3, which might be due to a higher proportion of aromatic/aliphatic amino acids exposed on its surface. As far as we know, no studies have used this gel with such high concentrations of ammonium sulphate in the equilibration/elution buffer. By raising the salt concentration considerably, we would promote hydrophobic-like interactions, and salt may allow the protein, by displacement of water, to come into close proximity to the ligand (the sulfone-thioether group), where short-range interactions could play a role. It was suggested that an electron donor–acceptor or proton-transfer mechanism may be involved [35–37]. It can generally be assumed that the specificity of the interaction was inversely proportional to the concentration of salt where Equ c2 had the highest affinity towards the thiophilic matrix, followed by Equ c1 and finally by Equ c3.

In this report we have shown that (i) thiophilic adsorption chromatography has broader potential applications than those for which it has hitherto been used (i.e., immunoglobulins), and (ii) that the ‘‘T-gel’’ can also be efficient for the purification of other proteins, such as allergens, thus revealing their thiophilic properties. We propose that the applicability of such thiophilic gels could be extended to a mixed thiophilic/hydrophobic mode which, in certain situations, might be useful in separating closely related proteins.

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