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STUDIES ON ALLERGENS OF MAMMALIAN ORIGIN

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SUMMARY

Cat and dog saliva and extracts obtained from cat and dog hair have been examined by high-performance liquid chromatography (HPLC) to identify the likely allergenic molecules. Separation of the materials by size exclusion HPLC showed that several components in the range of apparent molecular weight from 35,000 to 200,000 had inhibitory activity in the radioallergosorbent test (RAST) when tested against pooled serum from donors hypersensitive to dogs and cats. One of these components active in our test system was identified as albumin. Despite differences in gross composition of the extracts no significant difference in the position of the active fractions was observed between cat hair and saliva, between dog hair and saliva or between hair extracts from different breeds of dog. Fractionation by anion-exchange HPLC, although promising, was complicated by problems of sensitivity.

INTRODUCTION

Between 10 and 30% of all patients with allergies display sensitivity to domestic animals, notably dogs and cats[1]. For some time, efforts have been made to determine whether the responsible allergens originate from the epidermis, saliva, serum or urine[2–4] and to assess individual cross-sensitivity to both dogs and cats[5,6]. However, most of these studies have depended on biological techniques and only a few attempts have been made to define the allergens in physico- and immuno-chemical terms[6–8]. We have previously reported[9,10] some investigations on insect and plant allergens and demonstrated that high-performance liquid chromatography (HPLC) has advantages over conventional chromatographic methods for identification within complex extracts of constituents possessing activity in the radioallergosorbent test (RAST), employed as an immunological screening procedure. In this paper, we describe the application of HPLC, primarily in the size exclusion mode, together with RAST, for the identification of potentially allergenic components in extracts of hair/dander and saliva from cats and dogs.

EXPERIMENTAL

Materials

Reagents were of the highest grade available commercially. Size exclusion col-

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umns were calibrated with a protein kit (Boehringer, Mannheim, F.R.G.) covering a molecular weight range from 12,500 to 450,000. Dog albumin powder was obtained from Sigma (Poole, U.K.). An extract of dog hair was prepared as follows. Unwashed dog hair (500 g mixed from several breeds) cut into short lengths, was immersed in 0.125 *M* ammonium bicarbonate (10 l, pH 8–9, containing 0.02% sodium azide) and stirred intermittently for 22 h at 4°C. After sieving to remove fibres, the extract was centrifuged (10,000 *g*, 1 h, 4°C) and the resulting supernatant filtered through Millipore filters (final pore size 0.45 μm). The filtrate was concentrated using an ultrafiltration cell (Model 8400, Amicon, Danvers, U.S.A.) and membrane UM 10 with a molecular weight cut-off at 10,000. Concentration took place under nitrogen, at approx. 5 bars, for 15 days at an initial flow of 4 ml/min. The concentrate (550 ml) was dialysed in two aliquots against 0.005 *M* ammonium bicarbonate (twice) and distilled water (once), pooled, submitted to membrane filtration (0.22 μm) and lyophilised in four portions. The weight of lyophilised material was 400 mg (0.08%).

Small samples of hair from individual breeds were processed similarly. Three commercial, lyophilised dog hair extracts were also examined. Dog saliva was obtained from a single anaesthetised beagle at the Clinical Research Centre (Harrow, U.K.) and was filtered (0.22 μm) before use. Lyophilised cat saliva and cat fur extract (code 79/554) were from this Institute.

Chromatography

Size exclusion. The chromatographic system has been described previously[9]. Separations were performed using a G3000 SW column (300 × 7.5 mm, Toyo Soda, Tokyo, Japan), 0.1 *M* sodium phosphate or 0.05 *M* ammonium acetate buffer pH 7.0 as eluent at a flow-rate of 0.5 ml/min, and a detection wavelength of 210 nm.

Ion-exchange. The chromatograph consisted of a Spectra-Physics SP 8700 solvent delivery system and SP 8750 organizer module, a Cecil 2112 UV monitor and Tekman TE 200 recorder. Separations were performed using an Aquapore AX 300 anion-exchange column (250 × 4.6 mm, Brownlee Labs., Santa Clara, CA, U.S.A.). The solvent gradient was formed from (A) 0.02 *M* sodium phosphate pH 7 and (B) 0.5 *M* sodium phosphate pH 7, with an increase from 0 to 50% B in 30 min and from 50% to 100% B in 10 min. The flow-rate was 1 ml/min and detection at 280 nm.

Electrophoresis

Isoelectric focussing. Ampholine polyacrylamide PAG plates pH 3.5–9.5 (LKB Produkter, Bromma, Sweden) were used according to the manufacturer's instructions and stained as described previously[10].

Immunological methods. Potencies of the extracts, and of fractions obtained from them by HPLC, were assessed by inhibition of the radioallergosorbent test (RAST)[10,11] and the results are expressed as percentage inhibition of binding. The human serum used was a lyophilised pool (coded 79/530) of sera from patients with known sensitivity to cats and dogs.

RESULTS AND DISCUSSION

Size exclusion HPLC has been used as a screening procedure to identify and concentrate the immunologically active components in extracts from dog and cat

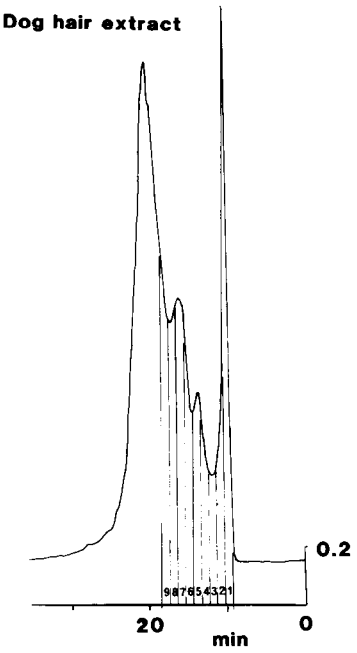

Fig. 1. Separation of dog hair extract by size exclusion chromatography. Conditions: column TSK G3000 SW, 300 × 7.5 mm; mobile phase 0.05 *M* ammonium acetate pH 7; flow-rate 0.5 ml/min; ambient temperature (approx. 20°C); UV detection at 210 nm. Amount applied: 500 μg.

hair/dander and saliva. The composition of individual fractions was further investigated by isoelectric focussing, immunoelectrophoretic methods and by ion-exchange chromatography.

Fig. 1, shows the size exclusion analysis of the extract of mixed breed dog hair. The HPLC profile indicates that the extract has a complex composition. To examine the components further, 500 μg of material were fractionated in three injections of 50 μl, using ammonium acetate as the buffer. The appropriate fractions were combined and lyophilised, the buffer volatilizing during the process. Isoelectric focussing (Fig. 2) of the whole extract and the fractions show that fractions 7, 8 and 9 contain several proteins and that fraction 5 gives a band corresponding to that of dog albumin.

The dog hair extract was also fractionated in 0.1 *M* sodium phosphate pH 7, giving a chromatogram shifted slightly to lower molecular size (Fig. 3a), and the fractions were tested directly by RAST. The higher the percentage RAST inhibition, the greater the binding of the IgE, indicating that the material has allergenic potential. Maximum RAST activity was found for peaks eluting at 15.5 and 18 min, although some inhibition was spread across several fractions. The individual RAST determinations have reproducibility of ± 10% and the phosphate buffer was found not to interfere. The active components had apparent molecular weights in the range 35,000–200,000. The elution position of the fraction containing most RAST activity corresponded to that of dog serum albumin (15.5 min).

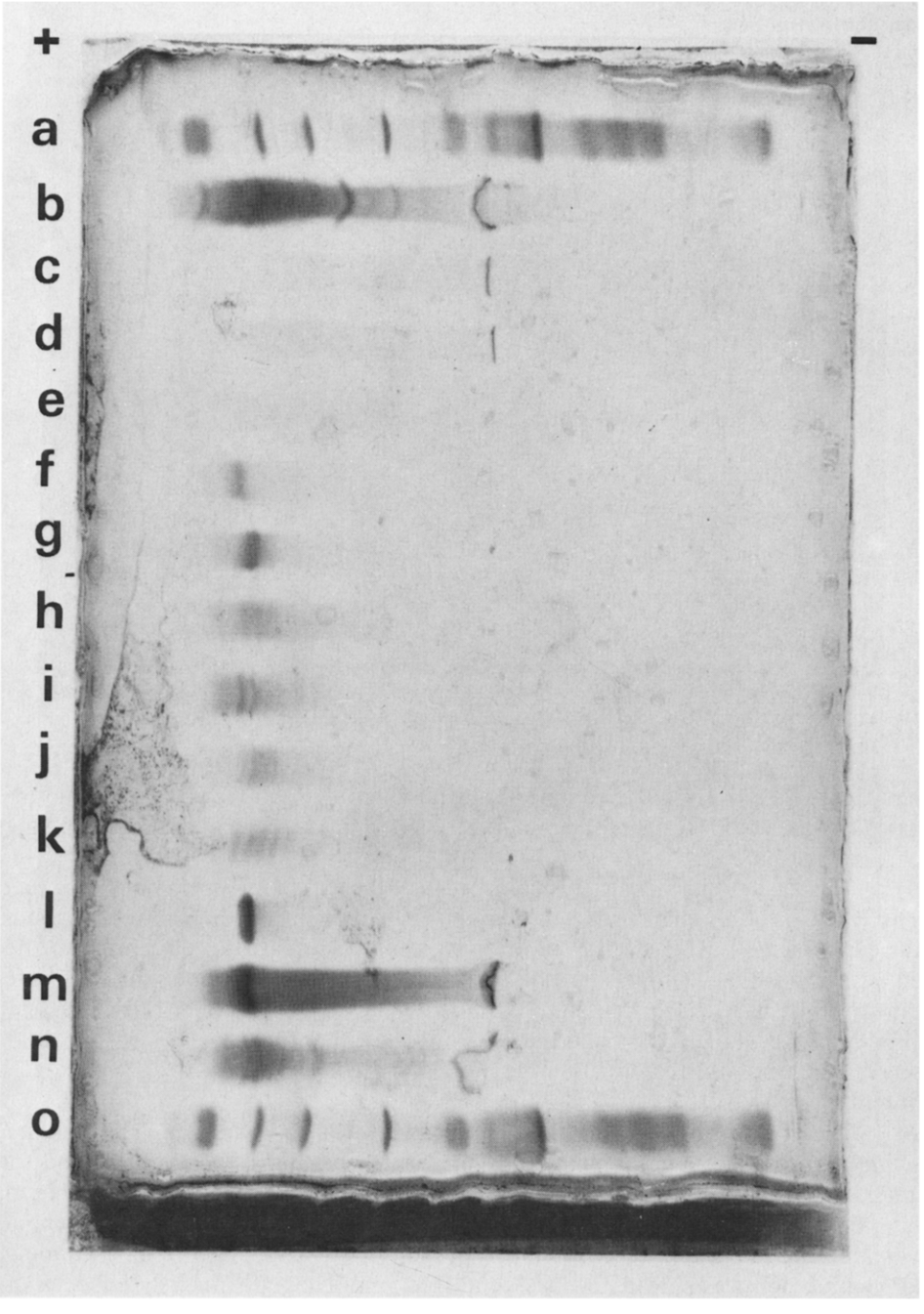

Fig. 2. Separation by isoelectric focussing of dog hair extract and fractions derived from size exclusion separation (Fig. 1). Conditions: Ampholine PAG plates pH 3.5–9.5. Samples: a and o = p*I* markers; b = dog hair extract; c = fraction 1; d = 2; e = 3; f = 4; g = 5; h = 6; i = 7; j = 8; k = 9; l = dog albumin; m = dog hair extract C; n = dog saliva.

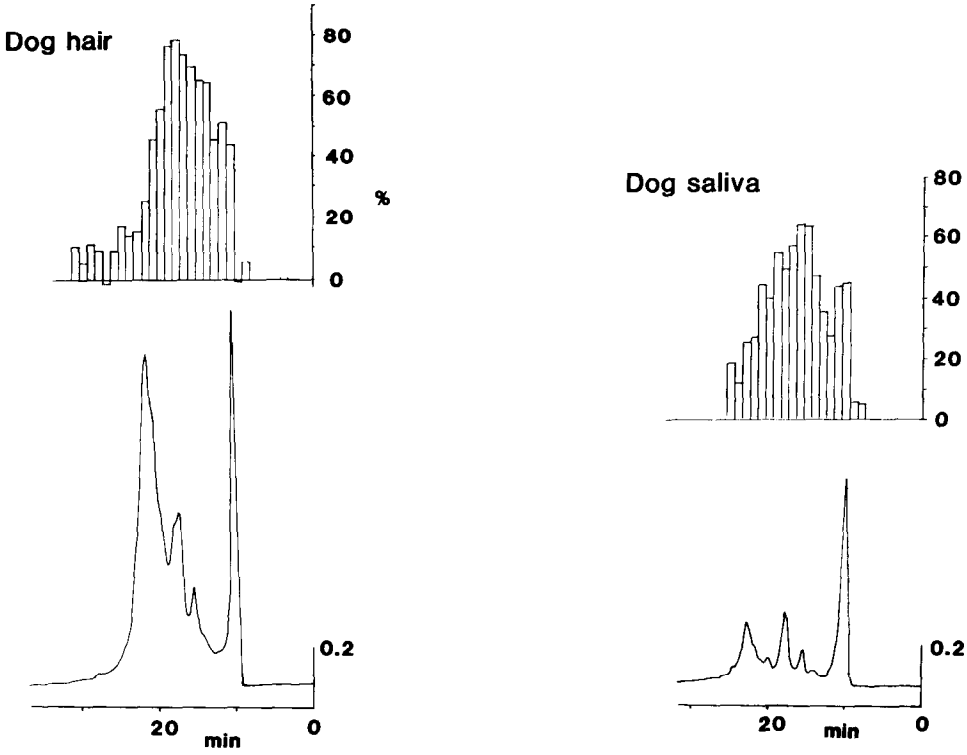

Fig. 3. Lower traces: high-performance size exclusion chromatography of dog hair extract (2 mg) and dog saliva filtered (50 μl). Conditions: 0.1 *M* sodium phosphate buffer pH 7. Other conditions as Fig. 1. Upper traces: percentage RAST inhibition of the fraction obtained from the column.

Dog saliva was filtered and injected directly onto the column, giving the chromatogram shown in Fig. 3b. Although significantly more high-molecular-weight material was present, maximum RAST activity was found in a similar position to that for the hair extract. From the animals' grooming behaviour, salivary components might be expected to appear in the fur.

Since albumin appeared to be present in the fractions showing highest RAST inhibitory activity, authentic dog albumin was also tested by this method and found to be highly active at concentrations ranging from 1 mg down to 10 ng per ml. However this albumin alone, even at high concentrations, was not capable of producing 100% inhibition of the binding between IgE antibody and dog hair allergens, indicating that dog hair extracts contain other molecules with IgE-binding activity. This is supported by observation of a number of additional precipitin bands in immunoelectrophoresis systems[12]. The presence of albumin in trace amounts in fractions adjacent to its main elution peak in the fractionated extract could have resulted in their raised RAST inhibiting activity.

Because of the heterogeneity of the size-exclusion fractions revealed by the charge-based separation (Fig. 2), attempts were made to separate the components by anion-exchange HPLC. (Fig. 4) However, the gradient employed produced a rising baseline and a less sensitive wavelength (280 nm) was necessary than that for the size

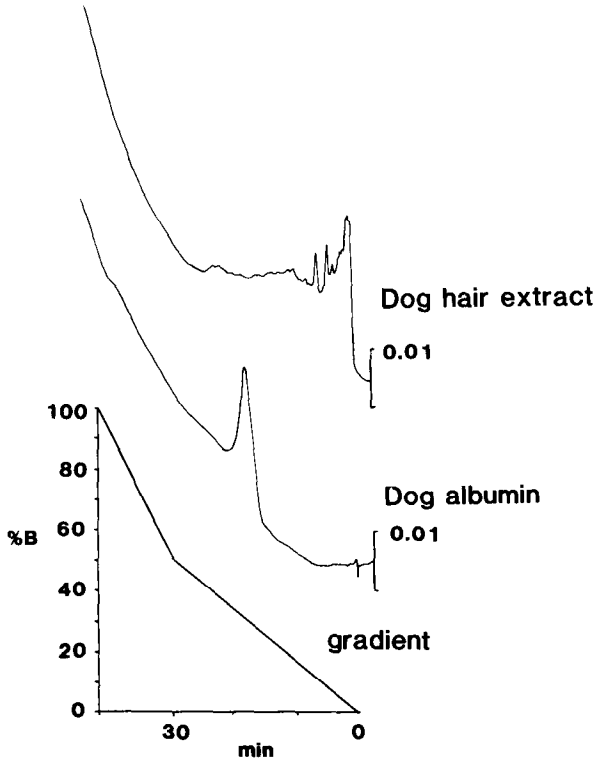

Fig. 4. Anion-exchange HPLC of dog hair extract (upper trace), and dog albumin (middle trace). Conditions: column Aquapore AX 300 25 × 4.6 mm; UV detection at 280 nm; ambient temperature (approx. 20°C); solvent system: A, 0.02 *M* sodium phosphate pH 7.0; B, 0.5 *M* sodium phosphate pH 7.0. Gradient composition shown in lower trace, flow-rate 1 ml/min.

exclusion procedure (210 nm), making detection of the individual peaks from the fractions difficult. However, the complexity of the whole extract is still apparent (Fig. 4, upper trace) since it produced a series of overlapping chromatographic peaks, preventing use of this procedure as the sole isolation step. Dog albumin alone eluted as a single peak (Fig. 4, middle trace).

Extracts of limited amounts of hair from individual breeds of dog were also examined, and the HPLC traces with appropriate RAST results are shown in Fig. 5. The results obtained are consistent with those for the mixed hair, and do not suggest that any significant inter-breed variation exists.

The complexity of the extracts from animal hair and the difficulties of interpreting the immunological results, particularly by RAST, have emphasised the need for a suitable reference material for calibration of assay systems. Three dog hair extracts from different sources, coded A, B and C, proved to have marked differences in composition (Fig. 6). C contained much more material of high molecular weight than A or B. The chromatographic fractions containing highest RAST inhibitory activity have been indicated and are comparable with those in the mixed hair extracts (Fig. 3a), since they contain several antigenic components, including albumin. The

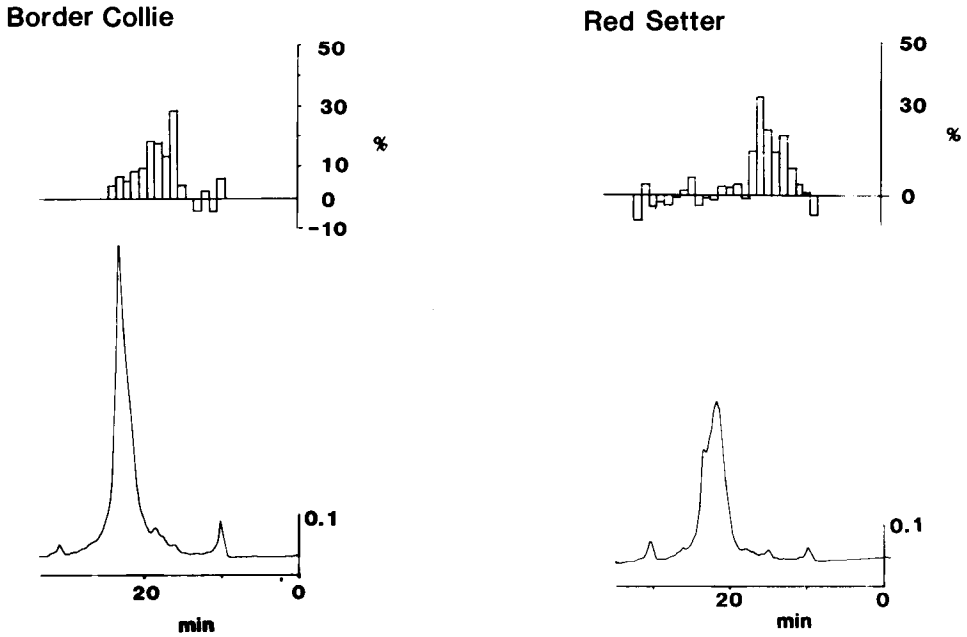

Fig. 5. Chromatographic comparison of hair extracts from individual breeds of dog: (a) border collie, (b) red setter. Conditions as Fig. 1. Upper traces show percentage inhibition by fractions in the RAST.

extent to which other material, of varying composition from one to the other sample, may influence the immunological behaviour is not yet known.

Fig. 7 shows the behaviour of cat fur extract on size exclusion and the corresponding RAST results. The cat fur yielded a high proportion of material of low molecular weight. Maximum RAST activity was obtained for peaks at 16 and 18.5 min. Examination of cat saliva (not shown) indicated RAST activity in similar fractions to these of cat fur. The peak at 16 min eluted in the position expected for cat albumin, based on its known molecular weight. Other authors have found the major cat dander allergen (cat allergen 1) to have the electrophoretic mobility of pre-albumin and a molecular weight of 30,000–60,000 and consider cat albumin itself to represent a minor allergen[8,13].

CONCLUSIONS

The combination of size exclusion HPLC and RAST is a useful means of screening extracts for immunologically-reactive components and for their preliminary separation. In extracts as complex as those from animal hair or saliva, further procedures are necessary to isolate individual components. Although anion-exchange HPLC would appear to be a good choice for this purpose, practical difficulties arise because of limitations in sensitivity imposed by gradient elution. The results obtained nevertheless indicate that albumin is an important antigen in our assay method, and is apparently present in all the extracts from cat and dog hair and saliva examined. Other active components are present with apparent molecular weights in the range

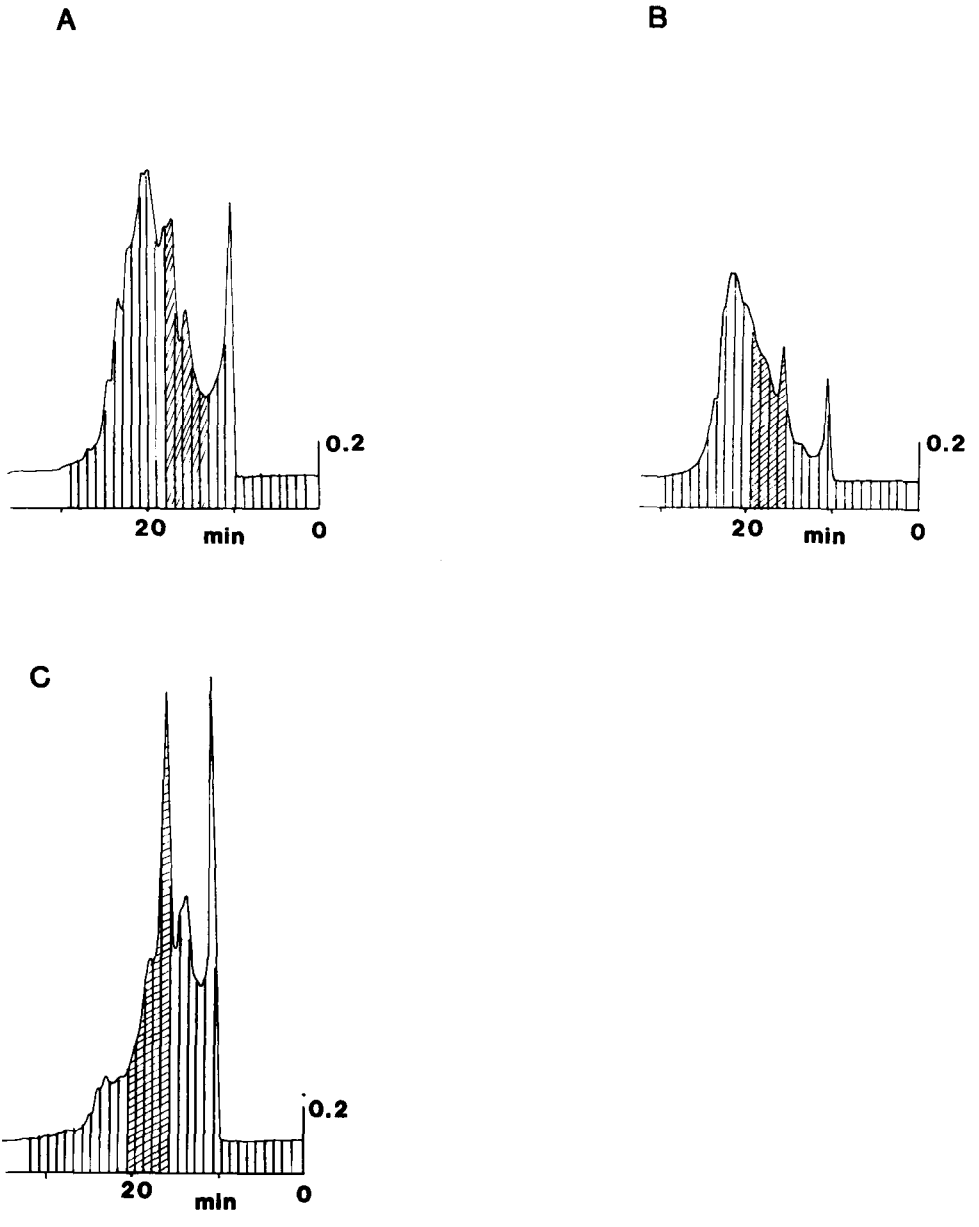

Fig. 6. Chromatographic comparison of three dog hair extracts. Conditions as Fig. 1. The shaded areas indicate fractions with highest inhibition in the RAST. Amounts applied: (A) 200 μg, (B) 250 μg, (C) 350 μg.

35,000–200,000. The gross composition of extracts from hair and saliva varies but the active fractions show consistent behaviour, providing no evidence for significant difference between breeds of dog or between hair and saliva.

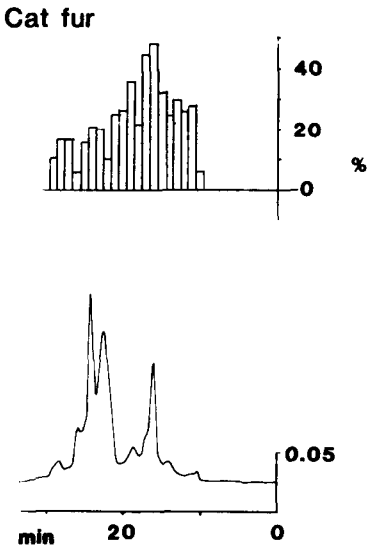

Fig. 7. Lower trace: high-performance size exclusion chromatography of an extract of cat fur. Conditions as Fig. 3. Upper trace: percentage RAST inhibition of fractions.

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