

Molecular analysis of allergenic proteins in bovine dander

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An analytic procedure was established to characterize bovine dander proteins with allergenic properties. The proteins from dander extract were separated by size-exclusion gel filtration, and the fractions were studied with SDS–PAGE followed by immunoblotting. An 11-kDa allergen was found in the same gel filtration fractions as 20- and 22-kDa allergens, and this suggests that the 11-kDa allergen is a dimer in its native form. Our method also detected two separate 22-kDa allergens. The primary structure of the major bovine dander allergen (BDA20) was also studied. A protein sequencer was used to determine the amino acid sequences of enzymatically cleaved peptides. The homology searches revealed that BDA20 is not a previously known bovine protein.

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Bovine dander is a source of potent allergens responsible for various occupational allergic disorders. Numerous allergenic proteins have been identified in bovine dander extracts (1, 2). On the basis of the early studies of Prahl et al. (3), the two main allergens were named as Bos d 1 (molecular mass 24–25 kDa, pI 3.6) and Bos d 2 (20–22 kDa, pI 4.2). In our patients with cattle-associated asthma, the highest prevalences of IgE antibodies were found against 20- and 22-kDa proteins, as determined by immunoblotting after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The relationship of these proteins to Bos d 1 and Bos d 2 is not yet known, although certain similarities between Bos d 2 and bovine dander allergen 20 (BDA20) were observed (4, 5).

Cloning of cDNAs coding for allergens is an approach to the detection of the primary structures of allergens. Using this strategy, we have already been able to define one of the minor allergens in bovine dander as oligomycin-sensitivity-conferring protein of the ATP synthase complex (6). To associate a cDNA clone with an allergen detected by immunoblotting, one must purify the native protein to the level allowing determination of the primary protein structure. Our preliminary tests showed that SDS–PAGE did not resolve bovine dander proteins well enough for amino acid sequencing. In order to improve the purification of dander proteins and to

keep the procedure as simple as possible, we decided to combine a separation based on size-exclusion gel chromatography with conventional SDS–PAGE. Proteins separated by this procedure were subjected to amino acid sequence analysis. Our experiments produced new information on the molecular arrangement of bovine dander allergens. We also present in this paper the amino acid sequences of three enzymatically cleaved peptides from the major bovine dander allergen BDA20.

Material and methods

Antigen preparation

Bovine dander antigen (BDA) extract was prepared essentially as described previously (5). The freeze-dried raw material was purchased from Allergon AB (Ängelholm, Sweden). The protein concentration of the antigen preparation was measured, according to the method of Lowry et al., with bovine serum albumin as a standard (7). BDA extract was lyophilized and stored at –20°C.

Gel-filtration liquid chromatography

Lyophilized BDA extract was dissolved in phosphate-buffered saline (PBS). A 2-ml sample of BDA extract was injected into the XK 16/70, Superdex 75 column (Pharmacia Biotech, Uppsala, Sweden).

PBS was used as the eluent, and HiLoad™ Pump P-50 (Pharmacia Biotech) was used to control the flow rate (1 ml/min). The monitoring system was Uvicord III, LKB, Sweden. Forty 2-ml fractions were collected, starting from the first peak.

Gel electrophoresis

SDS-PAGE was carried out in a 15% acrylamide gel with the discontinuous buffer system of Laemmli (8). Briefly, a 50- μ l sample from each fraction was reduced with 30 μ l of the sample buffer containing SDS and 2-mercaptoethanol, and the samples were boiled for 10 min. The samples and Pharmacia Low Molecular Weight calibration kit standards (Pharmacia Biotech) were loaded to the gels, and electrophoresis was carried out at 200 V for 45 min. The gels were stained with Coomassie brilliant blue.

The SDS-PAGE-separated proteins were transferred to nitrocellulose membrane (Millipore, 0.45 μ m, Bedford, MA, USA) by the method of Towbin et al. (9). The protein transfer and the detection of IgE- and IgG-binding bands were carried out as described previously (10).

Affinity chromatography

Monoclonal antibodies produced as described previously (5) were purified from the ascites fluid with a HiTrap Protein G affinity chromatography column (Pharmacia Biotech).

A HiTrap NHS-activated affinity column (Pharmacia Biotech) for purifying BDA20 from BDA extract was prepared according to the manufacturer's instructions. Briefly, a HiTrap NHS-activated 1-ml column was washed with 6 ml of ice-cold 1 mM HCl, and immediately 4 ml of purified antibodies (10 mg/ml) in the coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3) were injected into the column. The antibody solution was pumped back and forth for 30 min at room temperature with two syringes. The coupling solution was washed away with 3 ml of the coupling buffer. The excess active groups that had not coupled to the ligand were deactivated, and the nonspecifically bound ligands were washed away with three cycles of 6 ml 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3, followed by 6 ml 0.1 M acetate, 0.5 M NaCl, pH 4. Finally, 6 ml of PBS, pH 7.0, was injected to equilibrate the column.

BDA solution diluted 1:3 in PBS was passed three times through the column. The column was washed with 10 ml PBS followed by 10 ml PBS with a higher NaCl concentration (0.5 M). Bound protein was eluted with 100 mM glycine-HCl, pH 2.5. The BDA20-containing eluate was collected in 0.5-ml fractions and neutralized by 50 μ l of 1.0 M

Tris HCl, pH 8.0. Absorbance was measured at 280 nm and protein-containing fractions were pooled. Tris and glycine were removed with NAP-25 gel filtration columns (Pharmacia Biotech). The preparation was filtered through a 0.22- μ m filter and stored at -70°C .

N-terminal sequencing of proteins

Fractions 16–18 from gel filtration were pooled and concentrated by ultrafiltration (Centricon 3, Amicon, Beverly, CA, USA) to the final volume of 400 μ l. SDS-PAGE was carried out as described above. Proteins from SDS-PAGE gel were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore) at 100 V for 30 min. The membrane was stained with 0.1% Coomassie brilliant blue and destained with 50% methanol. Stained 20-kDa bands from six lanes were excised with a scalpel and allowed to dry at room temperature. Applied Biosystems 477A Pulsed Liquid Phase protein sequencer was used to determine the N-terminal amino acid sequence of protein.

Proteolytic digestion and amino acid sequencing of BDA20

Affinity-purified BDA20 was further purified with the gel-filtration equipment described previously. Fractions at the highest peak were pooled, and the sample was desalted with NAP-25-column. Lyophilized BDA20 (100 μ g) was reduced with dithiotreitol, carboxymethylated with iodoacetamide (11), and cleaved with endoproteinase Lys-C (Boehringer Mannheim, Germany) at enzyme/substrate mass ratios 1:25 for 12 h at 37°C in 0.4 M NH₄HCO₃, pH 8.0. Generated peptides were separated by reversed phase high-performance liquid chromatography (RP-HPLC) (Vydac C-18 column) with a linear acetonitrile gradient (0–60% acetonitrile in 0.1% trifluoroacetate) and lyophilized. Applied Biosystems 477A pulsed liquid phase protein sequencer was used to determine amino acid sequences of the peptides.

Results

Separation of bovine dander proteins

Bovine dander antigen extract contains numerous proteins which are not well resolved in conventional SDS-PAGE. For separation of the proteins first according to their sizes in the native form, bovine dander antigen (17.6 mg) was injected into the gel-filtration column. The elution curve of the absorbance at 280 nm and the location of standards are shown in Fig. 1. The fractions were examined with SDS-PAGE (Fig. 2A). Most proteins were in fractions 10–20. Despite the high peak of

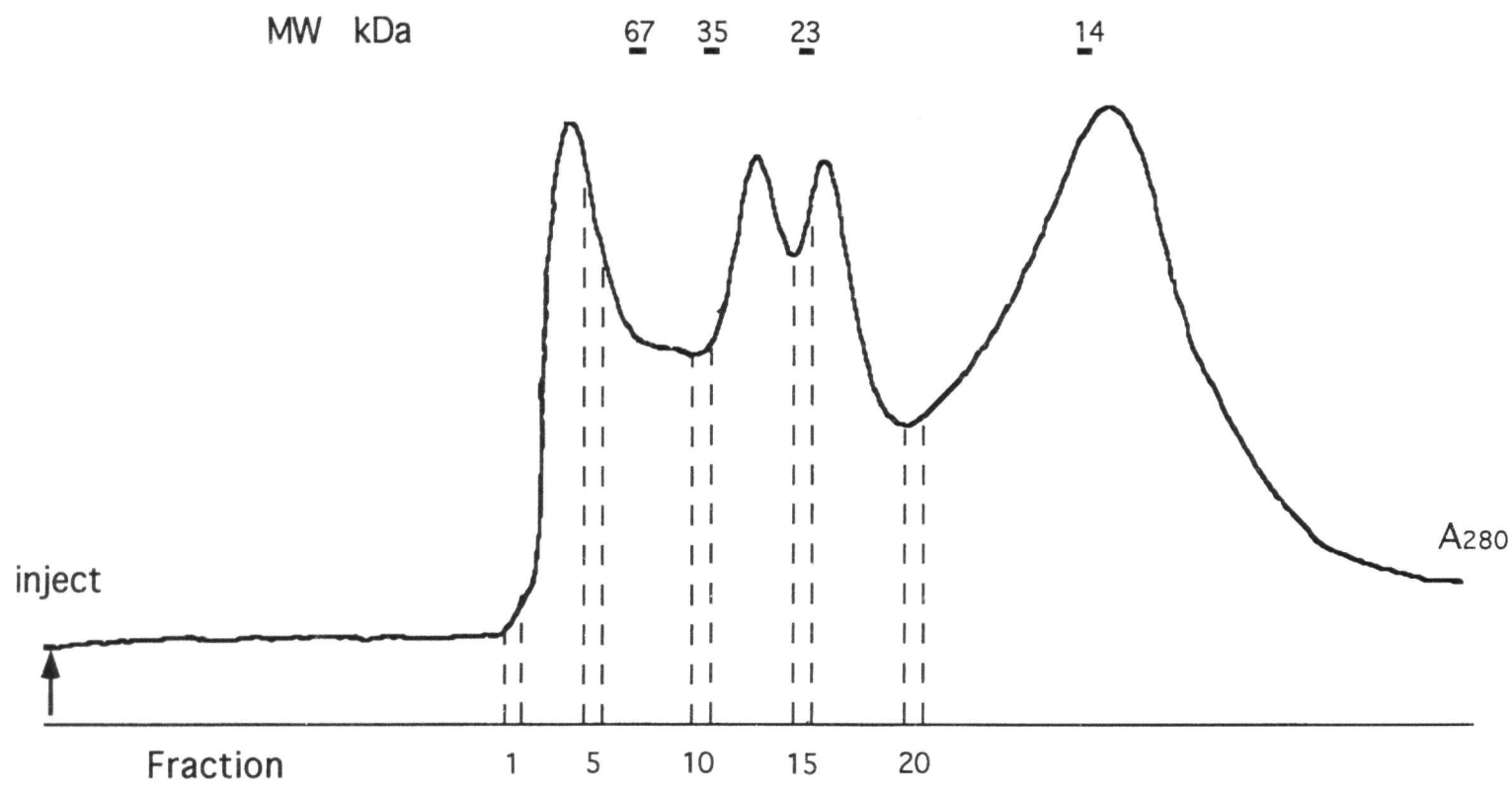


Fig. 1. Elution curve of bovine dander antigen (BDA) in size-exclusion gel filtration: 17.6 mg BDA was injected into column previously calibrated with following markers: bovine serum albumin (67 kDa), β -lactoglobulin (35 kDa), chymotrypsinogen (23 kDa), and lysozyme (14 kDa).

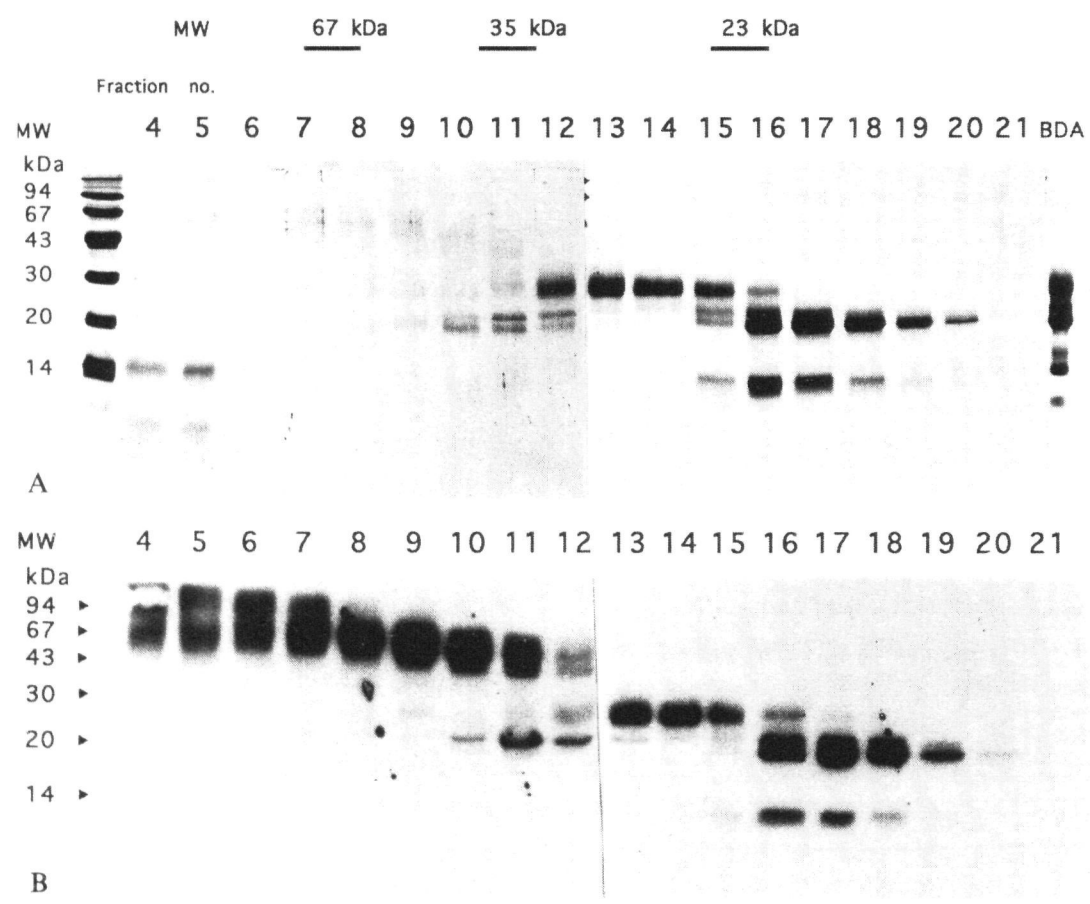


Fig. 2. A) Coomassie brilliant blue-stained SDS-PAGE gel. Lanes from left: MW standards (Pharmacia), fractions 4–21 from gel filtration and bovine dander antigen (BDA) (50 μ g protein/lane). Bars above fraction numbers indicate location of calibration markers in gel filtration. B) IgE immunoblot with serum from allergic patient. Samples and their order are identical with those shown in Fig. 2a.

absorbance corresponding to the size of 14 kDa, no protein was detected in these fractions (data not shown). Protein bands of 20 and 22 kDa by SDS-PAGE were found in both fractions 10–12 and 15–18. By gel filtration, these fractions would contain proteins of 35–40 and 20–22 kDa, respec-

tively. In the same way, an 11-kDa protein was found in fractions 15–18 corresponding to the size of about 22 kDa by gel filtration. A protein of 24 kDa by SDS-PAGE was eluted in gel filtration in fractions expected of proteins of that size (fractions 12–16).

Immunoblotting

To detect the IgE reactivity of proteins in different gel-filtration fractions, allergic patients' sera were used for IgE immunoblotting after SDS-PAGE. IgE from a highly allergic patient's serum reacted with numerous proteins of 11, 20, 22, 24, and >30 kDa (Fig. 2B). IgE reacted with the two 22-kDa proteins in fractions 10–12 and 15–18, respectively.

We have produced earlier a series of monoclonal antibodies against BDA20 (5). In this study, the reactivity of one of the antibodies against proteins in the gel-filtration fractions was also tested. There was a strong binding on the 20-kDa protein band in fractions 15–19 and a weak binding on two bands in fractions 10–12 which correspond to molecular masses of 35–45 kDa in SDS-PAGE (data not shown).

Amino acid sequencing analysis

According to our previous findings, BDA20 is the predominant bovine dander allergen. Because of the reactivity with the anti-BDA20 monoclonal antibody, it seemed likely that the 20-kDa protein in fraction 17 corresponded to the BDA20 allergen. The N-terminal amino acid sequencing of the 20-kDa protein was attempted from the concentrated gel-filtration fraction 17 without success, probably because the N-terminus of the protein was blocked. Sequencing attempts of the N-terminus of affinity-purified BDA20 produced the same result. Since it is important to determine the primary structure of BDA20, which we consider the predominant allergen in bovine dander, another approach was taken. A highly purified preparation of BDA20 was cleaved with endoproteinase Lys-C, and the peptide fragments were separated by RP-HPLC (Fig. 3). Two peptides of 22 and 12 residues, respectively, from well-separated peaks nos. 1 and 3 were sequenced (Table 1). In peak no. 2, two different sequences were obtained. One of the sequences was identical to the sequence from peak no. 1, and the other one had four residues. A sequence homology search using the BLAST program was done in the nonredundant (nr) protein sequence database (PDP, SwissProt, PIR, and GenPept databanks) (12), but no significant homologies between the BDA20 peptides and previously known bovine protein sequences were found.

Discussion

In this report, we describe the results of the application of a simple two-step procedure (gel filtration and SDS-PAGE) to obtain an expanded view of allergens present in bovine dander. We also present

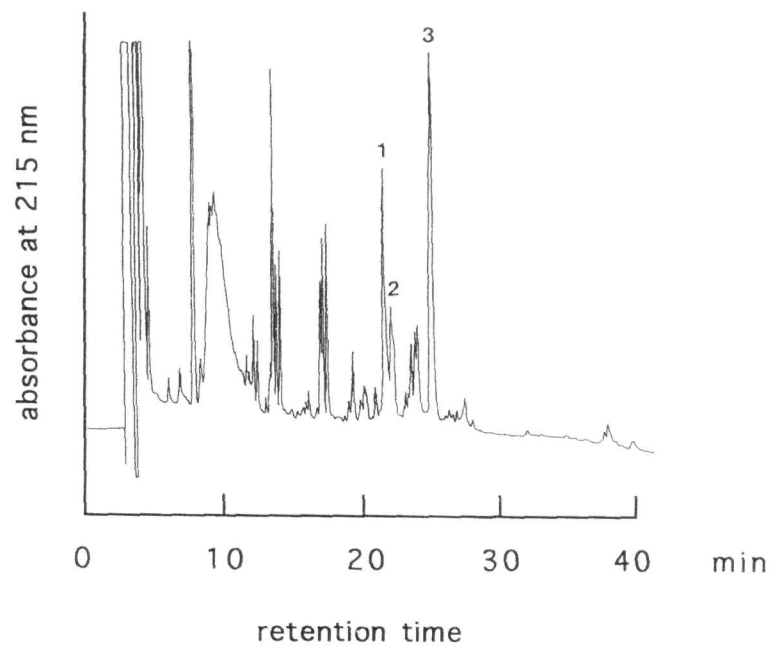


Fig. 3. RP-HPLC peptide map of endoproteinase Lys-C-digested BDA20. Peaks 1–3 were sequenced. Results are shown in Table 1.

Table 1. Amino acid sequences of three peptides from Lys-C-treated BDA20

Peptide 1	NH ₂ -YQQLNSERGVNENIENLIKTD
Peptide 2	NH ₂ -DQGT
Peptide 3	NH ₂ -IPGEWRIIYAAA

amino acid sequences of three peptide fragments from the major bovine allergen, BDA20.

IgE immunoblotting with patient sera is a common technique to detect allergens; the prevalence of IgE antibodies against a protein, as detected by immunoblotting, is used to define the "major" and "minor" allergens (13–15). We have earlier analyzed the allergen pattern of cow dander extracts revealed by immunoblotting after separation in SDS-PAGE (2). However, if allergens originate from a source such as cow dander containing numerous allergens, separation according to the size of the denatured polypeptides by SDS-PAGE does not necessarily reveal all allergenic components.

Two-dimensional (2-D) electrophoresis is a more powerful tool in resolving individual allergens present in a mixture. Proteins can be separated according to both the pI value and size of the denatured polypeptides (16–18). However, the minimal yield of individual proteins from a 2-D gel is a limiting factor for the subsequent analyses, and that was one of the reasons that we sought alternative methods. Depending on the size of the column, tens of milligrams of proteins can be fractionated by gel filtration according to their sizes in a nondenatured form. When this is followed by a preparative SDS-PAGE, it is possible to isolate enough of pure proteins for further studies.

The analysis of gel-filtration fractions of the cow dander extract by SDS-PAGE revealed a more complex pattern of allergens than that shown by

SDS-PAGE alone. One of the observations was the appearance of an allergen of 11 kDa in gel-filtration fractions corresponding to a molecular mass of about 22 kDa. The most likely explanation is that this allergen is present in the BDA extract as a dimer. Another interesting observation was the appearance of 22-kDa allergens in gel-filtration fractions of 35–40 and 20–22 kDa. At the moment, we do not know whether this is the same polypeptide occurring in bovine dander both as a monomer and as a homodimer, or as a heterodimer with a non-allergenic protein. A well-characterized example of allergens comprising different polypeptides is Fel d 1. It is a tetrameric molecule made up of two light chains (α -chain or chain 1) and two heavy chains (β -chain or chain 2) (19, 20).

It would have been important to determine the N-terminal amino acid sequence of the allergenic proteins because that would have determined the beginning of the coding site in cDNA. Unfortunately, we were not able to determine the N-termini of either the 20-kDa allergen (BDA20) or the 11-kDa allergen. Modification of the N-terminal amino group with different factors is very common; up to 50% of all proteins may be blocked in this way (21). However, by using another technique, we were able to determine the amino acid sequences of three internal peptides of BDA20.

The BLAST nr homology search revealed that BDA20 is not a previously known bovine protein. We have recently constructed from cow skin a cDNA library which contains numerous clones coding for allergens, including one encoding BDA20 (6, 22, Mäntyjärvi et al. submitted). By combination of the amino acid sequence data with the nucleotide sequences of cDNA clones, new information about the structure and function of bovine dander allergens will be obtained.

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