

Affinity purification of the major bovine allergen by a novel monoclonal antibody

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Abstract

A monoclonal antibody was developed to the 20 kd major allergen of cow by immunizing mice with crude dander extract. The monoclonal antibody did not exhibit cross-reactivity to cat, dog, and horse dander extracts when studied by ELISA inhibition. The antibody was used in affinity chromatography for the purification of the 20 kd allergen from cow dander extract. Purity of the allergen was estimated to be 88%, and allergenic reactivity was verified by IgE immunoblotting and skin prick tests. After further purification with size-exclusion chromatography, the allergen was almost 100% pure. The isoelectric point of the double-purified allergen was determined to be 4.1. The amino acid composition was characterized by the predominance of acidic amino acids. (J ALLERGY CLIN IMMUNOL 1994;93:851-8.)

BDA	Bovine dander antigen
BDA20	Twenty kilodalton component of bovine epithelial extract
CIE	Crossed immunoelectrophoresis
HPLC	High-pressure liquid chromatography
mAb	Monoclonal antibody
PBS	Phosphate-buffered saline
pI	Isoelectric point

Bovine allergens are significant inducers of occupational allergic diseases in the Finnish farming population.¹ The major allergens originate from the epithelium, and their molecular weights are in the range of 16 to 24 kd.²⁻⁴ Although three of the major bovine allergens were characterized and purified more than 10 years ago by Prahl et al.,² they have not been widely used for diagnostic or therapeutic purposes. Because the molecular weights and the isoelectric points (pIs) of cow allergens are very similar, purification has been difficult, and only limited amounts of pure allergen have been obtained.

Monoclonal antibodies (mAbs) have been demonstrated to be a powerful tool in allergy research. They have been used in epitope mapping^{5, 6} and in the purification and quantification of allergens in crude extracts.^{7, 8} They have also been used for measurement of allergens in environmental samples.⁹ Monoclonal antibodies appear to be especially efficient tools in the standardization of allergenic extracts.¹⁰ It has been recently suggested that the major allergen content of allergen preparations expressed in weight units could replace the use of biologic units as the measure of the potency.¹¹

In this report we describe the development of an mAb to the 20 kd component of bovine epithelial extract (BDA20). This component has been identified as the major allergen of cow by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with the sera of patients who have cow-induced asthma.⁴ The developed mAb was used for the purification of the 20 kd allergen. Almost 100% purity was achieved by including an additional purification step of size-exclusion high-pressure liquid chromatography (HPLC).

Methods

Antigen preparation

Bovine dander antigen (BDA), horse dander antigen, dog dander antigen, and cat dander antigen were prepared essentially as described previously.⁴ The freeze-dried raw materials were purchased from Allergon AB (Angelholm, Sweden). The materials were defatted in diethyl ether (1:10 wt/vol) at 4° C for 24 hours, dried at room temperature, and extracted then for 24 hours at room temperature in Coca's solution, pH 7.2 (1:10 wt/vol; 0.5% sodium chloride, 0.275% sodium hydrogen carbonate, 0.4% phenol). After centrifugation at 36,600g for 30 minutes, the supernatants were dialyzed (molecular weight cutoff = 3500) for 24 hours at 4° C against distilled water, lyophilized, and stored at –20° C. The protein concentrations of the antigen preparations were measured according to the method of Lowry et al.¹² with bovine serum albumin as a standard.

Monoclonal antibodies

BALB/c mice were immunized three times intraperitoneally with 100 µg BDA at 2-week intervals. Freund's complete adjuvant was used for the first injection, and Freund's incomplete adjuvant was used for the second injection. The last injection was given without adjuvant, and the mice were killed 4 days later. Monoclonal antibodies were produced according to the method of Galfre et al.¹³ Briefly, spleen cells of immunized mice were fused with NSI-1 mouse myeloma cell line with the use of polyethylene glycol (molecular weight = 1500). Fused cells were dispersed to several 96-well plates and cultured in Dulbecco's minimal essential medium supplemented with glutamine, penicillin, streptomycin, 10% fetal calf serum (Gibco, Grand Island, N.Y.) and 1 × hypoxanthine, aminopterin, and thymidine. Hybridoma supernatants were tested with ELISA and immunoblotting methods. A hybridoma-secreting antibody to the 20 kd component was cloned twice by the limiting dilution method. To make large amounts of the mAb, the cloned hybridoma was grown in the peritoneal cavities of BALB/c mice according to the method of Mueller et al.¹⁴ Mice were injected intraperitoneally with 0.5 ml of Freund's incomplete adjuvant. After 2 weeks, 10⁶ antibody-secreting hybridoma cells were injected intraperitoneally. After 1 week, ascites was collected three times at

3-day intervals. The antibodies were purified in a Protein-A Sepharose column (Pharmacia, Uppsala, Sweden) according to the method of Ey et al.¹⁵

The animal experiments were performed according to the guidelines of European Conventions for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg 18 March 1986).

ELISA

A microELISA plate (Nunc-Immuno Plate I; A/S Nunc, Roskilde, Denmark) was coated for 2 hours at 37° C with BDA at a concentration of 1 µg/ml of protein in a coating buffer (0.05 mol/L carbonate buffer, pH 9.6) in volumes of 100 µl/well. Next, the plate was washed and incubated for 2 hours at 37° C with a diluent (phosphate-buffered saline [PBS] containing 0.5% [wt/vol] Nonidet P40 [Merck Ltd., Diagnostics Div., Dorset, England] and 0.1% [wt/vol] casein [Merck Ltd., Diagnostics Div.]). After incubation, the plate was stored at –70° C. Before use, the plate was allowed to reach room temperature and was then washed. Next, 100 µl of 1:2 diluted hybridoma supernatant samples was added to the wells. Mouse anti-BDA antiserum was used as a positive control in each plate at a dilution of 1:1000. The ELISA plate was then incubated at 37° C for 1 hour. After washing, the substrate solution (tetramethylbenzidine, 0.1 mg/ml; Merck, Darmstadt, Germany) and urea hydrogen peroxide (BDH Chemicals Ltd.), corresponding to 0.05% H₂O₂ in 0.1 mol/L sodium acetate buffer (pH 6.0), were added, the reaction was stopped with 2 mol/L H₂SO₄, and the absorbance was measured at 450 nm with a Labsystems Multiskan Plus spectrophotometer (Labsystems, Helsinki, Finland).

Cross-reactivity of the mAb against horse and dog dander extracts was studied by ELISA inhibition. Briefly, an ELISA plate was coated with BDA20 at a concentration of 0.5 µg/ml. Several dilutions of the inhibiting extracts and 1:1000 diluted mAb were combined in a test tube and were then added to the ELISA plate. BDA20 and BDA were used at concentrations varying from 3.9 to 4000 ng/ml; and horse, dog, and cat dander antigen concentrations varied from 391 to 100,00 ng/ml. After 1 hour at 37° C, the plate was washed and incubated with the diluent. The bound mAb was detected as described above.

Western blot analysis

Antigens were first separated by means of SDS-PAGE (12.5% gel) with the discontinuous buffer system of Laemmli.¹⁶ The separated components were transferred to a nitrocellulose membrane (Millipore 0.45 µm; Millipore Corp., Bedford, Mass.) according to the procedure of Towbin et al.¹⁷ After transfer, the nitrocellulose membrane was blocked in PBS, pH 7.3, containing 0.1% casein, 0.05% Tween 20, and 0.05 wt/vol NaN₃ for 1 hour at 37° C. The membrane was cut into strips and incubated overnight at room temperature in the 1:10 diluted medium of ELISA-positive hybridomas. The strips were washed in Tween-PBS four times for 5 minutes and were then incubated in 1:200 diluted rabbit anti-mouse (Dako Z109; Dako Corp., Carpinteria, Calif.) for 3 hours. After an additional wash, strips were incubated for 3 hours with iodine 125–labeled anti-rabbit IgG from donkey (Amersham IM143; Amersham International, Amersham, UK). After washing, the reactive bands were detected with autoradiography. For human IgE immunoblots, strips were incubated overnight in 1:5 diluted serum from four allergic patients.⁴ Bound IgE was detected with ¹²⁵I-labeled anti-human IgE (Phadebas RAST [Kabi Pharmacia]).

Affinity chromatography

Purified antibodies from mouse ascites were dialyzed overnight against coupling buffer (0.1 mol/L NaHCO₃, pH 8.3, containing 0.5 mol/L NaCl). Two grams of CNBr-activated Sepharose 4B (Pharmacia) were washed with 1 N HCl in a sintered glass filter for 15 minutes and then briefly with the coupling buffer. The gel and the antibody solution (30 mg/20 ml) were combined in a stoppered vessel and rotated overnight at 4° C. Excess antibody was washed with the coupling buffer, and the remaining active groups were blocked with 0.1 mol/L Tris-HCl, pH 8.0, at room temperature for 2 hours. The gel was washed in three cycles of alternating pH with 0.1 mol/L acetate buffer, pH 4, containing 0.5 mol/L NaCl, followed by a wash with 0.1 mol/L Tris, pH 8.0, containing 0.5 mol/L NaCl. The gel was then transferred to a column, and BDA was passed three times by gravity through the column. After washing with 50 ml 0.1 mol/L Tris, pH 8.0, and 50 ml 0.01 mol/L Tris, pH 8.0, bound protein was eluted with 100 mmol/L glycine, pH 2.5. The eluate was collected in 1 ml fractions and neutralized immediately with 100 µl 1 mol/L Tris, pH 8.0. Absorbance was measured at 280 nm; and protein-containing fractions were pooled, dialyzed overnight against distilled water, and lyophilized.

HPLC purification

Affinity-purified allergen was further purified with a 2150 HPLC system (Wallac, Inc., Gaithersburg, Md.) with a Superose 12 column (Pharmacia) (50 cm × 2 cm). A 2 ml sample containing 1.8 mg of affinity-purified allergen was injected into the column in PBS. Fractions were collected according to the elution curve, and fractions at the highest peak were pooled and lyophilized.

Cross immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) was performed as described previously⁴ and is briefly summarized below. CIE was performed in 1% (wt/vol) agarose in 0.025 mol/L barbital buffer, pH 8.6, containing 0.03% NaN₃, according to the method of Hoiby and Axelsen.¹⁸ Antigen (25 µl with 5 µg protein) was loaded to the first-dimension gel and was separated at 4° C for 90 minutes with a constant voltage of 8 V/cm. A second-dimension gel containing 10% rabbit anti-BDA antiserum⁴ was used, and the second electrophoresis was carried out at 40° C for 16 to 18 hours at 2 V/cm. After electrophoresis, the gels were pressed four times for 5 minutes (filter paper–paper towel–glass plate–3 kg) and washed overnight in 0.9% NaCl, washed briefly in distilled water, and dried at 37° C. Gels were stained with Coomassie Brilliant Blue R-250 (Serva Feinbiochica GmbH & Co., Heidelberg, Germany).

Isoelectric focusing

The isoelectric point of the HPLC-purified allergen was determined with the Bio-Rad Mini IEF cell (Bio-Rad Laboratories, Chemical Div., Richmond, Calif.). Polyacrylamide gel was casted with BioLyte ampholytes (Bio-Rad Laboratories) 3/5. Pharmacia low pI markers (2.8 to 6.55) were used. Isoelectric focusing and Coomassie Brilliant Blue staining were performed according to the Bio-Rad instruction manual.

Amino acid analysis

The HPLC-purified allergen was hydrolyzed with 6 mol/L HCl at 110° C for 24 hours. The hydrolysate was lyophilized and the amino acid composition was analyzed with an LKB amino acid analyzer.

Skin prick test

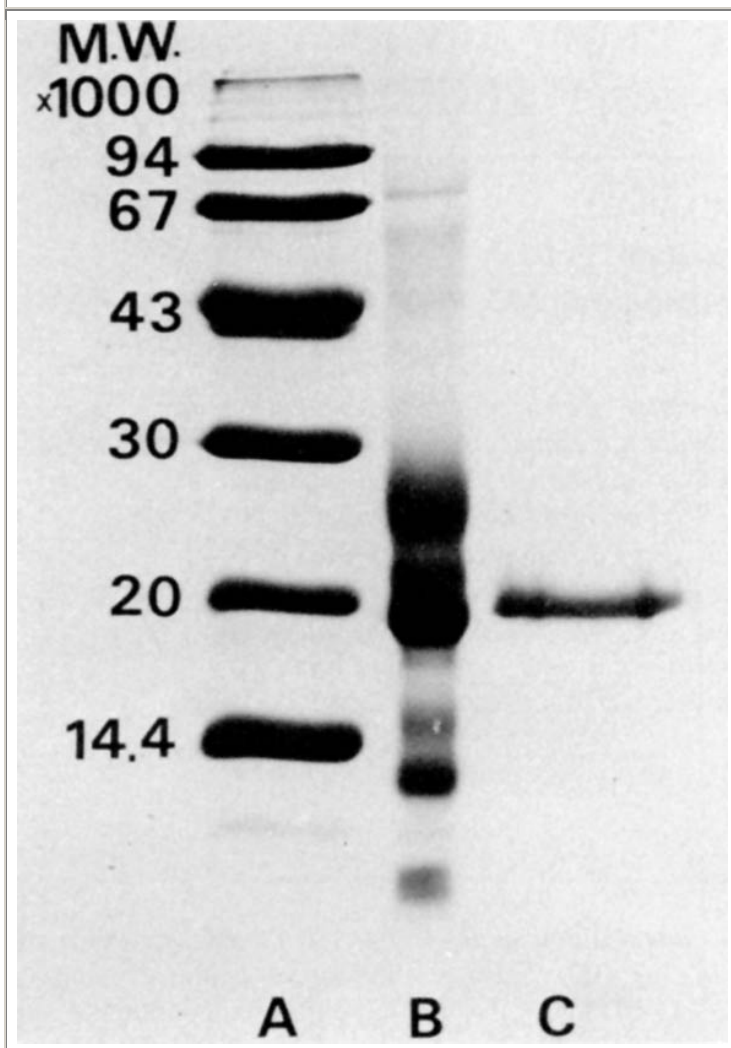
Skin prick tests with the cow dander preparation from Allergologisk Laboratorium A/S (ALK, Copenhagen, Denmark), crude allergen (BDA), and affinity-purified BDA20 were performed in the Department of Dermatology, Kuopio University Hospital. Results from six patients are included in the present study. They were persons participating in cattle tending who underwent testing because of work-related allergic reactions. The skin prick test results were interpreted according to the Scandinavian standards¹⁹ on a scale from 0 to 4 +, with 3 + being the reaction provoked by the histamine control (histamine hydrochloride, 1 mg/ml) and 2 + being one half and 4 + being twice the histamine control reaction.

Results

Monoclonal antibodies were made by immunizing BALB/c mice with crude cow dander extract. One hybridoma-secreting mAb to the 20 kd major allergen was found by means of immunoblotting (data not shown). The monoclonal antibody (mAb1) was coupled to CNBr-activated Sepharose and was used for single-step affinity purification of the allergen (BDA20). When 16.6 mg (protein) of BDA was applied to the column, 1.85 mg BDA20 was obtained. To confirm the purity of the affinity-purified major cow allergen, the BDA extract and the purified BDA20 were run in parallel in SDS-PAGE gel (Fig. 1). In the BDA20 lane, one intense band at 20 kd and a few barely detectable bands were seen in the Coomassie Brilliant Blue–stained gel.

FIG. 1. Coomassie Brilliant Blue–stained SDS-PAGE gel. Lane A, Molecular weight standards (Pharmacia); lane B, crude BDA (50 µg protein/lane); lane C, affinity purified

BDA20 (5 µg protein/lane). M.W., Molecular weight.



To verify the IgE reactivity of the purified protein, we selected four patients who were highly allergic to cows and tested their sera against the whole extract and the purified allergen by means of IgE immunoblotting (■ Fig. 2). As is evident, the patients' IgE reacted well with the BDA20 allergen. Skin prick test results of six persons participating in cattle tending are presented in ■Table I. As is obvious, the affinity-purified preparation was able to induce positive cutaneous reactions. The results seem to correlate well with the results obtained with crude extract and the commercial preparation from ALK.

FIG. 2. IgE immunoblots of four (1-4) farmers with cow-induced asthma. *Lane A*, BDA (25 µg protein/lane); *lane B*, BDA20 (5 µg protein/lane).

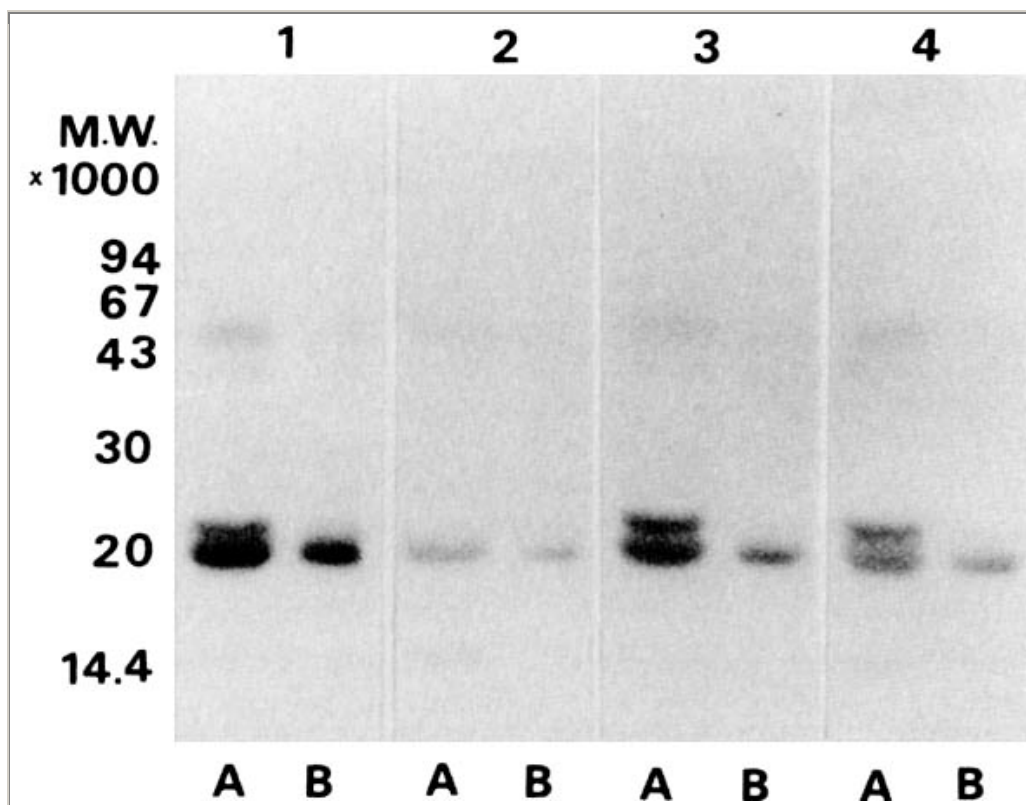


TABLE I. Skin prick results of six subjects who underwent testing for cow-associated allergy

	<u>Subject (age, sex)</u>					
	39F	45M	33F	28M	30F	52M
Histamine (1 mg/ml)	3 +	3 +	3 +	3 +	3 +	3 +
ALK negative control	—	—	—	—	—	—
ALK cow allergen	2 +	4 +	1 +	—	—	—
BDA (0.92 µg/ml)	—	2 +	—	—	—	—
BDA (92 µg/ml)	2 +	3 +	1 +	—	—	—
BDA (9200 µg/ml)	4 +	4 +	4 +	—	—	—
BDA20 (10 µg/ml)	—	2 +	1 +	—	—	—
BDA20 (50 µg/ml)	2 +	3 +	1 +	—	—	—

According to the stained SDS-PAGE gel, the affinity-purified BDA20 seemed to be quite pure. However, in CIE one large and five minor arcs were observed (■Fig. 3, A). Therefore we further purified the allergen with size-exclusion HPLC. Affinity-purified BDA20 (1.8 mg) was applied to the column. According to the elution curve (■Fig. 4), the sample was already approximately 90% pure. Fractions at the highest peak were pooled and shown to contain 1.2 mg of protein. The CIE analysis of the HPLC-purified BDA20 showed one large arc and one very small arc (■Fig. 3, B). The purification steps and yields are presented in ■Table II. The pI of the double-purified allergen was 4.1 as determined by isoelectric focusing (■Fig. 5). In agreement with the low pI, a predominance of acidic amino acids was observed in the analysis of amino acid composition (■Table III).

FIG. 3 CIE gels. **A**, Affinity-purified BDA20. **B**, Affinity- and HPLC-purified BDA20. The sample

wells are indicated in the figure. A 5 μ g sample of BDA20 was applied to both gels.

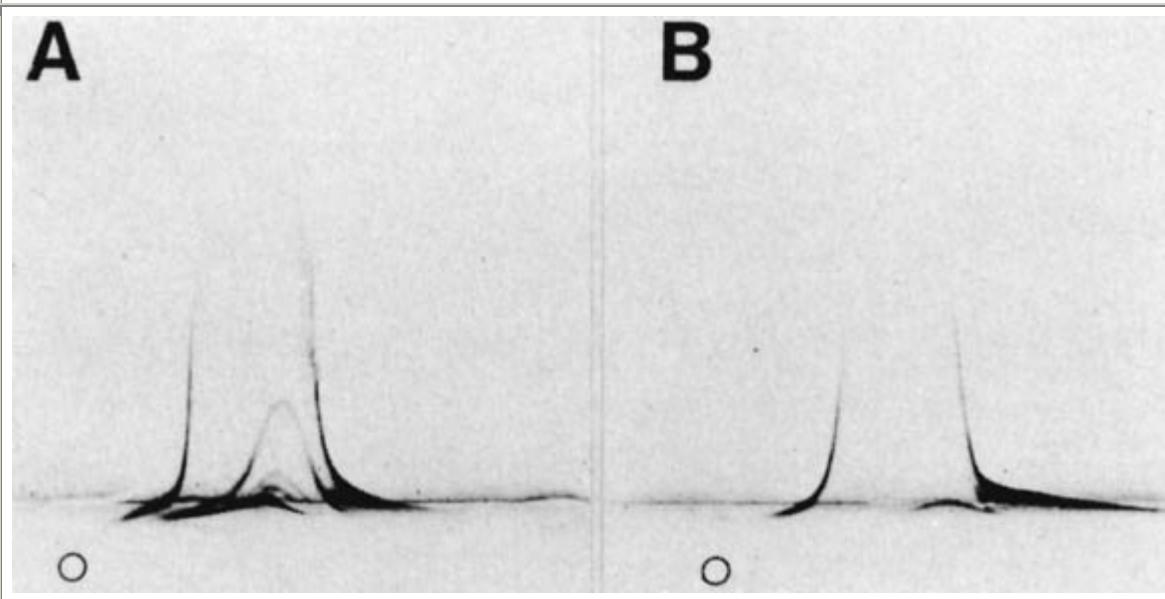


FIG. 4. Elution curve of affinity-purified BDA20 in HPLC. The fractions that contained BDA20 and were pooled are marked with arrows.

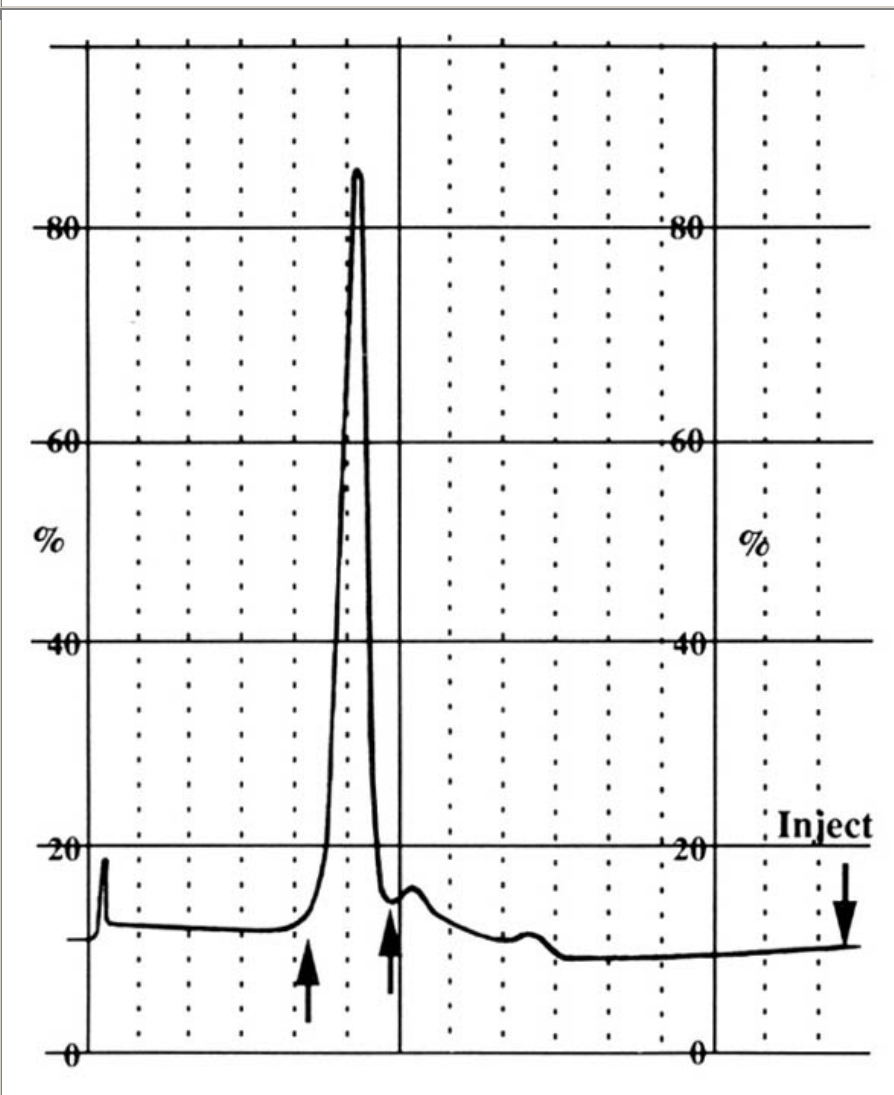


FIG. 5. Coomassie Brilliant Blue stained

isoelectric focusing gel. The pls of Pharmacia low pI standards are shown in the figure. The BDA20 band (3 μ g) is on the right.

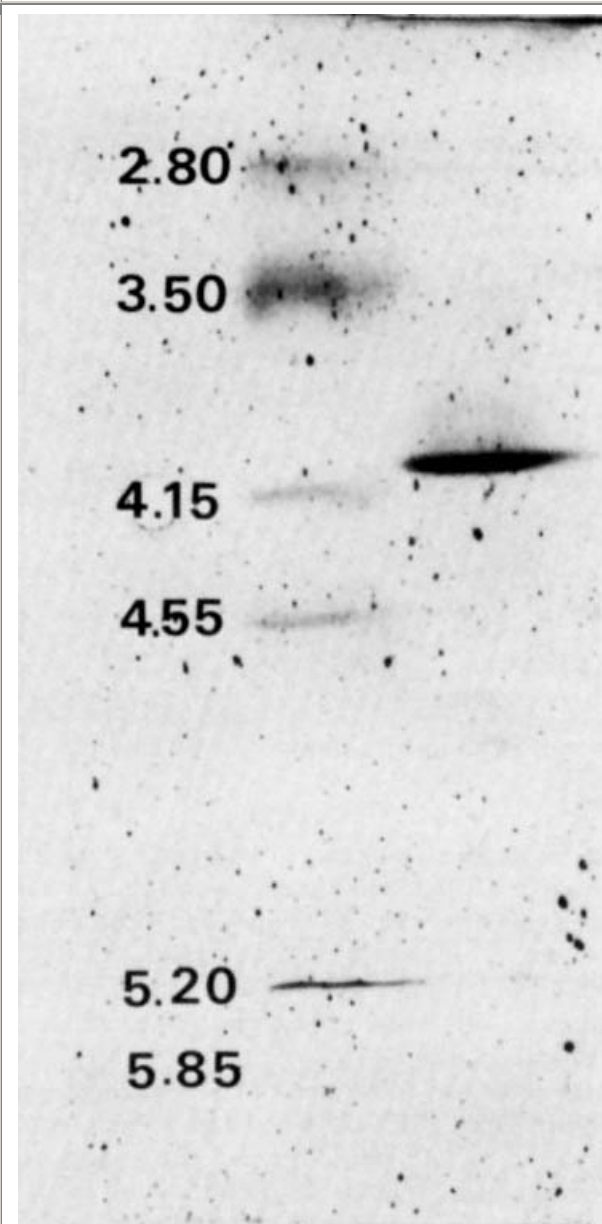


TABLE II. Purification yields and estimated purity of BDA20 at each purification step

	Protein (mg)	Estimated purity of BDA20
BDA applied to the affinity column	16.6	—
BDA20 eluted from the affinity column	1.85	88%
BDA20 after HPLC	1.2	99%

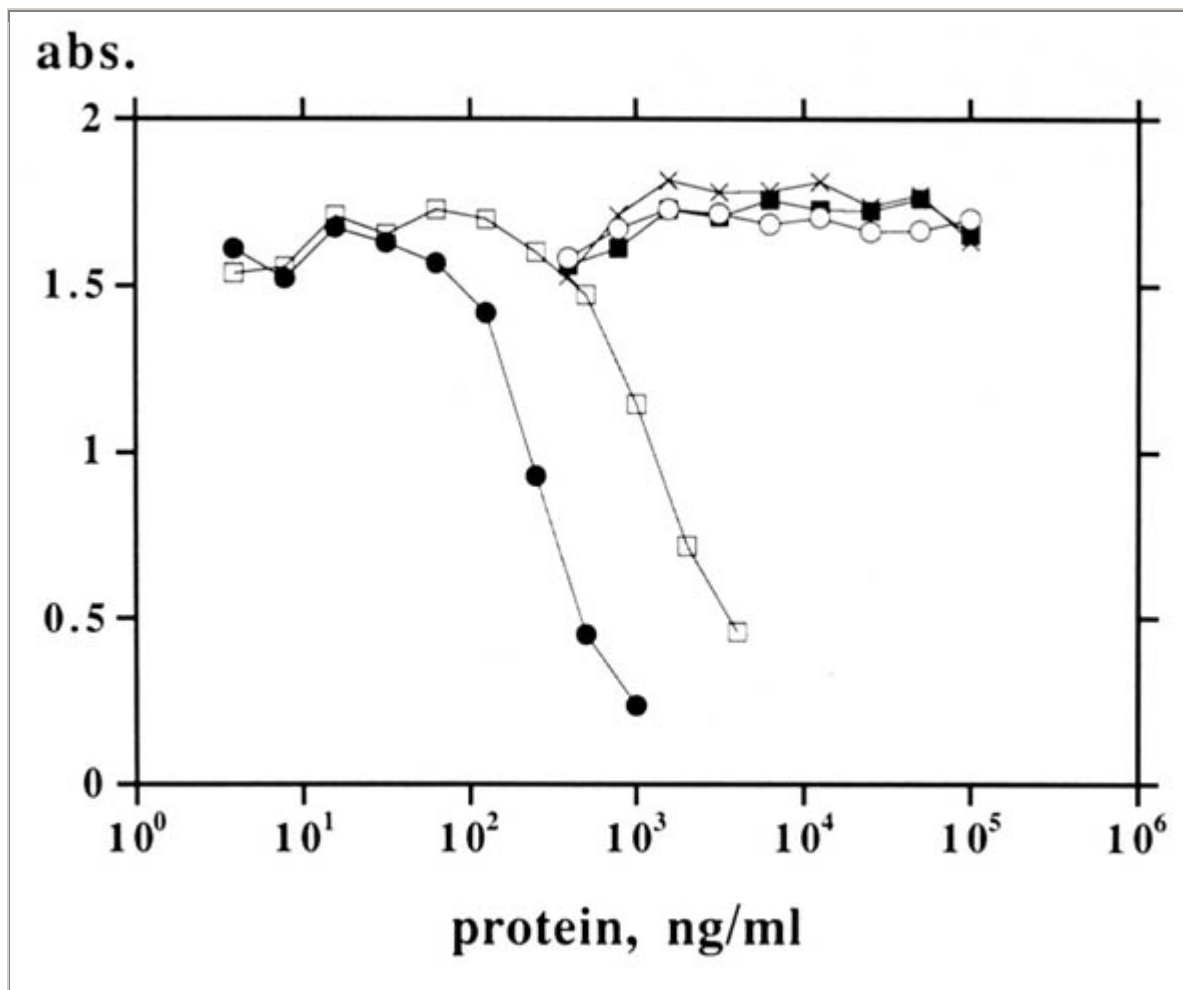
TABLE III. Amino acid composition of BDA20

Amino acid	Composition (nmol amino acid/nmol BDA20)
Aspartic acid	15
Threonine	10
Serine	5
Glutamic acid	27
Proline	10
Glycine	9
Alanine	6
Valine	10
Cysteine	0
Methionine	1
Isoleucine	8
Leucine	12
Tyrosine	7
Phenylalanine	3
Lysine	8
Histidine	3
Arginine	6

Twenty-four-hour hydrolysis with 6 mol/L HCl at 110° C.

The cross-reactivity of mAb1 between danDer preparations of different animal species was studied by ELISA inhibition. BDA and BDA20 and cat, horse, and dog dander extracts were tested for their inhibitory activity. As shown in ■Fig. 6, mAb1 is specific for cow allergen. Crude BDA was a strong inhibitor, as could be expected.

FIG. 6. ELISA inhibition curves. Plate was coated with BDA20. Varying amounts of BDA20 (solid circles), BDA (open squares), cat dander antigen (solid squares), horse dander antigen (x), and dog dander antigen (open squares) were tested for the inhibition of the mAb1-BDA20 reaction.



Discussion

The use of standardized allergens is considered necessary for the development of better methods for allergy diagnosis and therapy.¹⁰ Although the major bovine allergens were described and purified a long time ago,² they have not been widely used in clinical or research studies. This may be due in part to the laborious techniques that had to be used in the past. Affinity purification based on the use of specific antibodies has solved many of the problems of the former methods; the major advantage is that a highly purified product can be obtained with a single purification step.

For the affinity purification we decided to develop murine mAbs, which offer high specificity and can be produced in large quantities when necessary. Immunization of mice with a crude bovine epithelial extract and production of hybridomas resulted in the establishment of a stable clone that secretes mAb1 specific to the 20 kd bovine major allergen.

Animal allergens can have epitopes cross-reactive between species, as demonstrated by Blands et al.²⁰ for cow, cat, dog, guinea pig, horse, and sheep allergens and Viander et al.²¹ for cat and dog allergens. To characterize the specificity of mAb1, we tested it in ELISA inhibition, using as inhibitors horse and dog dander extracts, which are known to contain the major allergens of these animals.^{22, 23} As demonstrated in Fig. 6, even huge quantitative excesses of cat, horse, and dog epithelial extracts were unable to disturb the specific reaction.

When mAb1 was used for the affinity purification of the 20 kd bovine major allergen, a product of high purity was obtained. After the second purification step by size-exclusion HPLC, BDA20 was almost 100% pure. A major advantage of this procedure is that the affinity column is reusable. Therefore large amounts of the allergen can be purified. The BDA20 allergen was quantitatively one of the major components of cow dander. The affinity column bound approximately 10% of the total protein amount of BDA. The allergenicity

of the affinity-purified allergen was tested with two different methods. IgE immunoblotting with the sera of four patients with cow allergy showed strong reactions to BDA20. In skin prick tests of three persons with cow sensitivity, the BDA20 caused the same reaction as the crude extract that contained all allergenic components of cow epidermis. These tests confirmed that affinity purification did not damage the allergenic properties of the molecule.

Affinity purification has proven to be an efficient tool for allergen purification. For example, Chapman et al.⁹ have reported to have achieved a purity of 90% when the cat major allergen was purified from house dust. Lombardero et al.⁸ reported that they detected only one arc (*Olea europaea* major allergen) in CIE after single-step affinity purification with two mAbs that recognize nonoverlapping epitopes. It seems that an affinity purification method based on the use of a single mAb can be further improved by use of more complicated arrangements.

Prahl et al.² have previously reported some basic physical characteristics of cow allergens isolated by anion-exchange and hydrophobic chromatography. The molecular weight of *Bos d* I was 25 kd and the pI was 3.6. The respective figures for *Bos d* II were 20 kd and 4.2. As already mentioned, the molecular weight of our major cow allergen is 20 kd (determined by SDS-PAGE), but according to HPLC analysis it is 25 kd (data not shown). The pI turned out to be 4.1. Amino acid composition of BDA20 also seemed to have a closer resemblance to *Bos d* II than to *Bos d* I. In our previous studies we have demonstrated that in SDS-PAGE and immunoblotting, this 20 kd protein is the major IgE-binding component in cow dander and urine.^{3,4} More than 90% of the IgE-reactive patients had IgE antibodies to this component. On the other hand, *Bos d* I and *Bos d* II have demonstrated equal allergenic activity in RAST inhibition (67%).²

In this article we have described the development of an mAb to the major allergen of cow and the use of this antibody for the affinity purification of the allergenic protein. After final purification with HPLC, almost 100% pure allergen could be obtained. According to our experience, the procedure described is practical for large-scale purification. The opportunity to produce greater amounts of pure BDA20 makes possible future work in characterizing its structure and studying its effects on immune cells. When mAb1 was made, we obtained at the same time two additional BDA20-specific mAbs. One of them recognized the same epitope as mAb1, and the other recognized a different epitope. With these three mAbs we are now developing a double-antibody sandwich ELISA for quantification of airborne allergen in cowsheds. It can also be used for quantification of the allergen in allergen extracts used for clinical testing.

We thank Virpi Voutilainen for her skillful technical assistance.

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- Supported by the Finnish Allergy Research Foundation and the Emil Aaltonen Foundation.
- Received for publication Apr. 6, 1993.
- Accepted Oct. 6, 1993.
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- **1/1/52629**

Articles with References to this Article

This article is referenced by these articles:

Tissue localization of bovine dander allergen Bos d 2

Journal of Allergy and Clinical Immunology, The
March 1998 • Volume 101 • Number 3

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Recombinant allergen fragments as candidate preparations for allergen immunotherapy

Journal of Allergy and Clinical Immunology, The
December 1997, Part 1 • Volume 100 • Number 6

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BDA20, a major bovine dander allergen characterized at the sequence level, is Bos d 2

Journal of Allergy and Clinical Immunology, The
August 1997 • Volume 100 • Number 2

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