

Identification and Purification of a 16-kDa Allergen from *Psoroptes ovis* (Acarina: Psoroptidae)

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ABSTRACT *Psoroptes ovis*, (Hering), the sheep scab mite, is the causative agent of an allergic dermatitis of sheep and cattle. Recent studies of the host immune response to this ectoparasite have provided information that suggests control may be achieved by immune intervention. A significant effector in protection of the host from clinical lesions is host behavioral grooming. Host grooming is believed to be intensified by a pruritic immediate hypersensitivity response to mite allergens. Knowledge of potential *P. ovis* allergens is limited. This article reports on the identification and SDS-PAGE continuous elution purification of a 16-kDa polypeptide that elicits immediate type hypersensitivity in calves and has sequence homology with known group II mite allergens, *Lep d 2* of *Lepidoglyphus destructor* (Schrack), and *Der f II* of *Dermatophagoides farinae* (Hughes). This *P. ovis* allergen appears to be a good vaccine candidate for further study and cloning.

KEY WORDS *Psoroptes ovis*, allergen, vaccine

Psoroptes ovis (HERING) (Acarina: Psoroptidae), the globally distributed sheep scab mite, is an economically important parasite of domestic cattle and sheep, and a serious threat to wild populations of bighorn sheep (Boyce and Brown 1991). Mite infestation of susceptible cattle can result in the development of scabies lesions. The lesion is consistent with allergic dermatitis (Stromberg and Fisher 1986) characterized histologically by superficial perivascular dermatitis, marked edema in the superficial dermis, numerous eosinophils, and mast cells (Stromberg and Guillot 1989). The dermatitis can become extensive and lead to the death of cattle in poor condition and under extremes of environmental stress. It has been suggested that death may occur as a result of the high demand and consumption of neutrophils at the lesion site, leaving other tissues, such as the lungs, susceptible to secondary bacterial infection (Stromberg and Guillot 1987).

Stanchioned cattle repeatedly infested with *P. ovis* appear to develop some level of resistance, defined by a slower rate of lesion development and mite population growth (Stromberg and Fisher 1986). Guillot and Stromberg (1987) suggested that the slow progression of lesion development and mite population expansion on stanchioned, resistant cattle could be

attributed to a lowered ovipositional rate of the female mites by some unexplained mechanism. Losson et al. (1988) reexposed unstanchioned cattle to *P. ovis* and the cattle exhibited signs of immediate hypersensitivity including pruritus, scratching, and exudation. Scabies lesions developed on these cattle, but healed rapidly, and none of the cows developed clinical scabies. These studies suggest that the host immunological response to *P. ovis* may be protective and that vaccination may be used to control clinical scabies.

The potential for vaccination to prevent scabies lesions and limit mite transmission and population growth was further supported by results obtained in a recent vaccine/challenge study with unstanchioned calves (Pruett et al. 1998). Several candidate immunogens were identified in a partially purified fraction of *P. ovis* proteins. This study represents the purification and subsequent evaluation of the host response to one of those proteins.

Materials and Methods

Preparation of Antigens. *P. ovis* mites were reared on stanchioned calves and collected for protein extraction as described in Pruett et al. (1998). *P. ovis* soluble antigens (POI) were prepared by grinding age-mixed mites in a TenBroeck tissue grinder in 0.01 M Tris-HCl, pH 7.5 containing 1 mM PMSF. The solution of ground mites was centrifuged for 20 min at 4°C at 2,530 × g. The supernatant fluid was decanted and filtered at 5 µm (Gelman, Acrodisc, Ann Arbor, MI). The protein concentration of the supernatant fluid was determined by a modified Lowry (Bradshaw

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Institutional Animal Use and Care Committee (IAUCC) of the Knippling-Bushland U. S. Livestock Insects Research Laboratory, Kerrville, TX.

1966). The soluble antigen preparation was stored at -20°C .

DEAE Anion Exchange Chromatography. The FI protein fraction was prepared by ionexchange chromatography. POI proteins were applied to a column of DEAE Sephacel (Pharmacia, Uppsala, Sweden) and eluted with 0.01 M Tris-HCl, pH 7.5, over a linear gradient of NaCl ranging from 0.0 to 1.0 M.

SDS-PAGE and Western Blotting. POI and fractionated POI proteins were resolved on polyacrylamide gradient gels (3–22%) and stained with Coomassie blue for analysis as previously described (Pruett et al. 1998). Briefly, proteins were resolved using a modified method of Laemmli (1970) with reducing (2-mercaptoethanol, 2ME) sodium dodecyl sulfate (SDS) discontinuous polyacrylamide gel electrophoresis (2ME-SDS-PAGE) with an LKB 2050 Midget (LKB Produktor AB, Bromma, Sweden) electrophoresis unit. Resolved proteins were transferred to nitrocellulose paper for immunoblotting as described in Pruett and Thomas (1986). Blots were challenged with specific animal serum and developed as described in Pruett et al. (1998). Positive serum samples were obtained from an unstanchioned infested calf (week 6 sera, #211), a POI vaccinated calf [1 mg POI with complete Freund's adjuvant (CFA) primary + 2 boosters (3-wk interval) and 1 booster with incomplete CFA (ICFA), week 10 serum, #212], and an FI vaccinated calf (125 μg FI with CFA + 1 booster at week 3 with CFA, week 6 serum, #274). Negative control serum was made as a pool of 50 sero-negative calves.

Molecular Sieve Chromatography. POI proteins were fractionated by molecular size using a Sephacryl S-300 (Pharmacia Biotech, Piscataway, NJ) high resolution molecular sieve column. The column was calibrated using as molecular weight standards, ovalbumin (45 kDa), bovine serum albumin (68 kDa), bovine IgG (150 kDa), and blue dextran (1.5×10^6 kDa). Ten milliliter fractions were collected and pooled for SDS-PAGE analysis.

Reversed-Phase High-Performance Liquid Chromatography (HPLC). POI fractions were further fractionated using reverse-phase HPLC with a Waters μ Bondapak C18 column (3.9 by 300 mm) (Waters, Milford, MA). Sample elution was monitored at 280 nm with a Waters Lambda-Max model 481 LC spectrophotometer (Waters). Proteins were eluted at a flow rate of 1 ml/min over a 45-min linear gradient of 100% buffer A (0.1% trifluoroacetic acid in H_2O) to 100% buffer B (0.085% trifluoroacetic acid containing 70% acetonitrile in H_2O).

Continuous Elution SDS-PAGE Electrophoresis. The 16-kDa protein was purified from the molecular sieve fraction IV (FIV) using continuous elution SDS-PAGE with the Bio-Rad 491 prep cell according to the manufacturers instructions (Bio-Rad, Hercules, CA). FIV proteins were lyophilized and reconstituted with SDS-PAGE sample buffer containing SDS and 5% 2-ME (Pruett and Thomas 1986). A 37-mm-diameter column was used for polypeptide separation with a 6 cm high 16% polyacrylamide gel (30:0.0:8, acrylamide:

bis-acrylamide). The sample was loaded onto the stacking gel (10%) and the run conducted at 40 mAmps constant current. Continuous elution fractions (2.5 ml, 12S total) were collected and subjected to gradient SDS-PAGE analysis for purity.

Amino Terminal Sequencing. Purified 16-kDa protein was submitted to the Biotechnology Instrumentation Facility, Peptide and Protein Technologies, Department of Entomology, Texas A&M University, College Station, TX, for amino terminal sequencing. The 16-kDa protein was subjected to Edman chemistry by the method indicated (method 3.4) on the Sequence Sample Information Sheet for the Hewlett-Packard (Palo Alto, CA) GLOO5A Protein Sequencing System for 30 cycles. A 26 amino acid sequence segment of the 16-kDa protein (aa 2–27) was submitted to a computer assisted similarity search using BLASTP (Altschul et al. 1990) an algorithm that compares an amino acid query sequence against a protein database.

Immunization of Cattle with the 16-kDa Protein. Hereford steers (12 at 150–200 kg) were used in the 16-kDa immunization experiment. These calves were purchased from a local auction barn (Gillespie Livestock, Fredericksburg, TX). Upon arrival at the Knipling-Bushland U.S. Livestock Insects Research Laboratory (KBUSLIRL) the calves were in-processed as described previously (Pruett et al. 1998). Briefly, they were dewormed, vaccinated, and sprayed with 0.3% coumaphos to kill residual ectoparasites. The calves were assigned randomly to 4 experimental groups. Group 1 was vaccinated with a 25 μg /dose; group 2, a 10 μg /dose; group 3, a 5 μg /dose; and group 4, the sham vaccinated controls, received adjuvant only.

The 16-kDa protein, isolated with the Bio-Rad 491 prep cell, was stored in buffer containing 0.025 M Tris, 0.192 M glycine, pH 8.3, and 0.1% SDS. The protein concentration was determined using a micro-BCA kit according to manufacturer instructions (Pierce, Rockford, IL). The veterinary acceptable adjuvant used in this experiment was a mixture of alhydrogel and amphigen (A/A). The 16-kDa protein was adsorbed to alhydrogel (E. M. Sergeant Pulp and Chemical, Clifton, NJ, 12% by weight) as described previously (Pruett and Stromberg 1995). Briefly, the 16-kDa protein was adsorbed overnight at 4°C in a 0.01 M sodium phosphate buffer, pH 6.5. The following morning, amphigen (MRKS Marketing Services, Elkhorn, NE, 5% by volume) base (amphigen 1 volume: 3 volumes drakeol 5, Penreco, Dickinson, TX) was added to the adsorbed 16-kDa protein and emulsified. Injections were administered subcutaneously in a 2.0 ml volume. The primary injection was given on the 1st d of the experiment and a booster injection was given on day 21 of the experiment. All calves, vaccinated and sham vaccinated controls, were skin tested on day 14 after the booster injection.

Intradermal Skin Testing. Calves were injected intradermally with 0.1 ml containing 1 μg of 16-kDa protein in 0.010 M phosphate buffered saline (PBS, pH 7.2, 0.85% NaCl). Control injection sites included a

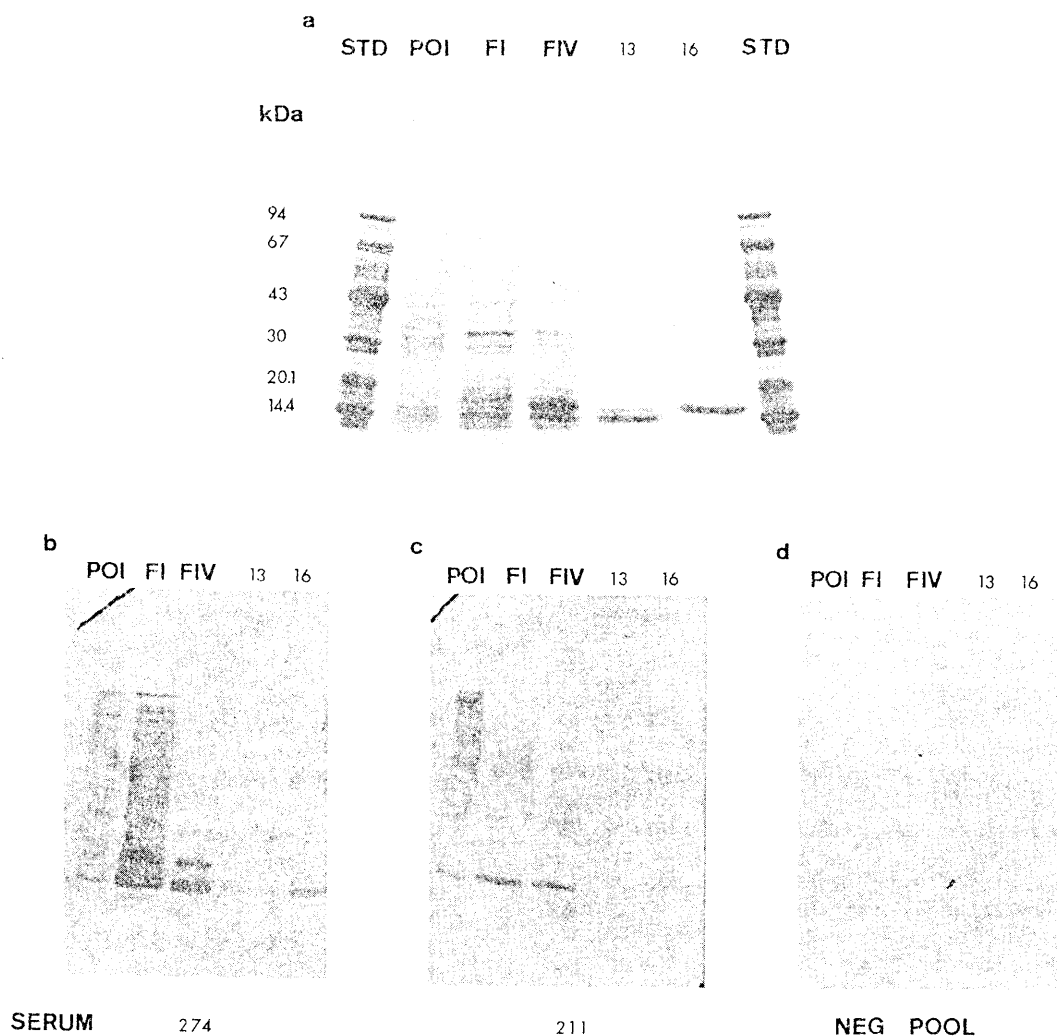


Fig. 1. 2ME-SDS-PAGE (3–22%) analysis of POI proteins to summarize the purification of the 16-kDa protein. Panel a, Coomassie stained proteins, lanes: molecular weight standards (STD), POI, FI anion-exchange fraction, FIV molecular sieve fraction, 13-kDa protein from Bio-Rad 491 Prep Cell fractionation of FIV, 16-kDa protein from Bio-Rad 491 Prep Cell fractionation of FIV, molecular weight standards (STD). Panel b, immunoblot of gel in panel a, with serum from an FI (anion-exchange fraction) vaccinated calf #274. Panel c, immunoblot of gel in panel a, with serum from an unstanchioned control infested calf #211 (week 6 serum). Panel d, immunoblot of gel in panel a, with serum from a negative pool.

PBS control and a separate PBS containing 0.1% SDS injection site. The diameter of the skin reaction was measured at 0.5, 1.0, 4, 24, and 48 h. The area of the skin reaction was determined by $A = \pi(r^2)$ and reported in square millimeters.

Results

Soluble protein extracts ($n = 22$) of homogenized mites (≈ 3 g) yielded $7.0 \pm 1.3\%$ protein by weight. SDS-PAGE (3–22%) gradient resolution of the polypeptides of the mite soluble protein extract (POI) yielded numerous, poorly resolved bands (Fig. 1a). These results suggest a low yield of any particular protein without further protein purification. POI was fractionated on a DEAE anion-exchange column using

a linear NaCl gradient from 0.0 to 1.0 M. The fractionated proteins were divided into 6 fractions (FI–FVI). FI proteins were representative of the fall through fraction (Fig. 1a). Several FI antigens were identified by immunoblotting with immune serum from an FI vaccinated calf (#274, Fig. 1b) and with immune serum from an unstanchioned, *P. ovis* infested calf (#211, Fig. 1c). Immunodominant antigens were noted at ≈ 43 , 30, and <20 kDa. SDS-PAGE and Ultrosan (LKB-Produkter, Laser Densitometer, Bromma, Sweden) analysis of the FI fraction revealed that the fraction contained at least 18 polypeptide bands that ranged in molecular weight from 208 to 14 kDa with isoelectric point (pI) values that ranged from 5.38 to 9.04. Seven of the polypeptides had pI values above 7.0 (data not shown).

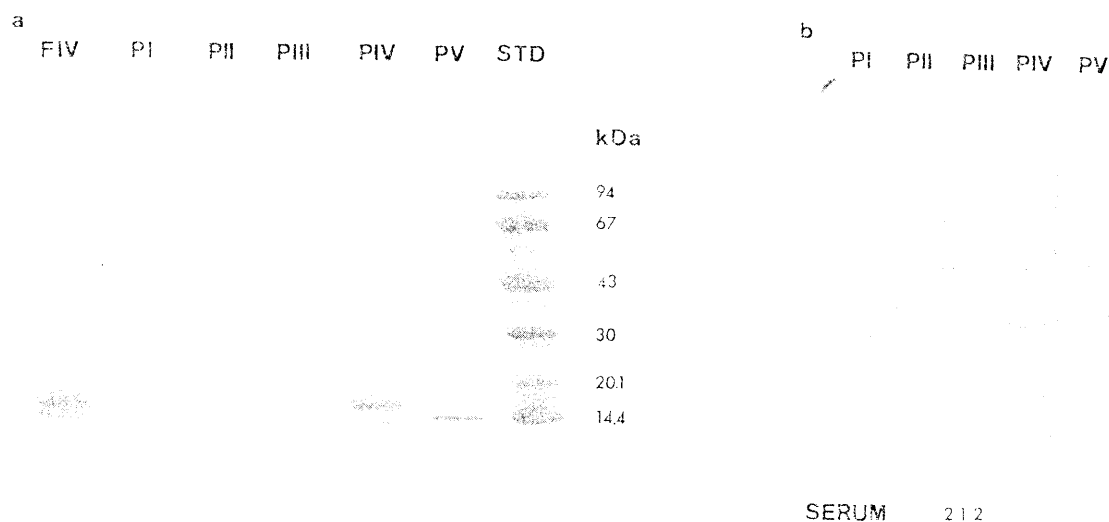


Fig. 2. 2ME-SDS-PAGE (3–22%) resolution of proteins in HPLC peaks of FIV, a S-300 molecular sieve fraction of POI. Panel a, Coomassie stained proteins, lanes: FIV molecular sieve fraction, HPLC peaks in order of elution, PI, PII, PIII, PIV, PV, molecular weight standards (STD). Panel b, immunoblot gel in panel a with serum from a POI vaccinated calf, #212.

Having determined that principal antigens were <40 kDa, POI was fractionated by S-300 column chromatography for the purpose of concentrating and separating, by molecular size, the proteins of interest from the many other *P. ovis* proteins in POI. POI was fractionated into 6 fractions (FI–FVI) using S-300 molecular sieve chromatography. These fractions were further analyzed by SDS-PAGE and immunoblotting. Fraction IV (FIV) was found to contain polypeptides of <40 kDa (Fig. 1a). Immunoblot results, with immune serum from a calf vaccinated with FI (#274, Fig. 1b) and immune serum from an unvaccinated infected calf (#211, Fig. 1c), demonstrated antibody binding to a polypeptide in the 16-kDa range.

Ultroscan analysis of SDS-PAGE separated FIV proteins revealed 9 principal peaks (data not shown). These proteins ranged in molecular weight from 55 to 13 kDa (Fig. 1a). The 16- to 17-kDa band represented 38% of the total protein. FIV proteins were further fractionated with analytical HPLC (C18). FIV proteins were separated into 5 principal peaks (PI–PV) by HPLC (Fig. 2a).

Immunoblot results, with immune serum from a POI vaccinated calf (#212), suggest that the 16-kDa protein was predominantly in peak 4 (PIV, Fig. 2b), but could not be obtained in pure form as a polypeptide of ~13 kDa co-eluted (Fig. 2a).

Purification of the 16-kDa protein was accomplished by preparative SDS-PAGE continuous elution gel electrophoresis of FIV (molecular sieve) proteins, with a Bio-Rad 491 Prep Cell apparatus (Fig. 3). Tubes (55–58) containing pure 16-kDa protein (determined by SDS-PAGE) were pooled and stored at -20°C for further analysis.

Pure 16-kDa protein was sequenced for 30 cycles with the Hewlett-Packard G 1005A Protein Sequencing System using Edman chemistry. The amino acid sequence of the first 30 amino acids of the 16-kDa

protein was reported as GDS>VKVKFQDCGKGE VESLVEGCGSDYCVLPIK. Glycine was called as amino acid 1, with isoleucine as a questionable call at amino acid position 25. The last 5 cycles were accompanied by considerable lag and background; thus, confidence in their call was reduced.

A 26-amino acid sequence segment of the 16-kDa protein (aa 2–27) was submitted to a similarity search using BLASTP. The 16-kDa amino acid sequence was compared with 260,575 different sequences deposited in GenBank, CDS translations, PDB, SwissProt, Spupdate, and PIR. Significant sequence homology ($P = 2.2 \times 10^{-7}$) was observed with the principal allergen *Lep d 1* of the storage mite *Lepidoglyphus destructor* (Schrack) and the *Der f II* house-dust mite allergen from *Dermatophagoides farinae* (Hughes), (Fig. 4). Alignment with *Lep d 1* [gi|999458 (X83575)] yielded 61% identities and 76% positives when considering similar amino acid substitutions. Alignment with *Der f II* [pir|A61501] yielded 36% identities and 63% positives ($P = 0.39$).

Intradermal skin test results of calves vaccinated with different dosages of 16-kDa protein are presented in Fig. 5. Tissue-fixed specific anti-16-kDa protein antibody was demonstrated by intradermal injection of purified 16-kDa protein. The 16-kDa protein formulated with A/A elicited immediate-type hypersensitivity responses in all vaccinated calves, regardless of immunization dose. Skin swelling at the injection site declined rapidly after 1 h.

Discussion

Knowledge of the immunogenicity/allergenicity of purified *P. ovis* proteins in cattle is limited. In an effort to qualitatively describe mite proteins to which cattle respond, Boyce and Brown (1991) electrophoretically resolved *Psoroptes* spp. crude soluble proteins from

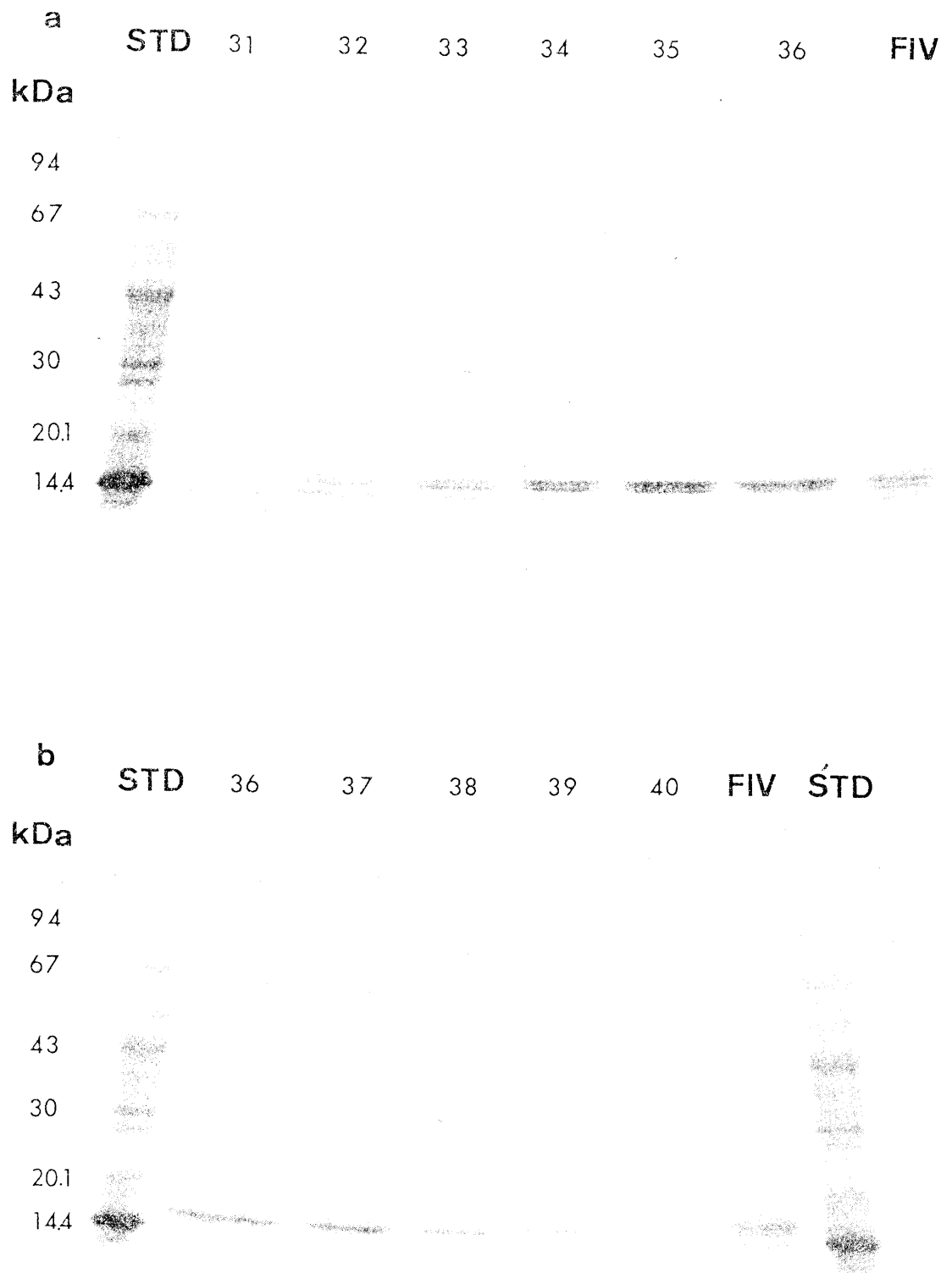


Fig. 3. 2ME-SDS-PAGE (3-22%) analysis for 16-kDa purity of continuous elution (2ME-SDS-PAGE) fractions from the Bio-Rad 491 Prep Cell fractionation of FIV, molecular sieve fraction of POI. Panel a, coomassie stained proteins, lanes: molecular weight standards (STD), fractions in order of elution, 31, 32, 33, 34, 35, 36, FIV. Panel b, Coomassie stained proteins, lanes: molecular weight standards (STD), continued analysis of fractions in order of elution, 36, 37, 38, 39, 40, FIV, molecular weight standards (STD). Fractions 35 through 38 were pooled as 16-kDa protein.

16 kDa	1 K V K F Q D C G K G E V E S L E V E G C S G D Y C V 26
	K + F + D C G G E V L + + G C S G D C V
Lep d I	18 K M T F K D C G H G E V T E L D I S G C S G D F C V 43
16 kDa	1 K V K F Q D C G K G E V E S L E V E G C S G 22
	+ V + D C E + + V + G C G
Der f II	2 Q V D V K D C A N N E I K K V M V D G C H G 23

Fig. 4. Comparison of amino acid sequences of *Lep d I*, *Der f II*, and the 16-kDa protein from *P. oris*. Sequence identities are noted by the one-letter symbol for the amino acid, and conservative changes (similar amino acid substitutions) are noted by +.

cattle-collected mites. These separated proteins were immunoblotted with serum from a moderately *Psoroptes* spp. infested cow. They found that <20 antigens were recognized by antibody in this serum, with bands at 10 and 116 kDa being most prominent. In the current study, Coomassie staining of SDS-PAGE resolved POI resulted in numerous poorly resolved polypeptide bands with several being antigenic as defined by immunoblotting with immune serum. Two immunodominant antigens were noted at molecular weights of <40 kDa, specifically 30 and 16 kDa.

In a recent vaccine/challenge trial conducted at this laboratory, naive calves vaccinated with a partially purified fraction of POI designated as fraction I (FI), were protected from infestation with *P. oris* (Pruett et al. 1998). A self-grooming behavioral response was believed to be the significant effector in protecting FI vaccinated calves. The grooming response among vaccinated calves was presumably heightened by a pruritic, immediate-type hypersensitivity response to FI allergens (Pruett et al. 1998). Antigens of molecular weights (30 and 16 kDa), similar to those immunodominant proteins described in POI, are found in FI (Fig. 1).

Before infestation in the FI trial, naive calves vaccinated with a high dose ($125 \mu\text{g} + \text{A/A}$) of FI proteins were skin tested with FI antigens intradermally. These

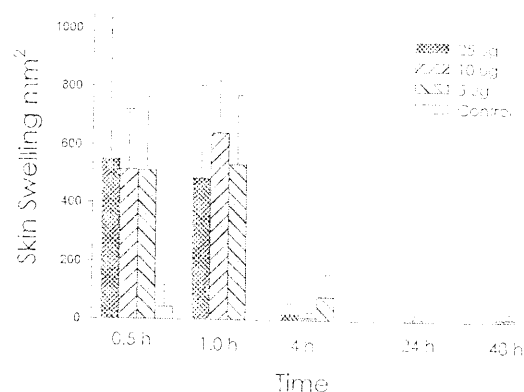


Fig. 5. Results of intradermal ($0.1 \mu\text{g}$ 16 kDa) skin tests (mean $\pm$ SD) conducted 14 d after booster immunization with the 16-kDa protein. Vaccination dosage listed in legend, adjuvant alhydrogel-amphigen for all groups.

calves responded with an immediate hypersensitivity response (0.5–1.0 h). Their skin response, as a group at 4–24 h, was heterogeneous. In contrast, naive calves vaccinated with a low dose ($10 \mu\text{g} + \text{A/A}$) of FI expressed comparable 0.5–1.0 h reaction, but had more intense and homogeneous 4–24 h, late-phase responses. Although the low-dose vaccinated calves had mite populations lower than control calves, they experienced dermatitis comparable to the control group. It was speculated that a late-phase host response to *P. oris* allergens may be responsible for host-induced dermatitis. Eosinophil accumulation and degranulation in the local region of late-phase inflammation is a characteristic of the late-phase response (Solley et al. 1976, Frew and Kay 1988). Degranulation of eosinophils and the subsequent release of cationic granular proteins can account for tissue damage (Frigas and Gleich 1986). Therefore, from these results it was reasoned that the best candidate vaccine antigen, one having the potential to reduce mite population and limit host dermatitis, should be an antigen that would elicit an immediate-type hypersensitivity response, encouraging host grooming behavior, without eliciting the subsequent late-phase response.

Of the 2 immunodominant proteins observed in FI (30 and 16 kDa) the 16-kDa protein was chosen for initial purification and immunological analysis as preliminary serological and immunoblot results suggest that the 30-kDa protein may be involved in cross reactions with antibodies elicited by host exposure to other ectoparasite species (data not shown). The 16-kDa protein is of relatively low concentration in FI. However, molecular sieve chromatography of the crude *P. oris* proteins and pooling of proteins of <40 kDa resulted in a fraction (FIV) that was $\approx 38\%$ of total protein as 16 kDa. Purification of the 16-kDa protein from FIV was attempted using reverse-phase HPLC (C18) chromatography. However, the 16-kDa protein could not be purified to single band purity with HPLC. A 13-kDa band consistently coeluted with the 16-kDa protein. Purification of the 16-kDa protein to single band purity was eventually accomplished with continuous elution SDS-PAGE using the Bio-Rad 491 prep cell apparatus.

The purified 16-kDa protein was found to be immunogenic in cattle when administered with A/A as adjuvant even at the low dose of $5 \mu\text{g}$. The results of intradermal skin testing with the 16-kDa protein elicited intense antibody-mediated immediate hypersensitivity reactions that subsided after 1 h. No measurable late-phase activity was observed. The allergenic nature of the 16-kDa protein was further supported by sequence homology with known mite allergens *Lep d I* from the storage grain mite *L. destructor* and *Der f II* from the dust mite *D. farinae* (Varela et al. 1991). The *L. destructor* allergen was originally named *Lep d I*, however recent sequence information revealed $\approx 50\%$ homology with the group II allergens of *Dermatophagoides*; therefore, *Lep d I* has been redesignated *Lep d 2* (Olsson et al. 1998). The function of the group II (*Der f II*) house dust mite allergens is unknown, however, a lysozyme-like activity has been proposed for

Der f II based on chemico-physical property homologies, but has not been proven (Stewart et al. 1992). There is $\approx 80\%$ conservation of identity of the amino acid sequence among the 25-kDa acidic-neutral proteins of the group I house dust mite allergens and 4 of the major group I house dust mite allergens are enzymes (Robinson et al. 1997). Considering the homology between the 16-kDa *P. ovis* protein and the group II allergens *Der f* II and *Lep d* 2, it may be that these proteins represent similar and conserved function. The function and source (tissue, gut, fecal) of the 16-kDa protein is unknown, but will be the subject of future studies.

These data suggest that the 16-kDa *P. ovis* protein is allergenic in cattle in that when formulated with A/A as adjuvant it elicits an immediate hypersensitivity response. In addition, it does not appear, in this formulation, to elicit the potential tissue destructive late-phase response. Therefore, the 16-kDa protein is considered a good candidate vaccine antigen for further study and subsequent cloning.

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