JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1992, p. 2662–2667
0095-1137/92/102662-06$02.00/0

Vol. 30, No. 10

Purification, Characterization, and Seroactivity of a 20-Kilodalton *Brucella* Protein Antigen

MICHEL S. ZYGMUNT,* FLORENCE B. GILBERT, AND GÉRARD DUBRAY

Laboratoire de Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, 37380 Nouzilly, France

Received 30 December 1991/Accepted 17 July 1992

An internal protein was purified from cell extracts of *Brucella melitensis* B115 by a combination of preparative isoelectric focusing and high-performance size exclusion chromatography. The protein has an apparent molecular mass of 230 kDa as determined by size exclusion chromatography. The protein was resolved to a single band of 20 kDa after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The native protein had an isoelectric point of 4.9. The N-terminal sequence of the 20-kDa protein was determined. The 20-kDa protein has been identified as antigen A-2 with a previously described anti-antigen A-2 serum (B. Stemshorn, K. Nielsen, and B. Samagh, Can. J. Comp. Med. 45:77–81, 1981). Antigen A-2 reacted with sera from infected sheep in immunoblotting and may be useful in developing diagnostic tests for brucellosis.

Brucellosis of bovines and small ruminants caused, respectively, by *Brucella abortus* and *B. melitensis* results in a marked decrease in reproductive efficiency owing to abortion, clinical disease, and infertility. Economic losses are high, and eradication schemes involving a combined program of vaccination, testing, and slaughter have been implemented (9). Vaccination programs have been instituted to prevent the disease. Cattle are vaccinated with live, attenuated smooth *B. abortus* 19, whereas small ruminants are vaccinated with attenuated smooth *B. melitensis* Rev1. The animals develop an immune response to the vaccine strain. Conventional serological tests, like the rose bengal plate test, milk ring test, and serum agglutination test, do not differentiate between sera of vaccinated and infected animals, because in these tests the smooth lipopolysaccharide (LPS) is the major immunodominant antigen (6, 10, 21). Research has been done in the development of new vaccines or vaccination methods which would induce protective immunity but still allow differentiation between vaccinated and infected animals. The use of lower doses of attenuated vaccine strains (1, 2) and other routes of vaccination, such as conjunctival inoculation, has been shown to give protection (14, 19, 20). Alternatively, the development of diagnostic tests based on antigens which may allow differentiation of vaccinated from infected animals has been proposed (12).

Nine distinct antigens in protein extracts of brucellae have been shown to be precipitated by sera of infected cows in the immunoelectrophoresis technique (15). Antibodies against one of these antigens, A-2, were found in the sera of infected cows and goats (24–26, 28). Antigen A-2 detected infected cattle with moderate sensitivity (55%) but good specificity (99%). However, antigen A-2 has been only partially purified (29). It was shown to be identical to *Brucella* E (18), Ea (27), and CO1 (29) and has a high molecular weight that has not been precisely determined.

In this study, we developed a procedure for extracting the *Brucella* 20-kDa protein by ultrafiltration and preparative isoelectric focusing (IEF). We then purified the protein by high-performance size exclusion chromatography. The protein was characterized by determining the apparent molecular mass, isoelectric point, and immunoreactivity with rabbit antiserum against antigen A-2 and with sera from infected sheep.

MATERIALS AND METHODS

Bacterial strain. Rough cells were obtained from a *B. melitensis* B115 isolate taken from a naturally infected goat by G. Alton (11). They were grown in a 20-liter fermentor by the method of the U.S. Department of Agriculture (2a). Briefly, cultures were grown at 37°C for 48 h on tryptic soy broth (BioMérieux, Marcy-l'Etoile, France) supplemented with 0.1% yeast extract (Difco Laboratories, Detroit, Mich.).

Preparation of CPE. Cytoplasmic protein extract (CPE; 31) from *B. melitensis* B115 was prepared by breaking cells with glass beads in a Dyno-Mill apparatus (Bachofen, Basel, Switzerland) and then initially ultrafiltering them with hollow-fiber cartridges with a 0.1-μm cutoff (Amicon, Grace S.a.r.l, Epernon, France). The ultrafiltrate was treated with RNase and DNase (Worthington Diagnostics, Freehold, N.J.) and subjected to a second ultrafiltration with a 100-kDa cutoff hollow-fiber cartridge (Amicon). The retentate was centrifuged at 50,000 × *g* for 16 h, and the cleared supernatant (CPE) was lyophilized.

Sera used to test seroactivity. Ten sera were collected from naturally infected sheep from an area where *B. melitensis* is endemic (they were a gift from A. Gomez Ferreira, Evora, Portugal). These sera were positive for *B. melitensis* antibodies in the rose bengal plate test and the complement fixation test described by Alton et al. (2a).

AGID. Agarose gel immunodiffusion (AGID) experiments were conducted by using a 1% (wt/vol) agarose gel at pH 8.6 with Veronal buffer. Glass slides were precoated with agarose and dried. Rabbit antiserum against antigen A-2 was a gift from B. Stemshorn (Animal Disease Research Institute, Nepean, Ontario, Canada). Undiluted antiantigen A-2 serum was tested against different fractions to reveal the presence of antigen A-2. Precipitin lines were recorded after incubation at 20°C for 24 h.

Purification of 20-kDa protein. The fraction that eluted at 80 min after chromatography on HiLoad Superdex 200 was separated on sodium dodecyl sulfate (SDS)-polyacrylamide

* Corresponding author.

gel electrophoresis (PAGE). This was followed by copper staining and electroelution of the 20-kDa protein from gel slices (17, 30).

ELISA procedure. Sera were tested for activity in an indirect enzyme-linked immunosorbent assay (ELISA) by using a modification of the procedure described by Nielsen and Wright (22). Ninety-six-well polystyrene plates (Greiner Labortechnic, Frickenhausen, Germany) were coated by passive adsorption at a concentration of 1 μg/100 μl of antigen (CPE and fractions P12 and P80 collected after gel filtration chromatography) per well, diluted in phosphate-buffered saline (PBS), pH 7.2, for 18 h at 25°C. The wells were emptied and washed five times with PBS containing 0.05% Tween 20. Anti-antigen A-2 serum was tested at dilutions of 1:50 and 1:100 in PBS–0.05% Tween 20 containing 1% skim milk, and 100-μl samples were applied in duplicate. Following incubation for 90 min at 37°C, the wells were again washed and filled with 100 μl of a 1:20,000 dilution of horseradish peroxidase-labeled goat antirabbit immunoglobulins G and M (heavy and light chain specific; Jackson Laboratory) in PBS–0.05% Tween 20. After incubation for 60 min at 37°C, the conjugate solution was discarded and the plates were washed with PBS without Tween. The wells were filled with 100 μl of substrate solution containing 1 mM ABTS [2,2-azino-di-(3-ethylbenzthiazoline-sulfonic acid)–4 mM H_2O_2 in 50 mM sodium citrate, pH 4.2], and the plates were then shaken continuously at 25°C for 1 h. Optical density values at 414 nm were then recorded with a Titertek Multiscan MC plate reader (Flow Laboratories, Les Ulis, France) inferfaced with a computer. An arbitrary optical density of 0.200 U, 2 standard deviations above the negative control values, was considered positive.

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (16), on 11% polyacrylamide slabs with a reservoir buffer at pH 8.6 containing 0.3% Tris, 18% glycine, 0.1% SDS. Antigen samples (1.25 mg/ml) were solubilized under reducing conditions in the sample buffer (62.5 mM Tris, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, pH 6.8), boiled for 3 min, and cooled. CPE samples were applied to a vertical slab gel apparatus (Bio-Rad SA, Paris, France) and electrophoresed at 30 mA per gel for 5 h. Standard molecular mass markers included a mixture of proteins ranging from 20 to 92.5 kDa (Bio-Rad). For protein detection, the gels were silver stained (23).

IEF. IEF was performed at 10°C by using an LKB 2117 Multiphor flat-bed apparatus with LKB Ampholine PAG plates (1804-102), pH 4 to 6.5, with 0.1 M glutamic acid in 0.5 M H_3PO_4 and 0.1 M β-alanine as electrode solutions at the anode and cathode, respectively. Samples (25 μl) of the CPE (1.25 mg/ml) were applied with individual wicks onto the gel surface. An LKB power supply was used at maximum settings of 25 W, 2,000 V, and 25 mA. IEF running time was 3 h. After focusing, proteins were fixed in a solution containing 11.5% (wt/vol) trichloroacetic acid and 3.45% (wt/vol) sulfosalicylic acid for 30 min. Afterwards, the gels were stained for 10 min at 60°C with 2% (wt/vol) solutions of Coomassie brillant blue R-250 in the destaining solution (ethanol-acetic acid-water, 1:0.2:1) and then destaining until a clear background was obtained.

Immunoblotting. For immunoblotting (5), electrophoretic transfer of gels was carried out on a semidry blotting apparatus (LKB). The nitrocellulose membranes were incubated for 2 h at 37°C in Tris-buffered saline (TBS) containing 1% skim milk to block any remaining protein-reactive sites. The nitrocellulose was then cut lengthwise into 0.5-cm strips, and each strip was placed in a separate tray. The strips were washed three times in TBS–0.05% Tween 20 and then incubated overnight with a 1:50 dilution (in TBS–1% skim milk) of sheep serum. The strips were washed three times for 10 min each time in TBS-Tween 20 and then incubated for 2 h with a 1:400 dilution of biotinylated donkey anti-sheep immunoglobulin G (heavy and light chain specific; Amersham SA, Les Ulis, France). After being washed, the strips were incubated for 2 h with a 1:300 dilution of streptavidin-peroxidase (Amersham International plc, Amersham, United Kingdom). After additional TBS washes, the strips were incubated in a substrate for horseradish peroxidase (development kit from Bio-Rad containing 4-chloro-1-naphthol). After color development, the strips were washed with distilled water and dried.

Preparative IEF. Preparative horizontal column IEF (7, 12) was performed within a pH 4 to 6 ampholine (LKB) gradient by using a Rotophor Cell (Bio-Rad). CPE (0.3 mg/ml) was mixed with 2.5 ml of ampholines and brought to a total volume of 50 ml. This mixture was loaded into the Rotophor electrofocusing cell for 4 to 6 h at 4°C at a constant 12 W by setting the maximum voltage to 2 kV using an LKB 2197 power supply. After focusing, 20 fractions were collected and analyzed by high-pressure liquid chromatography (HPLC) on a gel filtration column.

N-terminal sequence analysis. Edman recurrent degradation was done with an automated stepwise sequencer with an on-line 120A phenylthiohydantoin analyzer (Applied Biosystems, San Jose, Calif.). The sample was first desalted by reversed-phase HPLC on a 7-μm 300 A Aquapore RP 300 column (30 by 4.6 mm; Brownlee, Santa Clara, Calif.). Elution was done with a linear gradient from 100% solvent A (0.075% trifluoroacetic acid) to 100% solvent B (0.075% trifluoroacetic acid in acetonitrile-water, 60:40) in 30 min at 20°C at a flow rate of 200 μl/min.

HPLC. After Rotophor treatment, the different fractions were separated by gel filtration on a TSK G3000SW (600 by 7.5 mm) column (Toyo Soda Co.) or a HiLoad Superdex 200 column (Pharmacia-LKB Technologie, Saint-Quentin-Yvelines, France). The elution buffers contained, respectively, 250 mM phosphate (pH 7.2) and 20 mM Tris/HCl–250 mM NaCl (pH 7.2). The column was eluted at 1 and 0.75 ml/min, respectively. The eluate was monitored at 220 or 280 nm by using a 2140 rapid spectral detector and the LKB wavescan analysis program. The peaks were collected and analyzed by AGID or ELISA.

RESULTS

Extraction of the native form. The CPE is soluble, and antigen A-2 was detected in this extract by AGID (Fig. 1). In the first purification step, CPE (15 mg of protein) fractions were separated on the basis of their isoelectric points by Rotophor horizontal preparative column IEF in a pH 4 to 6 ampholine gradient. Twenty fractions were collected and analyzed by using the AGID test. Antigen A-2 was present in fractions 8, 9, 10, and 11 from pHs 4.2 to 4.9 (Fig. 2). Fractions 8, 9, 10, and 11 were subjected to size exclusion HPLC. Several peaks were obtained (Fig. 3), and the peak that eluted at 12 min (P12) was identified as containing antigen A-2 after this analysis by using AGID (Fig. 1) and ELISA. On the basis of the retention time determined with a standard protein kit (Bio-Rad) for gel filtration column calibration, the estimated molecular mass of P12 was 230 kDa. When P12 was subjected to SDS-PAGE, several bands appeared after silver staining: one major band with a molec-

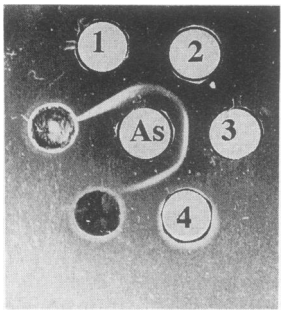

FIG. 1. AGID analysis of CPE fractions obtained at various stages of purification against anti-antigen A2 sera. Wells: As, anti-antigen A2 serum; 1, CPE; 2, P12; 3, P80; 4, 20-kDa protein.

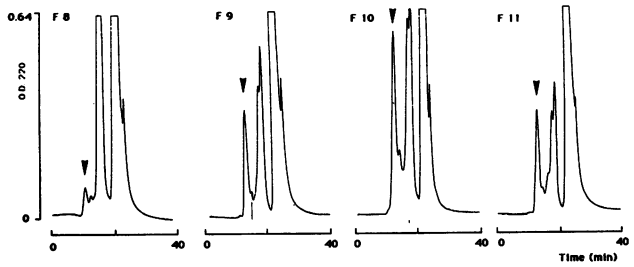

FIG. 3. Size exclusion chromatography elution profile of fractions 8, 9, 10, and 11 (Fig. 1) containing antigen A2. Arrowheads indicate the position of the P12 peak. Chromatographic conditions were as follows: TSK G 3000 SW column, 60 cm by 7.5 mm (inner diameter); eluant, 250 mM phosphate, pH 7.2; flow rate, 1 ml/min. The optical density (O.D.) of the eluate at 220 nm was monitored.

ular mass of 20 kDa and minor bands at 14, 25, 30, 46, and 70 kDa (Fig. 4). P12 was collected several times and rechromatographed on another size exclusion HiLoad Superdex 200 column. The main peak that eluted at 80 min (P80) corresponded to a molecular mass of 230 kDa after calibration of the column with a standard protein kit (Fig. 5). The ELISA response against P80 was positive with anti-antigen A-2 sera. This gel filtration chromatography step confirmed that P80, identified as antigen A-2 by AGID (Fig. 1), had a high molecular mass of 230 kDa. When P80 was subjected to SDS-PAGE, one band appeared at 20 kDa after silver staining, with only minor faint bands (Fig. 4). For further analysis, immunoblotting and preparative electrophoresis were carried out.

Immunoblotting analysis. Different fractions, CPE, P12, and P80, were examined by immunoblotting with anti-antigen A-2 serum and rabbit antiserum prepared against CPE (serum 14) (Fig. 6). When CPE was blotted, immune serum prepared against CPE reacted with several antigens of 20 to 70 kDa. In CPE, the intensity of the 20-kDa band did not seem be that of an immunodominant antigen. P12 give five bands, at 20, 28, 35, and 67 to 69 kDa, with anti-CPE serum, and P80 gave only one band. When CPE was analyzed with anti-antigen A-2 serum, an immunological reaction occurred with several proteins with molecular masses of 20, 30, and 46 to 48 kDa. These results indicate the nonmonospecificity of anti-antigen A-2 serum. When P12 and P80 were analyzed by SDS-PAGE and immunoblotting with anti-antigen A-2 serum, only one band at 20 kDa was observed.

CPE was not contaminated with a rough LPS, as demonstrated by a negative test with an anti-rough-LPS monoclonal antibody (data not shown).

To determine the pI of antigen A-2 after IEF analysis over a pH range of 4 to 6.5, we analyzed P80 (antigen A-2 in its native form). A single, well-resolved band appeared after Coomassie blue staining at pH 4.9 (Fig. 7), indicating the pI and that the aggregate could enter the gel.

Most efficient purification was achieved by submission of P80 to SDS-PAGE. After elution of the 20-kDa protein band, the purity of this 20-kDa electroeluted band was ensured by SDS-PAGE (Fig. 8). Only one band appeared after silver staining.

The specificity of this antigen was further analyzed by immunoblotting and confirmed with serum 14. The results obtained with the electroeluted 20-kDa band were identical to those obtained with P80 (Fig. 6). This serum, which reacted with many bands in CPE, reacted with only the single 20-kDa band. Analysis of the purified 20-kDa antigen with anti-antigen A-2 serum to test specificity gave only one reaction at 20 kDa. By AGID, the denatured 20-kDa antigen

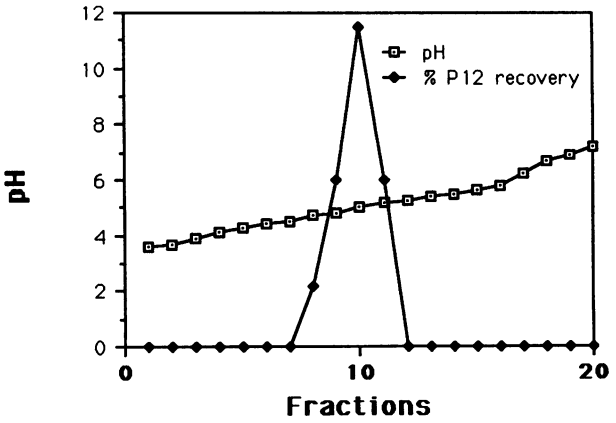

FIG. 2. Final pH and profile of P12 recovery from the 20-fraction IEF fractionation of CPE.

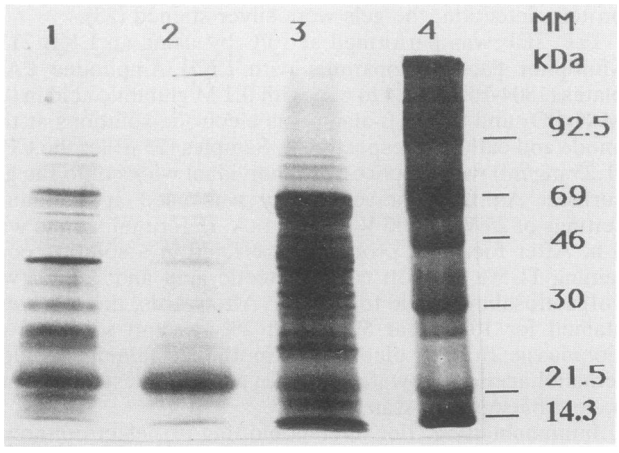

FIG. 4. SDS-PAGE and silver staining profiles of CPE fractions obtained at various stages of purification. Lanes: 1, peak eluted at 12 min after CPE fractionation on TSK G 3000 SW; 2, peak eluted at 80 min after CPE fractionation on HiLoad Superdex; 3, CPE; 4, molecular mass (MM) standards.

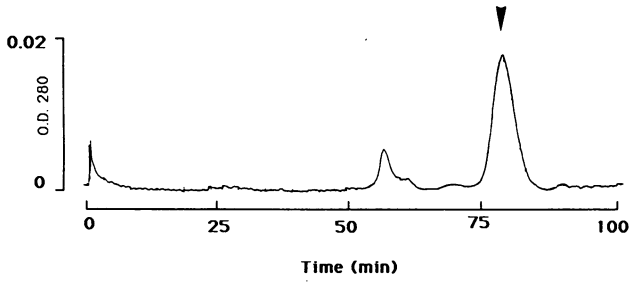

FIG. 5. Size exclusion profile of the P12 peak on HiLoad Superdex. The arrowhead indicates the position of the P80 peak. Chromatographic conditions were as follows: column, HiLoad 16/60 Superdex; eluant, 250 mM NaCl–20 mM Tris, pH 7.2; flow rate, 0.75 ml/min. The optical density (O.D.) of the eluate at 280 nm was monitored.

conserved immunoreactivity with anti-antigen A-2 serum (Fig. 1).

N-terminal sequence analysis. The purified 20-kDa protein was subjected to automated Edman degradation, which gave information on the sequence of the intact protein for seven residues and its purity. The N-terminal sequence was Met-Lys-Gly-Gln-Pro-Lys-Val.

Seroactivity. The seroactivity of the purified 20-kDa protein was tested by immunoblot analysis with 10 sera from infected sheep. Although the 20-kDa protein is not a major protein in brucellae, all of the sera tested reacted with this protein (Fig. 9).

DISCUSSION

This report describes a procedure for initial enrichment and further purification of the 20-kDa cytoplasmic protein. Several cytoplasmic molecules of brucellae were found to be antigenic (3, 6, 12). Antigen A-2 was described as a non-smooth-LPS antigen isolated from both smooth and rough *Brucella* cells (25). After sonication, it was recovered from the soluble fraction. Infected cattle were shown to develop anti-antigen A-2 antibodies (29). Antigen A-2 was shown to be heterogeneous, and by gel filtration on a G200 column, it eluted in the void volume, entering partially into the column (29). However, neither further physicochemical characterization nor purification controls were done.

We carried out an analysis of CPEs from rough *B. melitensis* B115 cells and found a 20-kDa protein which reacted with anti-antigen A-2 serum.

The necessity of two sequential preparative runs to purify the 20-kDa protein from CPE can be attributed to the

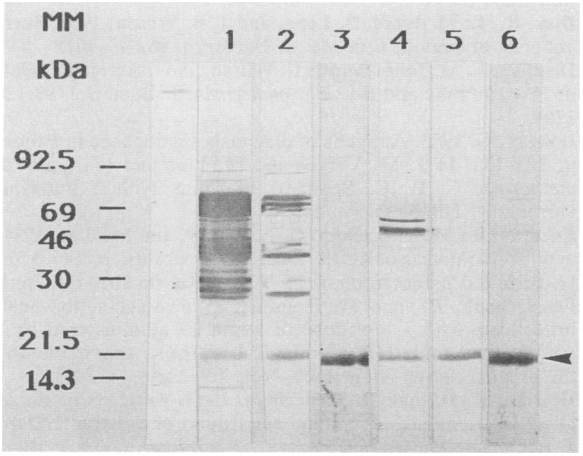

FIG. 6. Immunoblot analysis of CPE fractions obtained at various stages of purification with anti-CPE serum (lanes 1 to 3) and anti-antigen A2 serum (lanes 4 to 6). Lanes: 1 and 4, CPE; 2 and 5, P12; 3 and 6, P80. MM, molecular mass.

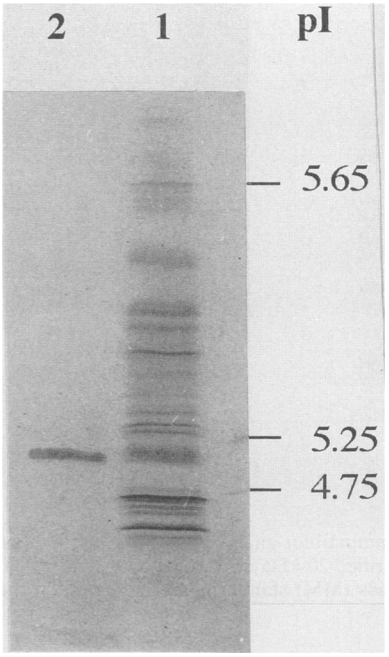

FIG. 7. IEF and Coomassie blue staining of the P80 peak. Lanes: 1, protein standards; 2, P80.

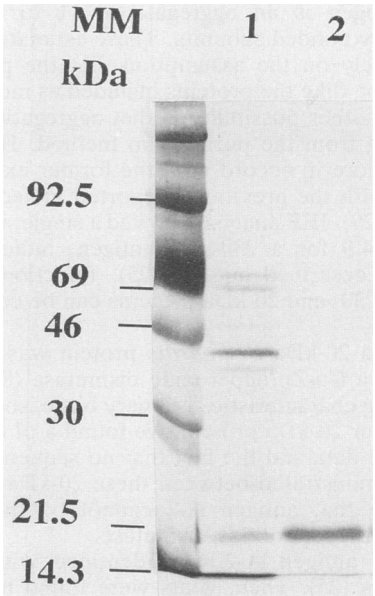

FIG. 8. SDS-PAGE analysis of CPE and the 20-kDa antigen. Lanes: 1, CPE; 2, 20-kDa antigen. MM, molecular mass.

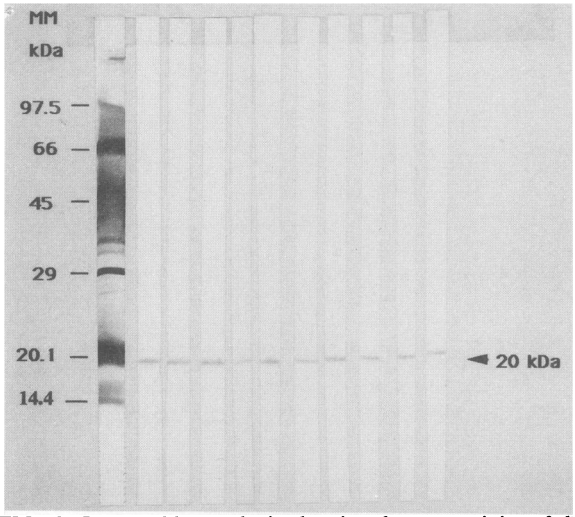

FIG. 9. Immunoblot analysis showing the seroactivity of the *B. melitensis* purified 20-kDa antigen with 10 sera from infected sheep. Molecular mass (MM) standards are indicated on the left.

extremely large amount of material contained in the initial CPE fraction (see SDS-PAGE and IEF analyses). First, the IEF preparative procedure was devised to separate and extract proteins from CPE on the basis of their pIs.

IEF is a high-resolution technique capable of resolving proteins that differ in pI by fractions of a pH unit (7, 13). By this technique we obtained four fractions, and after subsequent gel filtration the P12 fraction, which reacted with anti-antigen A-2 serum by AGID and ELISA, was obtained. After rechromagraphy of P12 by gel filtration, we obtained P80, which also reacted with anti-antigen A-2 serum and has an apparent molecular mass of 230 kDa. Several hypotheses may be advanced to explain the different molecular mass species. Our results suggest that in its native form, antigen A-2 could be an aggregate or a polymeric form of the 20-kDa protein found in SDS-PAGE. One can assume that the 230-kDa antigen is an aggregate of 11 to 13 identical, noncovalently bonded subunits. These estimates of molecular masses rely on the assumption that the protein has a globular shape (like the proteins included as molecular mass markers). Another possibility is that aggregation is an artifact resulting from the purification method. However, our results are more in accord with the former explanation, as they agree with the previously reported molecular mass of antigen A-2 (29). IEF analysis showed a single, well-resolved band at pI 4.9 for a 230-kDa antigen, indicating anodal mobility, as described initially (25). Functionally, antigen A-2 and the 230- and 20-kDa proteins can be considered the same antigen.

Recently, a 20-kDa *B. abortus* protein was isolated and identified as a Cu-Zn superoxide dismutase (8). One of its distinguishing characteristics is a very basic isoelectric point of 8.6. For our 20-kDa protein, we found a pI of 4.9. In the light of these data and the fact that no sequence homology has been demonstrated between these 20-kDa proteins (4), we conclude that antigen A-2 cannot be the previously reported Cu-Zn superoxide dismutase.

Originally, antigen A-2 was identified with sera from infected cows (25). Then, goats were found to respond to antigen A-2 only when infected with brucellae (24). Therefore, antigen A-2 could be used in a new diagnostic brucellosis test that is not dependent on smooth-LPS antigen if it can be demonstrated that most, if not all, infected animals produce antibodies to this antigen. Preliminary data obtained in our laboratory showed that antigen A-2 adheres well to microtiter plates, and immunoblotting showed that immunoreactivity is not affected by SDS-PAGE. Work is in progress to determine whether antigen A-2 can replace external cross-reacting polysaccharide antigens in the serology of brucellosis.

ACKNOWLEDGMENTS

This work was supported by the Commission of the European Communities under contract BAP: 1986–1989 no. 0157 F.

We are grateful to F. Gauthier and M. Brillard for N-terminal sequencing. We thank B. Stemshorn, Animal Disease Research Institute, for generously suppling anti-antigen A-2 serum and R. A. Bowden for critical reading of the manuscript.

REFERENCES

1. **Alton, G. G., and L. A. Corner.** 1981. Vaccination of heifers with a reduced dose of *Brucella abortus* strain 19 vaccine before first mating. Aust. Vet. J. **57**:548–550.
2. **Alton, G. G., L. A. Corner, and P. Plackett.** 1980. Vaccination of pregnant cows with low doses of *Brucella abortus* strain 19 vaccine. Aust. Vet. J. **56**:369–372.

2a. **Alton, G. G., L. M. Jones, R. D. Angus, and J. M. Verger.** 1988. Techniques for the brucellosis laboratory. Insitut National de la Recherche Agronomique, Paris.

3. **Baughn, R. E., and B. A. Freeman.** 1966. Antigenic structure of *Brucella suis* spheroplasts. J. Bacteriol. **92**:1298–1303.
4. **Beck, B. L., L. B. Tabatabai, and J. E. Mayfield.** 1990. A protein isolated from *Brucella abortus* is a Cu-Zn superoxide dismutase. Biochemistry **29**:372–376.
5. **Beisiegel, U.** 1986. Protein blotting. Electrophoresis **7**:1–18.
6. **Berman, D. T., B. L. Wilson, E. Moreno, R. D. Angus, and L. M. Jones.** 1980. Characterization of *Brucella abortus* soluble antigen employed in immunoassay. J. Clin. Microbiol. **11**:355–362.
7. **Binion, S. B., L. S. Rodley, N. B. Egen, and M. Bier.** 1982. Rapid purification of antibodies to single band purity using recycling isoelectric focusing. Electrophoresis **3**:284–288.
8. **Brickler, B. J., L. B. Tabatabai, B. L. Judge, B. L. Deyoe, and J. E. Mayfield.** 1990. Cloning, expression, and occurence of the *Brucella* Cu-Zn superoxide dismutase. Infect. Immun. **58**:2935–2939.
9. **Crawford, R. B., J. D. Huber, and B. S. Adams.** 1990. Epidemiology and surveillance, p. 131–151. *In* K. Nielsen and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.
10. **Diaz, R., L. M. Jones, D. Long, and J. B. Wilson.** 1968. Surface antigens of smooth *Brucella*. J. Bacteriol. **96**:893–901.
11. **Diaz, R., L. M. Jones, and J. B. Wilson.** 1967. Antigenic relation of *Brucella ovis* and *Brucella melitensis*. J. Bacteriol. **93**:1262–1268.
12. **Dubray, G.** 1985. Antigens of diagnosis significance in Brucella, p. 123–138. *In* J. M. Verger and M. Plommet (ed.), *Brucella melitensis*. C. E. C. Seminar. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
13. **Egen, N. B., W. Thormann, G. E. Twitty, and M. Bier.** 1984. A new preparative isoelectric focusing apparatus, p. 547–550. *In* H. Itirai (ed.), Electrophoresis '83. Walter de Gruyter, Berlin.
14. **Fensterbank, R., and M. Plommet.** 1976. Vaccination against brucellosis with a low dose of strain 19 administered by the conjunctival route. III. Serological response and immunity in the pregnant cow. Ann. Rech. Vet. **7**:9–23.
15. **Hinsdill, R. D., and D. T. Berman.** 1967. Antigens of *Brucella abortus*: chemical and immunoelectrophoretic characterization. J. Bacteriol. **93**:544–549.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **Lee, C., A. Levin, and D. Branton.** 1987. Copper staining: a five

minute protein stain for sodium dodecyl sulfate polyacrylamide gels. Anal. Biochem. **166**:308–312.
18. **McGhee, J. R., and B. A. Freeman.** 1970. Separation of soluble *Brucella* antigens by gel-filtration chromatography. Infect. Immun. **2**:48–53.
19. **Nicoletti, P., L. M. Jones, and D. T. Berman.** 1978. Adult vaccination with standard and reduced doses of *Brucella abortus* strain 19 vaccine in a dairy herd infected with brucellosis. J. Am. Vet. Assoc. **173**:1445–1449.
20. **Nicoletti, P., L. M. Jones, and D. T. Berman.** 1978. Comparison of the subcutaneous and conjunctival route of vaccination with *Brucella abortus* strain 19 vaccine in adult cattle. J. Am. Vet. Assoc. **173**:1450–1456.
21. **Nielsen, K., J. W. Cherwonogrodsky, J. R. Duncan, and D. R. Bundle.** 1989. Enzyme immunoassay for differentiation of the antibody response of cattle naturally infected with *Brucella abortus* or vaccinated with strain 19. Am. J. Vet. Res. **50**:5–9.
22. **Nielsen, K., and P. F. Wright.** 1984. Enzyme immunoassay: application to the detection of bovine antibody to "*Brucella abortus*," p. 1–121. Animal Diseases Research Istitute, Agriculture Canada, Nepean, Ontario, Canada.
23. **Oakley, B. R., D. R. Kirsch, and N. R. Morris.** 1980. A simplified ultra sensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105**:361–363.
24. **Schurig, G. G.** 1982. The immune response of goats vaccinated with low and high doses of *Brucella melitensis* Rev.1. Vet. Immunol. Immunopathol. **3**:311–324.
25. **Schurig, G. G., L. M. Jones, S. L. Speth, and D. T. Berman.** 1978. Antibody response to antigens distinct from smooth lipopolysaccharide complex in *Brucella* infection. Infect. Immun. **21**:994–1002.
26. **Schurig, G. G., A. T. Pringle, and S. S. Breese, Jr.** 1981. Localization of *Brucella* antigens that elicit a humoral immune response in *Brucella abortus*-infected cattle. Infect. Immun. **34**:1000–1007.
27. **Stemshorn, B., and K. Nielsen.** 1977. The bovine immune response to *Brucella abortus*. I. A water soluble antigen precipitated by sera of some naturally infected cattle. Can. J. Comp. Med. **41**:152–159.
28. **Stemshorn, B., and K. Nielsen.** 1981. The bovine immune response to *Brucella abortus*. IV. Studies with a double immunodiffusion test for antibody against antigen A2. Can. J. Comp. Med. **45**:147–153.
29. **Stemshorn, B., K. Nielsen, and B. Samagh.** 1981. The bovine immune response to *Brucella abortus*. III. Preparation of antisera against a *Brucella* component precipitated by sera of some infected cattle. Can. J. Comp. Med. **45**:77–81.
30. **Tessmer, U., and U. Dernick.** 1989. Preparative separation of poliovirus structural polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, copper staining and electroelution, and induction of monospecific antisera. Electrophoresis **10**:277–279.
31. **Zygmunt, M. S., J. C. Martin, and G. Dubray.** 1990. Analysis of immune response: comparison of immunoblots after isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis using cytoplasmic protein extract from *Brucella*. FEMS Microbiol. Lett. **70**:263–268.