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Isolation and Immunological Characterization of a 55-Kilodalton Surface Protein from *Salmonella typhimurium*

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Surface proteins of different *Salmonella* R mutants were labeled selectively by treating live bacteria with cycloheptaamylose-dansylchloride. The labeled proteins were extracted from the cells with 6 M urea and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. From the urea extract a 55-kilodalton protein common to numerous *Salmonella* strains could be isolated by ion-exchange chromatography and gel filtration free of lipopolysaccharide. Immunization of rabbits with isolated protein led to the formation of specific antibodies. Such antiprotein antisera could be employed in Western blots for the specific identification of the 55-kilodalton protein in bacterial extracts containing mixtures of different *Salmonella* proteins. The importance of this antigen is emphasized by antisera against acetone-killed *Salmonella* bacteria, showing a preferential interaction with the 55-kilodalton protein in Western blots. Active immunization of mice with the 55-kilodalton protein afforded significant protection against experimental infection with *S. typhimurium*.

Among bacterial infections salmonellosis is still a worldwide problem. Numerous attempts have been made to establish efficient vaccines and to get insight into mechanisms of antiinfectious immunity (1, 8, 17, 33). The immunoprotective components of salmonellae, however, have not yet been identified. Generally, the O antigens are considered to be responsible for the species-specific protection (8, 16). On the other hand, it is well known that *Salmonella* smooth strains of different serovars and even rough mutants devoid of the O antigen also can act as effective vaccines (7, 11, 26). Many components of the *Salmonella* cell have been claimed to evoke antiinfectious immunity. However, in most cases preparations used as vaccines were contaminated with other subcellular fractions. In preceding papers (3, 28) it could be demonstrated that the isolated and highly purified protein fraction from a *Salmonella typhimurium* smooth strain as well as those from various *Salmonella* rough mutants mediate protection against experimental *Salmonella* infection. Such preparations contained about 30 protein species (3). Species-overlapping protection became obvious. During natural infection of humans (3, 6, 14, 32), antibodies against *Salmonella* proteins are induced.

The aim of this study was to identify proteins exposed on the surface of *Salmonella* and to isolate relevant single proteins with potential importance for vaccine development. Antibody formation against a 55-kilodalton (kDa) protein as compared with that against whole bacteria, serological reactivity of this protein, and the protective capacity in comparison to a complex antigen extract are discussed.

MATERIALS AND METHODS

Bacteria and cultivation conditions. *S. typhimurium* his386 (Ra mutant), *S. typhimurium* SL1032 (Rd_1 mutant), and *Salmonella minnesota* R345 (Rb_2 mutant) derived from the culture collection of this Institute were used throughout this study. The organisms were grown in a complex medium consisting of 7.5 g of tryptone (Difco Laboratories) 7.5 g of tryptose (Difco), 10 g of yeast extract (Difco), 10 g of glucose, 3 g of NaCl, 8 g of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.3 ml of polyethylene glycol P 2000 per liter of deionized water. Cultivation was performed under aerobic conditions in a fermentor at 37°C and pH 7.2 (27). Bacteria were harvested by centrifugation in the logarithmic phase or immediately after cells reached the stationary phase, indicated by a sharp rise in oxygen partial pressure. The cells were used either for labeling or for preparative extraction of proteins. For preparation of flagella, growth was performed in shaking cultures overnight in the same medium with a reduced content of 0.3% glucose.

Cell labeling and analysis of labeled proteins. Labeling was performed with the cycloheptaamylose-dansyl chloride complex (CDC) described by Kinoshita et al. (12) with the modification of Legrum et al. (13).

About 10^{10} cells were washed three times with 3 ml of 5 mM phosphate buffer (pH 7.5) containing 140 mM NaCl and 10 mM $MgCl_2$. Bacteria were suspended in 2 ml of 50 mM phosphate buffer (pH 7.5). The 5 mg of CDC was added, and the mixture was stirred for 1 h at 10°C. The suspension was centrifuged for 10 min at 4,500 × *g*, and the supernatant was removed.

The cells were suspended in 6 M urea containing 10 mM Tris hydrochloride (pH 7.5) and 5 mM EDTA and stirred for 1 h (3). After centrifugation at 17,000 × *g* for 20 min, the supernatant was concentrated by ultrafiltration (centricon 10; Amicon Corp.) to a final volume of 70 μl. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels. Labeled molecules were visualized by UV illumination and photographed. Proteins were then stained with Coomassie blue.

Extraction and separation of proteins. Harvested bacteria were washed once in Tricine {*N*-[Tris(hydroxymethyl)-methyl]glycine} buffer (pH 7.2) and extracted with buffered 6 M urea (see above). The extract was dialyzed for 3 days against running tap water. The precipitated components were separated by centrifugation (34 800 × *g*, 60 min); both fractions, containing the soluble and insoluble material, were either kept at −20°C or lyophilized.

Isolation of 55-kDa protein. The 55-kDa protein was isolated from the soluble fraction of the extracts obtained from *S. typhimurium* his386 and *S. typhimurium* SL1032. All steps were carried out at 6°C with buffers without detergents.

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In a first step the soluble fraction was separated by anion-exchange chromatography (2) with DEAE-Sepharose CL 6B (Pharmacia Fine Chemicals). The extracted material, containing about 60 mg of protein, was dissolved in 100 ml of starting buffer (10 mM Tris hydrochloride [pH 8.0], 5 mM EDTA) and was loaded on a column of 2.4 by 11 cm. The column was washed with one bed volume of the starting buffer, and bound material was eluted with a linear gradient of 0 to 500 mM NaCl dissolved in starting buffer at a flow rate of 20 ml/h. Fractions of 5 ml were collected. Those containing 55-kDa protein were combined and dialyzed against buffer containing 20 mM Tris hydrochloride (pH 7.5)–5 mM EDTA–100 mM NaCl.

In a second purification step material was applied on a Sepharose CL-6B (Pharmacia) column of 2.6 by 100 cm. Elution was performed with Tris buffer (pH 7.5) (see above) at a flow rate of 20 ml/h, and 5-ml fractions were collected.

Analytical methods. Protein content was determined by the method of Bradford (5) with bovine serum albumin as a standard.

β-Hydroxymyristic acid, as an intrinsic component of lipopolysaccharide (LPS) was determined by gas-liquid chromatography (2, 9) with a Varian Aerograph 1400. Generally 2 to 3 mg of substance was used; the amount, however, was increased to 5 mg in samples expected to contain low amounts of LPS. The fatty acid methyl esters were dissolved in 5 μl of chloroform, from which 1.5 μl was injected into the column. The sensitivity of the aerograph was normally adjusted to $10^{-10/2}$; with samples expected to have low LPS content it was $10^{-11/2}$. The response factor was taken as 0.9. The minimum amount of β-hydroxymyristic acid measured by this method was 0.001%. Samples with a smaller content were considered to be free from LPS.

Stimulation of antibodies in rabbits. Outbred Chinchilla rabbits were used in all experiments. Samples (2 mg) of the 55-kDa protein isolated from *S. typhimurium* his386 and *S. typhimurium* SL1032 were dissolved in 1.0 ml of 0.25% $NaHCO_3$ solution, and 1.0 ml of Alu-Gel-S (Serva) was added. The mixture was kept at 4°C for 1 h; then 0.5 ml of sorbitan trioleate (Span 85; Serva) and 2.5 ml of paraffin oil DAB 7 (Roth) were added, and the suspension was mixed thoroughly with an Ultra-Turrax (Janke and Kunkel, Federal Republic of Germany) mixer. Two animals each were immunized three times subcutanously on days 1, 36, and 86 with the suspension described above containing 1.0, 1.0, and 1.5 mg of protein, respectively. Blood samples were taken on days 93 and 100, i.e., 7 and 14 days after the last immunization.

Another two animals were immunized intravenously with acetone-killed *S. typhimurium* his386 (26). The suspension contained 2×10^{10} cells per ml of 0.9% sodium chloride solution. Injections were performed on days 1, 8, 13, 18, and 49 with 0.25, 0.5, 1.0, and 2.0 ml, respectively. Blood samples were taken on days 21, 25, 49, 56, and 61.

Agar gel precipitation. Double diffusion tests by the method of Ouchterlony (22) were performed in 1% agarose gel (Litex) with 0.05 M barbiturate buffer (pH 8.2). An LPS-free 55-kDa protein isolated from *S. typhimurium* his386 was used as antigen with a concentration of 2 mg of protein per 100 μl of barbiturate buffer (pH 8.2). Antisera were used undiluted. The gels were kept at 4°C and observed for 5 days.

Preparation of flagella. Bacteria were suspended in 100 mM Tris hydrochloride buffer (pH 7.2). Flagella were sheared off by treatment with an Omnimixer (Sorvall) for 10 min at setting 2.8 in an ice-water bath. Bacteria and cell debris were removed by two cycles of centrifugation at 4,000 × *g* for 20 min and 8,000 × *g* for 15 min. From the supernatant of the second centrifugation step the flagella were spun down at 48,000 × *g* for 1 h, and the sediment was suspended in 2 ml of sterile water and kept at −20°C until use.

SDS-PAGE. SDS-PAGE was performed in a discontinuous gel system by the method of Lugtenberg et al. (15). Proteins were stained with Coomassie blue.

Western blots (immunoblots). Proteins were transfered electrophoretically from the SDS-polyacrylamide gel onto Immobilon transfer membrane (Millipore) by the method of Towbin et al. (31). The immune reaction was performed with antibodies obtained from immunized rabbits (see above). Bound rabbit immunoglobulin G was detected with alkaline phosphatase-conjugated goat antiserum as described by Blake et al. (4).

Vaccination and infection of mice. NMRI mice weighing about 20 g were randomized in groups of eight animals. Soluble urea-extracted material obtained from *S. typhimurium* SL1032 and 55-kDa protein separated from this strain were used as vaccines. Mice were immunized intraperitoneally two times at intervals of 14 days with single doses of 100 μg (3). Ten days after the booster vaccination the animals were challenged intraperitoneally with graded, 10-fold-diluted amounts of *S. typhimurium* C5. From the mortality values obtained 19 days after challenge, the 50% lethal dose was calculated by the method of Reed and Muench (23). Statistical significance was determined as described by Valtonen (34).

RESULTS

Identification of surface proteins. Urea-extracted proteins from *S. typhimurium* his386 and *S. minnesota* R345 showed very similar patterns when analyzed by SDS-PAGE and stained with Coomassie blue (Fig. 1). A somewhat different banding could be observed with *S. typhimurium* SL1032; in addition, the influence of the growth phase was apparent here (Fig. 1). Fluoresceing bands in extracts from all of these strains, however, were closely related. Bands of high intensity were those with molecular masses of about 55 kDa and close to 43 kDa. Some proteins with molecular masses of about 69 kDa and in the range of 24 kDa were also labeled. This indicates the presence of these proteins on the *Salmonella* surface and their possible importance as immunogens. One of them, with a molecular mass of 55 kDa, can be considered as a widespread component in *Salmonella* strains (3, 19) and as a most abundant component in the soluble fraction of urea extracts. Therefore this protein was selected for further investigation. Isolation of flagellae and their analysis by SDS-PAGE revealed a molecular mass of 52 kDa. This excluded the possibility that both proteins could be identical. Most probably the 55-kDa protein is an integral component of the outer membrane.

Separation of the 55-kDa protein. The soluble part of the urea extract obtained from *S. typhimurium* his386 was fractionated by ion-exchange chromtography. The elution profile is given in Fig. 2. The 55-kDa protein was eluted in two different ranges of salt concentrations, 110 through 165 and 235 through 290 mM, still in a mixture with other protein species (Fig. 3). According to elution ranges, the materials were designated as 55-kDa protein I and 55-kDa protein II, respectively. In further processing both were treated separately.

After dialysis, the 55-kDa protein was further purified by gel filtration. Part of the 55-kDa protein apparently formed

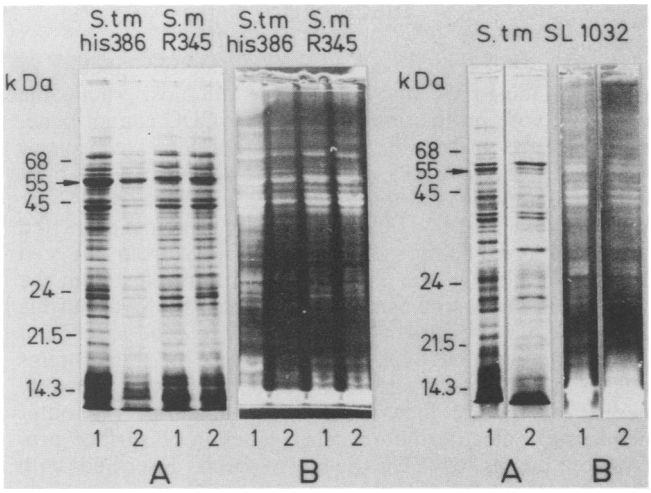

FIG. 1. SDS-PAGE of urea extracts from *S. typhimurium* his386, *S. minnesota* R345, and *S. typhimurium* SL1032. Patterns of proteins are demonstrated by Coomassie blue staining (A). Proteins exposed on the outside of the outer membrane are visualized by dansylation with CDC (B). Extracts were obtained from cells in the logarithmic growth phase (lane 1) and in the stationary phase (lane 2).

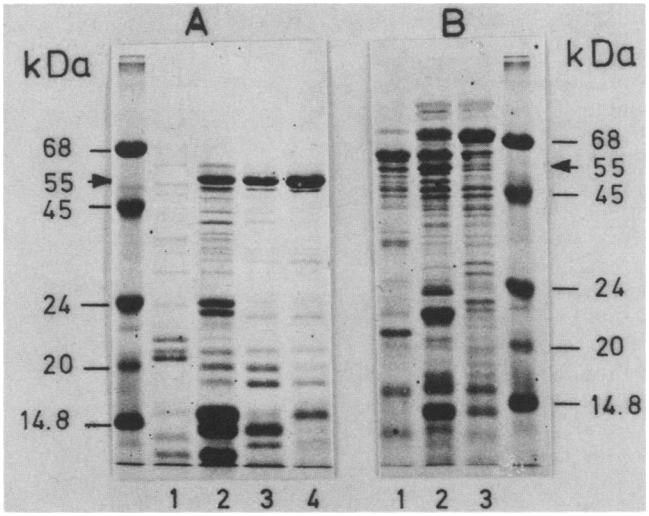

FIG. 3. SDS-PAGE (10% polyacrylamide) corresponding to the elution profile of Fig. 2. (A) Lanes: 1, 2, 3, and 4, fractions 23, 28, 31, and 35, respectively (peak I). (B) Lanes: 1, 2, and 3, fractions 51, 59, and 63, respectively (peak II). The arrow shows the position of the 55-kDa protein. Molecular weight standards were lysozyme, trypsin inhibitor, trypsinogen, ovalbumin, and bovine serum albumin.

polymers. These polymers were eluted from the Sepharose CL-6B column after 120 ml of buffer, which is comparable to the elution volume of the high-molecular-weight Dextran Blue 2000. Both 55-kDa proteins I and II obtained by ion-exchange chromatography showed identical behavior in gel filtration. Upon treatment with sample buffer the polymer molecules dissociated into their monomers as shown by SDS-PAGE. The 55-kDa protein was obtained by this procedure in an acceptably pure form, accompanied by small amounts of a second protein showing a somewhat higher molecular mass in the case of 55-kDa protein I and with a slightly lower molecular mass in the case of 55-kDa protein II (see Fig. 6). The remaining 55-kDa protein was eluted from the column in a mixture of other proteins (Fig. 4 and 5).

The LPS content of the soluble part of urea extracts from Salmonella R mutants is generally low (3). The starting material used here contained 0.06% β-hydroxymyristic acid. All 55-kDa protein fractions obtained by the procedures described above were free from LPS as determined by gas-liquid chromatography.

Material extracted from *S. typhimurium* SL1032 was first purified by two gel filtrations to eliminate contaminating LPS and then processed by ion-exchange chromatography as described above for *S. typhimurium* his386. In contrast to the latter, *S. typhimurium* SL1032 yielded only minimal amounts of polymeric 55-kDa protein, which was not used for further experiments. The separation resulted in a monomeric 55-kDa protein accompanied by a second protein of higher molecular mass, as obtained with the I 55-kDa fraction from *S. typhimurium* his386, but in greater amount. Further, some contamination was present with a 31-kDa protein that was not a labeled surface protein.

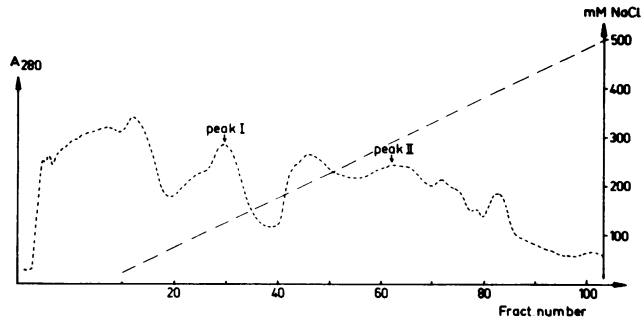

FIG. 2. Ion-exchange chromatographic fractionation of soluble proteins of *S. typhimurium* his386 extracted with urea. Proteins were eluted with a linear gradient of 0 to 500 mM NaCl. The peaks designated I and II contain the 55-kDa protein.

Immunogenicity and serological reactivity of the 55-kDa protein. Antisera obtained from rabbits immunized with either 55-kDa protein II or with acetone-killed *S. typhimurium* his386 were checked for their reactivity against *Salmonella* protein by agar gel precipitation. The strongest reactions were obtained in the first group with sera from day 93 and in the second group with sera from days 21 and 56. Such antisera were chosen for Western blot tests. No hemagglutinating activity against the corresponding rough LPS could be detected in the antisera against the 55-kDa proteins (titers below 1:2).

Figure 6 demonstrates banding patterns of the protein preparations used and immune reactions of antisera raised against 55-kDa protein II and acetone-killed bacteria. With the 55-kDa protein II antiserum (Fig. 6B), corresponding reactivity was observed with the soluble fraction of a crude

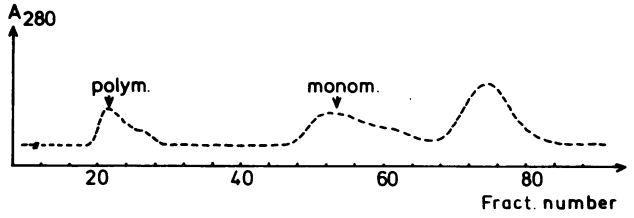

FIG. 4. Elution profile of the polymeric and monomeric 55-kDa protein after gel filtration on a Sepharose CL-6B column.

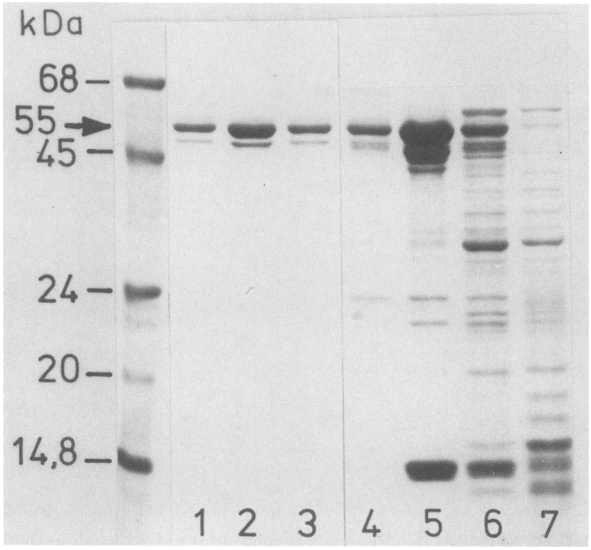

FIG. 5. Overview control SDS-PAGE corresponding to the elution profile of Fig. 4. Lanes: 1, 2, and 3, fractions 20, 24, and 28, respectively; 4, 5, 6, and 7, fractions 48, 52, 56, and 60, respectively. After treatment with SDS the polymers dissociate and migrate on the gel as monomers.

urea extract before and after removal of LPS (lanes 1 and 2) as well as 55-kDa protein fractions I and II (lanes 3 and 4). Remarkably, the immune reaction occurred not only with the 55-kDa protein but also with the accompanying protein of the 55-kDa protein I fraction not present in discernible amounts in the material used for immunization. Reactions with other proteins were negligible in all cases. The 55-kDa protein antiserum can be also used to identify the 55-kDa protein or the serologically related proteins in extracts obtained by other procedures (lanes 5 and 6).

Antiserum against whole cells of *S. typhimurium* his386 predominantly contained antibodies against the 55-kDa protein and the accompanying proteins (Fig. 6C). As expected, reactivity to other *Salmonella* proteins could be observed; the strength of those reactions, however, was moderate. Antibodies raised against the separated 55-kDa protein from *S. typhimurium* SL1032 exhibited strong reactivity with the 55-kDa protein from *S. typhimurium* his386.

Protection tests. The protective capacity of the 55-kDa protein against infection with *S. typhimurium* in mice is given in Table 1. The 50% lethal doses demonstrate a 200-fold decrease in mortality when compared to nonimmunized animals; the difference is statistically significant. The urea extract from which the 55-kDa protein had been separated brought about a somewhat higher protection. Materials for vaccination were obtained from stationary-phase cells like the whole-cell vaccine used in previous experiments (3).

DISCUSSION

The use of the CDC complex for selective labeling of surface proteins proved to be a simple and most practicable procedure with plasma membranes (12). CDC cannot penetrate the outer membrane pores of gram-negative bacteria because of its size (1,405 Da). Outer membrane pores limit uptake of molecules to those under 600 to 700 Da (20). As we demonstrated earlier (18), *Salmonella* proteins extracted with 6 M urea do not derive exclusively from the cell surface. In addition the extracts contain proteins from the periplasmic space and perhaps even from the cytoplasmic membrane. The results of this study are in good agreement with what could be expected: only some of the proteins present in the extracts showed fluorescence. Most probably these proteins are located on the outside of the outer membrane. Selective fluorescence labeling of surface proteins by means of CDC has also been described with *Neisseria gonorrhoeae* (10).

Dansylchloride, the staining component of the CDC-complex, is known to react with free α- and ε-amino groups of proteins. The fluorescence intensity of a given labeled protein depends on its total amount as well as on its content of lysyl groups accessible to the outside of the membrane. When dansylchloride was incorporated to lecithin-cholesterol vesicles (24), proteins could not be labeled without preconditioning bacteria with EDTA or certain antibiotics. This was not necessary in our experiments. Besides differences in bacterial strains, growth conditions, and composition of the dansylation buffer, the lower size of the carrier and absence of any electric charge might have had a beneficial influence on the access to the surface proteins. Nevertheless the relation between Coomassie blue staining and fluorescence intensity was quite different among the labeled bands. Therefore fluorescence has to be considered independently of its strength only as an indication of a location of proteins on the cell surface. The immunological importance of such proteins has to be checked by other suitable methods.

In our protection tests with whole cell vaccines, acetone-killed salmonellae were used (25). Immunization of rabbits with this type of vaccine leads to pronounced antibody formation against the 55-kDa protein. In the mouse protection test a 55-kDa protein separated from a *S. typhimurium* R mutant evokes significant protection against wild-type *S. typhimurium* infection. These responses indicate that protection obtained with *Salmonella* R mutant whole-cell vaccine is not caused by a nonspecific stimulation of the immune system but most probably is based on its proteins. Previous investigations show that rough LPS does not act as a protective antigen and that the R chemotype of the LPS does not determine the protective capacity of R-mutant vaccines (24, 27). However, by its lipid A part LPS functions as a strong immunoenhancer (29). The protein preparations used

TABLE 1. Protection against infection with *S. typhimurium* C5 mediated by the soluble fraction of a urea extract obtained from *S. typhimurium* SL1032 and by the 55-kDa protein separated from this extract[a]

Immunization with:	Single dose (μg of protein)	LD_{50} after challenge with *S. typhimurium* in:		Significance of difference to nonimmunized mice (*P*)
		Nonimmunized mice	Immunized mice	
Urea extract	100	1.0×10^2	1.3×10^5	<0.01
55-kDa protein	104		2.2×10^4	<0.01

[a] NMRI mice were immunized intraperitoneally twice at intervals of 14 days and infected intraperitoneally 10 days after the second immunization with 10-fold-graded amounts of *S. typhimurium* C5. The 50% lethal dose (LD_{50}) (22) indicates the final mortality on day 19 postinfection. Survival rates had become stable in both groups on day 13. Statistical significance was calculated as described by Valtonen (34).

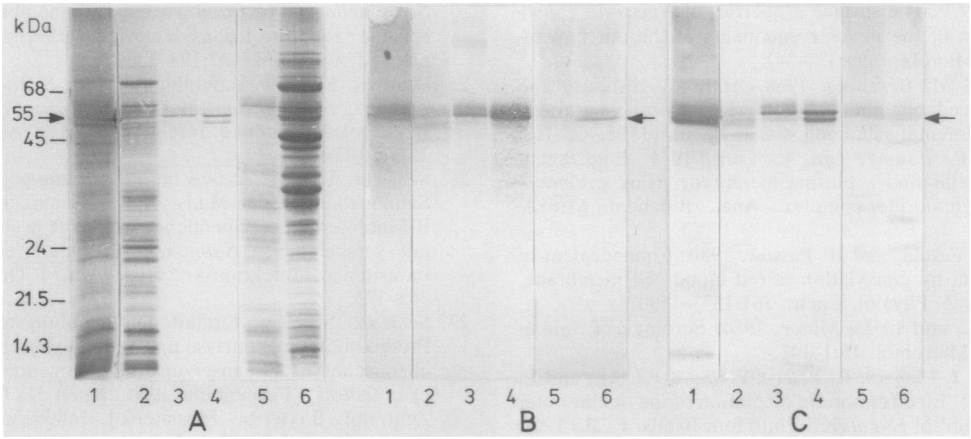

FIG. 6. Western blot analysis showing reactions of *Salmonella* proteins to rabbit antisera against the 55-kDa protein isolated from *S. typhimurium* his386 (B) and against acetone-killed *S. typhimurium* his386 (C). The banding pattern of the proteins obtained from *S. typhimurium* his386 and used as antigens is given (A). Lanes: 1, soluble fraction of the urea extract; 2, LPS-free protein separated from lane 1; 3, isolated 55-kDa protein I; 4, isolated 55-kDa protein II: 5, soluble fraction of an aqueous butanol extract; 6, insoluble fraction of the extract in lane 5.

in our investigations can be considered as free from LPS, as shown by sensitive chemical analyses and by immunological tests.

According to the similarity of labeling patterns in different *Salmonella* strains and to serological cross-reactions, the 55-kDa protein possibly belongs to those components of the *Salmonella* cell which are responsible for species-overlapping protection.

During the purification procedures the 55-kDa protein showed some unusual characteristics. In ion-exchange chromatography it was eluted in two clearly different maxima; thus it behaved like two different proteins. By the same procedure performed with buffers containing 6 M urea, it was detected only in one peak. Further, by gel filtration about one-quarter of the total amount of the 55-kDa protein was eluted in a highly polymeric form, whereas the bulk eluted according to its molecular mass.

It is well known that the urea-extracted material contains protein, LPS, and other components of the *Salmonella* cell (2). Probably, after removal of the urea, protein and LPS from complexes as well as polymers. Existing polymers again dissociate into monomers by treatment with an ionic detergent like SDS. LPS-protein complexes apparently show different binding affinities to the ion-exchange matrix, depending possibly on the number of LPS molecules and their conformation. This resembles to the behavior of the outer membrane proteins (20, 21, 30). The 55-kDa protein was separated together with an accompanying protein either of a slightly higher or lower molecular mass. Serological tests demonstrate a strong cross-reactivity of these protein bands, indicating their antigenic identity. Recent results of N-terminal sequence analysis obtained with the II 55-kDa protein from *S. typhimurium* his386 and with the 55-kDa protein fraction derived from *S. typhimurium* SL1032 have shown identity of all bands within the first seven amino acid residues. Further research has to be done on the origin of the accompanying protein bands.

Altogether, in the present study we identified and isolated from the *Salmonella* surface a protein that evoked antibody formation in rabbits and protected against experimental salmonellosis in mice. In further experiments the reactivity of our antiserum with homologous proteins of other strains and the importance of other surface-located *Salmonella* proteins will be studied.

ACKNOWLEDGMENTS

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