

Solubilization and Characterization of Surface Antigenic Components of *Erysipelothrix rhusiopathiae* T28

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The antigenicity of *Erysipelothrix rhusiopathiae* T28 (serotype 2) was investigated. Antigens were solubilized from the cell surface with detergents. By means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting it was shown that the molecular weight of the main antigenic component—a nonprotein—was 14,000 to 22,000. This major antigen was shown to be a polydisperse anionic polysaccharide located on the surface of *E. rhusiopathiae*. Affinity chromatography also revealed a number of immunologically active proteins with molecular weights of 78,000, 72,000, 68,000, and 48,000.

Erysipelothrix rhusiopathiae is known to induce erysipelas arthritis in swine. Due to the chronic progressive character of this arthritis of known bacterial origin, this disease has been looked upon as a model for rheumatoid arthritis, a chronic progressive inflammatory arthritis of unknown origin in humans. Using *E. rhusiopathiae* T28, Schulz et al. (21, 22) have established a standardized arthritis model in rats with a typical two-phase course. Whereas the early acute phase shows all of the features of a generalized bacterial infection, bacteremia disappears during the chronic phase, which is characterized by a chronic, progressive ankylosing type of arthritis. During this latter phase living bacteria can be found for months in the joints, but bacterial antigenic components persist even longer (27). Little is known, however, about the nature of such persisting antigenic materials.

Protective antisera against *E. rhusiopathiae* for passive immunization have been available for many years (17, 24), and approaches to the characterization of the protective antigen have been published (19, 20, 25, 26). However, comparatively little work has been done toward a systematic analysis of the antigenic components of this organism (18). There is evidence that the antigenic moieties of *E. rhusiopathiae* are chemically heterologous components; proteins, mucopeptides, and lipids have been described (9, 10). Regarding the chronic arthritis models, no information is presently available as to the number and nature of bacterial components responsible for the chronic inflammatory process, so that the mechanism of chronicity in this model has remained unresolved.

For all of these reasons, a systematic approach to an antigenic analysis of *E. rhusiopathiae* seemed necessary. Characterization of cell surface antigens solubilized with different detergents has been initiated. Results concerning the location and chemical composition of the major antigens are presented in this communication.

MATERIALS AND METHODS

Culture methods. *E. rhusiopathiae* T28, serotype 2 (Tierärztliche Hochschule), was grown from lyophilized cells in 100 ml of nutrient broth (E. Merck, Darmstadt, Federal Republic of Germany) for 24 h at 37°C. A 10-ml sample of the resulting culture suspension was used to inoculate a 2-liter Erlenmeyer flask containing 1 liter of Feist bouillon (4). The

flasks were incubated for 48 h at 37°C, and purity was checked by subculturing on blood agar.

The cells were harvested by centrifugation and washed three times in sterile distilled water. Approximately 1 g of fresh weight was obtained per liter of bouillon. The washed cells were heat killed by incubation for 2 h at 56°C.

Surface antigen preparation. Cells (5 g, fresh weight) were suspended in 1 mM Tris buffer (pH 7.2) containing one of the following detergents: 0.25% Triton X-100; 10 mM sodium deoxycholate, 10 mM CHAPS [3-(3-cholamidopropyl)dimethylammonium-1-propane sulfonate], 10 mM OG (*p*-octyl- β -D-glucopyranoside), 10 mM sodium dodecyl sulfate (SDS), 0.25% Lubrol, or 1 mM EDTA. The antigens were found to be dissolved in the buffer after 30 min of incubation at 37°C. Cells were removed by centrifugation. The supernatants were dialyzed against Dowex W 1 \times 8 (Serva, Heidelberg, Federal Republic of Germany) for 48 h at 4°C, concentrated 100-fold by ultrafiltration (Amicon standard cell 402, membrane Y-M 10; Amicon Corp., Lexington, Mass.; the membrane was permeable for molecules with molecular weight [MW] <10,000), and filter sterilized.

Enzyme treatment of antigenic components. After solubilization with EDTA the samples were incubated with DNase and RNase (final concentration, 100 μ g/ml) for 30 min at 37°C (pH 5.0). Proteinase K (final concentration, 100 μ g/ml) was added, and the samples were incubated for 3 h at 60°C (pH 7.5).

Production of antisera and IgG fractions. Six female rabbits were used; preimmune sera were taken and used as controls to check for any preimmune nonspecific antibodies. The rabbits were then immunized with heat-killed bacteria and were bled on day 44. Sera were collected and stored at -20°C until use. Immunoglobulin G (IgG) fractions from the sera were prepared by affinity chromatography with protein A-Sepharose (Pharmacia, Uppsala, Sweden) as described by Goudswaard et al. (5).

Adsorption of antisera. Cell surface antigens were first identified through the comparison of antigens detected by adsorbed versus unadsorbed antisera. The polyspecific antisera were adsorbed by incubation with heat-killed or with pretreated cells (45 min, 37°C). Cells were removed by centrifugation at 8,000 \times g for 30 min. The supernatant was sterilized by filtration (0.2 μ m).

Immunoabsorbent affinity chromatography. Polyclonal rabbit immunoabsorbents were prepared with 20 mg of IgG

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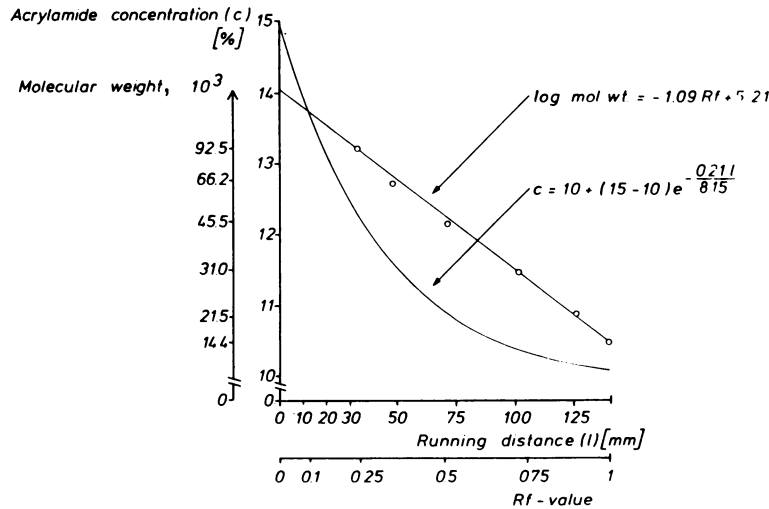


FIG. 1. MW estimation in concave SDS-polyacrylamide gels.

per ml of activated agarose (Affi-Gel 10; Bio-Rad Laboratories, Richmond, Calif.). Unbound IgG was eluted with 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 8.0). The pretreated gel was equilibrated with phosphate-buffered saline (pH 7.2) in a column, and 600 μ l of the sample was added. Unbound components were washed off with phosphate-buffered saline. Bound antigenic components

were eluted with 0.1 M sodium citrate (pH 3.0) and collected in 1-ml fractions. All fractions were dialyzed against phosphate-buffered saline (48 h, 4°C). They were then concentrated 20-fold by ultrafiltration.

SDS-PAGE. Antigenic components were separated by SDS-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli and Favre (12). In the stacking gel the acrylamide concentration was 4%. In the separating gel a concave gradient of 10 to 15% acrylamide was used (Fig. 1). The protein content per sample was 40 to 50 μ g. Gels were run at 20 mA until the tracking dye reached the separating gel. The current was then increased to 40 mA. Proteins were visualized with Coomassie blue (16).

Immunoblotting. The surface antigens were resolved by SDS-PAGE and transferred to nitrocellulose sheets by the method of Burnette (3). All following reactions were carried out at room temperature on a rotating platform. The nitrocellulose was incubated for 60 min in blocking solution, which contained 3% (wt/vol) gelatine in TTBS (20 mM Tris, 50 mM NaCl, 0.05% [vol/vol] Tween 20 [pH 7.5]), to block unreacted sites and then incubated for at least 60 min with

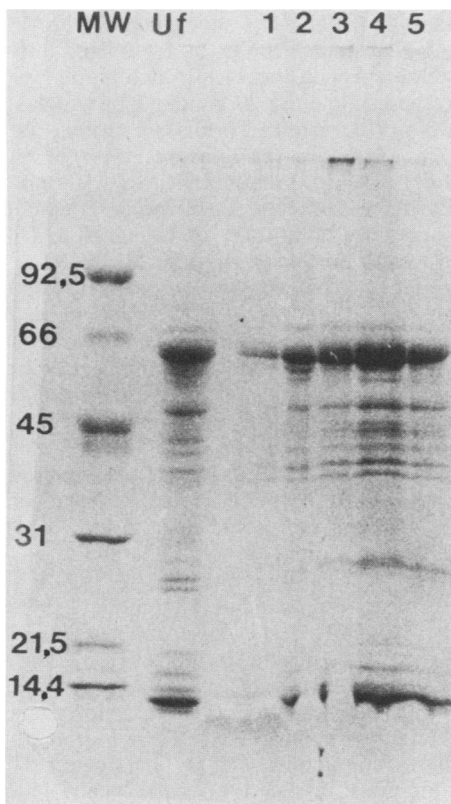


FIG. 2. SDS-PAGE of *E. rhusiopathiae* surface proteins, solubilized with different detergents. Lanes; MW, MW markers; Uf, EDTA; 1, CHAPS; 2, OG; 3, Triton X-100; 4, Lubrol; 5, deoxycholate.

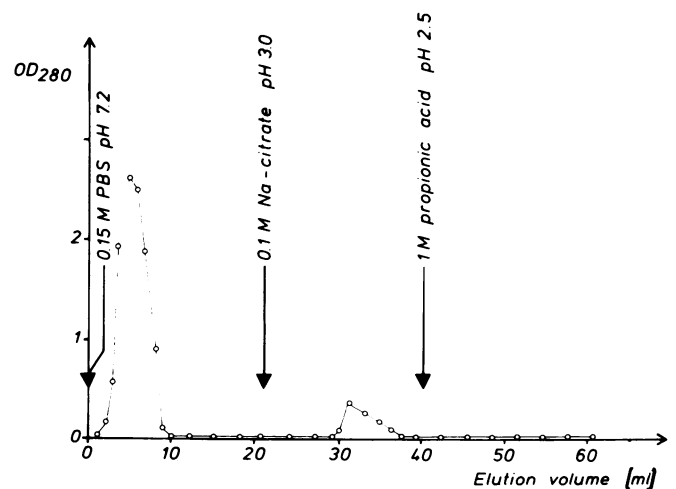


FIG. 3. Affinity chromatography of the EDTA-solubilized sample.

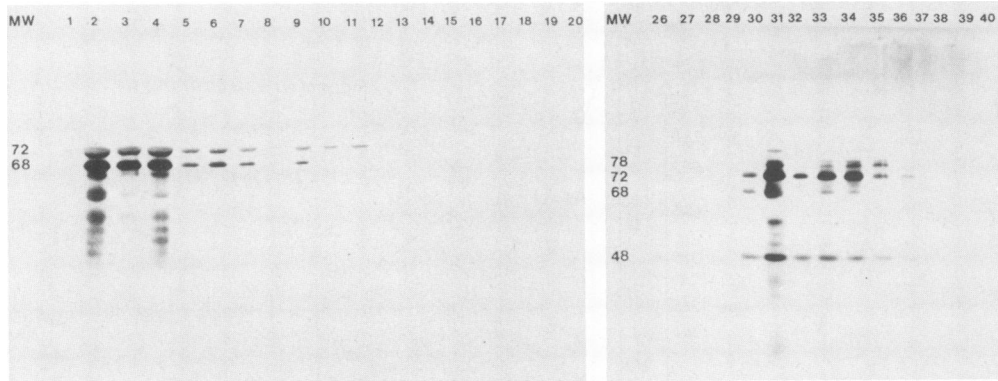


FIG. 4. Immunoblot of the affinity chromatography. Lane MW shows MW markers; lanes 1 through 40 correspond to the elution volume (milliliters) of the affigel 10 column.

antiserum that had been diluted 1:1,000 in TTBS. The nitrocellulose sheets were washed in TTBS and then incubated for 60 min with peroxidase-conjugated goat anti-rabbit serum diluted 1:3,000 in TTBS. After the addition of the substrate solution (containing 4-chloro-1-naphthol and hydrogen peroxide), color developed at the sites of antibody binding. The reaction was halted by immersing the strips in deionized water. MW standards and total protein profiles were visualized by staining the nitrocellulose strips with amido black (0.1% [wt/vol] in 45% methanol–10% acetic acid) and destained with 90% methanol–2% acetic acid.

Dot blotting. Prewetted nitrocellulose strips were air dried; 10 μ l of the sample was applied, and the membrane was allowed to dry completely before continuing to the next step (blocking of remaining reacting sites, etc.).

Chemical analysis. Soluble protein was determined by the method of Bradford (2) with the Bio-Rad protein assay. Particular protein was estimated by the method of Lowry et

al (13). DNA was determined by the method of Hanson and Philipps (7). RNA was quantified by the orcinol reaction (7). Total sugars were determined by the anthrone reaction (6) with glucose as the standard. Muramic acid was estimated by the method of King and White (11). Periodate oxidation was carried out as described by Manners (15). Acid hydrolysis was carried out with CH_3COOH (final concentration, 1%) for 30 min at 100°C. Alkaline hydrolysis was carried out with NaOH (final concentration, 0.25 N) for 1 h at 56°C.

RESULTS

To solubilize antigens of *E. rhusiopathiae*, the organism was treated either with Triton X-100, Lubrol, SDS, OG, deoxycholate, or EDTA. The supernatants were resolved in an SDS-PAGE system. Profiles of solubilized antigens prepared by treatment with the various detergents revealed the presence of approximately 20 bands, 2 of which were consistently more prominent in Coomassie brilliant blue-stained gels (Fig. 2). The major proteins had MW of 64,000 and 48,000. The following immunoblot revealed an identical pattern of antigens independent of the detergents used. An additional antigen with an MW of 14,000 to 22,000 did not correspond to any protein in the SDS-PAGE.

To determine whether antigens detected by Western blotting were exposed on the surface of *E. rhusiopathiae*, polyspecific antisera were adsorbed with heat-treated cells. That the polyspecific antisera contained antibodies to cell surface antigens was indicated by their specific ability to agglutinate heat-killed cells when compared with preimmune sera. Antigens that absorb antibodies can be identified by Western blotting, since there will occur no or a decreased enzymatic reaction. All antibodies could be adsorbed with intact cells, whereas pretreated cells failed to adsorb any antisera, indicating complete solubilization of all antigenic components from the cell surface.

To enrich the major antigenic components, affinity chromatography was carried out. The supernatant of EDTA-treated cells was put onto the column (Fig. 3). The collected fractions were resolved in an SDS-PAGE system and then immunoblotted (Fig. 4). Whereas antigenic proteins could be seen after elution with phosphate-buffered saline, the antigen with nonprotein character could not be detected. The elution of bound antigens revealed a single peak with marked back-tailing, pointing to a low dissociation constant of one of the bound components. The immunoblot of this peak revealed an enrichment of antigenic proteins with MW of 78,000 and 48,000. Other major antigenic components with

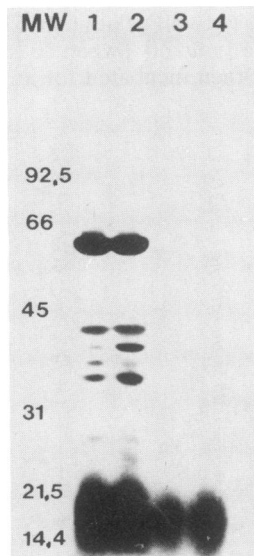


FIG. 5. Immunoblot of solubilized antigen before and after enzyme treatment. Lanes: MW, MW markers; 1, antigens solubilized from the cell surface with EDTA; 2, antigens after DNase and RNase treatment; 3 and 4, antigens after successive DNase, RNase, and proteinase K treatment.

MW of 72,000 and 68,000 were also present but could not be enriched by affinity chromatography. The antigenic nonprotein was bound very tightly to the column, so that only small amounts were released with citrate (Fig. 4). This antigen (MW, 14,000 to 22,000) was recovered after proteinase K treatment of the immobilized IgG, thus verifying its nonprotein character.

To isolate this latter component a successive enzyme treatment with DNase, RNase, and proteinase K was established (Fig. 5). Experiments aimed at preliminary characterization of the 14,000 to 22,000-MW cell surface antigen were undertaken next. The resulting antigenic moiety was checked for protein, sugar, nucleic acids, and muramic acid. Neither nucleic acids nor muramic acid could be detected. The protein content was below that accounted for by the added enzymes.

The anthrone reaction was positive; 600 µg of sugar per ml was estimated. We therefore concluded that this antigen was a polysaccharide located on the cell surface, not being part of the murein sacculus. The polysaccharide character of the antigen was further demonstrated by its stability against alkali and organic acids. Oxidation with meta-periodate destroyed the antigenicity, as revealed by dot blotting. Taken together, these results indicated that the epitopes recognized by this antibody were sugars or their derivatives.

DISCUSSION

This study was performed to identify and characterize the major antigens of *E. rhusiopathiae*. To accomplish this characterization, it was necessary to devise a reliable detergent system that would effectively solubilize the antigens. Upon SDS-PAGE, different detergents yielded similar and highly reproducible patterns. Among approximately 20 separate bands, 2 main proteins were found migrating at MW 64,000 and 48,000. Since surface proteins, i.e., proteins located below a microcapsule (6) of gram-positive organisms (1), have been implicated in various aspects of the pathogenic process, it was necessary to determine those *E. rhusiopathiae* antigens that were exposed on the surface. To identify such antigens, immune sera were preabsorbed with intact cells to remove specifically those antibodies directed against surface antigens. The immunoblot was utilized to investigate which of the proteins were immunogenic. The results indicate that there are a number of surface-expressed protein antigens. The most prominent antigens isolated by affinity chromatography migrated at MW 78,000, 72,000, 68,000, and 48,000. It remains to be determined precisely how many different proteinaceous antigenic specificities are present in these fractions.

An additional major surface antigen was found with a MW of 14,400 to 22,000, which did not correspond to any protein in the SDS-PAGE. Affinity chromatography showed that this antigen was tightly bound and could only be extracted by pronase treatment. This fact pointed to the nonprotein character of this moiety. Enzyme treatment and chemical analysis supported this assumption. The antigen could be identified as an acidic polysaccharide. Acidic polysaccharides are to be regarded as teichoic acids or as capsular components (23). Since for *E. rhusiopathiae* no teichoic acids have been described (14), it could be inferred that the major nonprotein antigen is a capsular polysaccharide. This conclusion was also supported by the absence of muramic acid. In contrast, the antigenic components described by Kalf et al. (9, 10), which were prepared by acid extraction at +100°C, contained muramic acid and were therefore characterized as part of the cell wall. A direct comparison

between these results, those described by others (18–20, 25, 26), and the findings reported in this paper is, however, precluded because of the fundamental differences in extraction and immunological methods.

Taken together, our results show that *E. rhusiopathiae* T28 contains one major polysaccharide capsular antigen and a number of additional proteinaceous antigenic components, which were readily detectable by sera from immunized rabbits. If antibodies play a major role in the defense against *E. rhusiopathiae* infection, one or more of these antigens may be important in the pathogenesis of the disease caused by *E. rhusiopathiae* T28. Moreover, with monoclonal antibodies against defined, distinct antigens which are now available, important questions concerning antigen persistence in the chronic polyarthritis induced by *E. rhusiopathiae* can now be approached.

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