Outer Cell Envelope Glycoprotein from Two Strains of Serratia marcescens

JOSEPH C. TSANG, SANDY TATTRIE, AND DENNIS KALLYVY
Department of Chemistry, Illinois State University, Normal, Illinois 61761

Received for publication 9 July 1970

Cell envelope glycoproteins were extracted with sodium dodecyl sulfate from isolated cell walls of two strains of Serratia marcescens and purified by gel filtration column chromatography on Sepharose 4B. There was no significant difference in the chemical composition. Both fractions contained approximately 50% proteins and 10% carbohydrates. Glucosamine and glucose were identified as the only sugar components in the carbohydrate moiety. Immunochemically they shared at least one common antigenic component with each other and possibly with their corresponding lipopolysaccharide fractions.

The cell envelope of gram-negative bacteria has been shown by electron microscopic studies (7) to consist of an inner rigid layer and an outer soft layer which has a membrane-like structure. Electron density measurements of the isolated cell envelope (4) provided further evidence that the outer membrane contains lipopolysaccharides, lipoproteins, and glycoproteins. The lipopolysaccharides are the major constituent of the endotoxin-complex (lipopolysaccharide-protein complex) and have been studied extensively (13, 15). However, the structure and function of the glycoproteins and lipoproteins are less clear. Weinbaum (21) reported the isolation of envelope glycoprotein from E. coli and determined its enzymatic activities (reduced nicotinamide adenine dinucleotide oxidase and adenosine triphosphatase) as well as its immunochemical cross-reactivity with animal and plant cell membrane proteins (14). No structural relationship of the glycoprotein to other cell surface macromolecules was investigated.

The purpose of this study is to isolate and characterize the envelope glycoprotein(s) from two strains of Serratia marcescens, namely, the wild-type chromogenic strain O8 and the mutant nonchromogenic strain Bizio. Formerly, S. marcescens was considered nonpathogenic, but recent reports indicated that the nonpigmented mutant was virulent and resistant to antibiotics (20). The variation of virulence between strains might be affected by the chemical differences of the various components of the cell surface. Indeed, the virulence of the wild-type Salmonella typhimurium has been attributed to the ability of the O-side chains of the lipopolysaccharides of the outer membrane to resist phagocytosis (12).

It might be of interest to study the possible roles of other surface components such as the envelope glycoproteins in the virulence of a bacterium.

MATERIALS AND METHODS

Bacteria. Serratia marcescens O8 (chromogenic wild-type) grown on an enriched medium and S. marcescens Bizio (nonchromogenic mutant) grown on an inorganic medium were supplied by General Biochemicals, Chagrin Falls, Ohio. The cells were harvested at the late log phase.

Preparation of cell wall and isolation of cell envelope glycoproteins. Cell walls were isolated by sonic treatment and centrifugation of the whole cells by the method of Williams (22; Fig. 1). The pigmented cell wall preparation (fraction CW08) and the nonpigmented cell wall preparation (fraction CW-B) were submitted to extraction with 0.5% sodium dodecyl sulfate (SDS) at 50 C for 1 hr. After centrifugation at 10,000 rev/min for 20 min, the supernatant and sediment fractions were separated. The dissociating agent was removed by dialysis against distilled water, and the supernatant fractions were recovered by lyophilization as fraction S-SO8 and fraction S-SB, respectively.

Preparation of endotoxins. Endotoxins (lipopolysaccharide-protein complex) were isolated from both strains by the trichloroacetic acid-extraction method of Boivin as reported previously (J. Tsang and J. Rilette, Trans. Ill. State Acad. Sci., in press).

Antisera. Antisera against whole cells of both strains were gifts from P. Alaupovic, Oklahoma Medical Foundation, Oklahoma City, Okla. Antisera against fraction S-SB was prepared by injection of white, New Zealand male rabbits (4 to 5 lb.) with saline suspension of fraction S-SB (2 mg per dose). The total dose of 9 mg was administered in four samples at intervals of 5 days. The last injection consisted of a dose of 3 mg. The animal was bled...
by cardiac puncture at the end of 20 days. The presence of antibodies was tested by double diffusion in agar gel (16).

**Gel filtration chromatography.** Purification of fractions S-SO8 and S-SB was performed by gel-filtration column chromatography in Sepharose 4B gel. Thirty milligrams of the samples were dissolved in 3 ml of 0.05 M phosphate buffer, pH 7.5, in 0.15 M NaCl which contained 0.2% SDS and eluted by the same buffer. Fractions were collected every 3 ml. Samples of 0.2 ml were taken for protein and carbohydrate analyses.

**Chemical analyses.** Anthrone-positive carbohydrates (hexoses) were measured by the method of Koehler (9) with beta-D-glucose (General Biochemicals) as the standard. Hexosamines were determined by the method of Rondle and Morgan (18) with D-glucosamine hydrochloride (Mann Research Laboratories) as the standard. Uronic acids were analyzed by the method of Bitter and Muir (5) with D-glucuronic acid (General Biochemicals) as the standard. Protein was determined by the method of Lowry et al. (11) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard. Phosphorus was analyzed by the procedure of Barlett (3) using KH2PO4 (Baker) as the standard. Total extractable lipids were removed by chloroform-methanol (2:1) extraction in a soxhlet apparatus and determined gravimetrically.

**Paper chromatography.** Samples were hydrolyzed in sealed tubes under two conditions: 2 N HCl at 110° C for 4 hr for detection of neutral sugars and 4 N HCl at 110° C for 6 hr for detection of amino sugars. Chromatographic analyses were performed by the descending method on Whatman no. 1 filter paper with the following solvent systems: (i) ethyl acetate-pyridine-water (3.6:1:1.5) and (ii) butanol-pyridine-water (11:3.6:5.5).

Reducing sugars were detected by spraying with ammoniacal silver nitrate, and amino sugars were detected by spraying with ninhydrin in 0.2% butanol and confirmed by Elson-Morgan reagents (17).

---

**Fig. 1. Procedure for the isolation of cell walls from Serratia marcescens strain O8 and strain Bizio. Precipitate ppt.**
RESULTS

Preparation of cell envelope glycoprotein. The yields of the SDS-extracted fractions (fractions S-SO8 and S-SB) from the corresponding isolated cell wall preparations (fractions CW08 and CW-B) were 95 and 80%, respectively (Table 1). Fraction S-SO8 differed from fraction S-SB in that it contained prodigiosin and was pink in color. Both fractions had similar solubility: insoluble in water, partially soluble in 0.2% sodium deoxycholate and 3 mM guanidine hydrochloride, but completely soluble in 0.5% SDS. Table 1 shows the chemical composition of fractions S-SO8 and S-SB. There is no significant difference between the extracts from two strains of *S. marcescens* with the exception of the amount of extractable lipids and the amount of phosphorus. There were twice as much lipids and phosphorus in fraction S-SB as in fraction S-SO8. It is possible that some of the extractable lipids contained phospholipids. The carbohydrate components of the extract were identified as glucose and glucosamine by paper chromatography and chemical analyses. The presence of glucosamine was confirmed by the following criteria: positive with ammoniacal silver nitrate, positive with ninhydrin, and positive with Elson-Morgan reagent (acetylacetone in sodium carbonate and p-dimethylaminobenzaldehyde). From the chemical compositions, it appears that the SDS extracts were glycoprotein in nature (50 to 58% protein and 10% carbohydrates; Table 1).

**Gel filtration chromatography.** Fractions S-SO8 and S-SB were purified by gel filtration on a Sepharose 4B column equilibrated with 0.2% SDS. The fractionation procedures were monitored by both protein and carbohydrate analyses. In both cases, most of the material (approximately 80%) was recovered in the void volume (Fig. 2 and 3). Since the exclusion limit of Sepharose 4B is about a molecular weight of 5 million for polysaccharide and 20 million for protein, the materials in the void column could be considered high-molecular-weight material.

After dialysis and lyophilization, the purified fractions (S-SO8C and S-SBC) were recovered with 75 and 80% yield, respectively. Table 2 summarizes the partial chemical composition of the two purified fractions. The protein content of fraction S-SO8C (53.7%) was slightly higher than that of fraction S-SBC (47.5%). Although the carbohydrate contents were essentially the same.

### Table 1. Chemical composition of glycoproteins extracted by sodium dodecyl sulfate from isolated cell walls

| Fractions | Yield (%) | Protein (%) | Lipid (%) | Total carbohydrate (%) | Hexose (%) | Uronic acids (%) | Glucosamine (%) | Phosphorus (%) |
|-----------|-----------|-------------|-----------|------------------------|------------|-----------------|----------------|---------------|
| LPS-08    |           |             |           |                        |            |                 |                |               |
| LPS-Bizio |           |             |           |                        |            |                 |                |               |
| S-SO8     | 85        | 50          | 18        | 41.6                   | 21.5       | 7.9             | 12.2           | 0.79          |
| S-SB      | 80        | 58          | 33        | 28.1                   | 18.7       | 6.9             | 2.5            | 1.19          |

**FIG. 2.** Column fractionation of fraction S-SO8 on Sepharose 4B. Volume collected at 3 ml per tube. Flow rate 16 ml per hr. Column was monitored by protein and carbohydrate analyses on 0.2-ml samples of every other tube. Solid line = protein analysis. Dotted line = carbohydrate analysis.

**FIG. 3.** Column fractionation of fraction S-SB on Sepharose 4B. For legend see Fig. 2.
in both fractions (10%), the glucosamine content was twice as much in fraction S-SO8C as in fraction S-SBC (2.3%). There was no significant difference in the phosphorus content.

**Immunochemical cross-reactivity.** The results of the immunochromatography are presented in Table 3. Fractions S-SB and LPS-Bizio gave two precipitin lines with anti-S-SB. However, the purified fraction (S-SBC) gave only one line. Similar results were obtained with fractions S-SO8, S-SO8C, and LPS-O8. Fraction S-SO8C gave an identity line with fraction S-SBC. One of the two lines given by LPS-Bizio was shown to have partial identity with fraction S-SBC. Since anti-S-SO8 was not available, fractions S-SO8, S-SO8C, and LPS-O8 were allowed to react with the anti-whole cell (08) serum. Two lines were observed in each case, and they were identical to each other. It is interesting to note that fractions S-SB and S-SBC gave only one line with anti-whole cell (08).

**DISCUSSION**

Extensive studies have been made recently on the effectiveness of various detergents in extracting and solubilizing membrane components. SDS in particular has been found to be efficient in disaggregating membranes of gram-positive bacteria (6) as well as membranes of mycoplasma (19) and erythrocyte membranes (10). Since the outer soft layer of the cell wall of gram-negative bacteria has a membranous structure, SDS might be useful for the extraction of outer membrane components of gram-negative bacteria. Indeed, Weinbaum (21) reported that SDS extracted envelope glycoproteins from *E. coli*. When a similar extraction method was applied to *S. marcescens*, the resulting extracts were also found to be glycoprotein in nature. Purification by gel-filtration column chromatography on Sephacryl S-4B did not change the chemical composition to any significant extent (50% protein and 10% carbohydrates). Glycoproteins with similar composition had been isolated from erythrocyte membrane (2). The fact that glucose and glucosamine were the only identifiable sugar components in both fractions S-SO8C and S-SBC suggested that contamination with lipopolysaccharide in the extracts was possible but not very likely. Other sugar components such as mannose, galactose, and heptose had been detected in lipopolysaccharides from both strains of *S. marcescens* in addition to glucose and glucosamine (1). The absence of these monosaccharides clearly indicated the absence of lipopolysaccharide contamination. Furthermore, the column chromatographic results with the overlapping protein and carbohydrate curves dismissed the possibility that the extracts were a mixture of carbohydrates and proteins (Fig. 2 and 3).

The immunochromatography results seemed to indicate that the glycoproteins extracted by SDS from the isolated cell walls of two strains of *S. marcescens* shared at least one common antigenic component to each other and possibly with their corresponding lipopolysaccharide fractions (Table 3). The cross-reactivity of the extracts with LPS fraction suggested that these fractions were either presented as a complex with the endotoxin-protein complex or containing a part of it. In any event, there is little doubt that SDS extracts originated from the outer cell surface. A similar pyocin cell wall protein from *Pseudomonas aeruginosa* cell surface was shown to possess a common specific antigen with the protein moiety of endotoxin (8). The protein moiety from endotoxins of *S. marcescens* has been recently isolated (W. Weber and P. Alaupovic, 1969, Amer. Chem. Soc. Nat. Meetings Abstr., no. 206). It would be interesting to study its cross-reactivity with the isolated cell envelope glycoproteins. For future immunochemical studies, it would be advisable to prepare antiserum with the purified fractions S-SO8C and S-SBC rather than with the crude extracts as is the case in this study.

**TABLE 2. Chemical analysis of purified cell envelope glycoproteins from two strains of *Serratia marcescens***

| Yield | Protein | Total carbohydrates | Hexoses (glucose) | Glucosamine | Uronic acid | Total phosphorus |
|-------|---------|---------------------|------------------|-------------|-------------|-----------------|
| Yield | 75      | 53.7                | 10.5             | 4.3         | 1.5         | 0.63            |
| Protein | 80     | 47.5                | 9.7              | 5.4         | 2.0         | 0.83            |

**TABLE 3. Immunochemical cross-reactivities of the cell envelope glycoproteins from two strains of *Serratia marcescens***

| Fractions | No. of precipitin lines formed with |
|-----------|-----------------------------------|
|           | Anti-S-SB | Anti-whole cell (08) |
| S-SB      | 2         | 1                  |
| S-SBC     | 1         | 1                  |
| LPS-Bizio | 2         | Negative           |
| S-SO8     | 1         | 2                  |
| S-SO8C    | 1         | 2                  |
| LPS-O8    | 1         | 2                  |
In conclusion, it appears that fractions obtained by SDS extraction of isolated cell walls and purified by gel filtration are high-molecular-weight glycoproteins. The chemical and immunochemical similarities of these materials from two strains of the same species seemed to indicate that these cell components may not be important in the consideration of the effect on the variation of virulence and antibiotic-resistance. In any event, the study of cell envelope components such as the glycoproteins should add important information to the study of structure and function of the outer cell membrane of gram-negative bacteria.

ACKNOWLEDGMENTS

We thank Petar Alaupovic of Oklahoma Medical Research Foundation, Oklahoma City, Okla., for the supply of anti-whole cell sera.

(Thia investigation was supported by Research Corp., Chicago Ill., and Illinois State University Research Committee, Normal Ill.

LITERATURE CITED

1. Alaupovic, P., A. C. Olson, and J. S. Tsang. 1966. Studies on the characterization of lipopolysaccharides from two strains of Serratia marcescens. Ann. N.Y. Acad. Sci. 133:546-565.
2. Bakerman, S., and G. Wasemiller. 1967. Studies on structural units of human erythrocyte membranes. I. Separation, isolation, and partial characterization. Biochemistry 6:1100-1113.
3. Barlett, G. R. 1959. Colorimetric assay methods for free and phosphorylated glyceral acids. J. Biol. Chem. 234:469-471.
4. Bayer, M. E., and T. F. Anderson. 1965. The surface structure of Escherichia coli. Proc. Nat. Acad. Sci. U.S.A. 54:1592-1599.
5. Bitter, T., and H. M. Mair. 1962. A modified uronic acid carbazole reaction. Anal. Biochem. 4:330-334.
6. Butler, T. J., G. L. Smith, and E. A. Grula. 1967. Bacterial cell membranes. I. Reaggregation of membrane subunits from Micrococcus l lysodeikticus. Can. J. Microbiol. 13:1471-1479.
7. De Petriss, S. 1967. Ultrastructure of the cell wall of Escherichia coli and the chemical nature of its constituent layers. J. Ultrastruct. Res. 19:45-83.
8. Homma, J. Y., and N. Suzuki. 1966. The protein moiety of the endotoxin of Pseudomonas aeruginosa. Ann. N.Y. Acad. Sci. 133:565-576.
9. Koehler, L. H. 1952. Differentiation of carbohydrates by anthrone reaction of rate and color intensity. Anal. Chem. 24:1576-1579.
10. Lenard, J. 1970. Protein and glycolipid components of human erythrocyte membranes. Biochemistry 9:1129-1132.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin reagent. J. Biol. Chem. 193:265-275.
12. Nakano, M., and K. Saito. 1969. Chemical components in the cell wall of Salmonella typhimurium affecting its virulence and immunogenicity in mice. Nature (London) 222:1085-1086.
13. Nowotny, A. 1969. Molecular aspects of endotoxic reactions. Bacteriol. Rev. 33:72-98.
14. Okuda, S., and G. Weinbaum. 1969. Immunologic cross-reactivity of E. coli B envelope glycoproteins with some animal and plant cell membrane proteins. J. Immunol. 181:869-871.
15. Osborn, M. J. 1969. Structure and biosynthesis of the bacterial cell wall. Annu. Rev. Biochem. 38:501-538.
16. Ouchterlony, O. 1962. Diffusion-in-gel-methods for immunological analysis, II. In S. Kenger (ed.), Progress in allergy, vol. VI. New York.
17. Partridge, S. M. 1948. Filter paper partition chromatography of sugars. I. General description and application to the qualitative analysis of sugars in applesauce, egg white, and foetal blood of sheep. Biochem. J. 42:238-250.
18. Rondle, G. J. M., and W. T. J. Morgan. 1955. The determination of glucosamine and galactosamine. Biochem. J. 61:586-589.
19. Rottem, S., O. Stein, and S. Razin. 1968. Reassembly of mycoplasma membranes disaggregated by detergents. Arch. Biochem. Biophys. 125:46-56.
20. Thornton, G. F., and V. T. Andrieo. 1969. Antibiotic sensitivities of nonpigmented Serratia marcescens to gentamicin and carbencillin. J. Infect. Dis. 12:393-394.
21. Weinbaum, G., and R. Markman. 1966. A rapid technique for distinguishing enzyme active proteins in the cell "envelope" of E. coli B. Biochim. Biophys. Acta. 124:207-209.
22. Williams, R. P., and M. Purkayastha. 1960. Association of pigment with the cell envelope of Serratia marcescens. Nature (London) 187:349-350.