ASSOCIATION OF PRODIGIOSIN WITH OUTER CELL WALL COMPONENTS

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ABSTRACT. — Prodigiosin was extracted along with outer membrane glycoprotein by sodium dodecyl sulfate and sodium deoxycholate from isolated cell envelopes of *Serratia marcescens* 08. Part of the pigment was separated from the glycoprotein by organic solvent extraction and by Sephadex G-200 column chromatography.

In *Serratia marcescens* there is a tripyrrole pigment, prodigiosin, which absorbs at 535-540 mu (red) in acid medium but becomes orange in color (470 mu) in alkaline medium. Prodigiosin has been studied by many workers because of its suspected biological properties. Castro (Castro, *et al.*, 1967) noted that prodigiosin was active against various pathogenic bacteria and fungi, while Allen (Allen, 1967) found that it acted as an auto-oxidizable electron acceptor and suggested its possible role in cellular respiration. Although the biosynthesis of the pigment has been worked out to some extent (Morrison, 1966), the exact location of biosynthesis and its site of association with cell particles remains unclear. Castro (Castro, *et al.*, 1959), speculated that prodigiosin was present in the cell as the salt of a fatty acid which associated closely with the lipid of cell membrane. However, the observation that the concentration of prodigiosin paralleled that of N-acetylhexosamine in the isolated cell envelope indicated that the pigment may be associated with the cell wall (Williams and Purkayastha, 1960). However, it is not certain whether the pigment is located in the outer or inner membrane of the cell envelope (DePetris, 1967). An extracellular glycoprotein containing prodigiosin has also been isolated and characterized (Yoshida, 1967). More recently, prodigiosin was found covalently-bound to a glycoprotein of high molecular weight (Cruz-Camrillo and Sanchez-Zuniga, 1968). This report represents the study of the association of prodigiosin with the outer soft layer of the cell envelope.

MATERIAL AND METHODS

The cell walls were isolated from *Serratia marcescens* 08 (grown on an enriched medium) according to the procedure of Williams (Williams and Purkayastha, 1960). The cell wall preparation (fr. CW08) was extracted with dissociating reagents such as sodium dodecyl sulfate (SDS), sodium deoxycholate (SC), and guanidine hydrochloride (GH). One hundred milligrams of fr. CW08 was stirred in 50 ml of 0.5% SDS (pH 7.5) for one hour at 50°. After centrifugation at 10,000 r.p.m. for 20 minutes, the supernate and sediment fractions were separated and SDS was removed by dialysis. After lyophilization, the supernate fraction

(fr. SDS-S) and the sediment fraction (fr. SDS-P) were recovered. Similarly, extraction with 1% sodium deoxycholate yielded fractions SC-S and SC-P. The condition for guanidine hydrochloride extraction varied slightly. Three molar guanidine hydrochloride solution was used at room temperature and fractions GH-S (supernate) and GH-P (sediment) were recovered. Protein (Lowry, *et al.*, 1951), glucosamine (Rondle and Morgan, 1955), hexoses (Koehler, 1952), and uronic acids (Bitter and Muir, 1962) were determined. Total lipids were extracted with chloroform; methanol (2:1 v/v) in a soxhlet apparatus and determined gravimetrically. Other organic solvents extractions were performed in a similar manner. Presence of prodigiosin was monitored with a Beckman DK-2A UV-Visible spectrophotometer after fractions SDS-S, SC-S, and GH-S were extracted with ethanol:0.1 N HCl (9:1 v/v) mixture. Double diffusion technique in agar gel (Ouchterlony, 1962) was used to study the immunological activities of the various fractions against anti-whole cell serum. Endotoxin was isolated according to the modified method of Boivin (Tsang and Rilett, 1970).

Column chromatography of fraction SDS-S was performed in Sephadex G-200 which was equilibrated with 0.5% SDS. Thirty milligrams of the sample were dissolved in 3 ml of 0.005 M sodium phosphate buffer, pH 7.4 in 0.2% SDS. The column was eluted by the same buffer and monitored by protein determination.

Results and Discussion

The yields and the results of the chemical analyses are presented in Table 1.

Table 1.—Chemical Composition of Extracts from Isolated Cell Envelope from *Serratia marcescens* 08.

Fractions	Yield Per Cent	Protein Per Cent	Total Lipid Per Cent	Total Carbohydrate Per Cent	Hexoses Per Cent	Hexosamine Per Cent	Uronic Acid Per Cent
SDS-S.....	85	58.0	17.5	9.4	4.6	2.6	2.2
SC-S.....	65	26.3	25.0	10.3	4.3	3.4	2.6
GH-S.....	16	65.0	N.D.	8.2	3.0	3.8	1.4

It appears that the extracts were all glycoprotein in nature. Although fraction GH-S has the highest content of protein (65%) and fraction SC-S has the highest total carbohydrate content (10.3%), the extracting effectiveness of sodium dodecyl sulfate was far superior to the other two reagents. Guanidine hydrochloride proved to be a relatively poor extractant for the surface material (16%), and it also failed to remove any of the prodigiosin. On the other hand, both sodium dodecyl sulfate and sodium deoxycholate completely removed the pigment

from the isolated cell envelope (Table 2). Despite the fact that previous evidence indicated that hexosamine and prodigiosin were present in the same cellular structure, such as the cell envelope (Williams and Purkayastha, 1960), our results of analysis with the extracts (fractions SDS-S, SC-S, and GH-S) do not show such parallel correlationship of content of hexosamine with the presence of pigment (Table 2). Both fractions SDS-S and SC-S were pigmented, but their hexosamine content was lower than their corresponding colorless sediment fractions (fr. SDS-P and fr. SC-P). This observation suggested that prodigiosin was associated with certain components which could be selectively removed from the isolated cell envelope by mild dissociating reagents. These components may possibly be the glycoproteins and/or other conjugated macromolecules, such as li,oglycoprotein complexes which are present on the outer soft layer of the wall. Indeed, glycoproteins have been removed from the outer cell membrane by sodium dodecyl sulfate (Weinbaum and Markman, 1966). It is surprising, however, that guanidine hydrochloride failed to perform the same function as the other two reagents since it has also been used for removing outer surface components from walls of other Gram-negative bacteria (Kushner, 1969).

In order to demonstrate the immunochemical similarities of the extracted fractions, namely fractions SDS-S, SC-S, and GH-S and lipopolysaccharide-protein complex (endotoxin) (Tsang and Rilett, 1970) from *S. marcescens* 08, anti-whole cell serum was allowed to react with these fractions by the double diffusion technique. All fractions tested with the exception of fr. GH-S gave one precipitate line which cross-reacted with each other. It is interesting to note that antigenicity parallels with the presence of prodigiosin (Table 2). It is possible that guanidine hydrochloride extracted the non-antigenic cellular glycoprotein rather than the outer-membrane glycoprotein, while the other reagents removed glycoproteins which have at least one common antigenic component with endotoxin.

In order to study the possible linkage between prodigiosin and the cell envelope glycoproteins, fractions CW08, SDS-S, and SC-S were extracted with acetone and ethanol, as well as chloroform-methanol (2:1 v/v). After organic solvent extractions, the residues were colorless, while the extracts gave the charac-

TABLE 2.—Correlationship between Pigmentation, Hexosamine Content and Immunological Activities of Extracts and Residues.

Fractions	Pigmentation	Hexosamine Per Cent	Immunological Activities
SDS-S	+	2.6	Positive, 1 line
SDS-P	—	5.6	N.D.*
SC-S	+	3.4	Positive, 1 line
SC-P	—	4.6	N.D.
GH-S	—	3.8	Negative
GH-P	+	5.3	N.D.
Endotoxin	+	12.2	Positive, 1 line

* N.D. = not done

teristic spectra of prodigiosin. Cruz-Camarillo claimed that prodigiosin was conjugated with a glycoprotein to form a soluble complex which could be broken down with ethanol (Cruz-Camarillo and Sanchez-Zuniga, 1968) into a colorless component and a colored compound of low molecular weight. Our observation does not agree entirely with the implication of a possible existence of covalent linkage only between the pigment and the glycoproteins. It is likely that at least part of the pigment was associated with the macromolecule by hydrophobic bonds and/or salt bridges.

In the column fractionation experiment we obtained a single peak which gave positive protein reaction. Figure 1 shows the elution pattern. After dialysis and lyophilization, a colorless amorphous material was recovered in 90% yield. The pigment remained bound to the column, and attempts to elute it failed. A similar result was obtained when the column was equilibrated with 1% sodium deoxycholate and eluted with 0.2% sodium deoxycholate in the same buffer. The results of these experiments strengthen our belief that not all of the prodigiosin is bound to the glycoprotein.

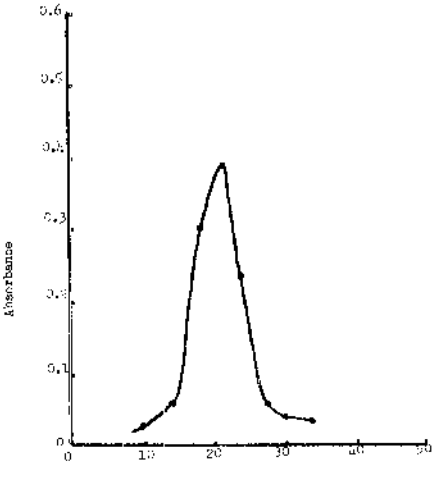

FIGURE 1. Sephadex G-200 Column Chromatography of Fraction SDS-S. Volume collected 3 ml per tube. Column was monitored by protein determination on 0.2 ml aliquots.

Thus, it appears that prodigiosin is associated with the outer cell wall component which is glycoprotein in nature. This glycoprotein shares at least one antigenic component with the lipopolysaccharide-protein (endotoxin) complex. Whether the pigment is synthesized by the membrane enzymes or synthesized in the cytoplasm and transported to the outer membrane of the cell wall remains to be elucidated.

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