Antigenicity of Solubilized Protein From Cells Infected with Pseudorabies Virus

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Introduction

Solubilization of biological membrane proteins for the investigation of their functional properties and structure is at the present time best achieved by using non-ionic detergents. Triton X-100 (polyoxyethylene p-t-octyl phenol), a non-ionic detergent, has been recognized as one of the most effective solubilizers of biological membrane (1, 5, 6, 7, 15, 17). Analysis of the solubilized proteins in the presence of the non-ionic detergent may cause difficulties, such as loss of biological activity. However, immunoelectrophoretic analysis in the presence of this detergent is possible because the antigenicity of the membrane protein is retained after solubilization with non-ionic detergent (2, 15). Although little exact data is presently available on the mechanisms by which this detergent acts, it is possible that due to selective binding of the non-ionic detergent to the hydrophobic part of the membrane protein, the antigenic determinants are left unaffected and free to react with antibodies.

Quantitative immunoelectrophoresis has been used for analysis of soluble proteins obtained from erythrocyte membranes (4). Crossed immunoelectrophoretic analysis of soluble proteins of herpes simplex viral infected cornea cells (rabbit) has also been described (3). This is to report on the identification and quantitation of a water soluble, membrane bound glyco-protein viral antigen.

Materials and Methods

Pig kidney cells (PKW2E) derived from 3-week-old pigs, at the 90th passage level, were used in the studies. The cells were grown in flasks with Eagle's medium containing 10% fetal calf serum (FCS), 100 U of penicillin per ml and 170 μg of steptomycin per ml at pH 7.4 and maintained in a similar medium containing 2% FCS. The strain of pseudorabis virus, PrV-FH, was used throughout the study. Plaque assay was done in PKW2E cells by determination of P.F.U. (Plaque forming units/ml) at 48 hours post exposure.

Triton X-100 (J. T. Baker Chemical Company, Phillipsburg, New Jersey 08865) was the detergent used. The cell debris after viral harvest was suspended in 0.01 M glycine — 0.0038 M Tris buffer pH 9.0, with and without detergent at a volume of six times that of the packed cellular debris. The mixture was sonically treated in an ice bath at 10 kc/sec three times for 15 seconds each (Branson Sonifer B12 with microtip, Danbury, Connecticut). The pellet obtained after centrifugation at 4° C in 60-T_1 rotor for 2 hours at 50,000 r.p.m. was dispersed in the same buffer and treated as above. This was repeated twice. The final pellet of cellular debris obtained after the third treatment was solubilized under special conditions (with or without detergent as shown in Table I).

TABLE I *Percent of Solubilized Protein from PrV-Infected Cells with Different Solubilization Procedures*

Solubilization procedure	Buffer without detertent		Buffer = 2% Triton X-100		Buffer without detergent (for extraction) Buffer + 2% Triton X-100 (for final pellet)	
	Solubilized protein (mg/ml)	Percent of solubilization (%)	Solubilized protein (mg/ml)	Percent of solubilization (%)	Solubilized protein (mg/ml)	Percent of solubilization (%)
First*: Sonication of 1 g. (wet wt.) cell pellet in 6 ml. fluid	4.0	41.1	5.5	50.0	5.0	39.2
Second*: Sonication of pellet of first in 2 ml. fluid	4.5	15.4	5.0	15.2	5.25	13.7
Third*: Sonication of pellet of second in 2 ml. fluid	4.25	14.6	4.5	13.6	5.0	13.1
Total extent of solubilization (%) in supernatant	—	~71	—	~79	—	~66
Fourth**: Dissolving the final pellet in 2 ml. fluid	0.42	28.9	0.35	21.2	0.65	34

*Fractions: First, Second, and Third are the supernatant fluid after centrifugation at 50,000 r.p.m. for 2 hr.
**Fraction: Fourth is the solution of final pellet dissolved in the fluid without centrifugation

Standard viral protein or other protein concentration was determined by biuret method (10) with bovine serum albumin as a standard.

After sonic treatment of viral infected cellular debris, the solubilized protein was purified by a modified method described by Ritzi et al (13). The soluble protein was applied to a washed column of concanavalin A-sepharose. The solubilized protein was eluted from the column with successive washes of 50 and 100 mg of methyl-D-mannoside (Sigma Chemical Company) per ml and dialyzed against 0.02 M Tris (pH 7.8). The dialyzed sample was then concentrated with Aquacide II (Calbiochem) and applied to a DEAE cellulose column. The protein was eluted from the column with increasing concentrations of NaCl (0-0.3 M). The protein fraction was finally dialyzed against distilled water and concentrated with Aquacide II. This purified protein was kept at -90°C and identified by immunodiffusion and rocket immunoelectrophoretic analysis.

A specimen of serum from immune swine was used as a source for antibody purification. A crude globulin fraction was separated from the serum by precipitation with concentrated ammonium sulfate (final molarity 1.77 M). The resultant slurry was centrifuged and passed through a column of coarse Sephadex G-25 as described by Kanitz (9). The eluent from the column was finally concentrated 10-fold with Aquacide II.

Immunodiffusion and rocket immunoelectrophoretic analyses were performed by using the procedure described by Sun et al (14).

Results

The amount of protein solubilized from the PrV-infected pig kidney cells by using different procedures is illustrated in Table I. The triton X-100 detergent treatment resulted in a higher total extent of solubilization in supernatant (~79%) as observed in percent of solubilization of column II (combined first, second and third fractions) in comparison to those of column I and column III (~71% and 66% respectively) which were extracted three times in buffer alone (without detergent). Treatment of the final pellet (fourth fraction) with 2% Triton X-100 also resulted in an additional (~5%) solubilization from cellular debris (34% in comparison to 28.9%). These results indicate Triton X-100 decreases protein-membrane binding affinity and releases protein.

Table II shows the difference in biological activity between proteins solubilized with and without the use of Triton X-100. A small amount of protein would also be solubilized in the presence of buffer alone as observed in Table I. Immunodiffusion analysis of the crude extract was used to compare the antigenicity of soluble fractions from the two solubilization procedures (with or without detergent). Extraction of antigenic determinants seemed to be a process requring Triton X-100. There was no immunoprecipitate formed in the agar gel when buffer alone was used in the solubilization procedure (Table II). On the contrary, well-defined immunoprecipitate was formed in several dilutions in the presence of anti-PrV swine serum when the detergent was used in the procedure. This study also indicates that the antigenic protein is tightly bound to cell membranes.

TABLE II *Comparable Analyses of Antigenicity of Fractions from Different Solubilization Procedures by Immunodiffusion*

Solubilization procedure		Immunodiffusion Dilution Undil.	1:2	1:4	1:8	1:16
Buffer without detergent	Supernatant	—	—	—	—	—
	Final pellet	—	—	—	—	—
Buffer + 2% Triton X-100	Supernatant	++	+	+	—	—
	Final pellet	—	—	—	—	—
Buffer without detergent (for extraction)	Supernatant	—	—	—	—	—
Buffer + 2% Triton X-100 (for final pellet)	Final pellet	+++	++	+	—	—

Immunodiffusion test was performed by filling the center well with anti-PrV serum and the surrounding wells with dilutions of materials obtained from different solubilization procedures.

+++ indicates a very strong positive precipitin band;
++ indicates a strong positive positive precipitin band;
+ indicates a weak positive precipitin band and
— indicates a negative reaction.

To analyze the soluble protein fraction further, purification of the crude extract through columns of concanavalin A-sepharose and DEAE cellulose was performed consecutively. Approximately 50% of the absorbing material of optical density at 280 nm (O.D. 280) from the solubilized crude extract was retained on DEAE cellulose column. By using a narrow NaCl gradient from 0 to 0.3 M, a single peak of 0.d. 280 absorbing material (fractions 3 through 7 in Fig. 1) was obtained in the NaCl gradient 0.047 M region (Fig. 1) with Gilford Instrument, Model 2400-S. This fraction was ready for assay of the antigenicity by immunodiffusion and rocket immunoelectrophoresis.

Analysis of the above single protein peak for the antigenic reactivity by immunodiffusion is shown in (Fig. 2). Anti-PrV swine serum in well 7 reacted with material in wells 2, 4, and 6 (filled with purified fraction of detergent solubilized protein) as it did with material in wells 1, 3, and 5 (filled with control positive antigen). This arrangement provided a control precipitin line on each side of the tested sample. The control line joined with the line of precipitation between the tested sample and specific serum forming a hexagonal ring. The antigenic protein in the Triton X-100 solubilized fraction was therefore identified as an antigen being homologous with or similar to the control positive antigen.

When normal pig serum was placed in the center well, lines of precipitation were not obtained, ruling out the possibility of non-specificity in tests of the antigen with antibody containing sera. The result realized when the solubilized

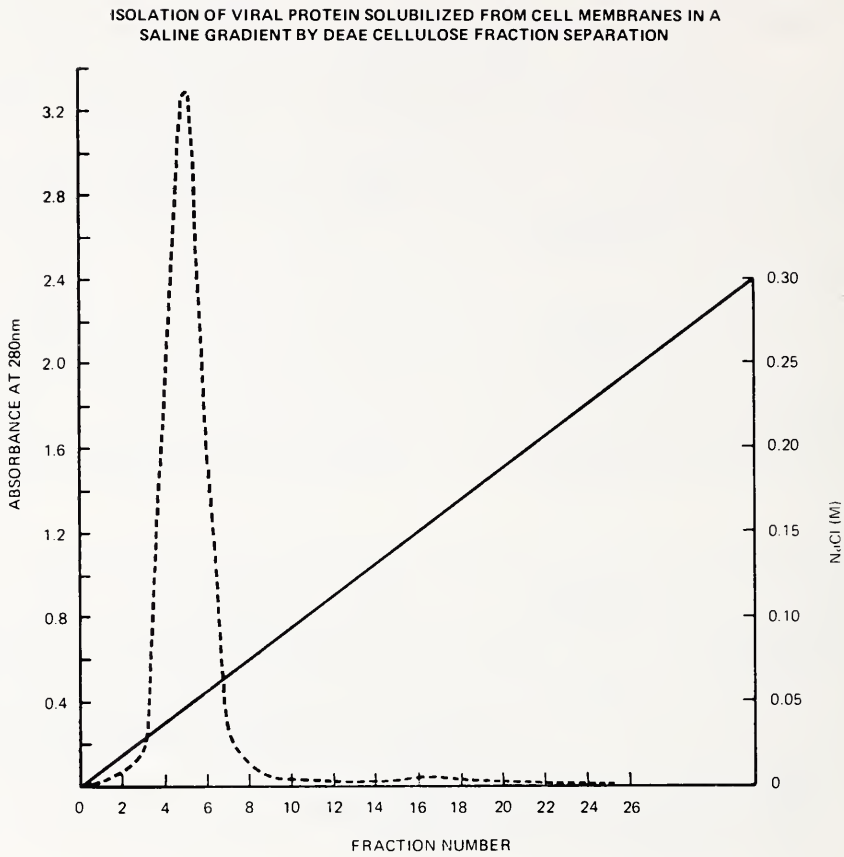

FIGURE 1. *Isolation of viral protein solubilized from cell membranes in a saline gradient by DEAE cellulose fraction separation. The solubilized crude extract was first applied to concanavalin A-sepharose column (1.5 by 25 cm) equilibrated in the buffer (methyl-D-mannoside, 100 mg/ml). 150 ml of eluent was collected after sample application. The eluent was dialyzed, concentrated, and applied to DEAE cellulose column with a 600 ml gradient of 0 to 0.3 M NaCl. The absorbancy at 280 mμ* was determined immediately.

protein obtained without the use of the detergent, as shown in (Fig. 3), was non specific since the precipitation lines between wells 2, 4, and 6 did not form a continuous line with those of the control wells 1, 3, and 5.

Antigenic determinants from above preparations (with or without Triton X-100) were investigated and quantitated with rocket immunoelectrophoretic techniques. Plot of immunoprecipitate migration distances of PrV dilutions from rocket immunoelectrophoresis was shown in (Fig. 4). This was used to provide a standard curve. The length of the precipitate is directly proportional to the concentration of the antigen. To obtain a quantitative estimate of the antigenic protein in the unknown sample, the length of the immunoprecipitate (E. G. the upper edge of the application well to the peak) was measured. By comparing the value obtained to that of the standard curve, the concentration of the antigenic determinant within the test sample was determined. (Fig. 5) shows that purified and solubilized protein extracted with Triton X-100 give a well-defined, rocket-shaped immunoprecipitate (well B). A similar phenomenon was not observed in well C which was filled with purified and solubilized protein

extracted without Triton X-100. The lenth of the precipitate shown in well B (50λ of test sample) compared with that of well A (54λ of standard control antigen) and the standard curve in (Fig. 4), shows the concentration of antigenic protein in well B to be 9.6 mg/ml.

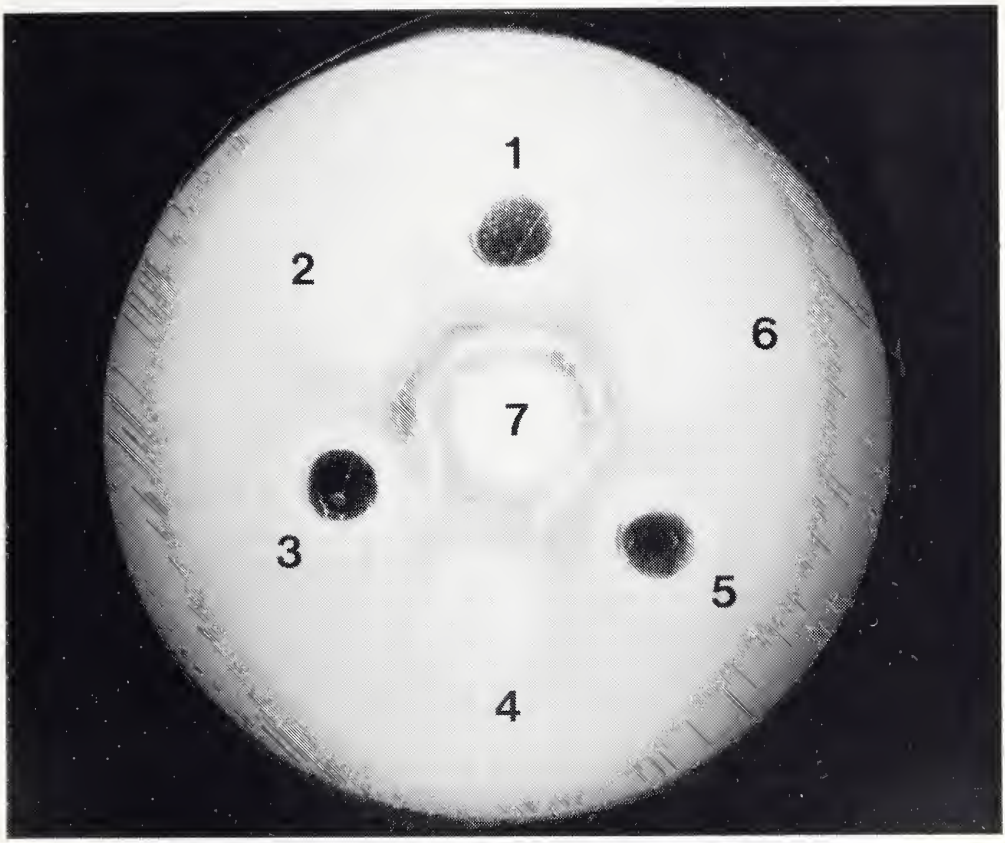

FIGURE 2. *Identification of the purified protein fraction of detergent solubilized membrane protein by immunodiffusion. An equal volume (40 microliters) of control positive antigen and test sample was present in wells. Wells represented in 1-7 are the following: (1), (3) and (5) control positive antigen; (2), (4) and (6) purified protein fraction of Triton X-100 solubilized membrane protein.*

Discussion

Presently, very little exact data are available on the mechanism by which non-ionic detergents act. Our studies using Triton X-100 as a membrane surfactant to solubilize tightly membrane-bound viral proteins and retain their antigenicity suggests that the detergent does not cause complete disruption of the membrane. The most likely mechanism involved is that the viral protein-cellular membrane binding affinity change is due to a conformational change in the structural proteins of the membrane.

In order to get optimum precipitate length reproducibility in rocket immunoelectrophoresis, the following factors are important: (a) antibody concentration, (b) field strength (volt), (c) buffer pH or ionic strength, (d) gel concentration, (e) time of electrophoresis, and (f) time between sample application and the initiation of electrophoresis. Variation in any of the above factors makes reproduction of the experimental data impossible.

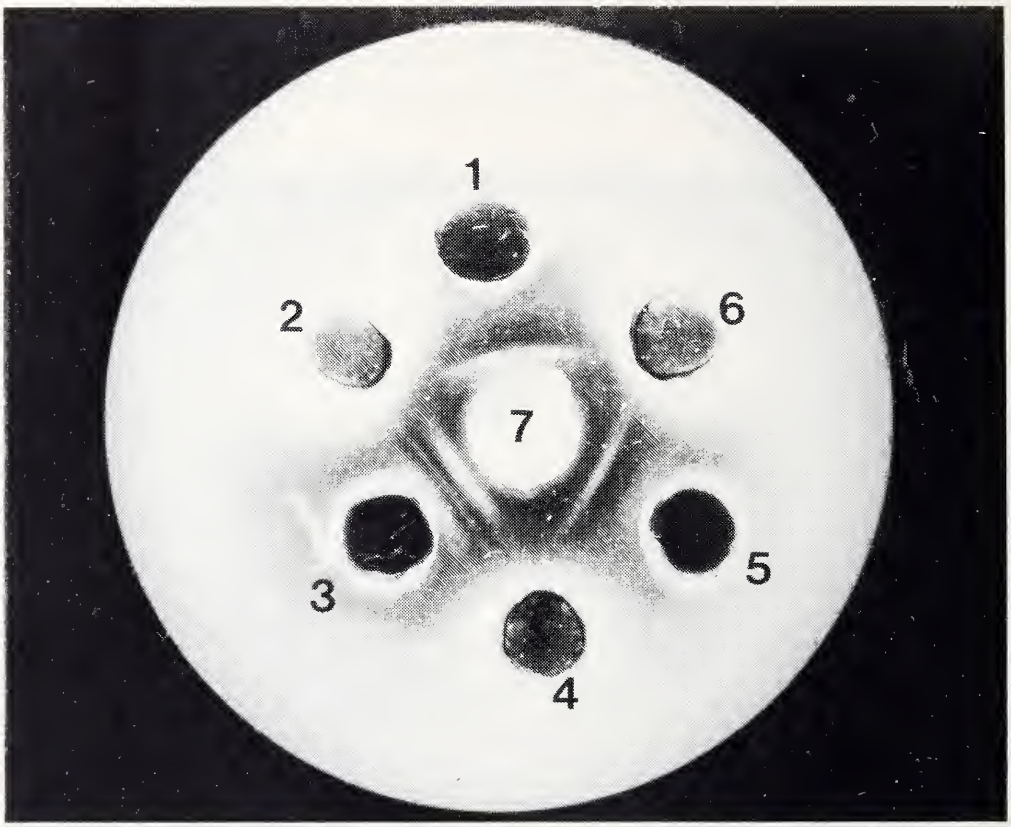

FIGURE 3. *Immunodiffusion analysis of purified protein fraction obtained with buffer alone (without detergent). An equal volume (40 microliters) of control positive antigen and test sample was present in the wells. Wells represented in 1-7 are the following: (1), (3) and (5) control positive antigen; (2), (4) and (6) purified protein fraction of buffer (without detergent) solubilized protein; (7) anti-Pr V swine serum.*

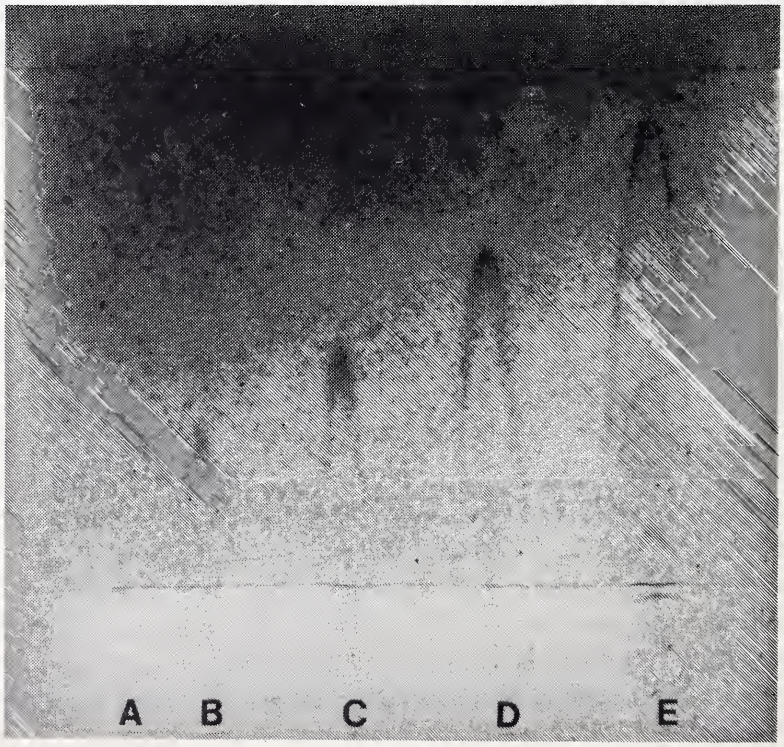

FIGURE 4.

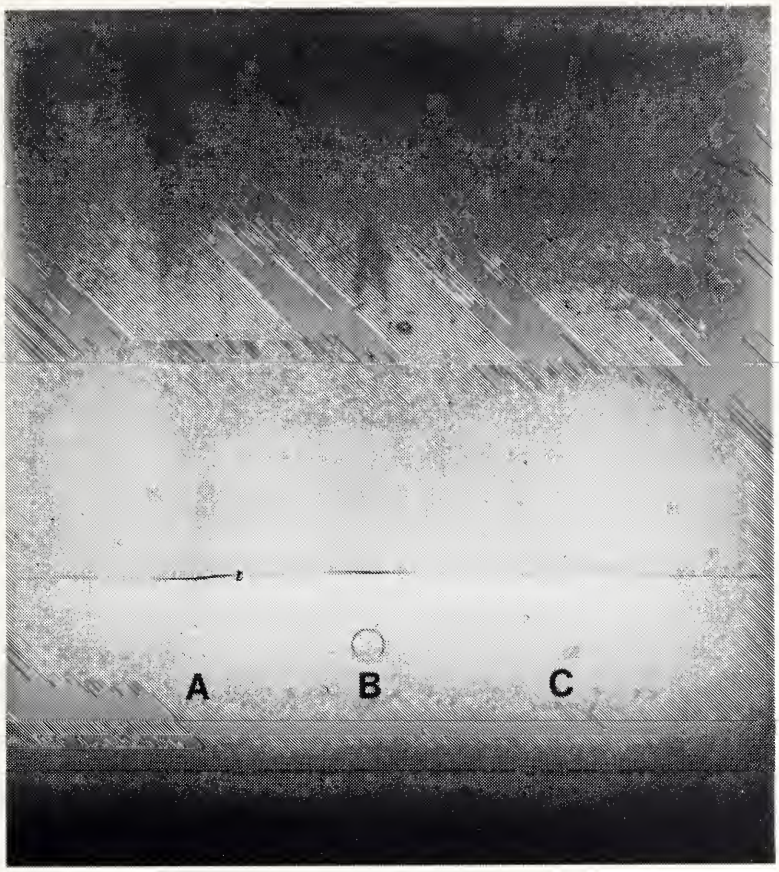

FIGURE 5.

Since the specificity of binding the detergent solubilized protein to anti-PrV swine serum appears to be the same as that observed with positive control antigen (Fig. 2 and Fig. 5), it is postulated that Triton X-100 required soluble protein represents either all or some portion of receptor for anti-PrV gamma-globulin. A similar phenomenon was observed by others previously (15, 11) in herpes simplex virus infected rabbit cornea cells and mouse tumor cells respectively. The fact that the O.D. (optical density) reading in 280 nm and 260 nm for the detergent solubilized protein has a ratio different from that of the purified standard virion (O.D. 280 nm/O.D. 260 nm = protein/nucleic acid = 1.33) indicates that this soluble protein is not the intact virion but a part or subunit of the virion.

Our investigation as well as Hoggs (8) disclose the existence of an antigenic protein on the cell surface which is derived from the replicative process for infectious virus used in the infection of cell culture. It is probably the result of adsorption of a viral component to the cell surface, although more complex interactions which result in changing the plasma membrane properties are possible. A similar mechanism was proposed previously by Phillips and Perdue (12) in which the ability of binding foreign protein to cell surface resulted in a plasma membrane characteristic which permitted transformed and untransformed cells to be distinguished from one another.

The application of detergent solubilized herpes simplex viral antigen to produce neutralizing antibody in rabbits has been reported (15). It may be

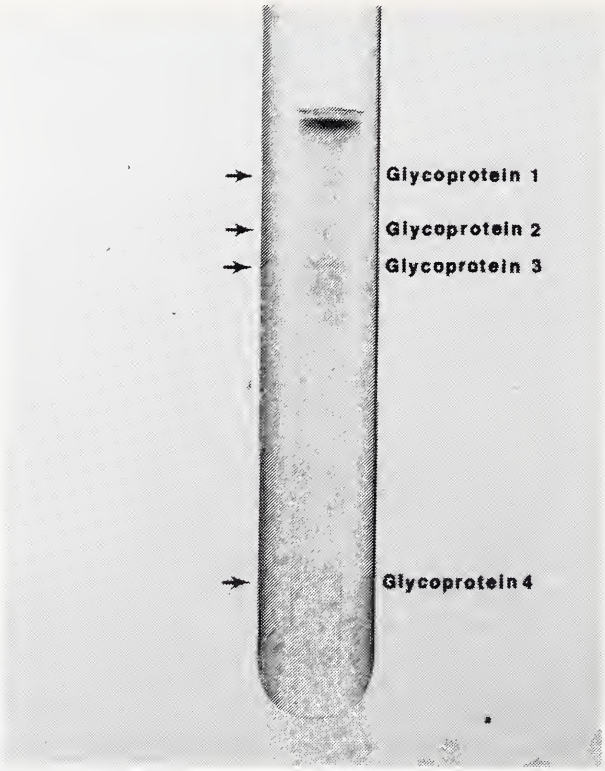

FIGURE 6. *SDS polyacrylamide gel electrophoresis of DEAE cellulose column fraction. The gel was stained with Coomassie brilliant blue and the protein sample present in the gel was 100 μl of protein solution (1 mg/ml).*

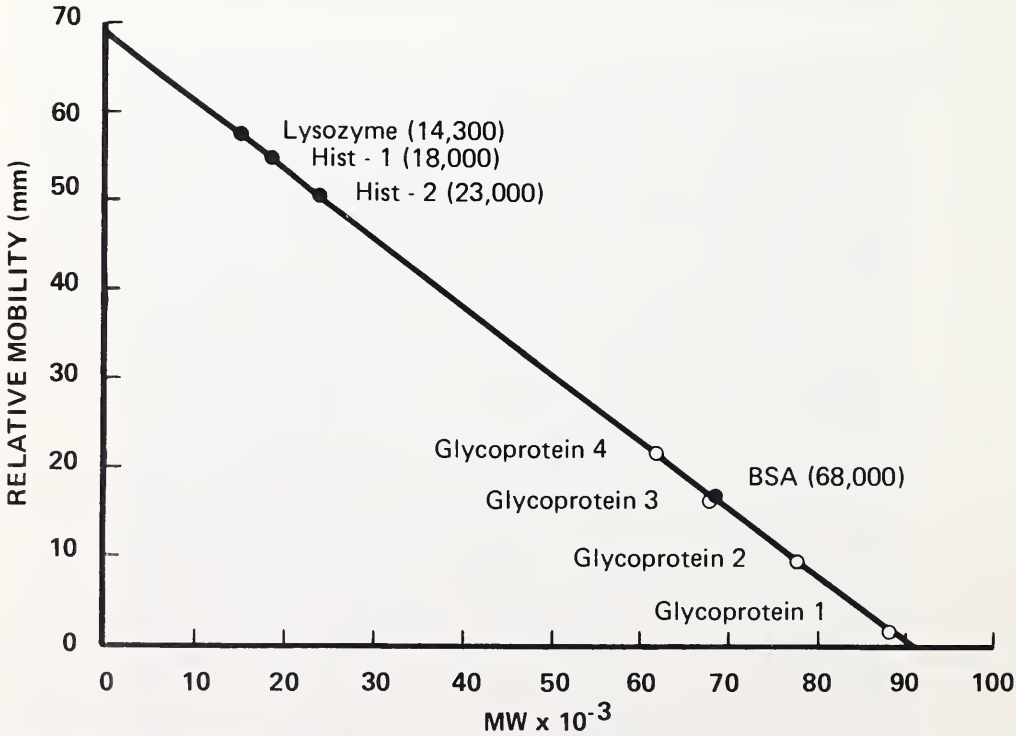

FIGURE 7. *Relation between relative mobility and molecular weight for standard protein markers and the solubilized protein in SDS gel. Standard protein markers used for molecular weight determination included bovine serum albumin (68,000), histone-1 (23,000), histone-2 (18,000) and lysozyme (14,300).*

possible to use the solubilized and Triton X-100 requiring PrV antigen as a subunit vaccine in animals.

There has been little experimental evidence to prove or detect persistent PrV infections in swine. Detection of persistent infections in swine may be possible by identifying the detergent solubilized protein extracted from cells of CNS ganglia through rocket electrophoresis.

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