Purification and Immunogenicity of Fusion VP1 Protein of Foot and Mouth Disease Virus†

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ABSTRACT: A procedure has been developed to purify foot and mouth disease virus (FMDV) VP1 surface antigens from recombinant *Escherichia coli*. The VP1 antigens are expressed as fusion proteins derived from the *E. coli* Trp operon and VP1 surface protein of FMDV. The procedure is capable of recovering >96% of the desired product at a purity of >96%. The resulting antigens induce significant levels of virus-neutralizing antibody in guinea pigs and cattle as determined by a mouse protection assay [Skinner, H. H. (1952) *Proc. Int. Vet. Congr., 15th 1*, 195]. *E. coli* contaminants have a deleterious effect on ion-exchange chromatography as well as immunogenicity of the expressed fusion VP1 antigens. The method presented removes significant *E. coli* contaminants, yielding fusion VP1 proteins which are immunogenically potent. In particular, virus neutralization titers at 100-μg dosage of the fusion VP1 proteins of the O1 and A24 serotypes are similar to that induced by the natural VP1 proteins isolated from FMD virions.

Foot and mouth disease (FMD)[1] is a highly contagious viral disease which is caused by a picornavirus (FMDV) whose structure, replication, and immunochemistry have been reviewed (Sanger, 1979; Bachrach, 1977; Haresnape & McCahon, 1983; Haresnape et al., 1983). Immunologically, FMDV is extremely diverse, having been serologically characterized by complement fixation into 7 serotypes and at least 61 subtypes (Bachrach, 1977; Brooksby, 1982; Pereira, 1977). FMDV afflicts mainly cloven-hoofed animals, and although mortality is low, the loss of productivity due to debilitation from the disease is estimated to be as high as 25% (Brown, 1981). The disease, which is endemic on every continent except Australia and North America, is controlled by vaccination, quarantine, and slaughter. The vaccines are produced by chemically inactivating virus after large-scale culture on bovine tongue epithelium (Frenkel, 1950) or growth in BHK cells (Mowat & Chapman, 1962; Chapstick et al., 1962; Whiteside et al., 1983). Outbreaks of the disease have been traced to vaccines which were not sufficiently inactivated (Pay et al., 1971; King et al., 1981). Furthermore, the current vaccines require refrigeration for stability, and large variations in vaccine potency of lots are not uncommon. The use of recombinant DNA technology can produce a vaccine which circumvents these problems.

There is evidence that an antigenic determinant(s) of the exposed capsid protein (VP1)[2] is (are) capable of raising a neutralizing antibody (La Porte et al., 1973; Bachrach et al., 1975; Haresnape, 1980). Although this may not be the sole determinant responsible for induction of virus neutralizing antibody (Strohmaier et al., 1982; Meloen et al., 1983), it has been demonstrated that the VP1 isolated from the A12 FMDV serotype as well as a 13-kdalton CNBr peptide fragment of the VP1 is capable of inducing a neutralizing antibody response in guinea pigs and a protective response in cattle and swine (Bachrach et al., 1979).

The cDNA cloning and sequencing of the VP1 gene for the FMD serotype A12 have been previously described (Kleid et al., 1981). This initial construction produced a 44-k dalton protein (termed "long fusion") consisting of 190 amino acids from the *Escherichia coli* LE′ protein fused to residues 7–211 of the VP1. The LE′ protein in this construction was also a fusion protein consisting of the Trp L peptide fused to the last third of the Trp E protein (Yansura & Kleid, 1982). The long fusion FMD protein was expressed in *E. coli* (strain W3110) and purified by preparative SDS–polyacrylamide gel electrophoresis. The protein was excised from the gel, emulsified with an incomplete Freund's oil adjuvant, and used to vaccinate guinea pigs, cattle, and swine (Kleid et al., 1981). All test animals developed neutralizing antibody titers, and in a challenge test, cattle and swine were protected against FMDV. In later experiments, a shorter version of the A12 VP1 fusion construction (termed "short fusion") was used which contained only 15 amino acids from the LE′ fusion proteins. The neutralizing titers in guinea pigs and cattle were similar to that produced by the long fusion VP1 and by VP1 isolated from FMDV (Moore, 1983). However, when the same procedures of extraction from SDS preparative gels were used to prepare fusion protein antigens for two other virus serotypes, A24 Cruzeiro and O1 Campos, satisfactory levels of neutralizing antibodies in swine were not observed (D. M. Moore, unpublished results).

This paper describes the methods and purification schemes we have developed to obtain FMD fusion protein antigens from *E. coli* which are capable of inducing significant levels of neutralizing antibodies in test animals.

Materials and Methods

Molecular Biology. The cDNA cloning, nucleotide sequencing, and strategy for expression in *E. coli* of the VP1 long fusion and short fusion proteins have been previously

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PEB, phosphate extraction buffer consisting of 50 mM NaH_2PO_4, 5 mM EDTA, 0.5 M NaCl, and 0.1% 2-mercaptoethanol at pH 7.0; Tris, tris(hydroxymethyl)aminomethane; UTB, urea–Tris buffer consisting of 8 M urea, 14 mM Tris, and 0.1% 2-mercaptoethanol at pH 8–9; FMD, foot and mouth disease; FMDV, foot and mouth disease virus; dpv, days postvaccination.

[2] The capsid protein which has neutralizing antigenic determinants is now termed VP1 by all researchers in the field. Previously, the American group at the USDA facility at Plum Island (Greenport, NY) referred to VP1 as VP3.

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reported (Kleid et al., 1981; Yansura & Kleid, 1982). The work presented here describes purification of short fusion although it is applicable to the long fusion protein. Hereafter, the term fusion protein refers to the short fusion construction.

Chemicals. All chemicals used in buffer preparations were reagent grade. Urea solutions were filtered and deionized on mixed-bed resin (Barnstead, ultrapure) and stored at 4–8 °C.

Chromatography. Sephacryl S-300 (Pharmacia Fine Chemicals) and DE-52 cellulose (Whatman Inc.) were preequilibrated according to methods suggested by the manufacturer. All chromatography was performed at room temperature.

Ultrafiltration and Dialysis. Ultrafiltration was performed in stirred cells equipped with YM-10 membranes (Amicon Inc.), and dialysis was performed with Spectrapor 2 tubing (Spectrum Medical Industries, Inc.) with a molecular weight exclusion of 10 kdaltons.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE), Electrotransfer, and Immunoblotting. Discontinuous SDS electrophoresis using 12.5% acrylamide gels was run according to the procedure of Laemmli (1970). Samples obtained from chromatography in guanidine hydrochloride (Gdn·HCl) buffer systems were dialyzed against 8 M urea (BRL Model 1200MD microdialyzer) before being loaded into SDS sample buffer. All samples were reduced with 1% 2-merceptoethanol prior to electrophoresis. The amount of protein represented by a particular Coomassie Blue stained band was estimated by scanning densitometry of the gels. After electrophoresis, the SDS gel was placed onto nitrocellulose paper and placed between two electrode grids with the anode adjacent to the nitrocellulose paper. Transfer of proteins to nitrocellulose paper, termed "western blot" (Burnette, 1981), was effected by applying a 2.5-A current transversely to the SDS gel–nitrocellulose sandwich in an EC blotting apparatus (E.C. Instrument Co.) filled with phosphate buffer (20 mM, pH 6.5). Antisera raised in guinea pigs to VP1 isolated from A12 virions and ^{125}I-labeled protein A were used for detection of transferred proteins by autoradiography.

Vaccination of Animals. All samples were emulsified 1:1 with Freund's incomplete oil adjuvant (Morgan et al., 1980), and 2 mL was injected subcutaneously at one site on the back of the neck of guinea pigs. The animals were bled and given a single boost 28 or 36 days postinitial vaccination (dpv), and final bleedings were taken either 42 or 56 dpv. FMDV neutralizing titers were determined from pooled postvaccinate sera from five animals.

Determination of Virus Neutralizing Antibody Titer. The titers of FMD neutralizing antibodies in test animals sera were determined by the suckling mouse protection assay of Skinner (1952). A virus inoculum containing 200 LD_{50} units was combined with an equal volume of diluted serum, and the 0.03-mL test dose was administered intraperitoneally to each of eight suckling mice. The titer is expressed as the negative logarithm of the final dilution required to protect 50% of the mice. The errors in the titers are estimated to be less than ±0.5.

Results

Chromatographic Purification of FMDV VP1 Fusion Protein. (A) Cell Growth and Harvest. E. coli transformed with altered pBR322 plasmids containing the fusion VP1 nucleic acid sequence were grown to a cell density corresponding to an absorbance of 30–50 OD units at 550 nm (~40 g wet cell paste per L). The cells were killed by adding phenol and toluene to the level of 0.25% each and incubated for at least 30 min.[3] The cells were harvested by centrifugation and stored frozen. The expression of the VP1 fusion protein results in bacteria containing one or two optically dense bodies when viewed by phase contrast microscopy. SDS–PAGE of acetone-precipitated proteins from the harvested fusion FMD O1 VP1 cell paste indicates that 20–30% of the total stained proteins electrophorese at a molecular weight of about 31 000 (data not shown). Acetone extracts of *E. coli* (strain W3110) without the O1 VP1 expression plasmid lack this protein band at the 31-kdalton position on the polyacrylamide gel (data not shown). Identical results were obtained with the fusion VP1 A24.

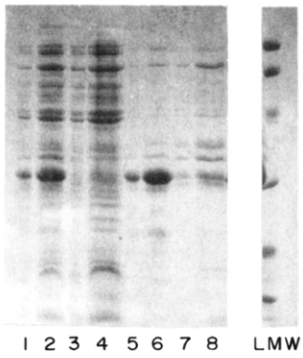

FIGURE 1: SDS Coomassie Blue stained PAGE of initial lysis and extraction from *E. coli* extracts of short fusion O1 VP1. Loading as follows: lanes 1 and 2, lysed cell suspension in PEB; lanes 3 and 4, supernatant after centrifugation of lysed cell suspension; lanes 5 and 6, resuspended pellet in PEB after centrifugation of lysed cell suspension; lanes 7 and 8, supernatant after centrifugation of resuspended pellet. LMW, molecular weight standards at 92.5K (phosphorylase *b*), 66.2K (bovine serum albumin), 45.0K (ovalbumin), 31.0K (carbonic anhydrase), 21.5K (soybean trypsin inhibitor), and 14.4K (lysozyme), respectively.

(B) Lysis and Extraction Procedure. Thawed *E. coli* cell paste was dispersed into 10 volumes of cold phosphate extraction buffer (PEB: 50 mM NaH_2PO_4, 5 mM EDTA, 0.5 M NaCl, and 0.1% 2-mercaptoethanol, pH 7.0). The resulting cell suspension was passed twice through a prechilled Gaulin cell homogenizer (type 15M equipped with a heat exchanger) at a flow rate of 1 L/min and a pressure of 6000 psi. The lysed cell suspension was centrifuged at 4400*g* for 30 min at 4–8 °C, and the pellet was redispersed into 20 volumes of PEB. Initial extraction with PEB resulted in a 65–75% loss of pellet mass. SDS–PAGE analysis of the fusion O1 serotype VP1 demonstrated that the PEB extraction procedure resulted in a pellet in which 60–80% of the total protein is the VP1 fusion protein (Figure 1, lanes 5 and 6). The abundant protein at 31kdalton present in the cell lysate (lanes 1 and 2) is barely detectable in the supernatant after centrifugation (lanes 3 and 4) whereas the contaminating *E. coli* proteins appear to be present at the same level as found in the lysed cell suspension.

A second extraction of the fusion VP1 pellet followed by centrifugation resulted in a supernatant containing the proteins shown at different loading concentrations in lanes 7 and 8 (Figure 1). Although a band is present at 31 kdalton, a large number of *E. coli* contaminating proteins were also removed in this second PEB extraction. The amount of VP1 fusion protein in the final resuspended pellet is estimated to be between 60% and 80% of the total Coomassie Blue stained proteins, yielding a 2-fold protein purification on extraction. When viewed by phase contrast microscopy, the resuspended pellet reveals a suspension of optically dense bodies with no

[3] The cells are killed in compliance with NIH guidelines for greater than 10-L containment of recombinant organisms.

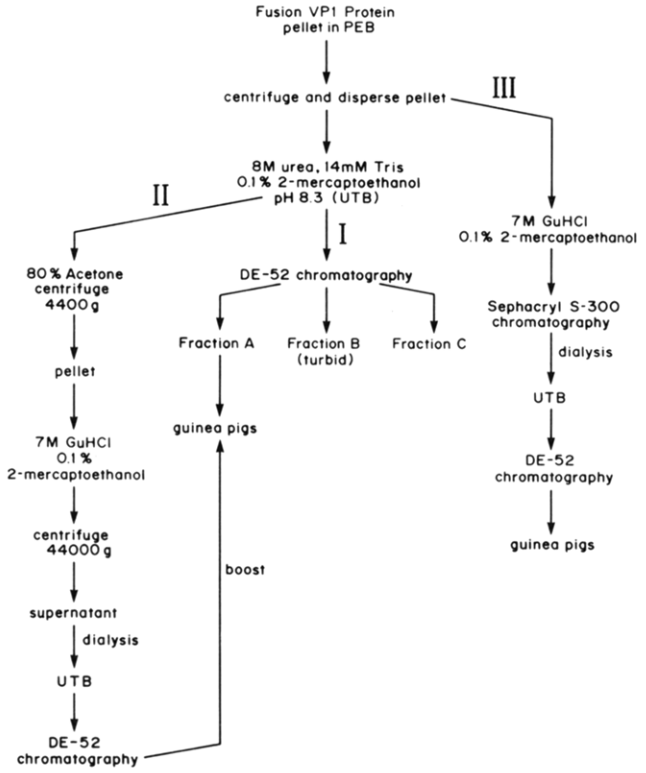

FIGURE 2: Schematic flow diagram for VP1 fusion protein purification. Starting material (I) is a pellet resulting from lysis of recombinant *E. coli* cells (see Results). Phosphate extraction buffer (PEB) and urea–Tris buffers (UTB) are as described in the text. The preparations used in the animal vaccinations are labeled schemes I–III under Results.

apparent cell debris. The same lysis and extraction procedure has been successfully used to obtain the FMD VP1 fusion protein of the A24 serotype.

Although the resuspended pellet in PEB could be solubilized with a variety of detergents and chaotropic agents, vaccination of guinea pigs with this crude solubilized protein did not result in satisfactory levels of virus neutralization antibody. Therefore, the VP1 fusion protein pellets were further purified by chromatography. A schematic flow diagram showing the various preparations (labeled I–III) is shown in Figure 2.

(*C*) *Scheme I—Solubilization of the Fusion VP1 Pellet in 8 M Urea and Ion-Exchange Chromatography* (*Figure 2*). The resuspended pellet of the VP1 fusion protein of the O1 virus subtype was centrifuged for 30 min at 4400*g* and the pellet dispersed into 20 volumes of a urea–Tris buffer at pH 8.3 (UTB: 8 M urea, 14 mM Tris, and 0.1% 2-mercaptoethanol). The resulting turbid solution was diluted 1:1 with UTB, and the total volume of 400 mL at 8.4 mg/mL total protein [as determined by the Bio-Rad Bradford assay (Bradford, 1976)] was chromatographed on a DE-52 column (5 × 9 cm). The chromatogram (Figure 3A) monitored at 280 nm consisted of an initial clear eluant (fraction A) followed by a sharp increase in turbidity (fraction B) with a subsequent clear eluant (fraction C).

The SDS gels of the pooled DE-52 column fractions, A, B, and C (Figure 3A), indicate that these fractions contain mainly one protein (90% of total stained Coomassie proteins) at a molecular weight of 31K which binds to VP1 A24 antisera in an immunoblot (results not shown). These fractions appear to be identical with each other within the limits of protein detection (Figure 3B). Clarification of the turbid fraction (fraction B) by centrifugation caused a 50% reduction in the total protein concentration but did not change the apparent distribution of protein species. Although the molecular weight determined by SDS gel electrophoresis is somewhat greater than expected on the basis of the amino acid composition deduced from the cDNA sequence, chromatography of purified fusion VP1 in UTB on a calibrated Sephacryl S-300 column

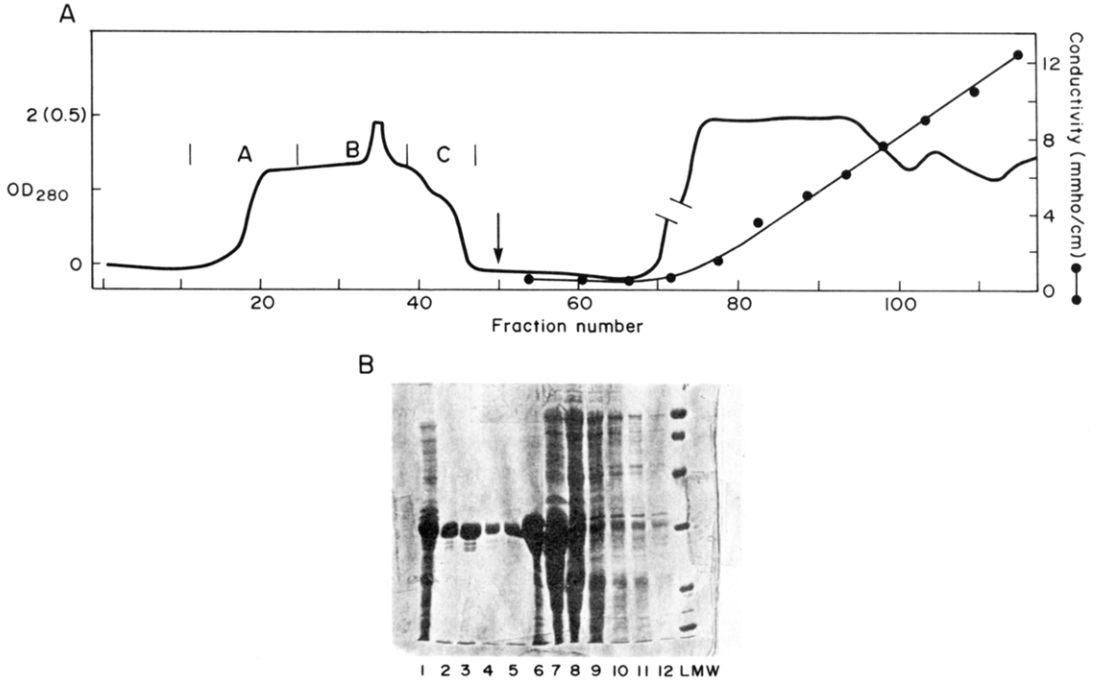

FIGURE 3: (A) DE-52 chromatography (5 × 9 cm) of VP1 O1 short fusion protein. The equilibration and elution buffer was 8 M urea, 14 mM Tris, and 0.1% 2-mercaptoethanol, pH 8.3. Detection was by absorbance at 280 nm, and fractions A–C were pooled as indicated on the chromatogram. The absorbance value in parentheses refers to fractions before the break point on the curve. The arrow denotes fraction position when a linear 0.0–0.5 M NaCl gradient was first applied to the column. The solid circles are measured conductivity values of the column effluent (units on the right ordinate). (B) SDS Coomassie Blue stained PAGE of DE-52 fractions. Lane 1, DE-52 load; lanes 2, 3, and 4, pooled fractions A, B, and C, respectively (Figure 4A); lanes 5, 6, 7, 8, 9, 10, 11, and 12, fractions 72, 75, 82, 88, 92, 98, 102, and 105, respectively; same molecular weight standards as in Figure 1.

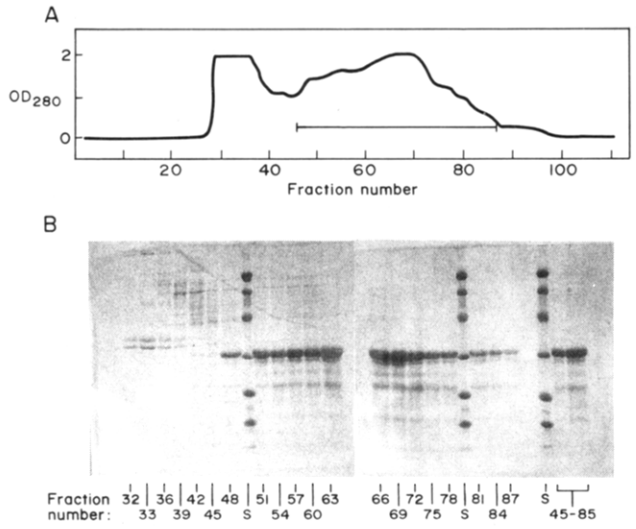

FIGURE 4: (A) Sephacryl S-300 chromatography of VP1 O1 short fusion protein (5 × 70 cm) using 7 M Gdn·HCl, 50 mM NaH_2PO_4, pH 7.0, and 0.1% 2-mercaptoethanol as the elution buffer. Detection was by absorbance at 280 nm, and the horizontal bar indicates pooled fractions. (B) SDS Coomassie Blue stained PAGE of Sephacryl S-300 fractions as indicated. S indicates molecular weight standards which are identical with those used in Figure 1. The last two lanes are pooled fractions 45–85 indicated by the horizontal bar in Figure 4.

yields the expected molecular weight of 25K (results not shown). A linear 0.0–0.5 M NaCl gradient resulted in further elution of proteins which had been absorbed to the DE-52 column. SDS–PAGE of selected fractions indicates that essentially all contaminating *E. coli* proteins are bound to the resin at pH 8.3 at low ionic strength. Moreover, most of the VP1 fusion protein is also bound and elutes with the remaining proteins at the beginning of the salt gradient (at about 1.0 mmho cm^{-1}). An identical experiment with the A24 short fusion protein yielded similar results.

(D) Scheme II—Solubilization of VP1 Fusion Protein Pellet in 7 M Gdn·HCl and Ion-Exchange Chromatography (Figure 2). O1 fusion VP1 pellet resuspended in PEB was solubilized with UTB, filtered, and then precipitated by adding 4 volumes of acetone. The centrifuged pellet was then redissolved in 20 volumes of 7 M Gdn·HCl and 0.1% 2-mercaptoethanol, and contaminating insoluble material was removed by centrifugation at 44000*g* for 45 min. The resulting pellet consisted mainly of two proteins with molecular weights of 37.5K and 34.0K as determined by SDS–PAGE analysis (50–60% of the total protein in the pellet). The supernatant was dialyzed into UTB at pH 8.3 and the clear solution chromatographed on DE-52 resin. The flow through was clear, and by SDS–PAGE analysis, one major band at 31.0kdalton accounted for 95% of the total Coomassie Blue stained proteins (preparation II, Figure 2). Elution of the column with a 0–0.3 M NaCl gradient resulted in the elution of *E. coli* proteins as well as bound VP1 fusion protein.

In later experiments, longer centrifugation of the 7 M Gdn·HCl extract (~12 h at 44000*g*) followed by dialysis into UTB yielded samples in which none of the VP1 fusion protein was absorbed onto the DE-52 resin at low ionic strength.

(E) Scheme III—Sephacryl S-300 Gel Filtration Chromatography of 7 M Gdn·HCl-Solubilized VP1 Fusion Pellet and Ion-Exchange Chromatography (Figure 2). An O1 VP1 fusion preparation (Figure 1, lanes 7 and 8) was centrifuged at 4400*g* for 30 min and the pellet dissolved in 8 volumes of 7 M Gdn-HCl, 0.1% 2-mercaptoethanol, and 50 mM NaH_2PO_4, pH 7.0. Fifty milliliters of the guanidine extract was loaded onto a 5 × 72 cm Sephacryl-300 column equilibrated with the Gdn·HCl buffer. This buffer was then used to elute the column at a flow rate of 1 mL/min. The chromatogram and SDS–PAGE of selected 15-mL fractions are shown in Figure 4. A similar chromatogram and SDS gel profile were obtained with the A24 VP1 fusion protein. The void volume fractions were turbid and contained primarily the two proteins at 37.5 and 34.0 kdalton found in the pellet after high-speed centrifugation of the Gdn·HCl extract of the initial pellet in PEB. Prolonged high-speed centrifugation (~15 h) at 44000*g* reduced the amplitude of the void volume peak. Later fractions contained proteins with molecular weights higher than 37K, indicating that even in 7 M Gdn·HCl at 0.1% 2-mercaptoethanol the 37.5- and 34.0-kdalton proteins are highly aggregated. The VP1 fusion protein began to elute at fraction 45 (Figure 4). Gel filtration was repeated with two additional 50-mL aliquots of the Gdn·HCl O1 fusion protein extract. The fractions containing the VP1 fusion protein were pooled and concentrated by ultrafiltration to a total volume of 715 mL. The pooled sample was dialyzed exhaustively against UTB at pH 8.5 prior to being loaded onto a 5 × 19 cm DE-52 cellulose ion-exhange column. After the sample was loaded, the resin was washed with UTB at pH 8.5. Proteins which remained bound to the DE-52 resin were eluted with a 0.0–0.2 M NaCl gradient in UTB. The chromatograms and SDS–PAGE analysis of the DE-52 effluent are shown in Figure 5. Densitometry of the pooled flow-through fractions indicated that the VP1 was approximately 97% pure, and SDS–PAGE of the salt gradient fractions revealed the presence of very little fusion protein, suggesting almost quantitative recovery from the DE-52 column [see (B) under Purification of Fusion VP1 Antigen under Discussion].

Table I: Neutralizing Antibody Titer in Guinea Pigs Induced by FMD VP1 Immunogens

protein source	protein mass (μg)	formulation[a]	Skinner PD_{50}[b] 36 dpv	56 dpv
O1 virion	100	urea/oil	2.0	2.4
	100	urea/oil + 0.1% SDS	2.1	2.3
short fusion O1 VP1	100	urea/oil	1.6	1.8
	500	urea/oil	2.1	3.2
	100	urea/oil + 0.1% SDS	<0.3	1.9
	500	urea/oil + 0.1% SDS	2.1	2.6
A24 virion	100	urea/oil	3.3	3.7
	250	urea/oil	3.6	3.9
short fusion A24 VP1	100	urea/oil	3.2	4.0
	500	urea/oil	3.6	4.2
	100	urea/oil + 0.1% SDS	3.4	3.9
	500	urea/oil + 0.1% SDS	3.5	4.4

[a] Formulated in 8 M urea, 10 mM Tris, and 0.1% 2-mercaptoethanol and emulsified in Freund's incomplete oil adjuvant (Morgan et al., 1980). [b] PD_{50} is the negative log of the dilution required to protect 50% of the mice. Booster vaccination given 36 dpv.

Immunization of Guinea Pigs with VP1 Antigens. (A) Immunization with Fusion and Virion VP1 Antigens. Virus neutralizing antibody titers (Skinner, 1952) for the fusion antigens as well as the titers from "natural" VP1 isolated from type O1 and A24 virions by the ion-exchange preparative method of Bernard et al. (1974) are presented in Table I. Guinea pigs were vaccinated with short fusion O1 VP1 that was prepared by using schemes I and II of Figure 2. The pooled soluble fraction A (scheme I, figure 2) was used for primary injection, and antigen prepared by scheme II (Figure 2) was used for booster injection at 36 days. Both short fusion O1 preparations gave a satisfactory immunogenic response. Pooled turbid fraction B (scheme I of Figure 2 and Figure 3A) did not induce satisfactory levels of neutralizing antibodies in

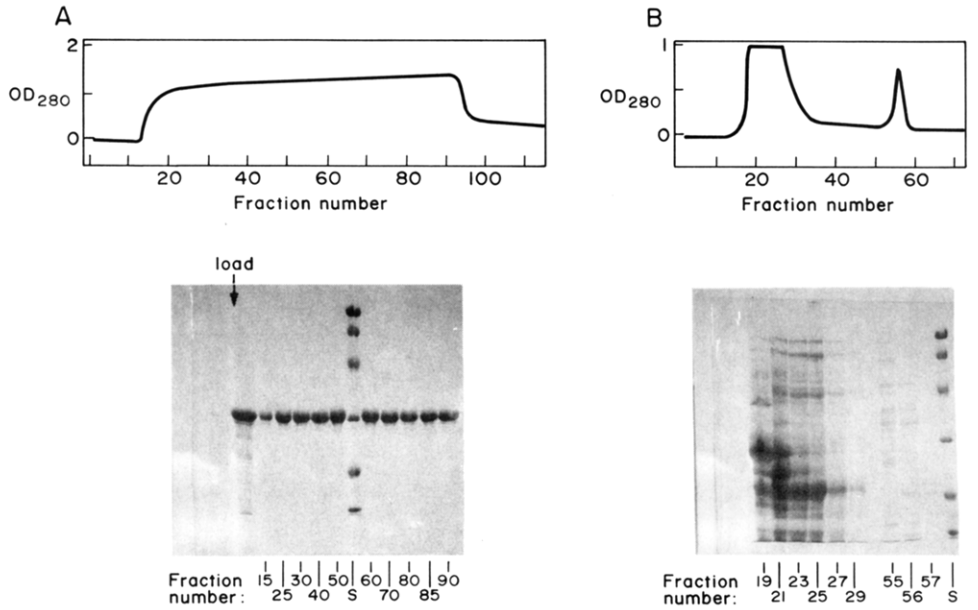

FIGURE 5: DE-52 chromatography (5 × 19 cm) of FMD O1 VP1 fusion protein after Sephacryl S-300 chromatography. (A) Load and elution using 8 M urea, 14 mM Tris, and 0.1% 2-mercaptoethanol, pH 8.5. SDS Coomassie Blue stained PAGE: lane labeled "load" is the column load, and subsequent lanes are labeled by fraction number. S is the molecular weight standard identical with that in Figure 1. (B) Salt gradient of DE-52 column from 0.0 to 0.2 M NaCl in pH 8.5 urea–Tris buffer. SDS Coomassie Blue stained PAGE of indicated fractions.

Table II: Bivalent FMD Short Fusion Antigen Induction of Neutralizing Antibody Titers in Guinea Pigs[a]

		Skinner PD_{50}[b]			
A24 mass (μg)	O1 mass (μg)	28 dpv vs. FMDV A24	28 dpv vs. FMDV O1	44 dpv vs. FMDV A24	44 dpv vs. FMDV O1
50		3.3	<0.3	3.1	nd
	250	<1	2.1	nd[c]	3.2
50	250	2.3	3.0	1.3	2.6

[a] Formulated in 8 M urea, 10 mM Tris, and 0.1% 2-mercaptoethanol and emulsified in a Freund's incomplete oil adjuvant (Morgan et al., 1980). [b] PD_{50} is the negative log of the dilution required to protect 50% of the mice. Booster vaccination given 28 dpv. [c] nd, not determined.

guinea pigs (data not shown). At the 100-μg dose, the titers raised against the A24 VP1 fusion antigen were similar to those raised against A24 virions. At the same dose, the O1 VP1 fusion antigen gave rise to slightly lower titers than VP1 from O1 virions (Table I). These data suggest that the A24 fusion antigen may be more potent as an immunogen than the O1 fusion protein. The addition of SDS to the final purified VP1 preparations (final SDS concentration at 0.1%) did not result in appreciable reduction in titers (Table I).

(B) Bivalent Immunization and Cross-Reactivity of Fusion VP1 Antigens. Guinea pigs were vaccinated with VP1 fusion proteins of the O1 Campos and A24 Cruzeiro FMDV serotypes prepared by scheme III (Figure 2). The results (Table II) show the response to each antigen given to separate groups of guinea pigs. Vaccination of the animals with both antigens at the same inoculation site induced significant neutralization titers to both serotypes as determined by the Skinner mouse protection assay (Skinner, 1952). A 5-fold higher amount of O1 fusion antigen was used since the A24 fusion protein was known to be a more potent immunogen.

Discussion

Purification of Fusion VP1 Antigen. (A) Effect of E. coli Contaminants on Purification of Fusion VP1. Certain *E. coli* contaminants adversely affect the behavior and purification of the fusion proteins on DE-52. The chromatograms and SDS–PAGE shown in Figure 3 indicate that some of the fusion protein passed through the DE-52 column unadsorbed. However, as much as 90% of the total VP1 fusion protein remained bound to the DE-52 resin and was eluted with a NaCl gradient. Theoretical protein hydrogen ion titration curves (Shire, 1983) predict a p*I* of 9.5 for both the O1 and A24 fusion proteins. These computations do not take into account interactions between ionized amino acid residues nor do they consider the influence of protein folding on the p*K* values of individual amino acid side chains. On the basis of the estimated p*I*, the fusion proteins would not be expected to bind to the DE-52 resin at pH 8.3. It is possible that asymmetric charge distribution on the protein surface could account for the observed binding to the resin. However, the results shown in Figures 3–5 strongly suggest that the VP1 fusion proteins are interacting with *E. coli* contaminants and that the resulting complexes bind to the resin. Centrifugation of a 7 M Gdn-HCl extract of the fusion pellet for greater than 15 h at 44000*g* (see Results) removed predominantly two proteins which are highly aggregated, and possibly bound to *E. coli* membrane or cell wall material. The resulting clear solution chromatographed as expected on DE-52 cellulose. In particular, essentially all the fusion protein passed through the column unadsorbed at pH 9.0, whereas at pH 10.0, some of the fusion protein bound to the resin and was eluted with increasing concentrations of Tris in UTB (data not shown).

Although 8 M urea is considered to be a strong protein denaturant, its effectiveness is temperature and pH dependent, and various proteins are resistant to denaturation in 8 M urea under a variety of conditions (Tanford, 1968). It is clear from the results presented in Figures 3 and 5 that solubilization of the fusion VP1 pellet with 8 M urea is not effective in eliminating interactions between the VP1 fusion protein and those *E. coli* contaminants which affect purification as well as immunogenicity of the VP1 fusion proteins. Solubilization with 8 M urea was originally expected to be the best method for

extraction since the solubilized fusion protein pellet could then be chromatographed directly on DE-52 resin. However (Figure 3), this procedure gave low yields and produced material having low immunogenicity. Attempts to solubilize the fusion protein pellet directly into 8 M urea buffers at various pH values also resulted in incomplete solubilization and hence further loss of material (data not shown). It was determined that 7 M Gdn-HCl could solubilize the pellet and that either high-speed centrifugation or S-300 Sephacryl chromatography of the solubilized fusion protein resulted in material which was easily purified by DE-52 ion-exchange chromatography. Gdn-HCl has been shown to be an extremely effective denaturant of proteins, capable of converting globular protein structure into a random-coil conformation (Tanford, 1970). Solubilization of the fusion protein pellet in 7 M Gdn-CHl apparently disrupts the interactions between the *E. coli* contaminants and the VP1 fusion protein. This permits their effective separation either by gel permeation chromatography or by prolonged high-speed centrifugation.

(*B*) *Estimates of Yield and Purity following Purification on Sephacryl S-300 and DE-52 Ion-Exchange Chromatography.* The theoretical recovery can be roughly estimated by making the following assumptions: (1) wet cell paste contains about 25% solids; (2) about 50% of the cell dry weight is protein (Ingraham et al., 1983); (3) about 25% of the cellular protein is the VP1 fusion protein (see Results). With these assumptions, we calculate that 1 kg of wet weight cell paste contains 31 g of VP1 fusion protein. A typical preparation for the A24 VP1 fusion protein resulted in a recovery of 30 g/kg of cell paste. This corresponds to an estimated recovery of 96% for the A24 fusion protein at 96% purity as determined by scanning densitometry of SDS gels.

Immunogenicity of Fusion VP1 Antigen. (*A*) *Effect of SDS on Immunogenicity of VP1 Antigens.* Although earlier immunization attempts with A12 fusion proteins purified by SDS–PAGE (Kleid et al., 1981) were successful, we did not observe satisfactory levels of neutralizing antibodies in swine with fusion VP1 antigens of the O1 and A24 serotypes purified in the same manner (data not shown). However, chromatographic purification without addition of SDS yielded antigens which did induce significant levels of neutralizing antibody (Table I). The results in Table I also suggest that SDS alone does not interfere with the ability of the VP1 antigens, regardless of the source, to raise a satisfactory level of neutralizing antibody in test animals. Thus, the inability to induce satisfactory levels of neutralizing antibody in vivo using antigens purified by SDS–PAGE and adjuvanted in acrylamide may be due to interactions with the acrylamide matrix possibly in combination with SDS binding.

(*B*) *Effect of E. coli Contaminants on Immunogenicity of Fusion VP1.* Further purification of solubilized fusion pellets by chromatographic techniques (see Results) yielded material which was greater than 96% pure as judged by scanning densitometry of SDS gels. These fusion protein preparations for both O1 and A24 serotypes induced significant antibody titers (Tables I and II). This strongly suggests that *E. coli* contaminants must be removed, since these contaminants severely interfere with the ability of the antigens to induce virus neutralizing antibody in test animals. The SDS gels and chromatograms shown in Figure 3 demonstrate that if these contaminants are proteins, they are present at levels below detection by SDS–PAGE, since DE-52 fractions A and B appeared to have identical composition whereas fraction A, but not B, induced significant antibody titers in guinea pigs. Alternatively, nonprotein contaminants such as lipids or lipopolysaccharides may cause the aberrant behavior. It is also possible that the inter- and intramolecular disulfide bonding in the fusion protein preparation is crucial to its immunigenicity. Although 2-mercaptoethanol is used to reduce the disulfides, it has been demonstrated that oxidation of proteins can occur in reducing media containing 2-mercaptoethanol because of contamination by 2-hydroxyethyl disulfide (Wetlaufer et al., 1983). This coupled with the volatility of 2-mercaptoethanol may result in preparations with different amounts of covalent aggregates of varying sizes. This may also contribute to the observed differences in immunogenicity between preparations which appear identical under SDS–PAGE reducing conditions (fractions A and B, Figure 3) and requires further investigation.

(*C*) *Comparison of Virus Neutralizing Antibody Titers for O1 and A24 Antigens.* O1 and A24 fusion VP1 antigens at 100 and 500 μg induced neutralizing antibody titers similar to that induced by the isolated virion O1 and A24 VP1 (Table I) at 100 μg. This demonstrates that the VP1 fusion proteins prepared by the chromatographic methods discussed (see Results) yielded a VP1 antigen with an immunogenicity essentially equivalent to the purified virion VP1. Furthermore, at a 100-μg dosage, the A24 fusion VP1 protein induced approximately 2-fold higher titers (on a logarithmic scale) than the O1 fusion VP1 protein. This result (Table I) is identical with that obtained for the natural virion VP1 and may reflect a difference in immunogenicity of the A24 and O1 amino acid sequences.

The antibodies induced by the A24 and O1 fusion VP1 proteins do not cross-neutralize as can be seen by the results of the mouse protection assay shown in Table II. Vaccination of guinea pigs with both antigens resulted in significant titers of antibody which neutralize both serotypes in the mouse protection assay of Skinner (1952). However, the inoculation of both antigens at one site does appear to result in a decrease in antibody titer for the A24 VP1 fusion antigen when compared to induced titers from vaccination with the A24 fusion protein alone. It is possible that there is an interaction between the antigens which resulted in this lower titer. Further work is required to clarify this point.

Conclusions

We have developed a simple and efficient procedure for isolating and purifying fusion VP1 antigens expressed in *E. coli*. The procedure is capable of recovering >96% of the crude fusion protein at >96% purity. The removal of the contaminants has been shown to be crucial since partially purified *E. coli* extracts containing VP1 fusion protein did not induce significant virus neutralizing antibody in guinea pigs as determined by the Skinner mouse protection assay (Skinner, 1952). Furthermore, *E. coli* contaminants interfere with purification on ion-exchange resins by virtue of their interactions with the fusion proteins. In particular, bacterial contaminants appar to bind to the fusion VP1 as well as to DE-52 resin and presumably also mask or alter antigenic sites, thus preventing the protein from efficiently raising a neutralizing antibody titer.

The experiments presented here demonstrate that it is possible to efficiently purify from *E. coli* extracts FMD VP1 fusion protein antigens of the O1 and A24 field serotypes which are capable of inducing significant levels of neutralizing antibody in guinea pigs. A24 fusion antigens prepared by this method have also successfully protected cattle from virus challenge (Kleid et al., 1984). Therefore, the procedure is capable of producing a protein which can present antigenic determinants to the immune system of cattle that are similar

to those of the whole virus.

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Raman Study of Reduced Nicotinamide Adenine Dinucleotide Bound to Liver Alcohol Dehydrogenase†

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ABSTRACT: We report the first Raman spectra of reduced nicotinamide adenine dinucleotide (NADH) when bound to an enzymatic active site, that of liver alcohol dehydrogenase (LADH). This was obtained by subtracting the Raman spectrum of LADH from that of the binary LADH/NADH complex. There are significant changes in the spectrum of bound NADH as compared to that in solution. The data indicate that both the nicotinamide moiety and the adenine moiety are involved in the binding. At least one of the two NH_2 moieties of NADH also participates.

Oxidized nicotinamide adenine dinucleotide (NAD^+)[1] and its reduced form (NADH) are coenzymes for hundreds of enzymatic oxidation–reduction reactions (Dalziel, 1975). The nature of the interaction of these cofactors with proteins is not understood. Some information on this interaction is available from X-ray crystallographic studies on several dehydrogenases (Brändén et al., 1975; Plapp et al., 1978; Eklund et al., 1981; Holbrook et al., 1975; Harris & Waters, 1976; Banaszak & Bradshaw, 1975; Rossman et al., 1975; Ohlsson et al., 1974). In these studies, electron-density differences were calculated to give the location of the ligand within the protein. Nevertheless, the nature of the interaction(s) between the ligand and protein can only be deduced and not studied directly with this technique. In contrast, Raman spectroscopy measures the vibrational normal modes and is sensitive to bonding changes that are likely to occur in binding of ligands to proteins. A

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[1] Abbreviations: LADH, liver alcohol dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; NAD^+, oxidized nicotinamide adenine dinucleotide; ADPR, adenosine diphosphate ribose.

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