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Purification and Characterization of a Family of High Molecular Weight Surface-Array Proteins from *Campylobacter fetus**

(Received for publication, November 10, 1987)

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A variety of Gram-negative and Gram-positive bacteria possess crystalline surface layers, although little is known of their function. We previously have shown that the high molecular weight surface-array proteins of *Campylobacter fetus* are important in both the pathogenicity and antigenicity of this organism. For biochemical and immunological characterization, we purified high molecular weight (100,000, 127,000, 149,000) surface-array proteins from three *C. fetus* strains using sequential gel filtration and ion exchange high performance liquid chromatography. These proteins are acidic with pI values between 4.12 and 4.25 and contain large proportions of acidic amino acids (19.7%–22.0%) in addition to hydrophobic amino acids (37.3%–38.5%). They share a novel amino-terminal sequence through at least 19 residues. Carbohydrate analysis using periodic acid-Schiff staining and treatment with trifluoromethanesulfonic acid shows no evidence of glycosylation. Antiserum to a purified M_r = 100,000 protein from *C. fetus* 82-40 LP cross-reacts with three other purified *C. fetus* surface-array proteins by enzyme-linked immunosorbent assay with titers >12,800. We conclude that: 1) there is a family of surface-array proteins of *C. fetus* with common structural and antigenic characteristics; 2) that these molecules have similar biochemical characteristics to surface-array proteins described for other bacteria; but however, 3) by amino-terminal sequence analysis these are unique.

It is now recognized that a wide variety of bacteria may possess surface layers of regularly arranged protein subunits (S-proteins) (1). These surface-arrays are noncovalently attached to the outer membrane of Gram-negative organisms or to the peptidoglycan of Gram-positive organisms. The ultrastructural basis of these layers has been well defined (2), but biochemical analysis has been limited. The S-proteins of *Aeromonas salmonicida*, a fish pathogen, and *Azotobacter vinelandii*, a nitrogen-fixing soil bacterium have been best characterized. The S-proteins of these bacteria have regular structures, are relatively hydrophobic, and are readily extracted from the cell envelopes (3, 4).

Campylobacter fetus subspecies *fetus* is an important veterinary pathogen causing abortion in cattle and sheep (5) and a human enteric pathogen that causes systemic infections in compromised hosts (6–8). Our previous work has demonstrated the presence of several high molecular weight surface proteins that are important virulence factors for these organisms (9). *C. fetus* strains encapsulated by these surface-array proteins (S-proteins) are usually resistant to normal human serum and to phagocytosis by polymorphonuclear leukocytes, whereas spontaneously appearing unencapsulated mutants are sensitive to these host defenses (9, 10). Prior studies of *C. fetus* S-protein by McCoy *et al.* (11, 12) using Coomassie Blue staining determined a M_r = 98,000 glycoprotein containing 3.8% carbohydrate which formed a hexagonal subunit capsular structure on the outer surface of the cell. The surface-exposure and pathogenic importance of the *C. fetus* S-proteins suggest that they will be potential candidates for a *C. fetus* vaccine. A vaccine prepared from bacteria-free high molecular weight protein-enriched supernatants of *C. fetus* broth culture protected pregnant ewes against challenge with live homologous *C. fetus* (13).

The present work is part of an investigation in the pathogenicity of *C. fetus* (14). Emphasis has been placed on developing a method to isolate and purify these S-proteins and to determine biochemical and immunological characteristics of S-proteins from different *C. fetus* strains.

EXPERIMENTAL PROCEDURES

Growth of Bacteria and Extraction of Surface Proteins—Five *C. fetus* strains (82-40 LP, 82-40 HP, 82-40 LP3, 84-86, and 84-112 from the culture collection of the Denver Veterans Administration Medical Center *Campylobacter laboratory*) were used in this study. Strains 82-40 LP, 84-86, and 84-112 originally were isolated from ill humans or animals. These strains had been passaged 5 to 10 times on artificial media. Strain 82-40 HP is a spontaneous mutant of 82-40 LP that lacks the M_r = 100,000 S-protein (9), and 82-40 LP3 is a spontaneous mutant in which the predominant form of the S-protein is as a M_r = 127,000 structure. All strains were maintained frozen at −70 °C in brucella broth (BBL Microbiology Systems, Cockeysville, MD) containing 15% glycerol for use as stocks. For studies, bacteria were grown on trypticase soy agar with 5% sheep blood (PASCO, Wheat Ridge, CO) in a microaerobic atmosphere (5% oxygen, 10% carbon dioxide, and 85% nitrogen) at 37 °C for 48 h for the first two generations, and for 24 h for the third generation. Cells were harvested and washed five times with distilled water by centrifugation at 3,500 × *g* for 15 min with the supernatants from each wash separately collected for subsequent comparisons. Acid-labile surface proteins were extracted with 0.2 M glycine hydrochloride buffer, pH 2.2, as previously described (10). Proteins were similarly extracted using 10 mM EDTA, 0.9% NaCl, or 100% hexadecane in place of the glycine

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hydrochloride buffer and using 5 mM calcium chloride in place of water in this procedure.

Analytical Procedures—Protein concentration was measured by using the Markwell *et al.* modification of the Lowry method (15). SDS-PAGE[1] was performed in a modified Laemmli gel system as described by Ames (16). Proteins were resolved using the modified silver stain of Oakley *et al.* (17). Proteins were stained for carbohydrate content with periodic acid-Schiff's reagent as described previously (18). Molecular weight standards (Bio-Rad) were assigned as follows: myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,000). Determination of the pI of the S-proteins was made by isoelectric focusing in Resolve™ thin layer agarose gels with pH range between 3 and 7 using the Resolve-Hb kit (Isolab Inc., Akron, OH) and the modified silver stain of Willoughby and Lambert (19). Isoelectric focusing standards (Sigma) were amyloglucosidase (3.55), trypsin inhibitor (4.55), and β-lactoglobulin A (5.13).

High Performance Liquid Chromatographic Procedures—Gel filtration HPLC was performed on a Superose 12 column (Pharmacia LKB Biotechnology Inc.) with a pH 7.5, 20 mM Tris, 50 mM NaCl at a flow rate of 0.15 ml/min. Ion exchange HPLC was performed on a Mono Q column (Pharmacia LKB Biotechnology Inc.) with a pH 7.5, 20 mM Tris buffer for the M_r = 100,000 protein from 82-40 LP and with a pH 5.0, 20 mM piperazine buffer for all other S-proteins at a flow rate of 0.5 ml/min and a linear gradient to 350 mM NaCl over 40 min. For all of these analyses, the column eluates were monitored for UV absorbance at 280 nm.

TFMS Deglycosylation—Deglycosylation with TFMS was performed by the method of Stewart *et al.* (20). Briefly, 1 ml of anisole and 2 ml of TFMS were mixed and cooled at 0 °C; 50 μg of lyophilized protein was dissolved in 40 μl of this mixture, and nitrogen was bubbled through the solution for 30 s. The mixture was agitated at 0 °C for 2 h, and the reaction was terminated by the addition of 250 μl of pyridine/water (4:1, v/v). The solution was dialyzed against water for 18 h at 4 °C and then lyophilized.

Amino Acid Analysis and Sequencing—Purified S-proteins were prepared for analysis by dialysis against water and lyophilization. Amino acid analysis was performed using the method of Jones (21). The purified proteins (400–500 pmol) were sequenced on an Applied Biosystems 470 A Protein Sequencer equipped with the 120A Autoanalyzer using the 03RPTH program. The column for the separation of the phenylthiohydantoin-amino acid derivatives was the phenylthiohydantoin C-18 (220 × 2.1 mm, 5 μm) with a gradient of: 0 min – 8% B, 120 μl/min; 0.1 min – flow to 200 μl/min; 11 min – 12%; 30 min – 30% B, flow to 230 μl/min; 37 min – 36% B; 37.1 min – 80% B; 40 min – flow to 300 μl/min; 48 min – 80% B. The buffers were: A, 5% tetrahydrofuran in H_2O with 28.8 ml/liter of 3 M sodium acetate, pH 3.8, and 6.7 ml/liter of 3 M sodium acetate, pH 4.6; and B, acetonitrile. The column was at 55 °C. Comparison of the amino-terminal sequences with known proteins was performed by an on line search of the National Biomedical Research Foundation database (Georgetown, Washington, D.C.).

Immunological Methods—Antiserum against the *C. fetus* 82-40 LP M_r = 100,000 S-protein was raised in adult New Zealand White female rabbits by three subcutaneous injections at 2-week intervals of 40 μg of purified protein in 1 ml of an equivolume mixture of antigen and adjuvant (67% 0.1 M PBS, 27% hexadecane, 6% glycerol). Fresh normal serum from the same rabbits was obtained prior to inoculation. Determination of antibody concentration and a comparison of the immunologic cross-reactivity of the purified S-proteins was performed by ELISA as previously described (22). To sensitize the ELISA plates (Immulon II; Dynatech Laboratories Inc., Alexandria, VA), purified proteins and the material from the second wash of 82-40 LP and 82-40 HP were diluted to 10 μg/ml, and whole bacterial cells were diluted to 40 μg/ml in 0.015 M carbonate buffer, pH 9.6. One hundred μl of these preparations were added to each well, and the plates were incubated at 4 °C overnight. The plates were washed once with 0.01 M PBS, pH 7.2, in 0.03% Tween 20 and 0.01% thimerosal (PBS-T-T) and were blocked with 200 μl/well of 0.1% gelatin in PBS-T-T overnight at 4 °C. The plates were washed twice with PBS-T-T, 100 μl of antisera was diluted with 0.5% bovine γ-globulin, and 0.1% gelatin in PBS-T-T was added in triplicate to each well. After a 1-h incubation at 37 °C, the plates were washed three times as before, 100 μl of peroxidase goat anti-rabbit IgG (1:10,000, Boehringer Mannheim Biochemicals, San Diego, CA) was diluted with 0.1% bovine γ-globulin, and 1% BSA in PBS-T-T was added to each well and incubated at 37 °C for 1 h. The plates were washed five times, and 100 μl of peroxidase developer (20 mg of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), 34 μl of H_2O_2, 9.35 ml of 0.2 M Na_2HPO_4, and 10.65 ml of 0.1 M citric acid) was added to each well, and the product of the peroxidase reaction was determined after 30 min at room temperature using a MR 600 microplate reader (Dynatech Laboratories Inc., Alexandria, VA) at 414 nm. The titer was defined as the reciprocal of the highest dilution giving a reading of 0.1.

RESULTS

Water Extraction of Surface Proteins—The purification of the S-proteins from *C. fetus* involves extraction of the surface layer from whole cells followed by gel filtration and ion exchange chromatography. The first extraction of an overnight culture of *C. fetus* strain 82-40 LP with water yielded a protein content that was more than 6 times that of the second, third, fourth, and fifth washes combined, but primarily contained small molecular weight proteins from the blood agar media as determined by SDS-PAGE (Fig. 1). Analysis of the third, fourth, and fifth washes demonstrated an approximate 80% purification of the M_r = 100,000 S-protein, with approximately 0.1 mg of protein per plate in the third wash. Similar results were found in water extracts of *C. fetus* strains 84-86, 82-40 LP3 and 84-112. Optimal harvesting of the M_r = 100,000 S-protein of 82-40 LP was in the third wash, and of the M_r = 100,000 S-protein of 84-86 in the second wash. Optimal extraction of the M_r = 127,000 S-protein of 82-40 LP3 was in pooled second and third washes and of the M_r = 149,000 S-protein of 84-112 in the second wash. Water extraction contained more M_r = 100,000 S-protein and less contamination with small molecular weight proteins (Fig. 1) when compared with acid extraction or extraction with 0.9% NaCl, 10 mM EDTA, or hexadecane (data not shown), and contained 10 times more M_r = 100,000 S-protein than similar extraction with 5 mM calcium chloride. The M_r = 100,000 S-protein was stable during 20 min boiling in water (data not shown).

Protein Purification—The M_r = 100,000 S-protein of 82-40 LP was further purified by HPLC (see "Experimental Procedures"). The M_r = 100,000 S-protein eluted from the gel filtration column between 11.7 and 12.6 ml. The protein was further purified to homogeneity by ion exchange separation on a Mono Q column with the M_r = 100,000 S-protein eluting between 18.4% and 20.1% of buffer B.

The M_r = 149,000 S-protein from the second wash of 84-112, the M_r = 100,000 S-protein from 84-86, and the M_r = 127,000 S-protein from second wash and third wash of LP3 was purified by sequential ion exchange HPLC on Mono Q and gel filtration HPLC on Superose 12 (Fig. 2).

Isoelectric Focusing—An experient formula of pI *versus* distance was generated by using three standard proteins of known pI between 3.55 and 5.13. All four S-proteins are acidic with pI values between 4.12 and 4.25 (Fig. 3). No relationship was found between molecular weight and pI.

Carbohydrate Analysis—To determine if any of the molecular weight differences of *C. fetus* S-protein was because of differential glycosylation, four *C. fetus* S-proteins were subjected to TFMS-mediated deglycosylation which removes *O*-linked and *N*-linked carbohydrates. After 120 min of incubation at 0 °C with TFMS, all four of the *C. fetus* S-proteins were decreased by about M_r ~ 1000. A similar decrease in the size of BSA (a nonglycosylated protein) was observed. In

[1] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; TFMS, trifluoromethanesulfonic acid; PBS, phosphate-buffered saline; PBS-T-T, thimerosal; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

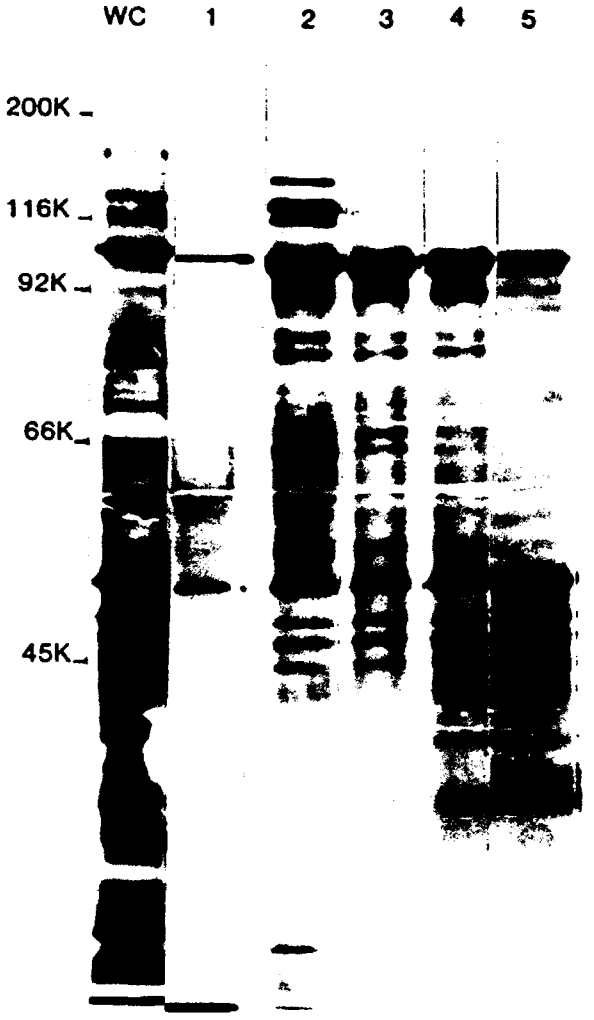

FIG. 1. **SDS-PAGE of water extracts of *C. fetus* 82-40 low passage (LP).** *Lanes* are from the whole cell preparation (*WC*), and after the first (*1*), second (*2*), third (*3*), fourth (*4*), and fifth (*5*) washes of whole cells in sterile distilled water. The amount of protein in these extracts is 29.1 mg (first), 2.1 mg (second), 1.4 mg (third), 0.6 mg (fourth), and 0.2 mg (fifth). In addition to the M_r = 100,000 S-protein, a second major S-protein in the second extraction migrated at M_r = 127,000.

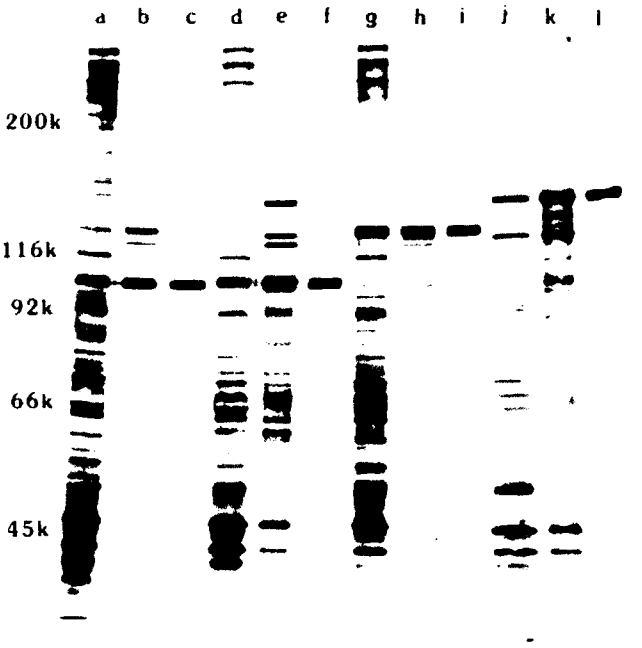

FIG. 2. **Purification of *C. fetus* high molecular weight S-proteins.** SDS-PAGE with 7% acrylamide. Lanes are: *a*, whole cell preparation of 82-40 LP; *b*, third water wash from 82-40 LP; *c*, purified M_r = 100,000 protein of 82-40 LP; *d*, whole cell preparation of 84-86; *e*, second water wash from 84-84; *f*, purified M_r = 100,000 S-protein of 84-86; *g*, whole cell preparation of 82-40 LP3; *h*, second and third water washes from 82-40 LP3; *i*, purified M_r = 127,000 S-protein of 82-40 LP3; *j*, whole cell preparation of 84-112; *k*, second water wash from 84-112; *l*, purified M_r = 149,000 S-protein of 84-112. Migration of marker proteins of known molecular weight are shown at *left*.

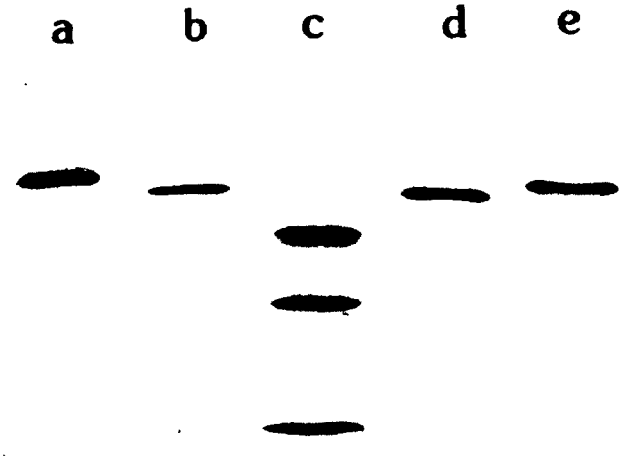

FIG. 3. **Isoelectric focusing of *C. fetus* proteins.** Lanes are: *a*, M_r = 100,000 S-protein of 82-40 LP (4.12); *b*, M_r = 100,000 S-protein of 84-86 (4.25); *c*, isoelectric standards from top, amyloglycosidase (3.55), trypsin inhibitor (4.55), β-lactoglobulin A (5.13), and bovine carbonic anhydrase B (5.85); *d*, M_r = 127,000 S-protein of 82-40 LP3 (4.21); *e*, M_r = 149,000 S-protein of 84-112 (4.20). The pI of unknown proteins was calculated by using an experient formula of pI *versus* distance, $y = 3.2162 + 0.1272x$; $R = 0.9995$. The difference between given and calculated pI values for standard proteins was <0.01.

contrast to the negative control of BSA and *C. fetus* S-proteins, TFMS treatment of fetuin caused a large decrease in the M_r of the major protein band. Partial degradation products in both control proteins and *C. fetus* S-proteins were observed. Because no larger changes were found in the size of *C. fetus* S-proteins than in that of negative control BSA, we conclude that S-proteins of *C. fetus* are glycosylated less than BSA, and the molecular weight differences of *C. fetus* S-proteins are not related to differential glycosylation. For confirmation, Schiff staining was performed with α_1-acid glycoprotein (41% glycosylated) as a positive control and BSA as a negative control. The Schiff staining procedure could detect as low as 0.15 μg of carbohydrate per lane for α_1-acid glycoprotein. No positive staining was found with 100 μg/lane of BSA or 100 μg/lane of four *C. fetus* S-proteins, indicating that glycosylation, if present, represented less than 0.15% of the molecular weight (data not shown).

Amino Acid Composition and Amino-terminal Amino Acid Sequences—The amino acid compositions of these four S-proteins were very similar to one another (Table I) and to antigen [a] described by Winter *et al.* (12). These four proteins are characterized by the absence of His, Cys, and Pro and by their relative (23) hydrophobicity (37.3–38.5%). A comparison of the amino-terminal amino acid sequences of three of these proteins indicated that this region is relatively hydrophobic containing 12 hydrophobic and only 3 charged amino acids

within the first 19 residues (Fig. 4). This region is also highly conserved. Thus, these structural and composition data strongly indicate the relatedness of these proteins and demonstrate that the primary structures are homologous at the amino-terminal.

The on line search for similar amino acid sequences identified one partial match (56.3% over 16 residues) with amino acids 396 to 411 of the fusion (F) glycoprotein of human respiratory syncytial virus (24). The significance of this match is not clear.

Immunological Characterization—Results are summarized in Table II. Normal rabbit serum showed reciprocal ELISA titers of no greater than 100 against the 8 antigens used in this study. Antiserum to 82-40 HP whole cell served in this study as a purity control for *C. fetus* S-proteins, while 82-40 HP whole cells and second wash were used to evaluate specificity of the antiserum to the purified M_r = 100,000 protein of LP, as the HP strain differs from the parent strain (LP) by lacking only high molecular weight S-proteins (9). The purity of the *C. fetus* S-proteins and the specificity of the antiserum to M_r = 100,000 protein of LP were verified by evaluating cross-reactivity between antiserum to HP whole cells and the purified *C. fetus* S-proteins and between antiserum to the M_r = 100,000 protein and either HP whole cell or HP second wash antigens (Table II). The cross-reactions between LP whole cell and *C. fetus* S-proteins suggested that *C. fetus* proteins from different strains share common antigenic determinants on the surface of cells. This cross-reactivity was further demonstrated when antiserum to the M_r = 100,000 protein of LP showed a homologous reciprocal titer of 102,400 and heterologous reciprocal titers of 12,800 against three other *C. fetus* S-proteins.

TABLE I

Relative amino acid composition of high molecular weight proteins of C. fetus

Amino acid	M_r = 100,000[b]	Mol %[a] M_r = 100,000[c]	M_r = 127,000[d]	M_r = 149,000[e]	Antigen [a][f]
Lys	7.9	4.6	6.8	6.4	6.4
His	0.0	0.0	0.0	0.0	0.6
Arg	0.0	1.3	0.0	0.0	0.5
Asp	16.6	15.3	15.0	16.0	15.2
Thr	13.8	10.9	12.1	10.8	10.4
Ser	7.4	7.7	8.6	8.8	6.6
Glu	3.4	6.7	4.7	5.0	4.6
Pro	0.0	0.0	0.0	0.0	3.0
Gly	11.4	3.2	12.6	12.9	12.4
Ala	12.5	10.6	12.1	10.8	14.2
Cys	0.0	0.0	0.0	0.0	0.0
Val	7.0	7.0	7.7	6.8	7.4
Met	1.3	1.4	0.9	1.1	1.3
Ile	6.6	6.8	7.3	7.3	6.2
Leu	7.0	8.4	8.6	9.6	6.9
Tyr	1.9	3.0	1.6	1.7	1.2
Phe	3.0	3.1	1.9	2.6	3.0
Trp[g]	ND[h]	ND	ND	ND	0.3
Hydrophobic[i] residues (%)	37.4	37.3	38.5	38.2	42.3

[a] Based on mean of duplicate determinations.
[b] From 82-40 LP.
[c] From 84-86.
[d] From 82-40 LP3.
[e] From 84-112.
[f] From Ref. 12.
[g] Not determined.
[h] ND, not determined.
[i] Val, Met, Ile, Leu, Ala, Phe, Trp, and Pro (23).

DISCUSSION

Three *C. fetus* S-proteins have been purified and characterized in the present study. Amino-terminal sequence analysis and immunologic evaluation indicate that the S-proteins are highly related. It is possible that they represent a single protein that is processed to varying degrees at the carboxyl-terminal. Sequence of the entire proteins, which presently is ongoing in our laboratory, will help answer this question. Why various *C. fetus* strains preferentially produce or process one or another particular form requires further study.

Although isolation of S-layer proteins in most bacteria has included complicated treatment with urea, SDS, EDTA, EGTA, acid, or alkali (1), repeated extractions in distilled water were successfully used to isolate *C. fetus* S-proteins in the present study, and those of *A. vinelandii* by Bingle *et al.* (3). The mechanism of releasing S-proteins with water remains unknown. As suggested by Bingle *et al.* (25), the subunits of S-layers are held together by divalent cations, notably Ca^{2+} and Mg^{2+}, which slowly equilibrate into the distilled water allowing the subunits to disaggregate into monomers. The three *C. fetus* S-proteins have the following common characteristics that appear similar to other bacterial S-proteins; 1) they are easily extracted with distilled water, 2) they are highly acidic, 3) they have a hydrophobic amino acid composition while lacking cysteine, and 4) they have hydrophobic amino-terminal sequences.

For each of the *C. fetus* strains that possessed S-proteins, three distinct S-proteins of different molecular weight ranging from M_r = 100,000 to ~150,000 always appeared to be present. However, for each strain, one protein always was present in a major proportion; this property was generally stable after

```
                1          5          10         15         20         25         30

100 kDa of 82-40  Met-leu-Asn-lys-Thr-Asp-Val-Ser-Met-Leu-Tyr-Ile-Thr-Ile-Met-Gly-Met-Ala-Ser-Glu

127 kDa of 82-40  Met-Leu-Asn-Lys-Thr-Asp-Val-Ser-Met-Leu-Tyr-Ile-Thr-Ile-Met-Gly-Met-Ala-Ser-Glu-Lys-Asp-Pro-Asn-Lys-Tyr-Trp-Leu-Asp-Typ-Ala

149 kDa of 84-112 Met-Leu-Asn-Lys-Thr-Asp-Val-Ser-Met-Leu-Try-Ile-Thr-Ile-Met-Gly-Met-Ala-Ser
                (Gln)                                                                (Thr)
                (Pro)
```

FIG. 4. **The amino-terminal sequence of *C. fetus* high molecular weight surface proteins determined with 400–500 pmol of protein, 23-42 cycles each performed once, quantitative HPLC (see "Experimental Procedures"), 94.2%–94.6% repetitive yield.**

TABLE II

ELISA titer of rabbit antiserum to purified C. fetus M_r = 100,000 protein against C. fetus antigens

Titer is defined as highest dilution showing optical density greater than 0.100.

C. fetus antigens	Reciprocal titer for rabbit antibody in ELISA			
	Normal serum	Anti-M_r = 100,000 serum	Anti-82-40 LP serum	Anti-82-40 HP serum
82-40 LP cells	<100	3,200	25,600	25,600
82-40 LP second wash	<100	51,200	102,400	6,400
82-40 HP cells	<100	<100	12,800	102,400
82-40 HP second wash	<100	<100	12,800	25,600
M_r = 100,000 S-protein of 82-40 LP	<100	102,400	51,200	<100
M_r = 100,000 S-protein of 84-86	<100	12,800	51,200	<100
M_r = 127,000 S-protein of 82-40 LP3	<100	12,800	51,200	<100
M_r = 149,000 S-protein of 84-112	<100	12,800	12,800	<100

repeated *in vitro* passages of the strain. The S-protein with highest molecular weight was extracted in the earlier water washes while those with lower molecular weight were extracted later. In ion exchange HPLC, the major S-protein was eluted off the column by a lower salt concentration than the minor one.

The S-proteins of *C. fetus* were thought to be glycoproteins by Myers (26) and Winter *et al.* (12), but we failed to confirm this. There are several possible reasons for this discrepancy. The earlier study did not employ HPLC methods for purification. Methods used to determine glycosylation of protein at that time included the Schiff stain and colorimetric methods. No special steps were designed in the Schiff stain used by Myers to elute interfering SDS from the gel and this may have caused a false positive stain based on a simple acid-base interaction (18). Complete elimination of carbohydrate components in protein preparations is a prerequisite when using colorimetric methods to determine glycosylation because minor contamination by lipopolysaccharides and polysaccharides may cause a falsely positive result. Two independent methods were used in the present study to evaluate the purity of our S-protein preparations. Silver stain is 10 to 50 times more sensitive (17) than the Coomassie Blue stain used by Winter *et al.* (12), and immunodetection in ELISA can reveal minor contaminants that are not stained by silver. We failed to find any evidence of glycosylation in our protein preparations by using Schiff stain or deglycosylation with TFMS, and we conclude that the S-proteins of *C. fetus* are not glycoproteins.

Immunological studies by ELISA clearly show antigenic similarities of the four *C. fetus* S-proteins from different strains which support the hypothesis that these S-proteins of *C. fetus* could be used as the basis for vaccines against *C. fetus* infections.

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