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PURIFICATION AND CHARACTERIZATION OF BOVINE CD36 FROM HEART MICROSOMES

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By

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF BOVINE CD36
FROM HEART MICROSOMES

by Shailja Patel

Bovine PAS IV is an integral membrane glycoprotein, which has been purified from milk-fat-globule membrane and partially characterized. PAS IV is present in the capillary endothelial cells of heart tissue. The endothelial form (heart) of the protein has a higher molecular weight compared to the epithelial form (milk-fat-globule membrane). Peptide mapping procedures have shown that both the proteins are similar. The capillary endothelial form has been purified from bovine heart microsomes and is called bovine CD36. It has been partially characterized. It has a molecular weight of 85,000 D. it is hydrophobic in nature as it partitions in the detergent phase of Triton X-114. Amino acid sequences of four internal fragments have been obtained. The sequences are homologous to bovine PAS IV and human platelet membrane glycoprotein GPIV (CD36). Bovine CD36 may be a member of a group of cell adhesion proteins.

ACKNOWLEDGEMENTS

I would like thank Dr. Greenwalt for his support and guidance during my research. I would also like to thank Dr. Roger Biringer and Dr. Joseph Pesek for their suggestions on my thesis.

I am very grateful to my husband, Ashok for his patience and support which made this possible.

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I. INTRODUCTION

Very little is known about membrane-related proteins compared to soluble proteins, since X-ray crystallography and other biophysical tools cannot be easily applied. Due to lack of knowledge about these proteins, models for the functions of membrane proteins are not as advanced as compared to models for various biological processes mediated by proteins in aqueous surroundings. The major difficulty involved in determining structures of membrane proteins has been in growing crystals needed for diffraction studies (Eisenberg, 1984).

Bacteriorhodopsin (the purple membrane protein) was the only membrane protein whose two dimensional structure was known to moderate resolution, until recently. Now the three-dimensional structure of the photosynthetic reaction center from the bacterium Rhodopseudomonas viridus is known. The current understanding of the function of the reaction center was developed by combining structural information with information from other experimental techniques. (Deisenhofer & Michel, 1989).

An important model membrane is the milk-fat-globule membrane system This apically derived plasma membrane which envelops the fat droplets is known as the milk-fat-globule membrane (MFGM). This milk-fat-globule membrane can be isolated from bovine milk conveniently available and a homogeneous preparation of pure plasmalemma can be readily obtained (Mather & Keenan, 1975). The

MFGM contains peripheral proteins loosely bound to the extracellular surface and integral membrane proteins embedded within the phospholipid bilayer and can be considered a suitable model for studying membrane proteins.

MFGM from several species shows the presence of seven major proteins (Mather, 1987). Two of these proteins, xanthine oxidase, a protein with a molecular weight about 155 kD (Mather et al. , 1980) and the membrane glycoprotein butyrophilin with an apparent molecular weight about 66 kD (Johnson & Mather, 1985) have been purified from the milk-fat-globule membrane.

MFGM preparations from human, cow and guinea pig show the presence of a glycoprotein with a molecular weight of 75-80 kD. Recently, this protein called PAS IV has been purified to homogeity from the bovine MFGM (Mather et al. , 1980). It constitutes 5% of the MFGM protein. Several characteristics of this protein revealed by previous work indicate that it is an hydrophobic integral membrane glycoprotein: 1) The amino acid analysis of the pure protein shows a high percentage of non-polar residues; 2) It is resistant to proteolysis when embedded in the membrane; 3) The protein remains associated with the MFGM when washed with 0.1M Na_2CO_3, pH 11.5, a procedure known to remove the loosely bound peripheral proteins; 4) This protein can be extracted from membrane by solubilization with detergents.

This protein is found in other tissues as well. Immunolocalization studies

indicate an uneven distribution of PAS IV in various tissues. It was detected in endothelial cells of heart, liver, spleen, pancreas, salivary gland, small intestine, in both the epithelial and capillary endothelial cells of mammary tissue as well as the epithelium of the bronchioles. This protein was absent in the capillary endothelial cells of lung, brain and kidney (Greenwalt & Mather, 1985).

The vascular endothelium consists of a single layer of flattened cells which line the inner surface of blood vessels. The capillary endothelial cells play an important role in transporting ions and macromolecules between the blood and extravascular tissue. PAS IV can be considered a specific membrane marker of the endothelial cells on the basis of its tissue distribution. Previous work has shown that capillary endothelial form of PAS IV (heart) is similar to the epithelial form (fat-globule membrane). However, the endothelial protein has a higher molecular weight (Greenwalt & Mather, 1985). Despite these differences in molecular size peptide mapping techniques have shown that PAS IV obtained from epithelial cells and endothelial cells were similar (Greenwalt et al. , 1985). The observed difference in molecular size may be due to post-translational modifications.

Recently an 88 kD membrane glycoprotein has been identified and isolated. This glycoprotein, known as GP IV, is present in the platelets, endothelial cells, monocytes and a variety of human tumor cell lines. This protein is the membrane binding site for thrombospondin, a glycoprotein which is secreted upon platelet

stimulation and supports platelet aggregation (Asch et al. , 1987). GP IV may have an important function as a cell adhesion molecule (Tandon et al. , 1989). GP IV also functions as a specific receptor for collagen on human platelets (Tandon et al. , 1989). CD36, an 88 kD leucocyte differentiation antigen, is a receptor for malaria-infected erythrocytes (Ockenhouse et al. , 1989), is immunologically related to GP IV (Shaw, 1987) and is considered to be identical to GP IV. GP IV (CD36) may represent a new group of cell adhesion proteins. The function of these cell adhesion proteins depends on the type of cells involved (Tandon et al. , 1989).

Recent data from this laboratory suggests that PAS IV is similar to GP IV, based on the comparision of protein sequence data and immunological cross-reactivity studies. Also PAS IV, like GP IV, binds thrombospondin (Greenwalt, manuscript in preparation). Recently, the cDNA was cloned and the amino-acid sequence of human CD36 was obtained. There is some similarity in the protein sequence of bovine PAS IV and human CD36. Affinity purified antibody to PAS IV cross reacts with bovine CD36. These results indicate that bovine PAS IV, CD36, and GP IV may represent a new group of cell adhesion proteins. Confirmation of this hypothesis can be done by cloning and sequencing the cDNA of the proteins. However, the function of PAS IV in the mammary epithelial cells is still not known.

The purpose of the present research is to study an integral membrane

protein associated with the capillary endothelial cells called bovine CD36. Very little is known about the integral membrane proteins associated with the endothelial cells. It was assumed that CD36 constitutes a very small percent of the total microsome fraction proteins obtained from the heart tissue. In order to purify CD36, monoclonal antibodies to PAS IV were obtained.

II. MATERIALS AND METHODS

Materials

Tris base, tris-HCl, Triton X-114, Triton X-100, amino-n-caproic acid, phenylmethylsulphonylfluoride (PMSF), nitroblue tetrazolium, barbital buffer, sodium dodecyl sulphate (SDS), aprotinin, glycine (free base anhydrous), sodium thiocyanate, Freund's adjuvant incompelete, 2-aminoethanol, 2,2' azino-bis(3-ethyl benzthiazoline-6-sulfonic acid) diammonium salt, calf serum(donor), r globulins (Bovine Cohn Fraction II), anti-mouse IgG (whole molecule) peroxidase conjugate, ethylene glycol-bis-(B-amino-ethyl ether)N,N'-tetra acetic acid (EGTA), and cyanogen bromide activated sepharose 4B were purchased from Sigma Chemical Co. (St. Louis, MO). Affinity purified goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate, coomassie brilliant blue R-250, nitrocellulose membrane bis(N,N'-methylene-bis-acrylamide), and affinity purified goat anti-mouse IgG (whole molecule) peroxidase conjugate were purchased from Bio-Rad (Richmond, CA). CM-Sepharose CL-6B (cation exchange 45-165 um) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Recombinant Protein G-sepharose 4B conjugate was purchased from Zymed Laboratories, Inc. (So. San Francisco, CA). Methanol, sodium chloride, and N,N-dimethyl formamide were purchased from E M Science (Cherry Hill, NJ). Acrylamide and ammonium persulfate were purchased from Spectrum Chemical MFG. Corp. (Redondo Beach, CA). Bicinchoninic acid was obtained from the Pierce Chemical Company (Rockford,

IL). Gel bond was purchased from the FMC Corporation (Rockland, ME). Gel gard 2500 film was purchased from Hoechest Celanese (Charlotte, NC). 5-Bromo-4-chloro-3-indoyl phsphate p-toluidine salt was purchased from United States Biochemical Corp. (Cleveland, OH). 2-Mercaptoethanol was purchased from Aldrich Chemical Company (Milwaukee, Wis.). RPMI 1640, L-glutamine (200mM), hypoxanthine/aminopterin/thymidine (HAT), hypoxanthine/thymidine (HT), and penicillin/streptomycin (5,000 I.U./ml; 5,000 mcg/ml) were purchased from Mediatech (Washington, DC). Centricon 30 microconcentrators were purchased from Amicon Division, W.R. Grace & Co., (Danvers, MA). Standard cellulose dialysis tubing with a molecular cut off at 12,000D to 14,000D was purchased from Spectrum Medical Industries, Inc. (Los Angeles, CA).

Methods

Preparation of membrane.

Fresh bovine cream was obtained from Claravale Guernsey Farm in Monte Sereno, CA. Bovine milk fat globule membrane (MFGM) was prepared by diluting unwashed cream 1:1 with 0.1M Citrate buffer, pH 7.0, containing 0.15M NaCl, followed by stirring in a Waring blender connected to a speed controller with a speed setting of 35, at 4 ^{o}C until butter was formed. The aqueous buttermilk was filtered through four layers of cheese cloth and centrifuged at 26,500 rpm (98,000 x g) for 1 hour at 4 ^{o}C in a SW 27 swinging bucket rotor. The membrane pellet was

homogenized (protein concentration 5mg/ml) in 10mM Tris, pH 7.4, containing 0.15M NaCl, 0.5mM PMSF, 1% (v/v) aprotinin, 1mM aminocaproic acid and 1mM EGTA. After stirring the above mixture for half an hour at 4°C, Triton X-114 (11%) was added to 1% (v/v) final concentration. The suspension was stirred for 1 hour at 4°C, and then centrifuged at 26,500 rpm for 1hour at 4°C . Traces of white lipid material were removed from the top of the solution and the volume of the supernatant was noted (Mather et al. , 1980). A BCA protein assay was performed to determine the amount of protein solubilized from the membrane (Smith et al. , 1985). The supernatant was subjected to phase partitioning (Bordier, 1981). The supernatant was warmed to 30°C and then centrifuged at 1000 x g for 10 minutes, at room temperature. The detergent phase was diluted five fold with 10mM Tris buffer containing 0.15M NaCl (TBS), pH 7.4. A BCA protein assay was done to determine the protein content of the detergent phase.

Purification of PAS IV.

The diluted detergent phase was dialyzed overnight against 10mM Tris, pH 8.0, containing 0.1% (v/v) Triton X-100 at 4°C. The dialyzed detergent phase was fractionated on CM-cellulose cation exchange resin equilibrated with 10mM TBS, pH 8.0, containing 0.1% (v/v) Triton X-100. After washing the ion-exchange column (13.0 x 2.0 cm) with several volumes of equilibration buffer, the column was eluted with 75mM and 1.0M NaCl in 10mM Tris, containing 0.1% (v/v) Triton X-100, pH

8.0. Five mL fractions were collected and dialyzed against 10mM Tris containing 0.1% (v/v) Triton X-100, pH 8.0. SDS-PAGE (under reducing conditions [sodium dodecyl polyacrylamide gel electrophoresis]) was run on each fraction to determine which fractions contained PAS IV. Fractions showing significant amounts of PAS IV were pooled together and concentrated using a Centricon 30 microconcentrator. A BCA protein assay was done to determine the protein content.

Proteins that did not bind to the ion-exchange column in the absence of NaCl were pooled together and dialyzed overnight against 10mM Tris containing 0.1% (v/v) Triton X-100, pH 7.0. The dialyzed fractions were loaded on the column as noted above after equilibrating the column to pH 7.0. The column was eluted using 25mM and 200mM NaCl in 10mM Tris buffer containing 0.1% (v/v) Triton X-100, pH 7.0. Fractions collected were treated as above.

Proteins that did not bind to the ion-exchange column in the absence of NaCl at pH 7.0 were pooled together and dialyzed overnight against 10mM Citrate buffer containing 0.1% (v/v) Triton X-100, pH 6. The ion-exchange column was equilibrated with 10mM Citrate buffer containing 0.1% (v/v) Triton X-100, pH 6.0. The column was eluted using 50mM, 100mM, 200mM, 500mM NaCl in 10mM Citrate buffer containing 0.1% (v/v) Triton X-100, pH 6. Fractions collected were treated as mentioned above.

Immunization of mice.

BALB/c mice were immunized by intraperitoneal injections of 60 ug of pure PAS IV emulsified in Freund's incompelete adjuvant. The mice were tagged as left, right and none. At 4 and 7 weeks after the initial immunization mice were boosted with 60 ug of PAS IV emulsified in Freund's incompelete adjuvant. The mice were bled from the tail before boosting, to determine the immune response of the mice to the antigen by an enzyme-linked solid-phase immunoassay. The mice were boosted the second time 3 days before hybridization.

Hybridization techniques and production of monoclonal antibodies.

Spleen cells ($1x10^{8}$) from two immunized mice were fused with SP2/0 myeloma cells ($1x10^{7}$, {Shulman et al., 1978}), with 42% (w/v) poly(ethylene glycol) as fusing agent as described by Kaetzel et al. (1984). After fusion the cells were centrifuged and resuspended in hypoxanthine/aminopterine/thymidine (HAT) supplemented medium (Littlefield, 1964). The cell suspension was distributed in 0.1 mL portions into 96-well tissue culture plates. Supernatants from positive clones were tested by enzyme linked immunoassay. Selected hybrid clones were sub-cloned two times by limiting dilution.

Enzyme linked immunosorbent assay (ELISA).

The ELISA was done according to procedures described by Mather et al.

(1982), with slight modifications. A 96-well plastic plate (Corning glass works,Corning, NY) was coated overnight at 4°C with PAS IV (0.25 ug/50 uL in 0.1M sodium bicarbonate buffer, pH 9.0). The plates were washed three times with ELISA wash (0.15M NaCl containing 0.05% (v/v) Triton X-100). Pre- and post-immune sera obtained from immunized mice were used as negative and positive control, when testing the clones. Supernatants from hybridomas (50 uL) to be screened were added to the wells. The plate was incubated at room temperature for one hour in a humidity chamber after adding the supernatant. The plate was then washed three times with ELISA wash. After washing, 200 uL of horseradish peroxidase- conjugated goat anti-mouse antibodies was added (1:1000 dilution in ELISA diluent {50mM Tris buffer containing 0.15M NaCl, 0.1% BSA [w/v] 0.05% [v/v] Triton X-100, pH 7.4}). The plate was incubated for 30 minutes at room temperature and washed as before and 100 uL of substrate (0.05M Citrate, pH 4.0 containing 0.4mM 2,2'-azino-di{3-ethylbenthiazoline sulfonic acid} diammonium salt , and 1.5mM hydrogen peroxide) was added. After color had developed at room temperature for one hour, the absorbance was measured with a Bio-Tek EL-310 plate reader (Bio-Tek Inc. , Burligton, VT) at dual wavelengths (A405, A450).

In order to determine the specificity of the antibody secreted , an ELISA was performed. A 96-well plastic plate was divided into four sections and coated with different antigens: 1) 10mM Tris buffered saline, pH 7.4 (50 uL); 2) Bovine serum

albumin (0.25 ug/50 ul); 3) Ovalbumin (0.25 ug/50 uL); 4) PAS IV (0.25 ug/50 uL). The procedure for the ELISA was same as described above.

Cell-line E-1, which secretes antibody to PAS IV was obtained from Dr. I. H. Mather, (University of Maryland, College Park, MD, U.S.A.). Antibody-secreting tumors were produced by subcutaneous inoculation of BALB/c mice with hybridoma cells. In order to obtain large amounts of monoclonal antibody, mice were primed with pristane (2,6,10,14, tetramethylpentadecane) and innoculated with tumor cells (2.5×10^6) into the peritoneal cavities. Ascities fluid was harvested after a period of 10 days from pristane primed mice.

Purification of monoclonal antibodies from ascites fluid.

Monoclonal antibody E-1 was partially purified from ascites fluid by precipitation with saturated $(NH_4)_2SO_4$ at $4^{\circ}C$. The ascites fluid was diluted 1:1 with 20mM Tris containing 0.15M NaCl pH 7.4. Saturated $(NH_4)_2SO_4$ was added to the diluted ascites fluid, dropwise with constant stirring until a final concentration of 40% (v/v) was reached. The suspension was stirred for 45 minutes and then centrifuged at 10,000 x g for 30 minutes. The pellet was resuspended in 20mM Tris containing 0.15M NaCl, pH 7.4, and dialyzed overnight against 20mM Phosphate buffer containing 0.15M NaCl (PBS), pH 7.0, at $4^{\circ}C$.

The monoclonal antibody was further purified by fractionation on a Recombinant Protein G Sepharose-4B conjugate column that had been previously equilibrated with 20mM PBS, pH 7.0. After washing the column (7 x 1 cm) with several volumns of equilibration buffer until the absorbance of the fractions collected measured almost zero at 280 nm, E-1 was eluted from the column using 100mM Glycine buffer, pH 2.7. Four mL fractions were collected and immediately neutralized using 1.0M Tris buffer containing 0.15M NaCl, pH 8.9. Fractions showing high absorbance at 280nm were pooled together and dialyzed overnight against 20mM Tris buffer containing 0.15M NaCl, pH 7.4. A BCA protein assay was done to determine the concentration of the purified immunoglobulin using r globulin as a reference standard. SDS-PAGE (under reducing conditions) was also run on the purified immunoglobulin to determine its purity.

Immunoaffinity chromatography.

Monoclonal antibody E-1 purified from ascites fluid was coupled to CNBr-activated Sepharose-4B (5.0 mg of purified immunoglobulin/gm of gel) in the presence of 100mM Sodium bicarbonate buffer (coupling buffer {5.0 mL/gm (dry weight) of the gel}) containing 0.5M NaCl, pH 8.0, by gentle stirring for one hour at room temperature. The unbound antibody was removed by washing with coupling buffer and the remaining active groups were blocked with 1.0M Ethanolamine pH 9.0. The coupled gel was washed with three alternate cycles of

100mM Acetate buffer containing 0.5M NaCl, pH 4.0 and 100mM Tris buffer containing 0.5M NaCl, pH 8.0. The immunoadsorbents were equilibrated and stored at 4°C in 10mM Tris buffer containing 0.5M NaCl, pH 7.4.

To test the specificity of the immobilized antibody, the immunoadsorbent was packed into a small chromatography column (7 x 1.5 cm) and equilibrated with 10mM Tris buffer containing 0.15M NaCl, 0.1% (v/v) Triton X-100 , pH 7.4. Bovine fat globule membrane proteins from bovine milk were solubilized in Triton X-114 (Bordier, 1981), and then fractionated on the column. Specifically bound material was eluted with 100mM Glycine buffer containing 0.1% (v/v) Triton X-100, pH 2.7. Four mL fractions were collected and were immediately neutralized with 1.0M Tris buffer containing 0.15M NaCl, 0.1% (v/v) Triton X-100), pH 8.9. Fractions collected were dialyzed overnight against 10mM Tris buffer containing 0.5M NaCl, 0.1% Triton X-100 (v/v), pH 7.4. Fractions were analyzed by SDS-PAGE (under reducing conditions). A BCA protein assay was done on specific dialyzed fractions showing significant amounts of PAS IV to determine the protein concentration.

Purification of CD36 from bovine heart tissue using the immunoaffinity column.

Bovine heart tissue was obtained from the Ferrara Meat Company (San Jose, CA). Microsomal membrane fractions were prepared by differential centrifugation. The bovine heart tissue was homogenized (10 gm/20 mL buffer) with 10mM Tris

buffer containing 0.15M NaCl, 0.5mM PMSF, 1% (v/v) aprotinin, 1mM aminocaproic acid and 1mM EGTA, pH 7.4, in a Waring blender connected to a speed controller "Powerstat" made by the Superior Electric company (Bristol, Conn.) with a setting of maximum for 10 seconds twice at ten second interval. The homogenized tissue was filtered through two layers of cheese cloth and the filtrate was centrifuged at 1000 x g for 10 minutes at 4°C. The resulting supernatant was centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant obtained was further centrifuged at 98,000 x g at 4°C for one hour and the pellet obtained at this stage was called the "microsomal membrane fraction." CD36 was purified from the microsomal membrane fraction by solubilization with Triton X-114 and chromatography on the immunoaffinity column as described before (under preparation of immunoaffinity column). After phase partitioning, 3 phases were obtained: aqueous, detergent, and an insoluble pellet. In order to determine the effectiveness of phase-partitioning, a Western blot was done before immunoaffinity chromatography. After chromatography of the detergent phase on the immunoaffinity column, fractions showing significant amount of CD36 were pooled together after dialysis against 10mM Tris buffer containing 0.15M NaCl, 0.1% (v/v) Triton X-100, pH 7.4, and were concentrated in a Centricon 30 microconcentrator. A BCA protein assay was done to determine the protein content.

Western blotting.

The presence of CD36 in the detergent phase and the pellet was determined by Western blotting. Protein samples were separated by SDS- PAGE as described by Laemmli (1965) and electrophoretically transferred to nitrocellulose paper, essentially as descibed by Towbin et al. (1979). The nitrocellulose paper after transfer was treated essentially as described by Greenwalt et al. (1985), with some modifications. The primary antibody affinity purified rabbit anti-PAS IV was prepared in our laboratory and was used at a dilution of 1:200. Specificaly bound primary antibodies were detected by using goat anti-rabbit IgG alkaline phosphatase conjugate (1:1000 dilution). The substrate used contained 0.1 mL 5-bromo-4-chloro-3-indolyl phosphate at a concentration of 5.0 mg/mL in dimethyl formamide, 1.0 mL nitro-blue tetrazolium at a concentration of 1.0 mg/mL in barbital buffer, 20 uL of 2.0M magnesium chloride, and 9.0 mL of barbital buffer at pH 7.0.

Densitometry.

In order to determine the purity of PAS IV eluted from CM-cellulose column SDS-PAGE (under reducing conditions) showing the fractions eluted from the column at pH 8.0, pH 7.0, and pH 6.0 were dried on a peice of gel supporting film (Gel bond), after staining with coomassie blue and densitometric studies were done. The recovery of PAS IV using the immunoaffinity column was also determined by

densitometric studies. SDS-PAGE (under reducing conditions) of whole membrane homogenates and fractions obtained at various steps during purification were treated as mentioned above. The bands on each lane of the dried gel were scanned individually with a Beckman "Appraise" densitometer at 600nm.

Amino Acid Sequence of Bovine CD36.

Purified bovine CD36 (170 ug) was sent to Dr. Ken Watt of Cetus Corporation (Emeryville, CA) for amino acid sequencing. The amino acid sequence was obtained through Edman degradation (Edman & Begg, 1967; Naill, 1973) using the Applied Systems gas phase sequenator.

III. RESULTS

A. Purification of PAS IV

1) Membrane preparation

In order to obtain milk-fat-globule membrane, one quart of fresh bovine cream was used for membrane preparation. The butter-milk fraction obtained (procedure described under materials and methods) was centrifuged and a membrane pellet was obtained. After homogenizing the pellet in 10mM Tris buffer containing 0.15M NaCl (TBS), pH 7.4, a BCA protein assay was performed. The membrane contained 1.22 gm of total MFGM protein.

2) Extraction with Triton X-114.

The homogenized pellet was resuspended in TBS to obtain a final concentration of 5 mg/mL total protein. The hydrophobic proteins were extracted with a detergent, Triton X-114. A BCA protein assay was done on the extract to determine the total proteins solubilized. Total membrane protein present in the extract were 345.13 mg.

3) Phase partitioning

The supernatant obtained from the membrane Triton X-114 extract was subjected to phase partitioning in a 37^{o}C water bath (procedure described under materials and methods). When the protein solution turned turbid due the formation

of protein-detergent micelles, it was maintained at that temperature for 10 minutes. After centrifugation at room temperature two phases were obtained: an upper aqueous phase and a lower detergent phase. There were 97.88 mg of protein in the detergent phase and 167.36 mg of protein present in the aqueous phase. Figure 1 shows a SDS-PAGE (under reducing conditions) of various stages of the solubilization. Lane 2 contains a sample of the whole membrane homogenate. The hydrohobic proteins including PAS IV partitioned in the detergent phase as shown in lane 3. Hydrophilic proteins including significant amounts of xanthine oxidase and butyrophilin were present in the aqueous phase as shown in lane 4.

4) Ion exchange chromatography

The detergent phase was diluted five-fold with 10mM Tris buffer, pH 8.0, and dialyzed overnight against 10mM Tris buffer containing 0.1% (v/v) Triton X-100, pH8.0. The diluted detergent phase was chromatographed on an ion-exchange column equilibrated to pH 8.0 with 10mM Tris buffer containing 0.1% (v/v) Triton X-100. The bound proteins were eluted with 75mM NaCl in 10mM Tris buffer containing 0.1% (v/v) Triton X-100, pH 8.0. Three fractions (5 mL each) showing significant amounts of PAS IV were concentrated to 2.10 mL using a Centricon 30 microconcentrator. The fractions containing PAS IV were determined by SDS-PAGE (under reducing conditions). The total proteins eluted from the ion-exchange column were 280 ug as determined by a BCA protein assay.

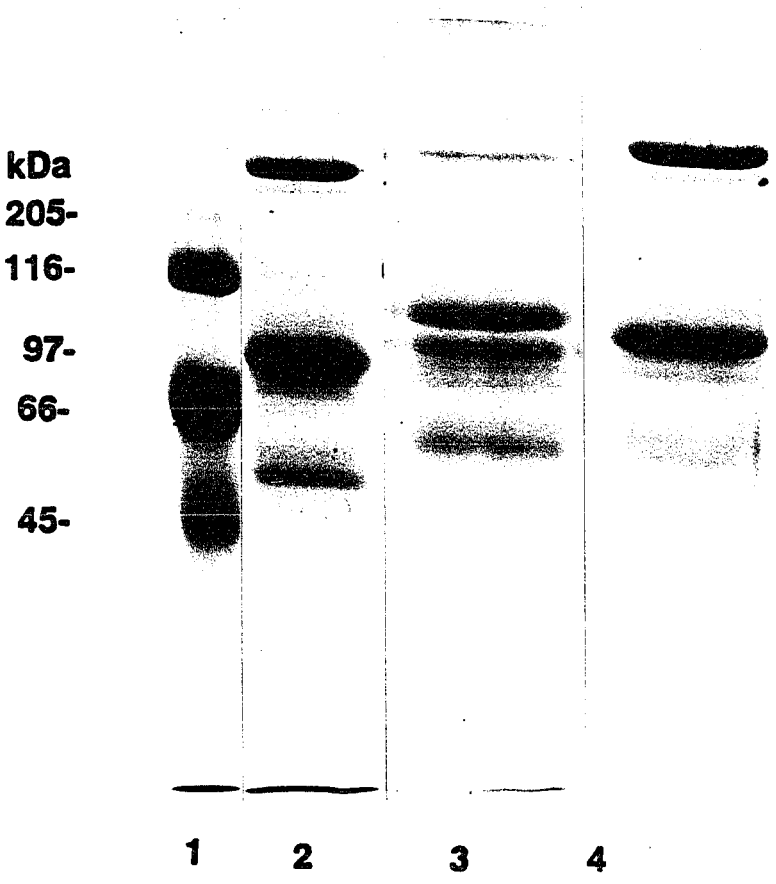


Figure 1. Distribution of proteins present in the milk fat globule membrane after phase partitioning in Triton X-114.

lane 1, molecular weight markers;

lane 2, whole membrane homogenate;

lane 3, detergent phase of Triton X-114 extract;

lane 4, aqueous phase of Triton X-114 extract.

At pH 7.0, 25mM and 200mM NaCl in Tris buffer containing 0.1% (v/v) Triton X-100, pH 7.0, was used for eluting the bound proteins. All the bound proteins were present in the two fractions (5 mL each) eluted by 200mM NaCl, as determined by SDS-PAGE (under reducing conditions). These two fractions were concentrated to 2.0 mL before doing a BCA protein assay. A total of 1.90 mg of proteins were eluted from the column.

At pH 6.0 the bound proteins were eluted with increasing salt concentrations (50mM , 100mM, 200mM, 500mM) in 10mM Citrate buffer containing 0.1% (v/v) Triton X-100. All the bound proteins were eluted with 50mM and 100mM NaCl, as determined by SDS-PAGE (under reducing conditions). A total of 17.50 mg of proteins were eluted from the column.

Densitometry of the dried gel showing the eluted proteins from the CM-cellulose column at pH 8.0, pH 7.0, and pH 6.0, indicate that the percentage purity of PAS IV recovered was 62.3%, 95.9%, 100% respectively (Figure 2).

B. Monoclonal antibody production.

1) *Immunization of mice.*

Three BALB/c mice were each immunized with 60 ug of PAS IV emulsified in Freund's incomplete adjuvant. At 4 weeks after immunization, mice were bled from the tail and serum samples were collected, before boosting the mice with 60 ug

A

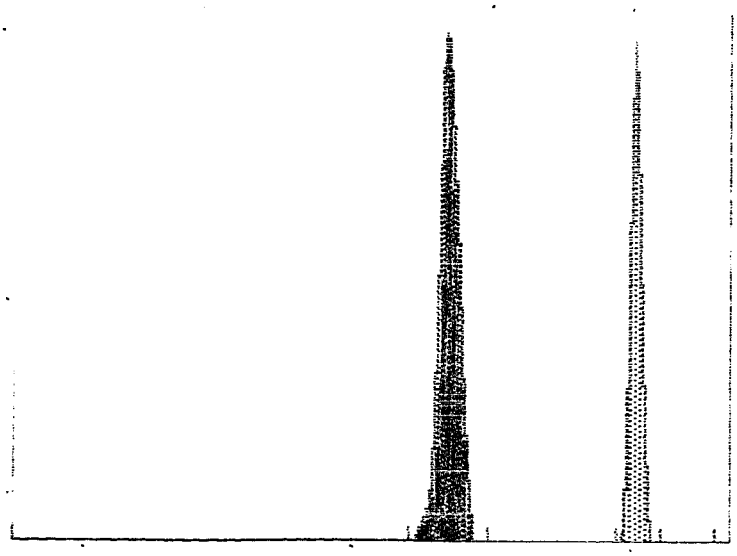


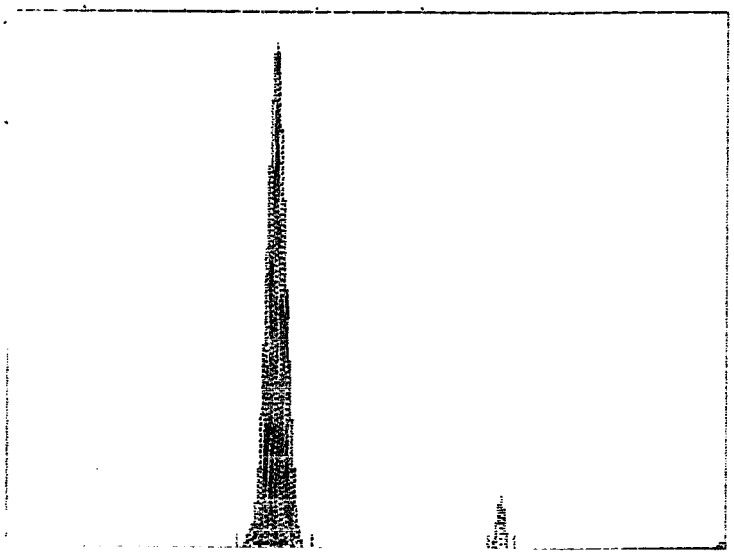
Figure 2. Densitometry results of the protein eluted from the CM-cellulose column. The darkened area represents the percentage of PAS IV present in the protein eluted from the column.

A, PAS IV eluted at pH 8.0 was 62.3% pure;

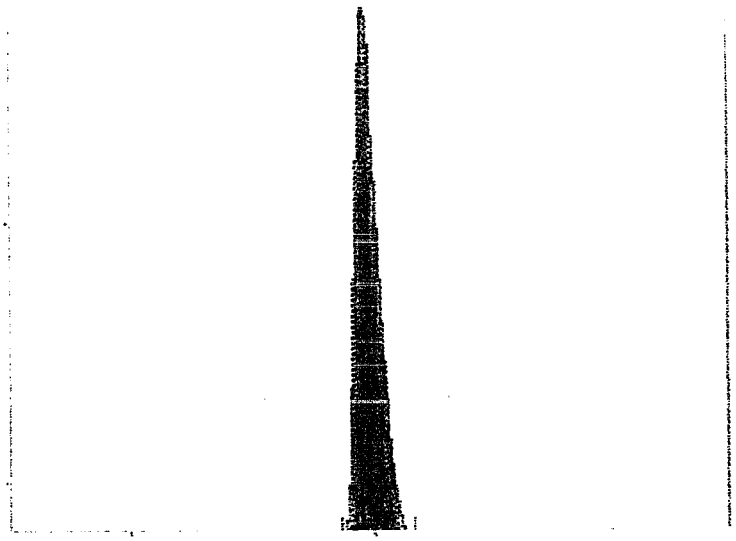
B, PAS IV eluted at pH 7.0 was 95.9% pure;

C, PAS IV eluted at pH 6.0 was 100% pure.

B



C



of PAS IV emulsion. An ELISA was done on the serum obtained to determine the immune response of mice to the antigen. Significant titres of 1:128000, 1:32000 and 1:8000 were obtained upon dilution of the serum samples from the mice tagged left, right and none respectively with respect to the pre-immune serum sample.

2) Fusion

Spleen cells from mice tagged left and right were used for fusion procedures. About 12-15 days after fusion colonies became visible in the culture plates. An ELISA was performed on the supernatants to identify the clones secreting the antibody. Of the 238 clones obtained, 44 clones tested positive. An ELISA was done to determine the specificity of the 44 clones. One cell line, 6-9H, was found specific for PAS IV (Figure 3). This hybridoma was subcloned twice by limiting dilution. However, after the second subcloning the hybridoma was lost due to fungal contamination.

3) *Propogation and purification of E-1*

Cell-line E-1 secreting antibody to PAS IV was propogated as tumors in BALB/c mice. Tumor cells (2.5×10^6) were injected in mice 10 days after priming the mice with pristane. Eleven mL of ascites fluid was harvested the peritoneal cavity. Monoclonal antibody E-1 was purified from ascites first by precipitation using saturated ammonium sulphate.

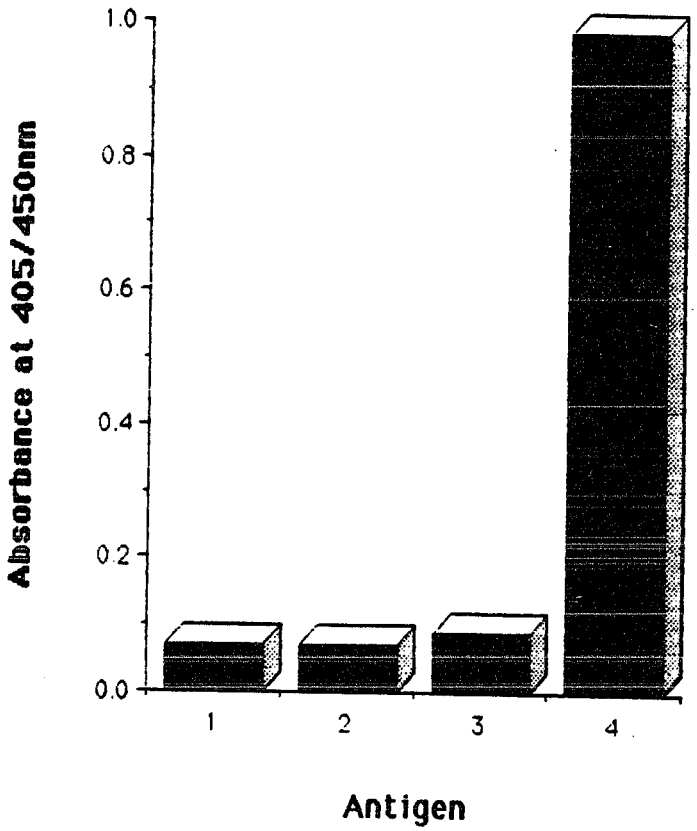


Figure 3. ELISA to determine the specificty of 6-9H.

The wells of an ELISA plate were coated with different antigens:

1: 10mM Tris buffered saline (50 uL/well);

2: Bovine Serum Albumin (0.25 ug/well);

3: Ovalbumin (0.25 ug/well);

4: Purified PAS-IV (0.25 ug/well).

The partially purified monoclonal antibody was then chromatographed on a Recombinant Protein G Sepharose-4B column. After loading the sample, the column was washed with several volumns of 0.1M Phosphate buffer containing 0.15M NaCl, pH 7.0, until the absorbance of the fractions collected measured almost zero at 280nm. The specifically bound monoclonal antidody was eluted by using 100mM Glycine buffer, pH 2.7 and 4 mL fractions collected were immediately neutralized with 1.0M Tris buffer containing 0.15M NaCl, pH 8.9. The absorbance of the eluted fractions was measured at 280nm to determine fractions containing the monoclonal antibody (Figure 4). SDS-PAGE (under reducing conditions) of the purified monoclonals showed two distinct bands (heavy chain and light chain) (Figure 5). A BCA protein assay was done to determine the protein content of the purified monoclonal antibody, using r globulins (1 mg/mL) as the reference standard. A total 74.0 mg of monoclonal antibody was purified.

4) Preparation of an immunoaffinity column

Purified monoclonal antibody was coupled to CNBr activated Sepharose-4B. Ten gm of purified monoclonal antibody were coupled to 2 gm of gel (dry weight) in the presence of Bicarbonate buffer 0.1M, pH 9.0, and the immunoabsorbent was packed into a small chromatography column. Percentage coupling of the purified monoclonal antibody to the the gel was determined to be 99.4%.

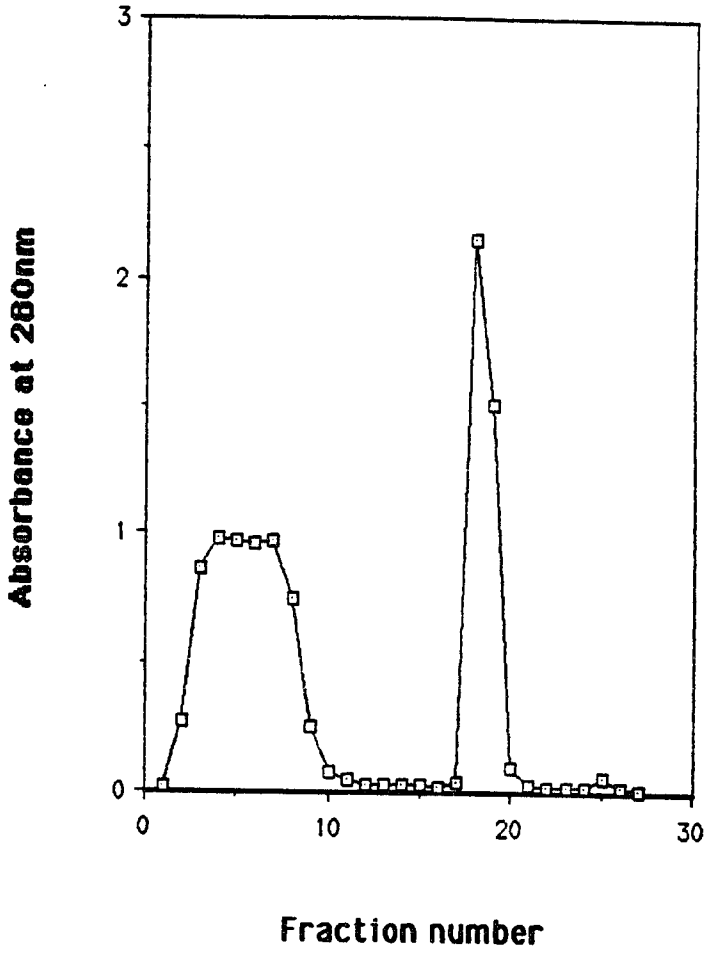


Figure 4. Elution profile of E-1 from Recombinant Protein G Sepharose-4B column. The column was washed with 0.1M Phosphate buffer containing 0.15M NaCl until the absorbance at 280nm reached baseline. The immunoglobulin was eluted from the column with 100mM Glycine buffer, pH 2.7. The absorbance of the fractions collected was monitored at 280nm.

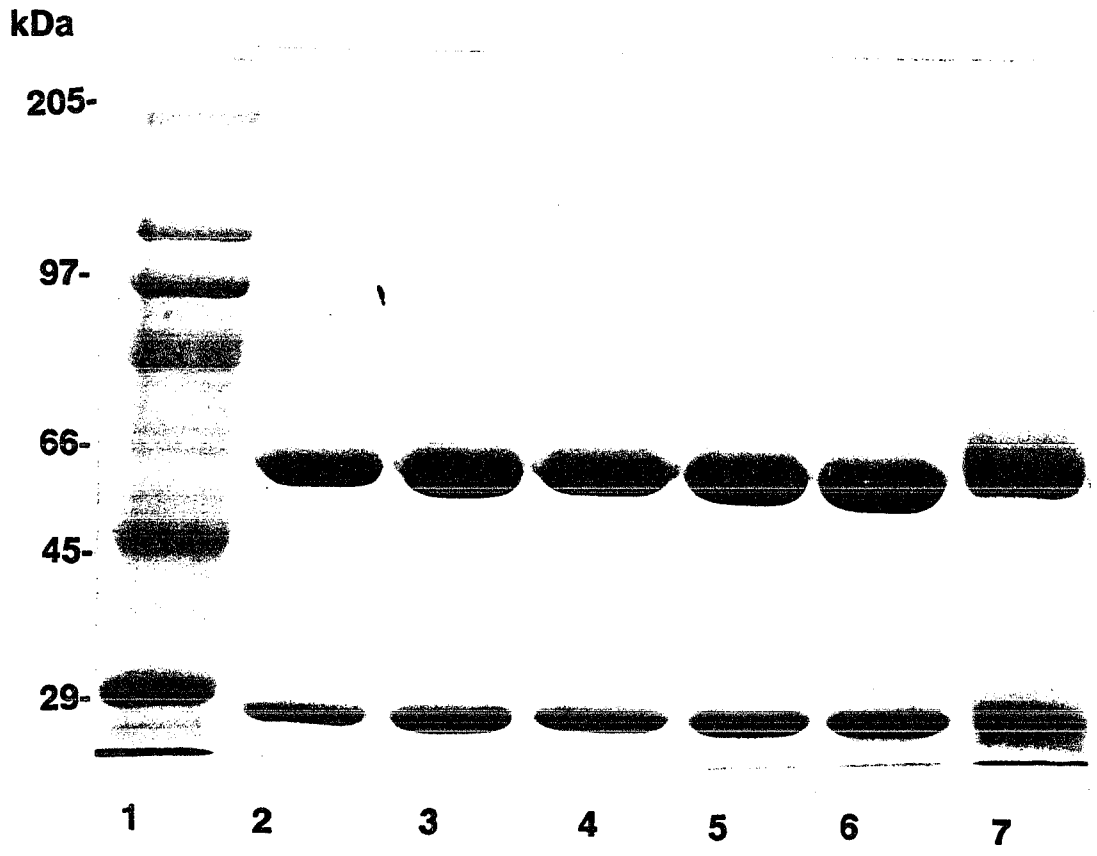


Figure 5. Analysis by SDS-PAGE to determine the purity of E-1.

lane 1, molecular weight markers;

lane 2 - lane 6, different batches of purified E-1;

lane 7, r globulin standard.

In order to determine the specificity of the immobilized antibody, 30 mg of the MFGM protein were extracted with Triton X-114. Twenty-one mg of protein were solubilized (Table 1). The detergent phase containing 10.80 mg of proteins was diluted five-fold with 10mM Tris buffer containing 0.15M NaCl (TBS), pH 7.4 and dialyzed overnight against TBS containing 0.1% (v/v) Triton X-100, pH 7.4. The dialyzed detergent phase was then chromatographed on an immunoaffinity column. Specifically bound material was eluted with 100mM Glycine buffer, pH 2.7. and the fractions were neutralized with 1.0M Tris buffer containing 0.15M NaCl, 0.1% (v/v) Triton X-100, pH 8.9. Three fractions showing significant amounts of PAS IV were identified by SDS-PAGE (under reducing conditions), and were pooled together (Figure 6). A BCA protein assay indicated 0.73 mg of protein present in the fractions eluted from the column. Densitometry of the dried gel demonstrated that PAS IV constituted 21.9 % of the proteins present in the detergent phase (Figure 7).

B. Purification of bovine CD36.

Inorder to purify CD36 from the bovine heart tissue, 333 gm of tissue was homogenized (described under materials and methods). The protein content of the microsomal membrane pellet was 1.10 gm, determined by BCA protein assay. The scheme followed for bovine CD36 purification is shown in Figure 8. The microsomal

Protein recovery	Total protein recovery (mg)	%total protein	PAS IV mg	PAS IV %	% PAS IV recovery
MFGM homogenate	30	100	--	--	--------
Triton X-114 extract	21.06	70.2	3.2*	15.4*	100
Detergent phase	10.8	36	2.37*	21.9*	74
Immunoaffinity chromatography	0.73	2.4	0.73	100*	31

Table 1. Proteins recovered during different stages of purification

* values may not represent true values.

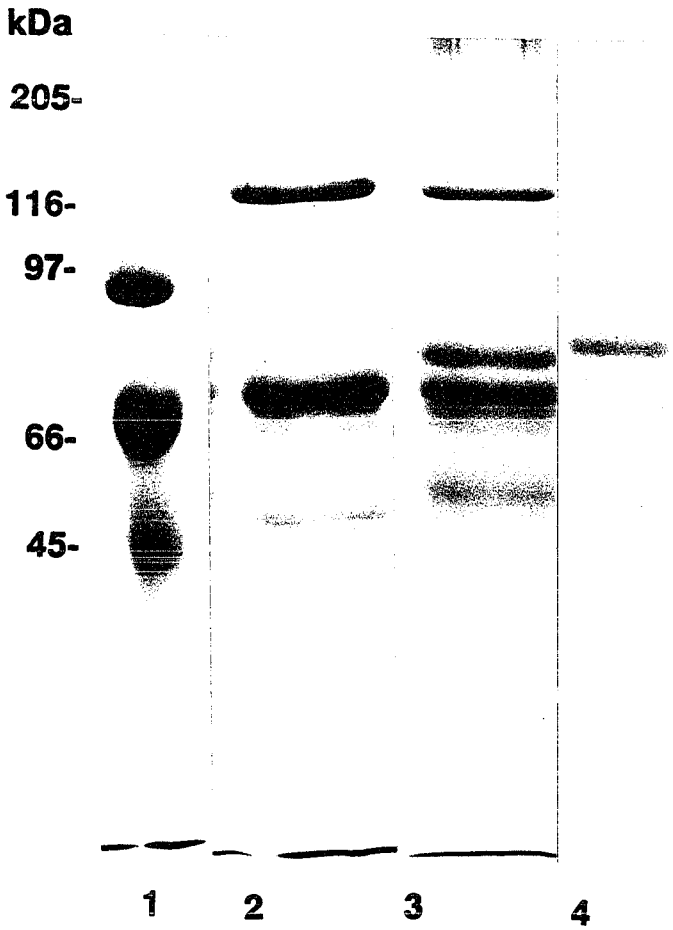


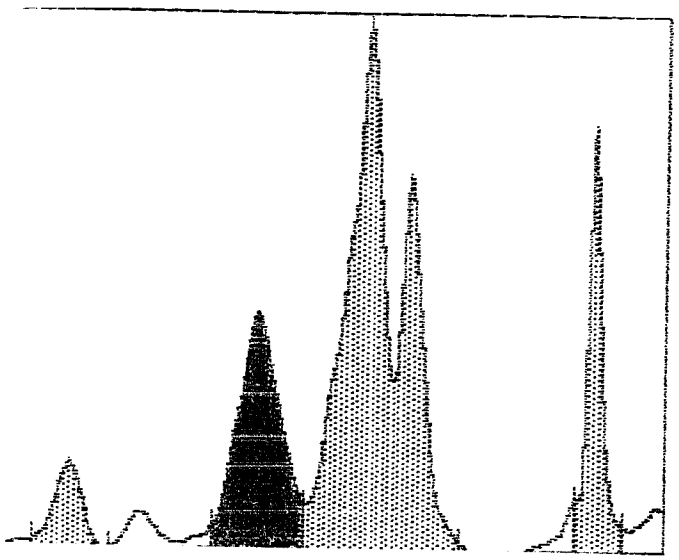
Figure 6. Immunoaffinity chromatography of detergent-solubilized milk-fat-globule membrane proteins on E-1 antibody covalently linked to CNBr activated Sepharose-4B.

lane 1, molecular weight markers;

lane 2, milk-fat-globule membrane homogenate;

lane 3, detergent phase of Triton X-114 extract;

lane 4, eluate from E-1-Sepharose 4B.



Fraction	Rel%
1	5.2
2	21.9
3	78.1

Figure 7. Distribution of PAS IV in the detergent phase.

PAS IV constitutes 21.9% of the total peak area. The darkened area represents PAS IV present in the detergent phase.

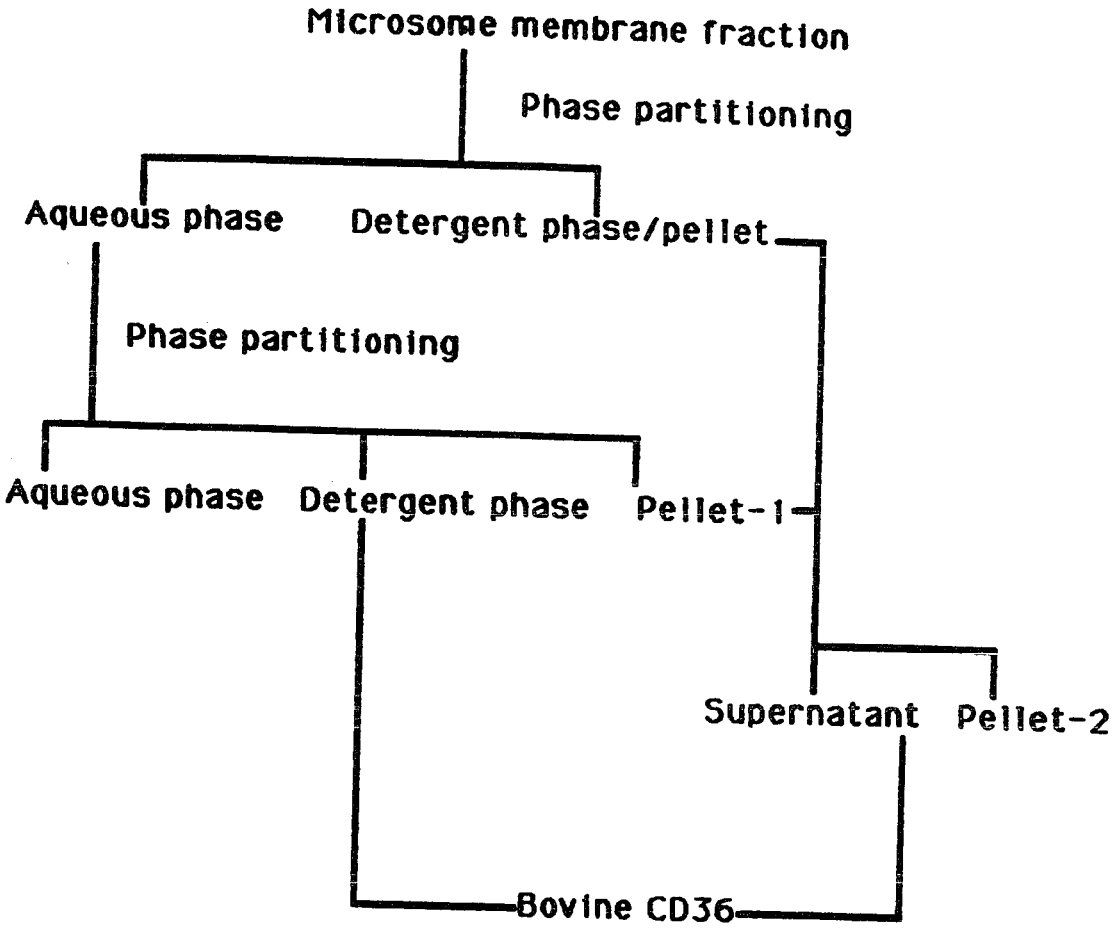


Figure 8. Schematic representation of the purification protocol of bovine CD36 from the microsomal membrane fraction obtained from the bovine heart tissue.

membrane pellet was resuspended in 10mM Tris buffer containing 0.15M NaCl, 0.5mM PMSF, 1% aprotinin, 1mM aminocaproic acid and 1mM EGTA, 1% (v/v) Triton X-114, pH 7.4, in order to obtain a protein concentration of 6 mg/mL. The supernatant obtained after centrifugation was subjected to phase partitioning in a 37°C water bath. Two phases were obtained after centrifugation at 1,500 rpm for 10 minutes at room temperature, an upper phase called the aqueous phase and a lower phase called the detergent phase/ pellet. No distinct detergent phase was obtained at this stage. The aqueous phase was again made 0.5% with respect to the detergent Triton X-114 (v/v) and subjected to phase partitioning as described before. Three phases were obtained: an aqueous phase, detergent phase and a pellet-1 (Figure 8).The protein content of the aqueous phase was 165 mg and the detergent phase was 56.9 mg. The pellet-1 obtained was combined with the original detergent phase/pellet. The protein content of the combined pellet-1 was 381 mg. Figure 9 shows the distribution of proteins present during various stages of purification of bovine CD36. A Western blot (as described under materials and method) was done to determine which phase contained bovine CD36. Primary antibody used was rabbit anti-bovine PAS IV (Figure 10A). Lane 1 contains the detergent phase, lane 2 the aqueous phase and lane 3 the combined pellet. A dark band for CD36 is seen in lane 1 and 2. Lane 4 contains pure PAS IV, the band for PAS IV is lower than the band seen for CD36, indicating the lower molecular weight of PAS IV. A negative

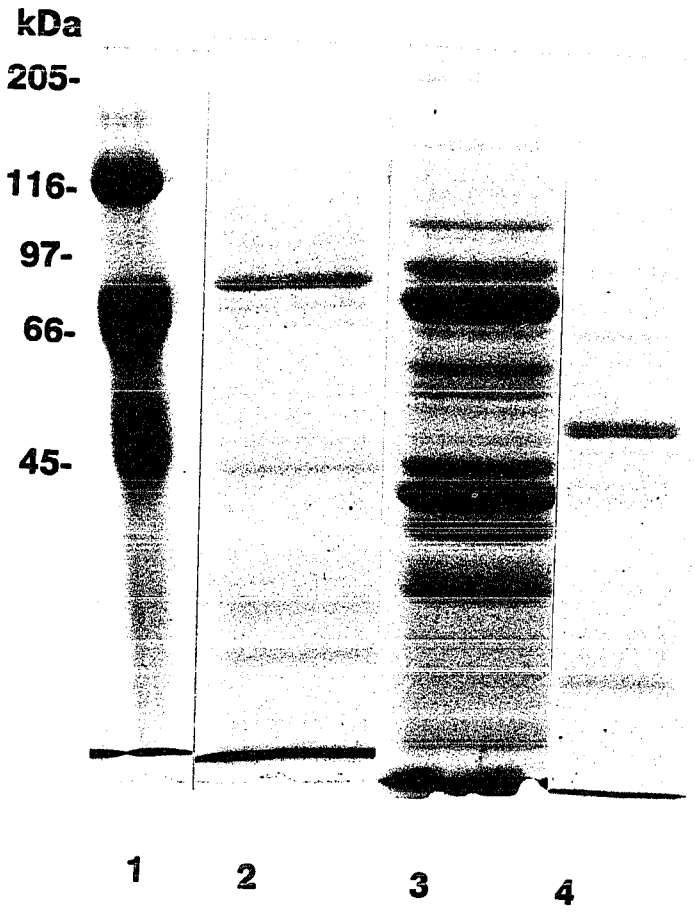


Figure 9. Analysis by SDS-PAGE of the proteins present in the three phases obtained after Triton X-114 phase partitioning of the heart microsome extract.

Lane 1, molecular weight markers;

lane 2, detergent phase of Triton X-114 extract;

lane 3, aqueous phase of Triton X-114 extract;

lane 4, pellet of Triton X-114 extract.

control showed no bands under similar conditions (data not shown). Pre-immune serum sample was used as the primary antibody for the negative control. The combined pellet-1 was resuspended in TBS and centrifuged at 26,500 RPM at 4 ^{o}C for one hour with a SW 27 swinging bucket rotor. The pellet-2 contained 353 mg of proteins and the supernatant contained 78.10 mg. In order to determine the presence of bovine CD36 a Western blot was done on the supernatant and the pellet obtained ({ Figure 10B} [procedure described under materials and methods]). Lane 1 contains the detergent phase and lane 2 contains the supernatant. Both the lanes show a bands corresponding to bovine CD36. Detergent phase was run as reference standard. Lane 3 contains pellet-2. No bovine CD36 was present in the pellet-2 and was discarded. Negative controls run under similar conditions did not show any band. The detergent phase after dialysis was chromatographed on the E-1 immunoaffinity column. The proteins bound to the column were eluted with 100mM Glycine buffer containing 0.1% (v/v) Triton X-100 and were immediately neutralized. Three fractions containing bovine CD36 were identified by SDS-PAGE (under reducing conditions) and were combined.

The supernatant after dialysis was chromatographed on the same immunoaffinity column. The bound proteins were eluted as described above and the fractions containing bovine CD36 were determined by SDS-PAGE (under reducing conditions). Fractions containing bovine CD36 were combined with the

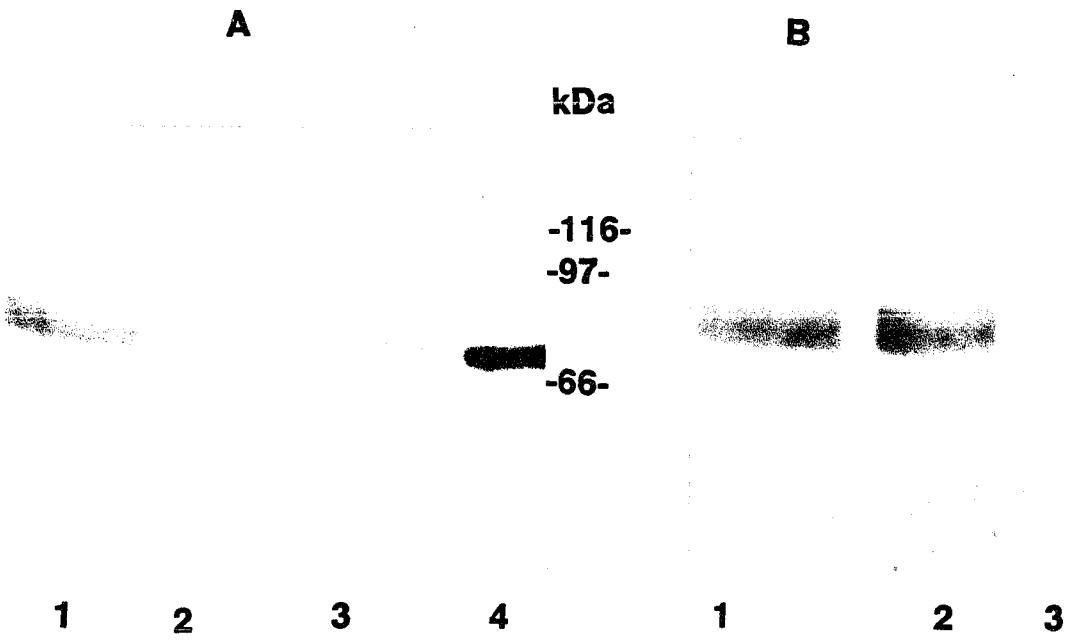


Figure 10A & 10B. Immunoblot analysis with anti-PAS IV to determine the presence of bovine CD36.

10A. lane 1, detergent phase of Triton X-114 extract;

lane 2, aqueous phase of Triton X-114 extract;

lane 3, pellet-1;

lane 4, bovine PAS IV.

10B. lane 1, detergent phase of Triton X-114 extract;

lane 2, supernatant;

lane 3, pellet-2.

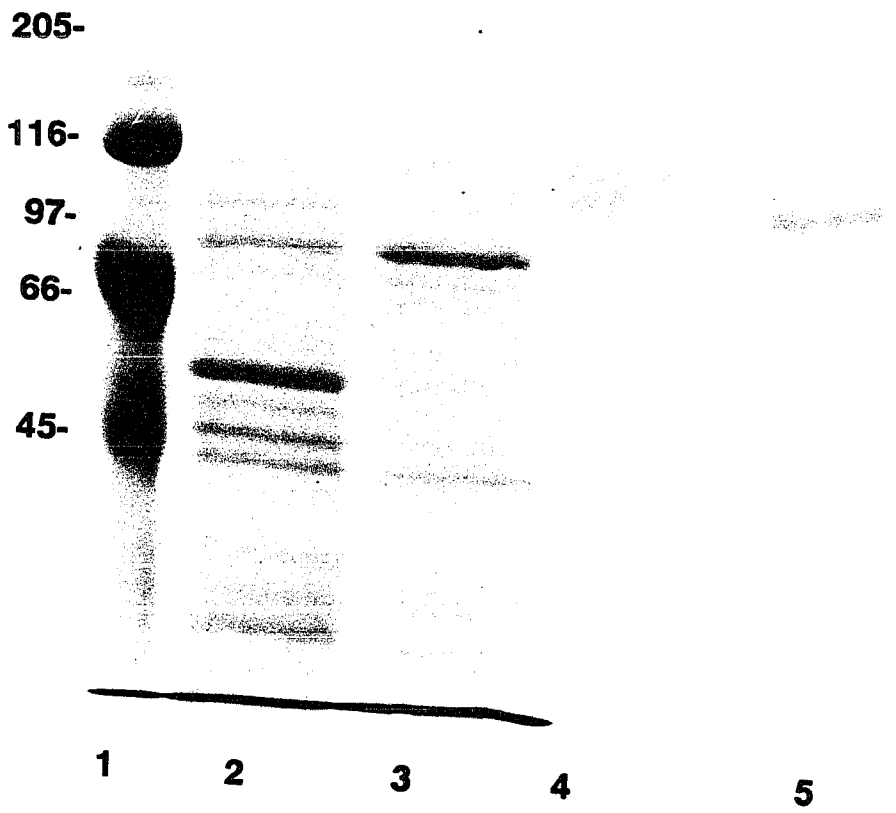


Figure 11. Analysis by SDS PAGE of the fractions obtained during the various stages of purification of bovine CD36.

lane 1, molecular weight markers;

lane 2, microsomal membrane fraction;

lane 3, detergent phase of Triton X-114 extract;

lane 4, purified bovine CD36;

lane 5, purified bovine PAS IV.

fractions obtained above. A BCA protein assay was done on the combined fractions and 1.10 mg of purified CD36 was obtained. SDS-PAGE (under reducing conditions) showed the presence of a single band for bovine CD36 (lane 4 , Figure 11).

C. Sequence Data

A sample containing 170 ug of pure bovine CD36 was sent to Cetus Corporation (Emeryville, CA) for amino acid sequencing. Sequences of four fragments numdered: #35, #36, #67A, #67B were obtained. The fragments were obtained by an endopeptidase: Lysylendopeptidase C which cleaves at the carboxy terminal of lysine residues (Figure 12). The amino acid sequence of the fragments was obtained by Edman degradation.

#35 K-S-S-M-F-Q-V-N-R-T-L-K

#36 K-V-A-I-I-D-T-Y-R

#67A I-I-S-K-N-C-T-L-Y-G-V-L-D-I-G-K-C-K-E-G-K-P-V-Y-I-S-L-P-H-F-L-H

#67B K-G-I-P-V-Y-R-F-I-L-P-S-F-A-F-A-S-P-F-Q-N-P-D-N-H-C-F-C-T-E

Figure 12. Sequence data of bovine CD36.

#35, #36, #67A, #67B are the four internal sequences. The underlined sequence is the N-linked glycosylation site

IV DISCUSSION

A membrane glycoprotein PAS IV has been previously purified from the MFGM and partially characterized. It has an apparent molecular weight of 78 kD and has been proved to be an hydrophobic integral membrane glycoprotein by its amino acid analysis, resistance to proteolysis when embedded in the membrane and its extraction by a detergent (Greenwalt & Mather, 1985). PAS IV constitutes 5% of the total coomassie blue stained proteins present in the MFGM (Mather et al. , 1980).

Immunoflorescence studies suggested the presence of PAS IV in the capillary endothelium of heart and mammary epithelial cells. However, the endothelial form of the protein was estimated to be several thousand daltons larger than the epithelial form. Peptide mapping studies with three different proteinases showed that the capillary endothelial form of PAS IV is similar to the epithelial form (Greenwalt et al. , 1985). The capillary endothelial form of PAS IV purified from the bovine heart tissue is known as bovine CD36.

The purification protocol for bovine CD36 was similar to PAS IV. However, since it was assumed that bovine CD36 constitutes only a small percent of the total proteins present in the heart microsome fraction, it was necessary to modify the purification protocol to increase the percentage recovery of the proteins during various stages of purifiation. CD36 was purified from the heart tissue by

solubilization of the microsome proteins in Triton X-114 and immunoaffinity chromatography of the detergent solubilized proteins.

Solubilization of membrane proteins with a nonionic detergent is an effective way to separate hydrophobic proteins from hydrophilic proteins. Integral membrane proteins generally form mixed micelles with nonionic detergents, in which the lipid molecules are replaced by the detergent and the hydrophobic domains of the proteins are embedded within the hydrophobic core of the micelle (Helenius & Simons, 1975). The Triton X series is a group of nonionic detergents each containing hydrophobic octylphenyl units and hydrophilic octylethylene units. The cloud point varies with the ethoxy groups present in the detergent. For eg. Triton X- 100 has a cloud point of 64 ^{o}C, whereas Triton X-114 has a cloud point of 20 ^{o}C. The decrease in ethoxy groups is sufficient to lower the cloud point of Triton X-114. The cloud point of Triton X-114 is more suitable for reversible condensation and to maintain the native form of the protein. Thus it is necessary to choose the right detergent for effective extraction and to minimize the degradation of the protein (Bordier, 1981; Goldfarb & Sepulveda, 1969).

Bovine CD36 being a hydrophobic membrane protein can be extracted with a non-ionic detergent, Triton X-114. Triton X-114 is a homogenous solution at 0 ^{o}C, as the temperature increases the protein-detergent micelles grow in size to form aggregates, and the solution becomes cloudy (Bordier, 1981). CD36 being

hydrophobic in nature partitions in the detergent phase depending on its hydrophobicity index. During the purification protocol for bovine CD36, all the CD36 was recovered in the detergent phase and the pellet as shown in Figure 10 (A & B). No CD36 was recovered in the aqueous phase.

In order to prepare an immunoaffinity column for further purification of bovine CD36, cell-line E-1 which secretes antibody to PAS IV was obtained from Dr. I. H. Mather (University of Maryland, College Park, MD). This cell-line was propagated as tumors in BALB/c mice and the monoclonal antibody E-1 was purified from the ascites fluid. E-1 was purified by ammonium sulphate precipitation and affinity chromatography on a Recombinant Protein G Sepharose-4B column.

The specificity of the purified monoclonal antibody E-1 was determined by immunoaffinity chromatography. PAS IV enriched detergent phase of Triton X-114 extract was chromatographed on the column. PAS IV constituted 21.9% of the proteins present in the detergent phase (Figure 7). The values obtained after densitometry do not necessarily reflect the true values, as they are determined on the basis of coomassie blue staining of the gels. As the concentration increases, the intensity of the blue color reflects the concentration of the protein, at higher concentration this relationship is not valid. Hence the percentage of proteins present at a lower concentration appear particulary high. The percentage recovery

of PAS IV from the detergent phase was almost 50% based on densitometry studies (Table1). However, the fractions that contained the proteins that did not bind to the immunoaffinity column before elution did not contain PAS IV. Hence all the PAS IV was recovered. Using the immunoaffinity column 1.10 mg of purified bovine CD36 was obtained. This constitutes 0.1% of the total proteins present in the heart microsomes.

Peptide mapping procedures have proved the similarity between PAS IV and bovine CD36 (Greenwalt & Mather, 1985). Also the monoclonal antibody to PAS IV shows specific binding for bovine CD36 indicating similarity between the two proteins.

There is difference in the molecular weight of the two proteins. Bovine CD36 has molecular weight of 85 kD and PAS IV has a molecular weight of 78 kD (Greenwalt & Mather, 1985). The difference in molecular weight can also be observed the immunoblot analysis (Figure 10A) and and the SDS-PAGE (Figure 11). However, the band for bovine CD36 appears diffuse compared to the sharp band observed for PAS IV, this may be due to the difference in the glycosylation pattern of the two proteins (Furukawa et al. , 1989).

Based on the hypothesis that bovine CD36 is larger than PAS IV it is difficult to compare the two proteins as the N-terminal sequence of bovine CD36 is not

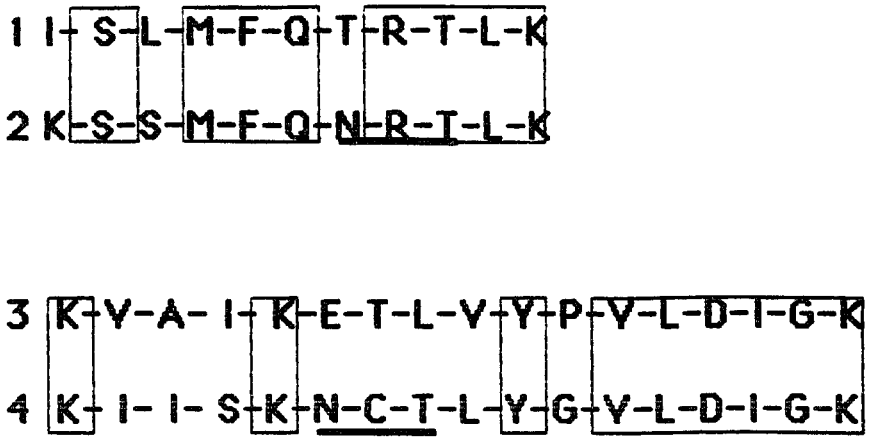


Figure 13. Sequence homology between bovine CD36 and bovine PAS IV.

The underlined sequence is the N-linked glycosylation site. The boxed sequence is similar. #1 and #3 are the bovine PAS IV internal sequences. #2 and #4 are bovine CD36 internal sequences numbered 35 and 67B.

known. The N-terminal sequence of PAS IV has been obtained by our laboratory. Hence it is difficult to determine whether the N-terminal of bovine CD-36 is extended by a few residues to account for the difference in molecular weight. Bovine CD36 internal sequence numbered 35 and 67B are homologous to PAS IV (Figure 13). An important observation made is that the bovine PAS IV sequence lacks the N-linked glycosylation site as seen in the bovine CD36 sequence and the substitution of valine present in PAS IV with leucine in the bovine CD36 sequence numbered 67B is conserved.

Glycosylation is a post-translational modification of protein synthesis, certain kinds of carbohydrate units are attached to the proteins at specific sites recognized by glycosylation enzymes (Marshall, 1974). The carbohydrate portion of the glycoprotein contain characteristic groups of monosaccharides which include neutral sugars (D-galactose, D-glucose and L-fucose), the amino sugars (D-glucosamine and D-galactosamine usually present in the N-acetyl form), and the amino sugar acids (the sialic acids). The carbohydrate content of glycoproteins may vary from 1 to more than 80% of the weight of the molecule (Spiro, 1966; Spiro, 1972).

Carbohydrate units are covalently linked to the proteins. This linkage may be N-glycosidic through the amide nitrogen of an asparagine residue or O-glycosidic through the hydroxyl group of serine and threonine. The minimum sequence

required for N-glycosylation is Asn-X-Thr/Ser where X can be any amino acid except proline (Beeley, 1974; Welpy et al. , 1983; Marshall, 1974).

The principal sugars associated with PAS IV include mannose, galactose, glucose, N-acetyl glucosamine and negatively charged sialic acid (Greenwalt & Mather, 1985). Nothing is known about the glycosylation pattern of bovine CD36. Based on SDS PAGE analysis (Figure 11) and sequence analysis (Figure 13) we can speculate that bovine CD36 is more glycosylated compared to bovine PAS IV.

Identical polypeptide chains may show different glycosylation patterns depending upon their tissue distribution. There is a difference in the sugar chains of r glutamyltranspeptidase purified from liver and kidney of mouse. Human collagen show differences in glycosylated hydroxy lysines in skin and bone. Also in the chicken, ovatransferrin and the serum protein transferrin differ only in the nature of their carbohydrate moieties (Marshall, 1972; Yamashita et al. , 1983; Pinnell et al. , 1971; Williams, 1968).

The type and the percentage of sugar moieties associated with proteins that influences the molecular weight of proteins and their rate of migration on SDS-PAGE (Leach et al. , 1980; Sergest et al. , 1971). In SDS-PAGE , SDS molecules bind to proteins, proportional to the surface area of the protein through hydrophobic interactions. The bound detergent molecules carrying negative charges

mask the native charge of the protein. The electrophoretic mobility of the SDS-protein complexes are influenced mainly by the molecular size.

In case of glycoproteins the sugar units and the negatively charged sialic acid groups attached to the proteins influence the binding of SDS onto the protein. The binding of SDS is no longer proportional to the polypeptide mass. As a result the migration profile of the SDS-protein molecules is determined by charge and size of the SDS-glycoprotein units. Thus a difference in glycosylation pattern may account for the difference in molecular weight of bovine CD36 and bovine PAS IV.

PAS IV and bovine CD36 are present in different tissues; hence proteins synthesized in different tissues may differ in the carbohydrate moieties. The degree of glycosylation depends on the availability of the lipid-linked oligosaccharide donor, the presence and the specificity of the appropriate glycosylation enzyme, and restrictions imposed due polypeptide folding. Also it has been noted that not all the Asn-X-Ser/Thr sequences are glycosylated (Marshall, 1972; Pless & Lennarg, 1977; Welpy et al. , 1983).

Recently human platelet GP IV (CD36) has been purified and partially characterized. Functional studies suggest that GP IV may play an important role as a cell adhesion molecule (Tandon et al. , 1989). Both bovine CD36 and human platelet GP IV are recovered in the detergent phase of Triton X-114 (McGregor et al. , 1989) indicating that both are hydrophobic integral membrane proteins.

The molecular weight of human GP IV is 88 kD, whereas the molecular weight of bovine CD36 is 85 kD (McGregor et al. , 1989; Greenwalt & Mather, 1985). The difference in molecular weight may be due to substitution of amino acid residues or difference in carbohydrate structure resulting from tissue specific biosynthetic pathways (Furukawa et al. , 1989). It has been reported that human prothrombin and bovine prothrombin differ only in their carbohydrate moieties (Mizuochi et al. , 1981; Mizuochi et al. , 1979).

The carbohydrate analysis of CD36 shows the presence of galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid (Tandon et al. , 1988). The carbohydrate composition of bovine CD36 is not known. Comparison of the human CD36 sequence with bovine CD36 is interesting (Figure 14). The bovine CD36 sequence shows the same glycosylation sequence as the cDNA derived sequence of human CD36. The cDNA derived sequence of human CD36 shows the presence of 10 N-linked glycosylation sites (Oquendo et al. , 1989). There is 88% similarity between the two sequences. In order to determine the homology between bovine PAS IV, bovine CD36, and human GP IV (CD36) it is necessary to obtain the cDNA sequence of the bovine proteins.

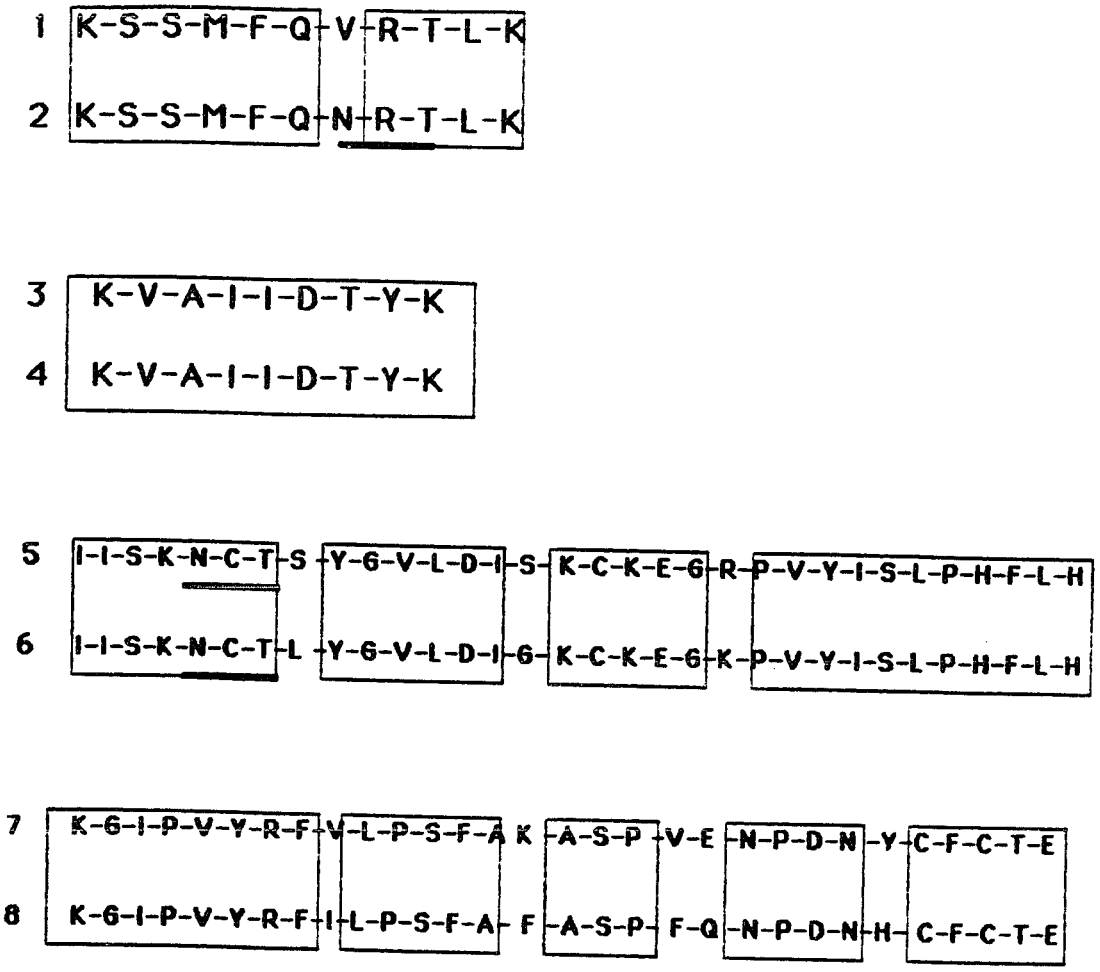


Figure 14. Comparison of the bovine CD36 sequence with cDNA derived sequence of human CD36. 1,3,5,7 represent the bovine CD36 internal sequence corresponding to fragments numbered 35, 36, 67A, 67B respectively. 2, 4, 6, 8 represent the cDNA derived sequence of human CD36. The underlined sequence is the N-linked glycosylation site. The boxed sequence is similar in both.

V. CONCLUSION

PAS IV is an integral membrane protein with an apparent molecular weight of 78 kD. It has been purified and partially characterized. PAS IV is present in the lactating mammary and bronchiolar epithelium and capillary endothelium of heart and lungs. The capillary endothelial from of PAS IV present in the heart tissue has a molecular weight of 85 kD (Greenwalt & Mather, 1985). I herein report the purification of the endothelial form of bovine PAS IV otherwise known as bovine CD36.

Bovine CD36 is a membrane glycoprotein, purified from the heart microsome fraction by solubilization with a detergent Triton X-114 followed by immunoaffinity chromatography. The amino acid sequence of four internal fragments of bovine CD36 have been obtained. Sequence comparision with bovine PAS IV and human platelet GP IV (CD36) show that they are homologous proteins. It is interesting to note that PAS IV lacks two glycosylation sites present in bovine CD36, since bovine CD36 is larger than PAS IV. This may be a result of post-translational modifications, including variable glycosylation (Greenwalt et al., 1985). The difference in molecular weight of PAS IV, bovine CD36, and human platelet GP IV (CD36) can be due to substitution of amino acid residues leading to variations in glycosylation sites or due to variation in post-translational modification

like glycosylation of proteins synthesized in different species or different cells (Furukawa et al., 1989).

Human GP IV (CD36), an 88 kD membrane glycoprotein has been purified and characterized. It plays an important role in platelet aggregation (McGregor et al., 1989), spreading of melanoma cells (Roberts et al., 1987), adhesion of platelets to collagen (Tandon et al., 1989) and also as a receptor for malaria-infected erthrocytes (Ockenhouse et al., 1989). Analogs of CD36 may play an important role in the treatment of acute falciparum malaria (Oquendo et al., 1989). The function of PAS IV and bovine CD36 is not yet known. Based on the new data reported here bovine PAS IV and CD36, and human GP IV (CD36) are members of a new family of cell adhesion proteins present in different tissues and having different functions based on their distribution. Cell adhesion proteins play an important role in mediating cohesive interactions between cells/ cell-tissue interactions. It may be necessary to obtain the cDNA sequence of the bovine proteins for further comparison.

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