PURIFICATION AND PROPERTIES OF BUFFALO (Bubalus bubalis) PLASMA FIBRONECTIN

BY
NANDINI CHOUDHURY

THESIS
SUBMITTED IN FULFILMENT OF THE REQUIREMENTS
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ABSTRACT

The study of fibronectins as a distinct class of proteins, started in the middle of this century when it was found that this protein co-precipitated with fibrinogen in the cold. The term, "fibronectin" (Latin: fibra-fiber, nectene-to connect or link), however, was adopted much later. Purification and characterization proved that fibronectin was a distinct entity, different from fibrinogen and other blood proteins. A protein, similar to fibronectin was also found to exist on the cell-surface of cultured fibroblasts. By late 1970s, to the general acceptance of all workers, fibronectins were classified as adhesive glycoproteins of high molecular weight. They exist in two forms:

1) Plasma fibronectin is the soluble form and is present, predominantly in the plasma.

2) The other form is insoluble and is called cell-surface fibronectin. This form is associated with basement membrane of connective tissues as well as a component of the extracellular matrix.

Both forms of FN share certain structural and physical properties. The proteins are made up of non-identical, but similar subunits of nearly equal sizes, joined at their carboxyl-terminal ends by two disulfide bonds. Each subunit is folded into discrete globular functional domains. These domains possess specific binding sites for biological macromolecules. Because of fibronectins' ability to bind these macromolecules, this protein mediates a large number of diverse and multiple roles, participating in cell adhesion, cellular morphology and spreading, cytoskeletal organization, wound healing, clot formation and phagocytosis.

Fibronectins have been studied from a large number of sources, viz., bovine, human, chicken, hamster and mice. Despite the wide diversity of sources, the overall structural and functional properties of this protein appear to be similar. Nevertheless, a few differences, specially in solubility have been observed. The secondary and tertiary structures of this protein

and the structure-function relationship have not yet been fully revealed.

The endeavour of this study was to isolate, purify and characterize fibronectin from buffalo plasma. The standardized isolation and purification process used was modified at certain steps to overcome a few difficulties encountered, especially with regard to storage of the protein. However, inspite of the modifications incorporated, the yield of plasma fibronectin was poor, and the tendency of the protein to aggregate could not be abolished altogether.

Purified buffalo plasma fibronectin (BpFN) yielded a single band on PAGE, both upon Coomassie Brilliant Blue staining as well as silver staining. Molecular weight of BpFN, determined by gel filtration was 442 kDa. In SDS-PAGE (homogeneous), BpFN migrated as a closely spaced doublet, the subunits corresponding to molecular weights of 216.2 kDa and 208.9 kDa, respectively. Two subunits were also obtained by SDS-PAGE in a PhastGel gradient 4-15, whose molecular weights were found to be 214 kDa and 204 kDa, respectively. The Stokes radius of the protein was calculated by two different plots and the values obtained were 64.8Å and 66.3Å, respectively. Although the molecular weight of the native protein tallied closely with that reported from other sources, the values of Stokes radius were significantly different from that of human pFN.

The amino-terminal end of BpFN was found to be alanine and the carboxyl-terminal end was phenylalanine for both the subunits. Human and bovine pFN have a blocked amino-terminal residue. The protein was found to contain 1.97 free sulfhydryl groups per subunit of BpFN and the sulfhydryl groups were not accessible to titration in absence of the denaturant. There were 73.08 moles of tryptophan residues per mole of BpFN. Carbohydrate content of the protein was 4.9%, a value lying well within the range of those reported from various sources.

The amino acid composition of BpFN showed close similarities with human and bovine pFN, except that the contents of Gly, Met and Leu, were significantly lower. The protein had an

isoionic pH of 6.12, showing that the protein is acidic in nature and this value is consistent with the values of pH range 5.5-6.3, reported from different sources. Two peaks were obtained in the UV absorption spectrum of BpFN, the first between 240-245 nm and the second between 277-278 nm. The fluorescence emission maxima was at 315 nm when excited at 280 nm and at 338 nm when excited at 300 nm. The fluorescence excitation maxima was centered at 320 nm when emitted both at 315 nm and 338 nm. The fluorescence spectrum of BpFN is characteristic of proteins containing tryptophan residues. The specific extinction coefficient (E_{1cm}) of the protein was 14 .02, higher than that reported for human pFN.

Polyclonal antibodies were produced when purified BpFN was injected in rabbits and a precipitin line was obtained upon cross-reaction of anti-BpFN antiserum with BpFN. Precipitin lines were also obtained when anti-BpFN antiserum cross-reacted with goat cFN, goat pFN and human pFN, showing that anti-BpFN antiserum obtained in this study shares immunological properties with FNs from other sources. Heating BpFN resulted in a decrease in the intensity of the protein's intrinsic fluorescence upto a temperature of 61°C, after which it underwent an irreversible transition with a mid-point between 61-62°C. Human pFN has also been observed to undergo a similar irreversible transition with a mid-point at around 63.5°C.

The effects of perturbants, DMSO, PEG 200 and PEG 400 on the intrinsic fluorescence of BpFN were studied at four different temperatures, 20°, 37°, 47° and 65°C. DMSO, at 20°C causes perturbation by progressive quenching of intrinsic fluorescence of the protein, because, being a small molecule, it is able to enter the interior of BpFN. At higher temperatures, the same extent of perturbation is achieved at lower DMSO concentrations. PEG 400, a large perturbant is unable to enter the protein interior at 20°C and 37°C. However, at 47°C, BpFN slowly unfolds and undergoes a conformational change at 65°C, with the result that the intrinsic fluorescence of BpFN is quenched at these temperatures. PEG 200 is a perturbant, intermediate in size between DMSO and PEG 400.

Thus, it generates an effect of perturbation also intermediate between the effects of DMSO and PEG 400. These responses suggest that, the conformational change that BpFN undergoes is restricted to a separation of its domains without significant loss in secondary structure.

A temperature of upto 50°C and a pH range of 6-9 caused no significant dissociation of BpFN from heparin-agarose affinity gel. BpFN, however, dissociated from heparin-agarose gel when the ionic strength was increased to 0.5 M NaCl at pH 7.4, indicating that the protein retains its heparin-binding property at high temperatures and between pH 6-9. Maximal elution of BpFN from heparin-agarose gel was found to occur at 0.65 M NaCl, in contrast to 0.5 M NaCl required for elution of human pFN. This might be indicative of the presence of a larger number of basic amino acid residues within the heparin-binding domains of the protein.

Thus BpFN shares most of its physico-chemical properties with its counterparts from human and bovine sources, although differing in certain aspects like extinction coefficient, amino-terminal end and a higher ionic strength requirement for elution from heparin-agarose affinity column. The findings support the postulation that multiple structural forms of FN, obtained under different conditions might play important roles in various FN-mediated physiological processes and provide suitable adaptation to cells and organs for better cell-cell interaction and other associated processes in organisms.

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CERTIFICATE

We certify that the thesis entitled **"Purification and properties of Buffalo (*Bubalus bubalis*) Plasma Fibronectin,"** submitted by **Miss. Nandini Choudhury** for the degree of **Doctor of Philosophy** of the **North Eastern Hill University**, Shillong, embodies the record of original investigations carried out by her under our supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of Ph.D. degree. This work has not been submitted for any degree of any other University.

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(Supervisor I)

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Nandini Choudhury.

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LIST OF ABBREVIATIONS

cFN	: Cell-surface/Cellular fibronectin
BpFN	: Buffalo plasma fibronectin
BSA	: Bovine serum albumin
DAB	: p-Dimethyl aminobenzaldehyde
DMSO	: Dimethylsulfoxide
DTNB	: 5, 5′-Dithiobis-(2-nitrobenzoic acid)
EDTA	: Ethylenediamine tetra acetic acid
FCA	: Freund's complete adjuvant
HPLC	: High performance liquid chromatography
IFA	: Incomplete Freund's adjuvant
NaCl	: Sodium chloride
NaN_3	: Sodium azide
PAGE	: Polyacrylamide gel electrophoresis
PEG	: Polyethylene glycol
pFN	: Plasma fibronectin
PMSF	: Phenylmethyl sulfonyl fluoride
R_f	: Relative front
R_m	: Relative mobility
SDS	: Sodium dodecyl sulfate
TEMED	: N, N, N′, N′,- tetramethylethylenediamine
TLC	: Thin layer chromatography
Tris	: Tris-(hydroxymethyl)-aminomethane

INTRODUCTION

Overview

Almost five decades ago, Morrison *et al.*,(1948) isolated a partially purified fraction of human plasma, which could be separated from the bulk of fibrinogen, owing to its cold-insolubility. They named the fraction "cold-insoluble globulin" (CIg). Later, CIg was found to be a mixture of several components, including fibrinogen (Edsall *et al.*, 1955). Because of the presence of a number of proteins in this preparation, further work on CIg was possible, only fifteen years later, when Mosesson and Umfleet (1970) purified and characterized CIg from plasma cryoprecipitate. They established that it was different from fibrinogen and other blood proteins. CIg was subsequently isolated from different sources by various groups and each named it differently (Yamada and Olden, 1978). In order to bring about an uniformity in nomenclature, the term fibronectin (Latin: *fibra*-fibre; *nectene*-to connect or link), was introduced for this class of proteins (Hynes, 1990).

Yamada and Weston (1974) isolated a cell-surface protein from fibroblasts, whose characterization later by several groups (Mosher, 1976; Vuento *et al.*, 1977; Matsuda *et al.*, 1978) showed that the protein derived from fibroblasts was similar but not identical to plasma fibronectin and the two proteins belonged to the same class. A method to isolate and purify fibronectin from both sources by gelatin-affinity chromatography was developed only in 1977 by Engall and Ruoslahti and has since been used as an established method of purification for this class of proteins. The idea of classifying fibronectins (FN) as a distinct class of proteins became clear by 1980, with the availability of adequate data on their basic structure and function.

Distribution of Fibronectin (FN):

FNs exist both as soluble and insoluble forms. The soluble form is predominantly present in the plasma at a concentration range of 260-380μg/ml and is termed as plasma fibronectin (pFN). All other body fluids also contain the soluble form of FN, albeit in varying concentrations. Some of these are distinct and originate from different cell types than do pFN (Hynes, 1990)

The insoluble form is called cell-surface fibronectin (cFN). The type synthesized by fibroblasts contributes 1-3% of total cellular proteins (Yamada *et al.*, 1977). Fibroblastic FN is either incorporated as fibrillar arrays on the cell-surface or secreted into the culture medium or both (Yamada *et al.*, 1977; Olden and Yamada, 1977; Mosher *et al.*, 1977). cFN extends between cells as well as between cells and tissue culture substratum. (Yamada and Olden, 1978).

Structure and Physico-chemical Properties of Fibronectin:

Both forms of FN are large glycoproteins and are made up of two disulfide-bonded subunits of 215-250 kDa, each (Chen *et al.*, 1977; Furcht, 1983).They have similar isoelectric points (Vuento *et al.*, 1977). pFN migrates in SDS-polyacrylamide gels as a closely spaced doublet whereas cFN migrates as one broad band (Balian *et al.*, 1979; Yamada and Kennedy, 1979). pFN is soluble at neutral pH (Mosesson *et al.*, 1975), whereas the cellular form is soluble at an alkaline pH (Yamada and Olden, 1978). The interchain disulfide bonds in both the forms of FN are located towards the carboxyl-terminal end of the protein (Iwanaga *et al.*, 1978; Keski-Oja *et al.*, 1977). Human and bovine pFN have blocked amino-terminal ends (pyrrolidone carboxylic acid and pyroglutamate residues) (Garcia-Pardo *et al.*,1983; Petersen *et al.*, 1983), while chick cFN has a free amino-terminal end. (Yamada and Olden, 1978). About 30 intrachain disulfide bonds are found in FN, concentrated around the amino-terminal

region of the protein molecule (Pearlstein *et al.*, 1980; Wagner and Hynes, 1980; McDonald and Kelley, 1980). Approximately, 1.3 to 1.6 free sulfhydryl groups are present in each subunit (Smith *et al.*, 1982). Sedimentation coefficient ($S_{20,w}$) values of FNs vary between 11 to 13.6 under normal physiological conditions but fall with an increase in ionic strength and changes in pH (Erickson and Carrell, 1983; Rocco *et al,* 1983). Intrinsic viscosity [η] of human pFN is 10.2 and axial ratio varies between 8-18:1. The Stokes radius is 10.4 nm at physiological salt concentrations and pH. The radius of gyration increases from 10.7 to 17.5, when the ionic strength is increased (Williams *et al.*, 1982; Rocco *et al.,* 1987). These biophysical parameters reinforced the early observations that pFN is a partially folded protein and that was further confirmed by studies with electron microscopy and NMR, where pFN molecules were found to have a length of 120 nm and a width of 2.4 nm, with an angle of 70° between the two equal subunits. Approximately 8 KJ/mol of energy is required to change pFN from a "V" shape to a linear form (Engel *et al.,* 1981; Erickson *et al.,* 1981; Odermatt *et al.,* 1982). pFN exists as a disc-shaped molecule under physiological conditions with a diameter of 30 nm and a thickness of 2 nm (Sjoberg *et al.*, 1987).

The amino acid composition of FN from various sources are found to be similar and have an acidic pI (Hormann, 1982; Tamkun and Hynes, 1983; Paul and Hynes, 1984). The cleavage of FN by various proteolytic enzymes has revealed the presence of distinct domains that are responsible for many of its important functions. These domains are separated by short stretches of polypeptide chains (Wagner and Hynes, 1980), which are susceptible to proteolysis. These functional domains consist of several homologous repeating sequences of three distinct types, I, II and III characterized by presence of conserved consensus sequences (Engel *et al.*, 1981; Skorstengaard *et al.*, 1986a, b; Petersen *et al.*, 1983). Type I and type II repeats are disulfide bonded loops that are about 50 amino acids long. There are 12 type I homologies, which are

25-50% identical with each other, 2 type II homologies and 16 type III homologies in the FN molecule. The first few amino acid residues and the last 25-30 amino acid residues of each type III repeat show strong homology amongst each other, whereas the central region of type III repeat do not show much homology between them (Hynes, 1985)

Modular Structure of Fibronectin:

As stated above, the FN molecule can be cleaved by various proteolytic enzymes into a number of localized regions or domains These domains are responsible for giving the protein a number of its important properties, primarily, ligand binding, adhesion and its role in cell motility.

The amino-terminal domain **:**

This domain can be generated by digesting FN with trypsin, thrombin, plasmin and thermolysin, as a 20-25 kDa fragment (Wagner and Hynes, 1980; Furie and Rifkin, 1980; Ehrismann *et al.*, 1982). The pI of this domain is basic (Varito, 1982; Sekiguchi and Hakomori, 1983a; Seidl and Hormann, 1983), and contains no carbohydrates or sulphydryl groups (Sekiguchi et al., 1981; McDonald and Kelley, 1980; Seidl and Hormann, 1983). The amino-terminal residue of pFN is pyrrolidone carboxylic acid in human, and pyroglutamate in bovine (Petersen *et al.*, 1983; Garcia-Pardo *et al.*, 1983). This domain binds to heparin and fibrin (Sekiguchi and Hakomori, 1983b; Smith and Furcht, 1982; Seidl and Hormann, 1983), on a complementary site at its own carboxyl-terminal region (Homandberg,1987) and participates in matrix assembly (Quade and McDonald, 1988). There are five type I homologies in the amino-terminal domain (Petersen *et al.*, 1983; Skorstengaard, 1984; Kornblihtt, 1985).

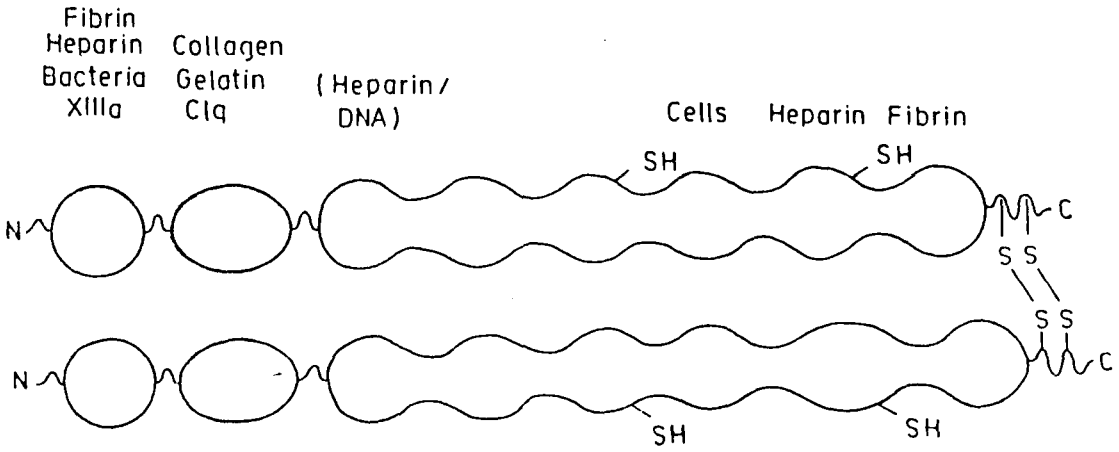


Fig.1. Schematic diagram of plasma fibronectin. The two subunits and the eight major binding domains are represented. The amino-terminal end is indicated by ~N and the carboxyl-terminal end by ~C. The two disulfide bonds at the carboxyl-terminal end are also shown. The top panel shows the macromolecules that bind to their respective domains (Hynes, 1990).

The gelatin-binding domain :

The amino-terminal domain is followed by a short stretch of 37 amino acid residues which separates the gelatin-binding domain (GBD) from the amino-terminal domain. GBD can be cleaved by trypsin, plasmin, thrombin and thermolysin and has a molecular weight of 40-45 kDa (Wagner and Hynes, 1979; Sekiguchi and Hakomori, 1980a; Ruoslahti *et al.*, 1981 ; Varito *et al.*, 1981). The GBD isolated from bovine pFN is 340 amino acid residues long (Skorstengaard *et al.*, 1984). In the entire FN molecule, this is the only domain in each subunit, which has gelatin-binding activity (McDonald and Kelley, 1980). GBD is highly resistant to proteolysis due to the presence of intrachain disulfide bonds (Williams *et al.*, 1983), and carbohydrate side chains (Bernard *et al.*, 1982), which are complex, branched and arginine-linked (Bernard *et al.*, 1984; Nichols *et al.*, 1986; Ingham *et al.*, 1995). In the absence of intrachain disulfide bonds, this domain does not bind to gelatin (Balian *et al.*, 1979; Williams *et al.*, 1983). However, the carbohydrate moieties are not essential for binding to gelatin (Olden *et al.*, 1979). GBD has an acidic pI and does not bind to heparin (Ehrismann *et al.*, 1982; Sekiguchi and Hakomori, 1983b; Sekiguchi *et al.*, 1983), or promote cell adhesion (Hahn and Yamada, 1979; McDonald and Kelley, 1980). This domain has four type I repeats and two type II repeats in the form of disulfide loops. The type II repeats are flanked on one side by one type I and on the other side by three type I repeats (Skorstengaard, *et al.*, 1984; Kornblihtt *et al.*, 1985).

The central domain :

A fragment, having its origin from the central region of FN has an approximate molecular weight of 60 kDa and binds to fibrin (Seidl and Hormann, 1983), FNs (Ehrismann *et al.*, 1982) and heparin (Griffin *et al.*, 1986). This fragment can be further cleaved into a 40 kDa subfragment, binding only gelatin and a

14–20 kDa subfragment, binding only to heparin (Calaycay *et al.*, 1985). This fragment contains highly conserved type III homologies, designated as type III 1-6 (Hynes, 1990). The fragment comprising the first three repeating units (30 kDa) is susceptible to proteolysis and shows heparin-binding activity (Calaycay *et al.*, 1985). The next three repeating units (30 kDa) can be generated as a DNA- and heparin-binding domain (Skorstengaard *et al.*, 1986a). The binding of DNA and heparin to this region however, occur only at low salt concentrations and its physiological relevance is yet to be ascertained (Calaycay *et al.*, *1985*; Siri *et al.*, 1986). Each type III repeat contains 90 amino acid residues which are highly conserved, especially an aromatic residue, which occurs at three positions in each repeat (Hynes, 1990). Amino acid sequences of this region of bovine (Skorstengaard *et al.*, 1986b) and human (Pande *et al.*, 1985; Calaycay *et al.*, 1985) pFN show that they are more than 90% identical. Of the two pairs of sulfhydryl groups in FN, one pair lies in the central domain (Smith *et al.*, 1982; Mosher and Johnson, 1983).

The cell-binding domain :

Another fragment obtained from the central region is a 12 kDa fragment, containing the arginine-glycine-aspartate-serine (RGDS) sequence (Pierbascher and Ruoslahti, 1984; Obara *et al.*, 1988), essential for cell-binding activity of pFN. This region was first purified from human pFN by Pierschbacher *et al.*, (1981), using a monoclonal antibody ($3E_3$), that blocked cell-attachment activity of intact FN. This fragment is 108 amino acids long in humans (Pierschbacher *et al.*, 1982) and is made up of a single type III repeat (designated type III-10). It has been predicted that the RGDS sequence lies as an exposed hydrophilic loop, sandwiched between two stretches of β-structures (Pierschbacher *et al.*, 1982; Odermatt *et al.*, 1985). The RGDS sequence is also found to be highly conserved (Hynes, 1990).

The heparin-binding domain :

The cell-binding central domain is followed by an exclusively heparin-binding domain (HBD), the third and strongest in the FN molecule (Richter *et al.*, 1981; Hayashi and Yamada, 1983). This domain can be isolated by the action of trypsin and cathepsin D (Click and Balian, 1985). Exclusive HBD was first isolated from chicken cFN as a 50 kDa domain. This segment, upon cleavage, is obtained as a globular monomer with a low content of cystine and carbohydrate moieties (Yamada *et al.*, 1980; Hayashi *et al.*, 1980). The pI of this region is basic (Richter and Hormann, 1982). The binding of heparin to this region is not affected by divalent cations (Hayashi and Yamada, 1982) or reduced pH (Siri *et al.*, 1986). Located towards the carboxyl-terminal end of FN (Garcia-Pardo *et al.*, 1987) this domain has three type III repeats, along with a variable region or the "V" segment. The "V" segment was first isolated from rat FN cDNA clones (Schwarzbauer, 1983) and its sequence varies in different types of FNs as well as FN from different sources (Kornblihtt *et al.*, 1984; Bernard *et al.*, 1985; Umezawa *et al.*, 1985). The "V" segment unlike type III repeats, contains a large amount of histidine and proline residues. A sequence, similar to the RGDS sequence is found in the variable segment of this domain, which is RGDV in rat and bovine FNs (Schwarzbauer *et al.*, 1983; Skorstengaard *et al* ., 1986) and REDV in human pFN (Bernard *et al.*, 1985) and hence, exhibits some amount of cell-binding activity (McCarthy *et al.*, 1986; Rogers *et al.*, 1987; Saunders and Bernfield, 1988).

The carboxyl- terminal fibrin-binding domain :

The fibrin-binding domain (FBD) is a small 21-37 kDa fragment, having its origin from the carboxyl-terminal end of pFN, which can be released by thermolysin (Sekiguchi and Hakomori, 1983a), trypsin (Smith *et al.*, 1982; Hayashi and Yamada, 1983) and plasmin (Petersen *et al.*, 1983). This region

is specific for fibrin and is the second FBD in the FN molecule. This domain does not bind to gelatin or heparin (Sekiguchi and Hakomori, 1983b; Hayashi and Yamada, 1983), and is not a substrate for activated clotting factor XIIIa (Mosher *et al.*, 1980; Sekiguchi *et al.*, 1981). This domain is obtained as a monomer and contains no carbohydrate moieties (Hayashi and Yamada, 1983). FBD is rich in cystine residues and has an acidic pI (Sekiguchi *et al.*, 1983a). FBD comprises of three type I repeats in the form of disulfide bonded loops (Hynes, 1990).

The carboxyl-terminal end :

The region containing the two interchain disulfide bonds can be fragmented by cathepsin D (Richter and Hormann,1982), plasmin (Petersen *et al.*, 1983) or trypsin (Garcia-Pardo *et al.*, 1984) into a short 6 kDa fragment, whose last 20 amino acid residues contain the two interchain disulfide bonds.

Ligand-binding Properties of Fibronectin:

FN possesses the distinct property of ligand-binding. It binds to collagen, gelatin (Engvall and Ruoslahti, 1977), fibrin (Seidl and Hormann, 1983), heparin (Sekiguchi and Hakomori, 1983a, b; Yamada *et al.*, 1980), sulfated glycosaminoglycans like heparin sulfate, chondroitin-4 sulfate, dermatan sulfate and heparan sulfate (Laterra *et al.*, 1980; Stamatoglou and Keller, 1982; McCarthy *et al.*, 1986; Saunders and Bernfield, 1988; Bidnaset *et al.*, 1992) It has affinities for bacteria, *Staphylococcus aureas, Candida albicans* (Mosher and Proctor, 1980; Nègre *et al.*, 1994), streptococci (Babu and Dabbous, 1986) and also for actin (Keski-Oja *et al.*, 1980), DNA (Siri *et al.*, 1986). Moreover, it binds sphingolipids (Klienman *et al.*, 1979), complement factor, Clq (Menzel *et al.*, 1981) and is a substrate for transglutaminase (Mosher and Proctor, 1980).

FN is able to bind, simultaneously multiple ligands such as glycosaminoglycan, collagen and the cell surface. Collagen

binding to FN has been found to be enhanced in the presence of heparin. This suggests that the binding of ligands to FN may be cooperative, the binding of one ligand being affected by the conformational changes induced in FN by the binding of other ligands (Osterlund *et al.*, 1985).

Heparin as well as sulfated glycosaminoglycans bind to pFN which has three distinct heparin-binding regions within itself (Saunders and Bernfield, 1988; Bidnaset *et al,* 1992). The binding of heparin to the amino-terminal domain of pFN is blocked by 0.25 M sodium chloride (Richter *et al.*, 1981; Sekiguchi *et al.*, 1983a). This site is not the strongest heparin-binding site under physiological conditions. The binding of heparin to the heparin-binding domain located in the carboxyl-terminal end of FN is the strongest which can be blocked by 0.5 M salt (Hayashi and Yamada, 1983).

Hayashi and Yamada (1982) identified three kinds of heparin-binding to FN:

a) calcium sensitive, exhibited by the 31 kDa amino-terminal fragment. The binding is insensitive to $MgCl_2$ or $MnCl_2$. 50% inhibition of binding can be achieved with 3 to 4 mM $CaCl_2$;

b) divalent cation sensitive, exhibited by 75 kDa fragment from the central region: $CaCl_2$, $MnCl_2$ and $MgCl_2$ inhibit binding of heparin to this fragment and

c) divalent cation insensitive binding, exhibited by the site located in the carboxyl-terminal end of the molecule (Hayashi and Yamada, 1982).

Investigations using fluorescence emission, intrinsic fluorescence polarization and CD spectroscopy in the presence of guanidine hydrochloride, elevated temperatures and solvent perturbants by Khan *et al.*, (1990b) have established that heparin-binding to both native pFN as well as its amino-terminal domain stabilizes the protein against unfolding, induced by heat. Binding of heparin decreases the accessibility of the perturbants to the chromophores within the protein, indicating that the protein becomes more compact. Thus both native FN and its amino-terminal domain undergo a definite

conformational change upon binding to heparin, although the type of change induced is variable in each case (Khan *et al.*, 1988; 1990b).

Consistent with the earlier finding that heparin-binding to the amino-terminal domain of pFN is calcium dependent (Hayashi and Yamada, 1982), Khan *et al.*, (1988) found that the heparin-binding induced conformational change in the amino-terminal domain of pFN by heparin is totally inhibited by 10 mM Ca^{2+}. In contrast, the change in native FN is not affected by the presence of calcium ions (Khan *et al.*, 1988).

The binding activity of heparin to the carboxyl-terminal end of the molecule has been localized within the type III repeat (repeat III_{13}) of the protein (Barkalow and Schwarzbauer, 1991; Kimizuka *et al.*, 1991). Major activity of this domain is contributed by the presence of arginine residues within repeat III_{13} sequence as confirmed from studies involving site-directed mutagenesis (Barkalow and Schwarzbauer, 1991). The importance of arginine residues in heparin binding was also confirmed by Jaikaria *et al.*, (1991). Modification of arginine residues reduces the capacity of pFN to bind to heparin with a corresponding decrease in the translocation-promoting activity of FN. Desulfation of heparin also inhibited binding of heparin to the amino-terminal end of pFN (Khan *et al.*, 1988; Jaikaria *et al.*, 1991). Modulation of heparin-binding to pFN may be responsible for *in vivo* regulation of FN interaction with extracellular glycosaminoglycans, its strength as well as the nature of cross -links between extracellular matrix. (Hayashi and Yamada, 1982).

Cellular responses of FN like cell-adhesion, spreading, migration and growth involve the interaction of the functional domains of the protein, namely, the heparin-binding domain and cell-binding domain (McCarthy *et al.*, 1986; Yoneda *et al.*, 1995). The recognition of RGDS sequence by integrins, specific cell-surface receptors (Bowditch *et al.*, 1994) is the best understood mechanism by which cells adhere to FN and to other

matrix and plasma proteins. Synthetic peptides containing this sequence promote cell attachment when immobilized on a suitable substratum and inhibit the adhesion of fibroblast cells to intact FN. Thus, this sequence has been postulated to be its physiological ligand (Fujita *et al.*, 1995). Laterra *et al.*, (1980) demonstrated that cell-surface glycosaminoglycans, especially the highly N-sulfated sequences of heparan sulfate and a small subset of dermatan sulfate bind FN in cell-substratum adhesion sites of murine fibroblasts. Sulfated proteoglycans including heparan sulfate and chondroitin sulfate have been shown to play a regulatory role in the adhesion and motility of cells (Makabe *et al.*, 1990). The formation of tight focal contacts with associated stress fibres upon adhesion of fibroblasts to FN require both reactions, binding to heparan sulfate proteoglycan on cell surface and to its unidentified receptor. Neither cell-, nor heparin-binding domain of cleaved FN is sufficient to promote spreading and focal adhesion formation. At least two stimuli from FN are required to achieve complete adhesion. Studies utilizing recombinant FN polypeptides have shown that the fusion polypeptide that contained both cell-binding as well as heparin-binding domains (CH-271) promotes the spreading of tumor cells (human fibrosarcoma cells HT1080) as effectively as FN and much more effectively than only the cell-binding polypeptide or their mixture (Yoneda *et al.*, 1995). Such spread-promoting activity of CH-271 was dose- and time-dependent and also induced accumulation of vinculin at focal contact in spread cells. Hence, the induction of full cellular spreading may be associated with interaction of cells with the heparin-binding domain of FN, in combination with the cell-binding domain (Yoneda *et al.*, 1995). Matrix-driven translocation assays and its biological relevance could well provide insight into cell-surface-extracellular matrix interactions (Jaikaria *et al.*, 1991), including controlling tumor metastasis, tumor cell-adhesion and motility by peptides containing various functional domains of FN (Yoneda, 1995).

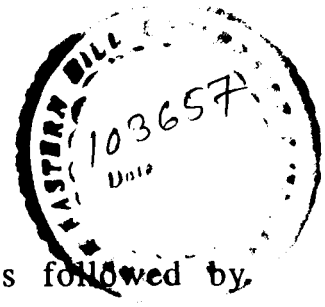
The affinity of pFN for fibrin (Stathakis *et al.*, 1978; Gold *et al.*, 1982) and integrin on platelets (Hynes, 1990) mediates wound healing The amino-terminal domain of FN, inspite of having a low affinity for fibrin under physiological conditions (Garcia-Pardo *et al.*, 1985), plays a critical role in the ensuing process of wound healing. It appears that the interaction of FN with fibrin takes place through the carboxyl-terminal end of the α-chain of fibrin (Kirschbaum and Budzynski, 1990). pFN is incorporated into the fibrin clot in two steps. First, a reversible non-covalent interaction between FN and fibrin occurs, after which, the two proteins are covalently cross-linked by activated Factor XIII. This activated Factor XIII acts by coupling the α-amino groups of lysine residues in fibrin to that of the γ-carboxyl group of specific glutamine residues in FN (Matsuka *et al.*, 1994).FN induces a dose dependent acceleration of both the conversion of fibrinogen to fibrin and fibrin polymerization. FN, within the developing blood clot, forms a substrate for binding collagen, and other glycosaminoglycans. These interactions stabilize the clot, promoting hemostasis (Rostagno *et al.*, 1994).

Such cross-links require that the two participating reactants are in their proper orientation and proximity. In order to localize the fibrin-binding site within the FN molecule, recombinant DNA technology was used to express five type I repeats from the amino-terminal domain [also called "finger" (F) modules], rF1, 2, 3, 4 and 5 and their pairs rF1-2, 2-3, 4-5, as fusion proteins in *Escherichia coli.* Affinity chromatography showed that only the recombinant fragment, rF4-5 bind to fibrin-Sepharose. Individual rF4 shows lesser stability in its melting behavior than rF4-5. This suggests a strong interaction between these two fingers which creates proper orientation of side chains to form the fibrin-binding site (Matsuka *et al.*, 1994). However, a proteolytic fragment composed of all five amino-terminal finger modules showed 4 to 5 fold higher fibrin-binding activity than F4-5, on a molar basis. Thus, although none of the individual modules or module pairs, apart from rF4-5, showed any binding to fibrin, the presence of contiguous five

Type I modules increased fibrin-binding activity. The tandem repeats enhanced binding either in a supplementary manner or by providing a conformationally suitable environment for enhanced binding. No other individual modules or module pairs other than F4 and F5 show any binding to fibrin (Rostagno *et al.*, 1994).

The carboxyl-terminal end of FN has three Type I finger modules (F10 - 12), which also exhibit fibrin-binding activity. To understand the role of this region, the fibrin-binding to FN was competitively inhibited in an ELISA by rF4-5 as well as by a monoclonal antibody to an epitope in this module pair (Rostagno *et al.*, 1994). The experiment was performed at physiological ionic strength and a maximum of 67% inhibition could be obtained. Since fibrin-binding activity could not be totally blocked, it was concluded that residual activity is conferred by the carboxyl-terminal domain, and that both amino- as well as carboxyl-terminal domains are functional in the native FN molecule. Fibrin may be bound by the domains either alone or simultaneously, resulting in a stronger affinity of molecular binding (Rostagno *et al.*, 1994).

Cell movement and behavior, at the molecular level, are mediated by FN fibrils, extending between cells and the substratum. FN fibrils are formed via interactions between FN molecules (Chernousov *et al.*, 1991) and multimerization relies on the FN dimer structure. The cell-binding domain and the RGDS sequence apparently initiate fibrillogenesis and stabilize the fibrils by interactions with cell-surface integrins (Schwarzbauer, 1991). Matrix assembly has been suggested to proceed through three stages: initiation, fibrillogenesis and stabilization. The secreted FN dimers bind through the RGDS sequence and also other domains, to the cell surface. The FN subunits are in an anti parallel orientation and hence the association with other FN dimers are in a head-to-tail fashion. The onset of fibrillogenesis occurs where the fibrils are formed by association between subunits from different dimers. This is followed by lengthening and bundling of fibrils to generate a



linear and branched extracellular network. This is followed by the stabilization of the newly-formed matrix by concerted interactions between fibrils, FN and the cell-surface (Schwarzbauer,1991).

The two regions that are involved both in FN-binding as well as matrix-assembly are a 70 kDa amino-terminal region and a fragment containing the first two type III repeats, adjacent to the gelatin-binding domain (Schwarzbauer, 1991; Morla and Ruoslahti, 1992). The matrix-assembly activity of the 70 kDa amino-terminal region is conferred by the 5 type I repeats within this region. Deletion of any of the 5 repeats drastically reduces the ability of this domain to bind with fibroblasts. FN fragments lacking these repeats or recombinant proteins with deletions in the amino-terminal segment are not assembled into a fibrillar matrix. The role of this fragment in matrix-assembly is also supported by antibody and fragment blocking experiments and cross-linking studies (Aguirre *et al.*, 1994; Halliday and Tomasek, 1995). The RGDS sequence does not play a significant role in the overall incorporation of FN into the matrix and its deletion had only a slight effect on fibrillogenesis. Fibrils still formed and had a more punctate appearance than when the RGDS sequence was present (Schwarzbauer, 1991). One feature of FN which is absolutely required for fibrillogenesis is the disulfide-bonded dimer structure. A monomeric recombinant FN, whose carboxy-terminal cystines are deleted remain incapable of forming fibrils. Thus, in addition to domains involved in the intermolecular interactions within the fibrils, a dimeric structure of the protein is necessary for the propagation of fibrillar network (Schwarzbauer,1991).

Secondary Structure of Fibronectin:

Circular dichroic (CD) spectrum has been routinely used to elucidate the secondary structure of FN. Mosesson *et al.*,(1975) recorded a far UV CD spectrum, suggesting absence of α-helix and presence of β-structures, as also an unusual positive peak at

227-230 nm. Further work on FN from different sources revealed a similarity in secondary structure of cFN and pFN (Colonna *et al.*, 1978; Welsh *et al.*, 1983; Venyaminov *et al.*, 1983). However, certain inconsistency exists in the type of secondary structure of FN. Osterlund *et al.*, (1985) have reported about 79% β-sheet and 21% β-turn in the CD spectrum of human pFN, in contrast to reports of only 35% β-sheet, obtained from infra-red spectroscopic studies (Koteliansky *et al.*, 1981; Venyaminov *et al.*, 1983). The results of the analysis of far UV CD spectrum on the conformation of FN and its fragments (Brumfeld and Werber, 1995) are comparable with differences of interpretation to those obtained from previous studies (Osterlund *et al.*, 1985; Khan *et al.*, 1988).

Apart from the CD spectra, fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectra of FN also show characteristics of β-structures. Thus the unusual CD spectrum of FN reported earlier (1983, 1987,1989) belongs to a protein with a high amount of β-structure (Brumfeld and Werber, 1993).

Secondary structure estimations obtained from both FTIR spectra and far UV CD spectra also indicate significant differences between the two functionally different domains of FN viz, CBD and FBD. CBD possesses almost exclusive β-sheet conformation as the whole FN, the amino-terminal FBD alternates between aperiodic and β-turn structures (Brumfeld and Werber, 1993). This is in contrast to an earlier finding that the FBD is the most highly organized domain in the entire FN molecule with a greater degree of β-structure (Khan *et al.*, 1988).

Structural Transitions of Fibronectin:

pFN responds to changes in the environment by altering its shape. This characteristic can be attributed to its high degree of flexibility. It ranges from a compact form to a rod-like structure (Erickson and Carrel, 1983; Tooney *et al.*, 1983; Khan,

1990a). Intramolecular, non-covalent interactions between the domains within a subunit or between the domains of both the subunits allow the folding of pFN (Hormann and Ritcher, 1986; Homandberg and Erickson, 1986; Litvinivich *et al.*, 1991; Matsuka *et al.*,1994). Under physiological conditions, it assumes a compact form which undergoes a reversible transition to an expanded conformation with changes in pH, glycerol, ionic strength and urea concentrations (Rocco *et al.*, 1983; Khan, 1990a; Benecky *et al.*, 1991; Lai *et al.*, 1993). An increase in the conductance of the protein solution, upon increasing the ionic strength from 0.15 μ to 1μ, leads to an increase in the Stokes radius of the protein, proving a definite conformational change in pFN (Williams *et al.*, 1982). The transition induced by guanidine hydrochloride is multiphasic. The first phase involves the exposure of various domains to the denaturant, followed by the availability of each domain to the effects of the denaturing agent (Khan, 1990a). pFN undergoes a conformational change by interacting with ligands. Heparin induces a change in the secondary structure of the protein. Studies on the interaction of native as well as modified heparin with FN and its 31 kDa amino-terminal fragment have implicated a conformational change in the protein (Khan *et al.*, 1988; Khan *et al.*, 1990b). Analysis of CD spectrum proved an induction of structural change due to alteration in its conformational states. The alteration in the amino-terminal end of the protein is different to the change induced elsewhere in the molecule, by the same ligand (Khan *et al.*, 1988)

pFN , which otherwise binds poorly to cell types in suspension, binds well after deposition onto a substratum (Rennard *et al.*, 1981; Grinnel *et al.*, 1982). The molecule undergoes an "expansion" from its native conformation, when the protein comes in contact with a suitable surface (Ugarova *et al.*, 1995). Studies with site-directed monoclonal antibodies have revealed that the conformational change along with interactions with intercellular matrix components leads to an increase in the activity of the cell-binding domain. It has been suggested that

the RGDS sequence, located in the type III_{10} of the protein is more accessible to a conformational change upon attachment to a surface and may contribute significantly to the adhesive properties of pFN (Ugarova *et al.*, 1995).

Glycosylation of Fibronectin:

One of the major post-translational modifications of FNs is glycosylation. Both pFN and cFN from almost all sources have, predominantly, complex, biantennary asparagine-linked carbohydrate side chains, although there are differences in the structure of side chains in FNs derived from different sources (Fukuda *et al.*, 1982; Nichols *et al.*, 1986). In hamster FN, glycosylation is confined almost exclusively on the 44 kDa GBD, but in human glycosylation sites are distributed to other domains as well. Fuc $\alpha(1\rightarrow 6)$ linked to the innermost N-acetyl glucosamine residue of the chitobiosyl core is found only in cFN but is absent in pFN. Human pFN shows extensive $\alpha(2\rightarrow 6)$ sialylation of the terminal galactose residue of the biantennary side chain whereas human cFN is less sialylated with mainly $\alpha(2\rightarrow 3)$ linkages. These structural differences are present in both hamster and human FNs.

Three of the 6-7 N-linked carbohydrate moieties are located in the 42-44 kDa GBD, one carbohydrate chain is attached to type II_2 and the other two with the type I_8. The variation in the composition of carbohydrate groups alters the affinity of the protein for various ligands. The carbohydrate groups in the GBD of placental FN are elongated by polylactosamine and this modification decreases the affinity of GBD for gelatin and alters its tryptophan environment (Ingham *et al.*, 1995). FN, synthesized in the presence of tunicamycin lacks carbohydrates, rendering the protein especially the GBD, sensitive to proteolysis (Bernard *et al.*,1982), without affecting its gelatin-binding property (Ingham *et al.*,1995). Carbohydrate moieties, thus function to protect the GBD of FN from proteolysis and stabilize its structure.

Fibronectin Gene Structure:

Experiments with recombinant genomic clones have established that there is a single FN that codes for all known forms of FN. The FN gene is localized on chromosome 2 in rats. The FN gene is 70 kb long and has 50 exons of nearly equal sizes (Tamkun *et al.*, 1984; Patel *et al.*, 1986; Schwarzbauer *et al.*, 1987). Hirano *et al.*, (1983) have reported chicken FN gene as 50 kb long. The FN gene has also been reported to be located on chromosome 2 in humans (Prowse *et al.*, 1986; Jhanwar, 1986). Nearly all type I and type II repeats are encoded by precisely single exons, as evident from the sequence of rat FN gene (Patel *et al.*, 1986; Schwarzbauer *et al.*, 1987). Of the 17 type III repeats that have been analysed, 14 of them are separated from each other by introns. In contrast from the type I and type II repeats, most type III repeats that have been analysed are encoded by two exons rather than one (Hynes, 1990). The positions of the introns interrupting the type III repeats vary among repeats. Most of these introns lie in the central parts of the type III repeats, are the least well-conserved and apparently lack ordered structure.

Tissue-specific expression of the FN isoforms is regulated at both the transcriptional as well as post-transcriptional levels(Sporn and Schwarzbauer, 1995). Post-transcriptional modifications occur via alternative splicing (Schwarzbauer *et al.*, 1983). There are two forms of alternative splicing of the FN transcript. Exon skipping accounts for the presence or absence of the extra type III segment and thus the differences between cFN and pFN. In hepatocytes, this segment is not coded, leading to the synthesis of pFN. When it is coded, cFN arise with large FN subunits, characteristic of cFN only. Exon subdivision accounts for the difference between pFN subunits. The ratio of various splices in the region of exon subdivision varies in different cell types and the smallest subunits which lack inserts ("V" segment) are prevalent in pFN and hepatocyte FN, but rare in fibroblast FN (Hynes, 1990).

Transcriptional control also plays an important role in controlling FN levels in specific tissues and also in response to certain stimuli during cell migration, wound healing or severe tissue injury. FN gene transcription is induced by serum epidermal growth factor, platelet-derived growth factor, transforming growth factor, cAMP and gamma interferon (Sporn and Schwarzbauer, 1995).

The 5′ end of rat and human FN genes contain clusters of transcriptional regulatory elements, including TATAA and CCAAT promoter elements and SP1 binding sites. This region as well as the first intron of the gene contains matches to concensus sequences for heat shock, glucocorticoid and cAMP responses, serving as potential regulatory elements (Patel *et al.*, 1986; Dean *et al.*, 1987). Recently, it has been shown that two regions within 4 kb upstream of the rat FN gene increased transcription upon transfection into different cell types. The region between -1.08 and -2.6 kb showed tissue-specificity and was active in fibroblasts but not in hepatoma cells and was proved to be an enhancer while the second region between -3.2 and 3.9 kb was active in both cell types. This led to the identification of a novel DNA sequence at -1.68 kb which is involved in regulating transcription of the FN gene in fibroblasts (Sporn and Schwarzbauer, 1995).

Immunological Characterization of Fibronectin:

FN has been found to be strongly immunogenic. Both cFN and pFN show antigenic properties (Ruoslahti *et al.*, 1982). Anti-FN antibodies have been successfully obtained from amphibians, fish and reptiles, in addition to mammals and birds (Hynes, 1990). Antihuman pFN antisera show a wide range of cross-reactivity with FN from other sources (Repesh *et al.*, 1981; 1982; Schoen *et al.*, 1982), although the extent of cross-reactivity decreases with increase in phylogenetic distance.

The antigenic properties of FN have been utilized extensively to study FN structure and function (Click and Balian,

1985). Monoclonal antibodies have been useful tools in elucidating the domain structure of FN. Monoclonal anti-FN antibodies have also been successfully used in localizing the gelatin-binding site as well as the carboxyl-terminal heparin binding site (Atherton and Hynes, 1981; McDonald *et al.*, 1982; Smith and Furcht, 1982). Assembly of FN is inhibited by two monoclonal anti-fibronectin antibodies, L8 and 9D2. Studies with L8 and 9D2 have defined the functional unit of FN, responsible for deposition of FN onto extracellular matrices (Chernosov *et al.*, 1991; Halliday and Tomasek, 1994). Gold *et al.*, (1983) used antiFN antibodies to determine the number of heparin-binding sites in the FN molecule with their varying affinities and Smith *et al.*, (1982) used two monoclonal antibodies 2-8 and 180-8 to identify the two sulfhydryl containing fragments of human pFN . Expression of FN by cells in culture or by tissues have been analysed by immunofluorescence and immunoperoxidase staining methods (Hsieh *et al.*, 1980 Hynes *et al.*, 1982). Immunoprecipitation was used by Tamkun and Hynes (1983) to detect and quantify FN from different sources.

Despite extensive progress in fibronectin research, neither its secondary and tertiary structures nor the structure-function relationship and the mechanism of formation of FN-fibrils have been clearly understood. In an attempt to further elucidate the above properties of FN and contribute to the existing information available, we aimed to systematically study FN from an hitherto unstudied source, buffalo plasma, by isolating, purifying and characterizing it. The data, thus obtained have been compared with available data on FN from other sources.

EXPERIMENTAL

MATERIALS AND METHODS

Materials :

A. Proteins:

Fibronectin was isolated from the plasma of buffalo blood. Blood was obtained from the local abbatoir. Bovine serum albumin, ovalbumin, catalase, β-galactosidase, phosphorylase b, thyroglobulin, ferritin and carboxypeptidase A were purchased from Sigma Chemical U.S.A. High molecular weight marker proteins were purchased from Pharmacia Biotech, Uppsala, Sweden. Human plasma fibronectin was a gift from Dr. S. A. Newman, Department of Cell Biology and Anatomy, New York Medical College, New York.

B. Reagents used for end group analysis:

Dansyl chloride, iodoacetamide and standard kit of dansyl derivatives of amino acids were purchased from Sigma Chemical, U.S.A. Organic solvents (benzene, amyl alcohol, N-butanol, tertiary butanol, pyridine, toluene, acetone, methanol, ethanol, chloroform, formic acid and glacial acetic acid) were purchased from BDH, India. Micropolyamide sheets (5 × 5 cm) were obtained from Pierce Chemical, U.S.A.

C. Column chromatography media:

Gelatin-agarose, heparin-agarose, sephacryl S-300 HR, sepharose CL-6B, MB-3 were purchased from Sigma Chemical U.S.A. Blue dextran-2000 was purchased from Pharmacia Biotech, Uppsala, Sweden.

D. Reagents used for polyacrylamide gel electrophoresis:

Acrylamide, N-N′ methylene bisacrylamide, N,N,N′,N′-tetramethylenediamine, 2-mercaptoethanol, Coomassie Brilliant Blue R-250, glycine, tris-(hydroxymethyl)-aminomethane, sodium dodecyl sulfate were obtained from Sigma Chemical,

U.S.A. Ammonium persulfate, sucrose and glycerol were from E. Merck, India. Bromophenol blue was from Sigma Chemical, U.S.A. PhastGel gradient 4-15 was from Pharmacia.

E. Other regeants:

Standard buffer tablets of different pH, trichloroacetic acid, D-glucose, orthophosphoric acid, ethylenediamine tetraacetic acid disodium salt, polyethylene glycol 200 and 400, potassium ferricyanide, sodium nitrite, phenol, sodium citrate were products of Glaxo Laboratories, India. Sodium chloride, citric acid, ammonium sulfate and urea were purchased from E. Merck, India. Phenylmethylsulfonyl fluoride, Freund's complete adjuvant, incomplete Freund's adjuvant, dimethyl sulfoxide, 5, 5′-dithiobis-(2-nitrobenzoic acid), sodium azide, calibration mixture for amino acid analysis were obtained from Sigma Chemical, U.S.A. Brij-35, O-pthalaldehyde were from Serva Biochemicals, U.S.A. Agarose was purchased from Spectrochem, India. Ethanol was supplied by Bengal Chemicals and Pharmaceuticals, India. Methyl cellosolve and tryptophan were purchased from Sisco Research Laboratories, India.

F. Miscellaneous:

Dialysis tubings of different diameters were purchased from Sigma Chemical, U.S.A. Millipore filters (pore size 0.45 μM), filter papers of different diameters and pH papers were obtained from Whatman, England. Nitrogen gas was supplied by I.O.L. India. Rabbits were purchased from ICAR, Shillong and housed in the departmental animal room under standard conditions of temperature and light.

METHODS **:**

1. pH measurements:

pH measurements were carried out at room temperature, using a Control Dynamics digital pH meter, model APX 175 E/C. Routine calibration of the instrument was done using standard

buffer tablets of different pH.

2. Spectrophotometric measurements:

Light absorption measurements in the ultraviolet region as well as visible region were performed on either a JASCO UVIDEC-610 double beam spectrophotometer or on a Hitachi U-2000 double beam spectrophotometer, using quartz (in UV region) or glass (in visible region) cuvettes of 1 cm path length. All measurements were done at room temperature, unless otherwise stated.

3. Spectrofluorimetric measurements:

Fluorimetric studies were performed on a Shimadzu RF540 spectrofluorophotometer, fitted with a thermoregulated sample chamber. Fused quartz cuvettes of 1 cm path length were used for all experiments.

4. Centrifugation:

Centrifugation was carried out at 4°C in either a Beckman refrigerated J2-21 centrifuge or a Remi C-24 cooling centrifuge. Microcentrifugation was done in an Appendorf 5414S microcentrifuge.

5. Lyophilization:

Lyophilization of protein samples were done in LSL Secfroid, Lyolab BII, operated between -32° C to 30°C.

6. Determination of protein concentration:

Absorbance of different protein solutions were measured at 280 nm and the values were divided by their respective specific extinction coefficient ($E^{1\%}_{1cm}$). The quotient gave the

amount of protein in g/100 ml. Light scattering corrections were routinely done by measuring the absorbance of the protein solution in the wavelength range of 340-360 nm and extrapolating the values to the absorbing region.

7. Gel filtration analysis:

i). Sepharose CL-6B:

The pre-swollen gel was extensively washed with distilled water and was degassed to remove trapped air. Glass columns, previously washed with detergent, chromic acid and water, was mounted vertically. The gel was slowly poured into the column (1.5 × 50 cm), with the help of a glass rod and allowed to settle under gravity. Hydrostatic pressure was then applied through a peristaltic pump to settle the gel at a flow rate of 30 ml/hr. The column was equilibrated by passing 4 column volumes of equilibrating buffer (10 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF/0.02% NaN_3), at a constant flow rate of 20 ml/hr.

ii). Sephacryl S-300 HR:

The pre-swollen gel was washed thoroughly with distilled water and degassed to remove trapped air. The gel slurry was poured into a vertically mounted glass column (2.6 × 96 cm) with the help of a glass rod. The gel was allowed to settle under gravity. Hydrostatic pressure was then applied through a peristaltic pump to get a flow rate of 30 ml/hr. The column was then equilibrated by passing almost 4 volumes of above equilibrating buffer. The homogeneity of the packed gel bed was checked by monitoring a narrow band of Blue dextran 2000 and $K_3[Fe(CN)_6]$. The elution volume of the former gave the void volume (V_o) and the latter gave the total volume (V_t) of the packed column. Before loading the sample, the buffer from the top of the gel was first removed carefully by suction. 2-5 ml of the sample, containing 2-5 mg protein was then layered on top

of the gel with the help of a thin tubing. The sample was then allowed to enter under gravity and then connected to a reservoir of equilibrating buffer. The column was allowed to run with a constant flow rate of 30 ml/hr. Fractions of 3 ml were collected in a Gilson-type fraction collector and absorbance was recorded at 280 nm.

8. Affinity chromatography:

Pre-swollen gelatin-agarose and heparin-agarose gels were thoroughly washed with distilled water. They were separately poured into glass columns (1.7 × 12 cm and 1.3 × 20 cm, respectively). Once the gels had settled under gravity, they were connected to peristaltic pumps to run at a flow-rate of 30 ml/hr. The gelatin-agarose column was then equilibrated by passing an excess of 75 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF/0.02% NaN_3 at the same flow-rate and the heparin-agarose column was equilibrated by passing an excess of 20 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF/0.02% NaN_3, also at 30 ml/hr.

9. Thin layer chromatography:

Thin layer chromatography was performed on micropolyamide sheets (5 × 5 cm). Samples were applied repeatedly on the plates at a height of 5 mm from the bottom edge, using capillary tubes with finely drawn tips. Spots were not allowed to spread beyond 2 mm in diameter and dried after each application. Ascending chromatography was then carried out in a 150 ml chromatographic chamber, containing the appropriate solvent system [i)1.5% formic acid; ii) benzene: acetic acid :: 9 : 1 (^{v}/v); iii) ethyl acetate : acetic acid : methanol :: 20 : 1 : 1 (^{v}/v); iv) 0.05 M sodium phosphate in 25% aqueous ethanol.]. After completion of the run, the plates were removed, dried and the samples were detected as fluorescent spots under an UV lamp. R_f values were computed by dividing the distance

moved by the samples with that of the solvent front.

10. Gel electrophoresis:

Electrophoresis on polyacrylamide gels were done in the absence and presence of SDS, according to the method of Davies (1964) and Laemmli (1970) respectively, in a Bio-Rad Mini Protean II slab gel electrophoresis apparatus as well as Pharmacia Phast System.

i). Polyacrylamide gel electrophoresis (PAGE):

Clean glass plates were mounted on stands vertically. A gel mixture was prepared by mixing one part of solution A (containing a molar ratio of acrylamide : N,N′-methylenebisacrylamide of 29 :1), 2.5 parts of solution B (1.5 M tris-HCl pH 8.8, containing 0.11 ml of TEMED) and 2.5 parts of freshly prepared APS solution (2.8 mg/ml) to give a cross-link of 5%. The solution was degassed for 5 min and poured between the plates, followed by insertion of the comb. The gel was allowed to polymerize for 60 min and was pre-run by applying a current of 15 mA for 15 min. After completion of pre-run, 5-15 µl of sample(10-20 µg protein), containing 40% glycerol and a small amount of bromophenol blue was gently applied on to each slot with a Hamilton syringe. Electrophoresis was carried out with an anodic current of 30 mA for 45 min, using tris-glycine buffer, pH 8.3 (prepared by dissolving 2.88 g glycine and 0.6 g tris in 1000 ml distilled water). The gel was stained with Coomassie Brilliant Blue R-250 (prepared by dissolving 0.25 g of Coomassie Brilliant Blue in 100 ml of methanol/water/ acetic acid in a ratio of 4.5/4.5/1) for 45 min. Destaining was carried out in a solution of methanol/water/acetic acid in the above ratio until the background was clear.

Alternatively, the gel was stained with silver nitrate by the method of Sammons(1984). The staining procedure was done at room temperature. After electrophoresis, the protein

bands were fixed by immersing the gel in a solution of ethanol/glacial acetic acid/water in a ratio of 3/1/6 for 12 hr with gentle shaking. Next, the gel was immersed in 30% ethanol for 30 min This was followed by thorough washing of the gel in distilled water. Silver nitrate solution (0.1%, freshly diluted from a 20% stock), was added, incubated for 30 min with gentle shaking. After washing the gel a freshly made aqueous solution of 2.5% Na_2CO_3 and 0.02% formaldehyde was added and incubated until stained protein bands appeared. After obtaining the desired contrast, the reaction was quenched by adding 1% acetic acid, incubated for a few minutes and finally washed thoroughly with distilled water.

ii) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE of proteins was performed in presence of 10% SDS and a discontinuous buffer system. A 6% resolving gel was prepared by taking 2.08 ml water/0.8 ml 30% acrylamide/1.04 ml 1.5 M tris-HCl, pH 8.8/0.04 ml 10% SDS/0.04 ml 10% freshly prepared ammonium persulfate. After degassing the contents, 2 µl of TEMED was added, swirled rapidly and poured between the glass plates. The acrylamide solution was overlayed with water and polymerization was allowed to proceed. After polymerization, the water from the gel top was drained off and layered with 3% stacking gel, prepared by mixing 1.4 ml water/0.33 ml 30% acrylamide/0.25 ml 1.0M tris-HCl, pH 6.8/0.02 ml 10% SDS/0.02 ml 10% ammonium persulfate (freshly prepared). After degassing the mixture for 5 min, 2 µl of TEMED was added and polymerization was allowed to proceed at room temperature. Protein samples (10-20 µg) were prepared by heating them to 100°C for 5 min in SDS sample buffer (50 mM tris-HCl, pH 6.8, containing 100 mM β-mercaptoethanol/2% SDS/0.1% bromophenol blue/10% glycerol). The samples were carefully loaded onto the slots. Electrophoresis was performed in tris-glycine buffer, pH 8.3, containing 1% SDS, with a constant

current of 30 mA. The gel was stained with Coomassie Brilliant Blue, R-250, and finally destained with a destaining solution containing methanol 45%, water 45% and acetic acid 10%. The relative mobilities (R_m) of the electrophoresed protein samples were determined using the expression:

$$R_m = \frac{\text{Distance traversed by the protein band (cm)}}{\text{Distance traversed by the dye band (cm)}}$$

iii) Sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis:

SDS-PAGE of buffalo plasma fibronectin in a gradient gel (PhastGel gradient 4-15) was performed in a Pharmacia Phast System using pre-cast PhastGel gradient media. The gel had a 13 mm stacking gel zone and a 32 mm continuous 5-15% gradient gel zone with a continuous 1-2% gradient cross-linker. The buffer system in the gel was 0.112 M acetate (leading ion)/0.112 M tris pH 6.4. The gel was run with PhastGel SDS buffer strips containing 0.20 M tricine/0.20 M tris pH 8.1/ 0.55% SDS in 3% agarose. To the protein solution (1 mg/ml) in 10 mM tris-HCl, pH 8.0/1 mM EDTA, 2.5% SDS and 5% 2-mercaptoethanol was added and heated at 100°C for 5 min. Bromophenol blue, (stock solution was prepared by dissolving 1 tablet of Phast Gel Blue R in 80 ml distilled water and 120 ml methanol) was added to the sample at a concentration of 0.01%. The gel was run for an hr at constant voltage and at 15°C. After completion of run, the gel was transferred to to staining-destaining chamber and stained with 0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid in distilled water from the stock solution for 8 min. The gel was destained with a solution of methanol/acetic acid/water in a ratio of 3/1/6 for 23 min and preserved in a solution of glycerol/acetic acid/water in a ratio of 5/1/4 for 5 min.

11. Isolation and purification of plasma fibronectin:

a). Isolation :

Blood obtained from freshly slaughtered buffaloes was collected in glucose-citrate buffer, pH 6.1 containing 10 mM EDTA in a ratio of 9:1 (v/v). It was centrifuged at 9,000 × g for 30 min at 4°C. The plasma (supernatant) was collected with freshly prepared 0.2 M PMSF in 95% ethanol and was stored either at 4°C or processed immediately for purification as stated below.

b) Gel filtration on Sepharose CL-6B :

The plasma was passed through a sepharose CL-6B column (1.5 × 50 cm), equilibrated with 10 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF (in 95% ethanol)/0.02% NaN_3. The flow-through was collected and used for affinity chromatography.

c) Affinity chromatography with gelatin-agarose :

The plasma collected after gel-filtration was applied to a gelatin-agarose affinity column (1.7 × 12 cm) previously equilibrated with 75 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF (in 95% ethanaol)/0.02% NaN_3. After washing the column with the same buffer, the bound protein was eluted with 4M urea in 10 mM tris-HCl, pH 7.4/0.15 M NaCl/ 5 mM EDTA/2 mM PMSF/0.02% NaN_3. Protein fractions (3 ml) were collected monitored spectrophotometrically and pooled together.

d). Affinity chromatography with heparin-agarose :

The pooled fractions collected after affinity chromatography with gelatin-agarose were applied onto a heparin-agarose column (1.3 × 20 cm), prewashed with 20 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF/ 0.02% NaN_3. The column was eluted with 0.5 M NaCl in 20 mM tris-HCl, pH7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF/0.02% NaN_3. The eluted fractions (3 ml) were pooled together dialysed against 10 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM

PMSF/0.02% NaN_3 and was either used directly for experiments or concentrated by the addition of solid ammonium sulfate to 40% saturation. The solution was centrifuged at 2000 × g for 20min at 4°C. The pellet was redissolved in 10 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF/0.02% NaN_3, dialysed against the same buffer and was used for experiments or was lyophilized and stored at -70°C.

12. Chemical analyses:

a). End group analyses of plasma fibronectin:

i).Identification of amino-terminal residue :

Determination of amino-terminal residue of purified plasma fibronectin from buffalo was done according to the method of Gray (1967). To 1 ml protein solution (containing 250 µg of plasma fibronectin), urea and sodium bicarbonate were added to a final concentration of 8M and 5M, respectively and incubated at 37°C for 1 hr. Dansyl chloride, 1 ml(20 mg/ml in acetone) was then added to the above protein mixture and allowed to incubate overnight at 37°C. The entire mixture was then dialysed thoroughly against distilled water to remove unreacted dansyl chloride, salt and other low molecular weight substances.

The dansylated protein was taken in a tube with 5.7N freshly distilled HCl. The open end of the tube was sealed and protein hydrolysis was carried out at 105-110°C for 20 hr in a Pierce Reacti-therm heating/stirring module. The contents were evaporated to dryness and the residues were dissolved in 50% aqueous pyridine solution. Identification of the dansylated amino-terminal residue was done with thin layer chromatography (TLC) on polyamide sheets by comparing with standard dansylated amino acids.

ii). Identification of carboxyl-terminal residue :

The carboxyl-terminal amino acid residue of buffalo plasma fibronectin was determined by the enzymatic method of Narita (1970). To 1 ml of protein solution (1 mg protein), 0.24 g of urea, 6.03 mg of iodoacetamide and 10.05 µl of β-mercaptoethanol to give a final concentration of 6 M urea, 0.05 M iodoacetamide and 1% β-mercaptoethanol. The reaction mixture was incubated overnight at 37°C. The solution was extensively dialysed against tris-HCl buffer, pH 8, containing 6 M urea. A solution of carboxypeptidase A was prepared by suspending 10 mg (equivalent to 500 units) of the enzyme in 10 ml of distilled water. The suspension was centrifuged in Eppendorf microcentrifuge at 5000 rpm for 10 min at room temperature and the supernatant was discarded. This washing was repeated 5 times and the residual pellet was placed in a water bath with added 0.1 ml of 1% sodium bicarbonate. This was followed by gradual addition of 1 N NaOH to dissolve all the enzyme crystals and finally the pH of this solution was adjusted 8 by titrating with 0.1 N HCl. The concentration of the enzyme solution was determined using a molar extinction coefficient of 8.6×10^4 at 278 nm (Neurath, 1955). The molecular weight of the enzyme was taken to be 34 kDa (Narita, 1970).The enzyme solution was diluted to 1 mg/ml with tris-HCl buffer, pH 8.

The protein solution of purified plasma fibronectin and carboxypeptidase A were mixed in a molar ratio of 1 : 200 and the enzymatic reaction was allowed to proceed at 37°C. Aliquots (5 ml) of the reaction mixture were pipetted out at time intervals of 5, 15, 30, 45, 60, 120, and 150 min. The reaction was stopped by adding 0.1 N HCl upto pH 2. Control (0 min) was prepared in a similar manner, except that HCl was added to the enzyme prior to addition of plasma fibronectin. The acid precipitated protein was removed from each aliquot by centrifugation and the supernatant was analyzed for amino acid using TLC, after dansylation.

b). Determination of free sulfhydryl group(s):

The free sulfhydryl content of purified buffalo plasma fibronectin was estimated according to the method of Ellman (1959). Ellman's reagent was prepared by dissolving 10 mM 5, 5-dithiobis(2-nitrobenzoic acid), (DTNB) in 10 mM tris-HCl, pH 8, containing 0.1 mM EDTA. Samples of purified plasma fibronectin (1.5-2 mg/ml) in 10 mM tris-HCl, pH 8 were denatured with 6.3 M urea for 90 min at room temperature. A reaction mixture of 1 ml protein solution and 1.97 ml of the above buffer was mixed gently and placed in the sample cuvette. The reference cuvette contained 2.97 ml buffer. Then, at zero time, 0.03 ml of 10 mM DTNB solution was added to both the sample and reference cuvette to make up the final volume of the reaction mixture to 3 ml and the change in absorbance at 412 nm was determined. Free sulfhydryl groups were calculated using a molar extinction coefficient of 13,600 M^{-1}, cm^{-1} for the ρ-nitrothiophenol anion.

c). Determination of total carbohydrate content:

The total carbohydrate content of buffalo plasma fibronectin was determined by the standard procedure of Dubois et al. (1956). The total sugar content was expressed in terms of D-glucose equivalent , determined with the help of a standard curve prepared with glucose solution.

To 1.0 ml of the protein solution (containing varying amounts of the protein), 1.0 ml of 5% $^{w}/_{v}$ phenol solution was added and mixed thoroughly. Concentrated sulphuric acid (5.0 ml) was added carefully and the reaction mixture was stirred properly. After colour had developed at room temperature, the absorbance was measured at 485 nm against a suitable blank.

d). Determination of tryptophan residues:

Total tryptophan residues of plasma fibronectin was determined colorimetrically by the method of Spies and

Chambers (1949), using p-dimethyl aminobenzaldehyde (DAB). To 0.5 ml of the salt free protein solution (containing known amount of protein), 4.0 ml of 23.8 N sulphuric acid was added and mixed thoroughly. Freshly prepared 0.5 ml DAB solution (30 mg/ml in 2.0 N sulphuric acid) was then added to it and mixed well. The reaction was carried out for 18 hr at room temperature in the dark. Thereafter, the reaction was stopped by the addition of freshly prepared aqueous solution of 0.045% sodium nitrite. After further incubation for 45 min in the dark, the absorption of the solution was measured at 590 nm against a suitable reagent blank. The tryptophan content was calculated from the standard curve, prepared as described using known tryptophan concentrations.

13. Determination of specific extinction coefficient:

Buffalo plasma fibronectin was extensively dialyzed against deionized millipore water and passed through a column of mixed bed resin of Amberlite MB-3. The pH of the eluent containing the protein was then directly measured to get its isoionic pH. The absorbance of the protein solution, thus obtained, was measured at 280 nm. Known volumes of this solution were taken in different pre-weighed weighing bottles which were then heated to dryness at 105-110°C. The bottles were weighed repeatedly (after heating and cooling, each time), at fixed intervals of time until a constant weight was obtained. The weights of the empty bottles were subtracted from that of the respective bottles containing protein, to give the actual weight of the protein taken in the weighing bottle. The specific extinction coefficient ($E^{1\%}_{1cm}$) of the protein solution was calculated by dividing the absorbance of the protein solution by its weight (g/100 ml).

14. Amino acid analysis:

Amino acid analysis of purified plasma fibronectin was

done by high performance liquid chromatography (HPLC) using a Shimadzu LC-4A HPLC system fitted with a column oven (model CTO-6A), a fluorescence HPLC monitor (model, RF-530) and a chromatopac (model, CR-3A) with a single Li^+ type cation exchange column (Li ISC-07/S1504) from Shimadzu Co., Japan. The buffers used were:

1. Sample buffer: 0.2 M lithium phosphate buffer pH 2.2, containing 7% v/v ethanol. The pH was adjusted with 80% perchloric acid.

2. Solution A: 0.2 M sodium citrate buffer, pH 3.2, containing 7% v/v ethanol and 1% v/v perchloric acid.

3. Solution B: 0.6 M lithium citrate buffer, pH 10.6, containing 1.24% w/v boric acid and 3%, 4 M lithium hydroxide solution.
4. Solution C: 0.2 M lithium hydroxide solution.

5. Reaction reagent: Lithium carbonate (0.34 M), boric acid (0.12 M) and potassium sulfate (0.10 M), dissolved in distilled water.

6. OPA solution: 0.2 g o-pthalaldehyde (OPA) was dissolved completely in 3.5 ml ethanol. 0.25 g N-acetyl-L-cysteine and 1.0 ml of 10% Brij 35 solution were then added to it. The volume was made upto 250 ml with distilled water.

7. Sodium hypochlorite solution: 50 ml of sodium hypochlorite, dissolved in 250 ml of reaction reagent.

Preparation of sample for HPLC analysis:

In a hydrolysis tube, 0.3 ml protein solution (containing 0.5 mg of purified plasma fibronectin) was taken and dried under vacuum.0.5 ml freshly distilled 5.7 N HCl was added and sealed properly. Hydrolysis was performed at 110°C for 6, 12, and 24 hr, after which the HCl was evaporated by passing a stream of nitrogen through the protein-HCl solution. After the

HCl had completely evaporated,100 µl of HPLC sample buffer, pH 2.2 (described above) was added to the hydrosylate. The content was then centrifuged to remove any precipitate and finally filtered through a millipore filter (0.45 µM), before injecting into the HPLC column. The HPLC column was run at a temperature of 40°C and a pressure of 60 mm Hg. After injecting the sample, solution A was run at a maximum flow rate of 0.4 ml/hr for 40 min. Then, solution B was run, also at a maximum flow rate of 0.4 ml/hr.

Identification of amino acid residues:

Before injecting the sample, 0.3 ml of a mixture of standard amino acids was run through the HPLC column. The samples were detected by the fluorescence HPLC monitor after reaction with OPA solution and a chromatogram of the standard amino acids was obtained along with their retention times. The amino acid residues in the sample were identified by comparing their retention times with the retention times of the standard amino acids.

15. Immunological studies:

Polyclonal antibodies were raised in adult rabbits.The animals were immunized by administering 1 mg of purified buffalo plasma fibronectin with Freund's complete adjuvant subcutaneously. This was followed by two booster doses of 1 mg of plasma fibronectin with incomplete Freund's adjuvant after 30 day intervals. Serum was collected, 4 days after giving the second booster dose. The cross-reactivity of the antibody with buffalo plasma fibronectin as well as human plasma fibronectin, goat plasma fibronectin and goat cellular fibronectin was checked by Ouchterlony double diffusion technique (Ouchterlony, 1949).

Briefly, 1.5% ($^{w}/_{v}$) agarose solution in saline (0.9% NaCl with 0.02% NaN_3) was prepared by boiling it for 5-10 min. The agarose solution was uniformly layered on glass slides or on

petri dishes and was allowed to solidify at room temperature. After gelation of agarose on the slides or on petri plates, wells were made. The central well was filled with antisera. Cross-reaction with purified buffalo plasma fibronectin and other proteins were allowed to occur at 37°C, overnight and the slides/petri plates were examined for formation of precipitin lines. The slides/petri plates were then thoroughly washed with 1 M NaCl solution containing 0.02% NaN_3, stained with 0.25% Coomassie Brilliant blue R-250 and finally destained with a solution of methanol/acetic acid/water (3/1/6).

16. Thermal unfolding of plasma fibronectin:

The effect of increasing temperature at 3°C on the intrinsic fluorescence of buffalo plasma fibronectin in 10 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/0.02% NaN_3 was monitored at an excitation wavelength of 280 nm in a Shimadzu RF-540 spectrofluorophotometer, equipped with thermostated cell holders. Temperature was controlled by circulating water from a Shimadzu TB-85 thermobath. Transition temperature was calculated from a derived plot of fluorescence vs temperature.

17. Solvent perturbation studies:

Solvent perturbation on buffalo plasma fibronectin were performed using dimethyl sulfoxide (DMSO), polyethylene glycol 200 (PEG 200) and polyethylene glycol 400 (PEG 400) as perturbants at concentrations ranging from 0-25%. Samples were prepared according to the method of Herskovits (1967). Briefly, stock solutions (50% $^{v}/_{v}$) were prepared by mixing the perturbants in 10 mM tris-HCl buffer, pH 7.4/0.15 M NaCl/5 mM EDTA/0.02% NaN_3 and adjusting the pH to 7.4 with either HCl or tris base. Serial dilutions (0-25%) of the perturbant solutions were made from the stock solutions into a series of test tubes and the volumes were adjusted with the same buffer. A fixed volume of the protein solution (0.3 ml) was then added

to each of the test tubes and mixed gently. An appropriate buffer blank as well as a perturbing blank was also prepared in the same manner. Fluorescence emission was measured at an excitation wavelength of 280 nm in the presence of increasing concentrations of the three at four different temperatures, 20°, 37°, 47°and 65°C.

18. Heparin-binding studies:

a) Effect of temperature :

Heparin-agarose gel (0.4 ml) equilibrated with 10 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/ 0.02% NaN_3 was taken in a set of microcentrifuge tubes and to each tube 0.3 ml (0.21 mg) of buffalo plasma fibronectin was added. The heparin-agarose gel-protein mixture was incubated for 3 hr and then each tube was thoroughly washed with the above buffer to remove unbound plasma fibronectin. Affinity-bound fibronectin was, thereafter, incubated at various temperatures (0-50°C) for 20 min in the same buffer. The absorbance of released fibronectin, if any, was then measured at 280 nm and the amounts of plasma fibronectin was calculated in terms of µg.

b). Effect of pH :

Heparin-agarose gel (0.4 ml), equilibrated with 10 mM tris-HCl, pH 7.4/0.15 M NaCl/% mM EDTA/ 0.02% NaN_3 was taken in a set of microcentrifuge tubes and to each tube, 0.3 ml (0.27 mg) of buffalo plasma fibronectin was added. The heparin-agarose gel-protein mixture was incubated for 3 hr and then each tube was thoroughly washed with the above buffer to remove unbound plasma fibronectin. The tubes were then further incubated in 0.5 ml of the same buffer having different pHs (pH 6-9) for 60 min. The absorbance of released fibronectin, if any, was measured at 280 nm and the amounts of plasma fibronectin was calculated in terms of µg.

c) Effect of ionic strength :

Heparin-agarose column (1.3 × 15 cm) was equilibrated with 10 mM tris-HCl pH, 7.4/0.15 M NaCl/5 mM EDTA/0.02% NaN_3 and 0.2 ml of protein solution containing 2 mg protein was loaded onto the column and allowed to bind. After washing with the equilibrating buffer, the column was connected to a linear gradient of NaCl (0.15-1.00 M) in the same buffer to elute fibronectin bound to heparin-agarose. Absorbance of the eluted fibronectin was monitored at 280 nm. Ionic strength required to eluete fibronectin from heparin-agarose was calculated from the gradient elution profile.

RESULTS

A. Isolation and purification of buffalo plasma fibronectin (pFN):

Fibronectin from buffalo blood was isolated using the method of Hayashi and Yamada (1982) with certain modifications. Use of 75 mM tris-HCl buffer, pH 7.4, instead of 10 mM tris-HCl buffer, pH 7.4 increased pFN yields from the gelatin-agarose column. 4M urea in 10 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF/0.02% NaN_3 was used to elute the protein from gelatin-agarose affinity column. The elution profile is shown in Fig 2. The protein fractions obtained from the gelatin column were applied directly onto heparin-agarose affinity column, equilibrated with 20 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF/0.02% NaN_3. pFN was eluted from this column with 0.5 M NaCl in 20 mM tris-HCl (Fig 3). The fractions collected were measured spectrophotometrically at 280 nm and the protein containing fractions were pooled together and concentrated.

The polyacrylamide gel electrophoresis (5% gel at pH 8.3) of the protein thus obtained, resulted in a single band after staining with either Coomassie Brilliant Blue or silver stain (Fig 4).

B. Characterization of buffalo plasma fibronectin:

a). Molecular weight determination

i). Gel filtration :

The molecular weight of buffalo pFN was determined by gel filtration on sephacryl S-300 HR column (2.6 × 96 cm), calibrated with four standard molecular weight marker proteins (Fig 5). The elution profile of buffalo pFN is shown in Fig 6. The void volume, V_0 of the column was 196 ml as determined by

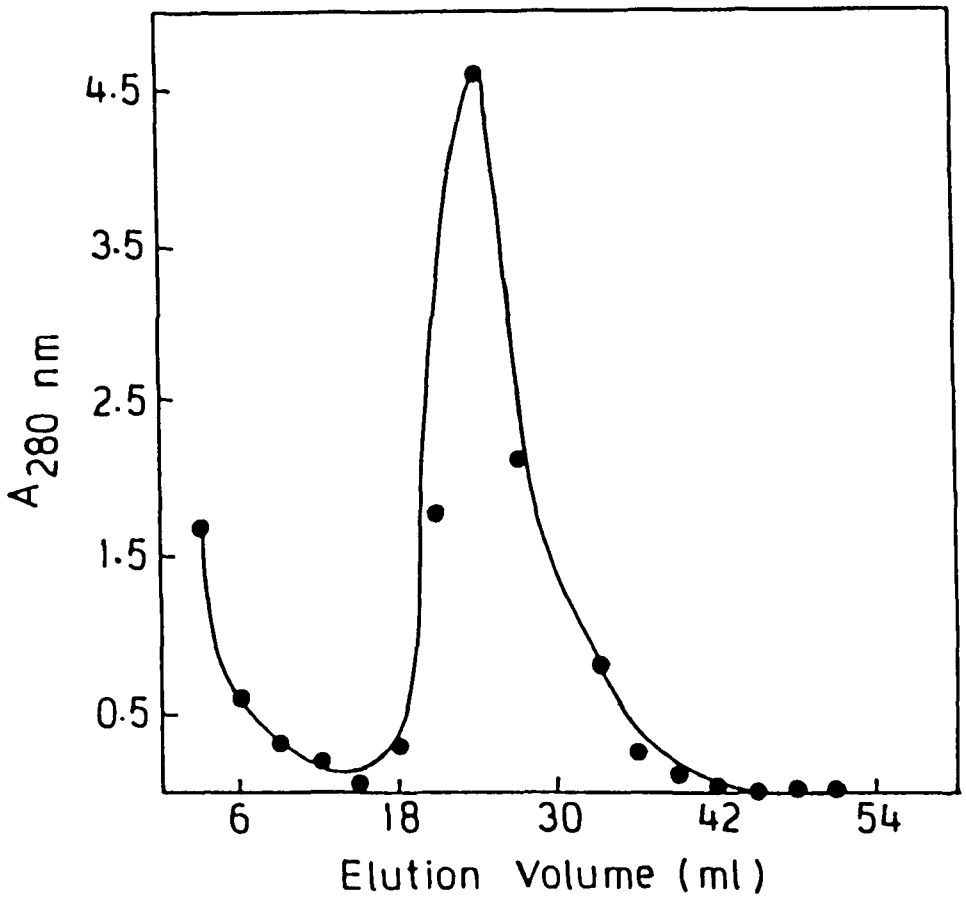


Fig 2. Elution profile of buffalo plasma fibronectin through gelatin-agarose affinity column. Protein absorbance monitored at 280 nm was plotted against elution volume. Details of experimental procedures are given in the Methods section A.

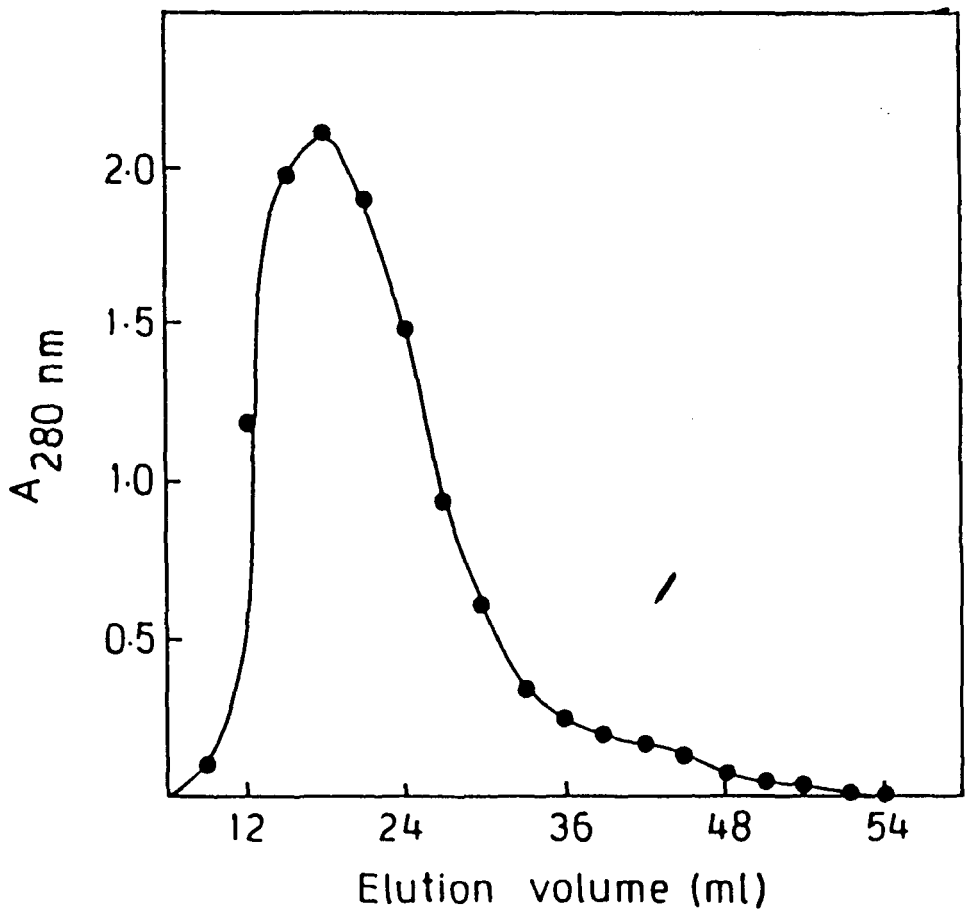
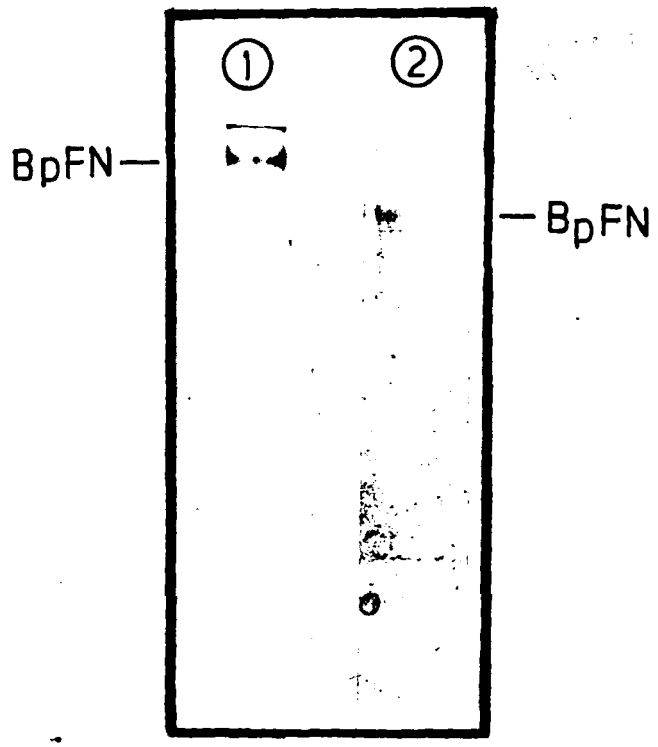


Fig 3. Elution profile of buffalo plasma fibronectin through heparin-agarose affinity column. Experimental details are as described in legend to Fig. 2.

Fig. 4. Polyacrylamide gel electrophoretic (PAGE) pattern of purified buffalo plasma fibronectin (BpFN) : Lane1 shows the position of BpFN stained with Coomassie Brilliant Blue stain. Lane 2 shows the position of BpFN stained with Silver stain.


①
②
BpFN—
—BpFN

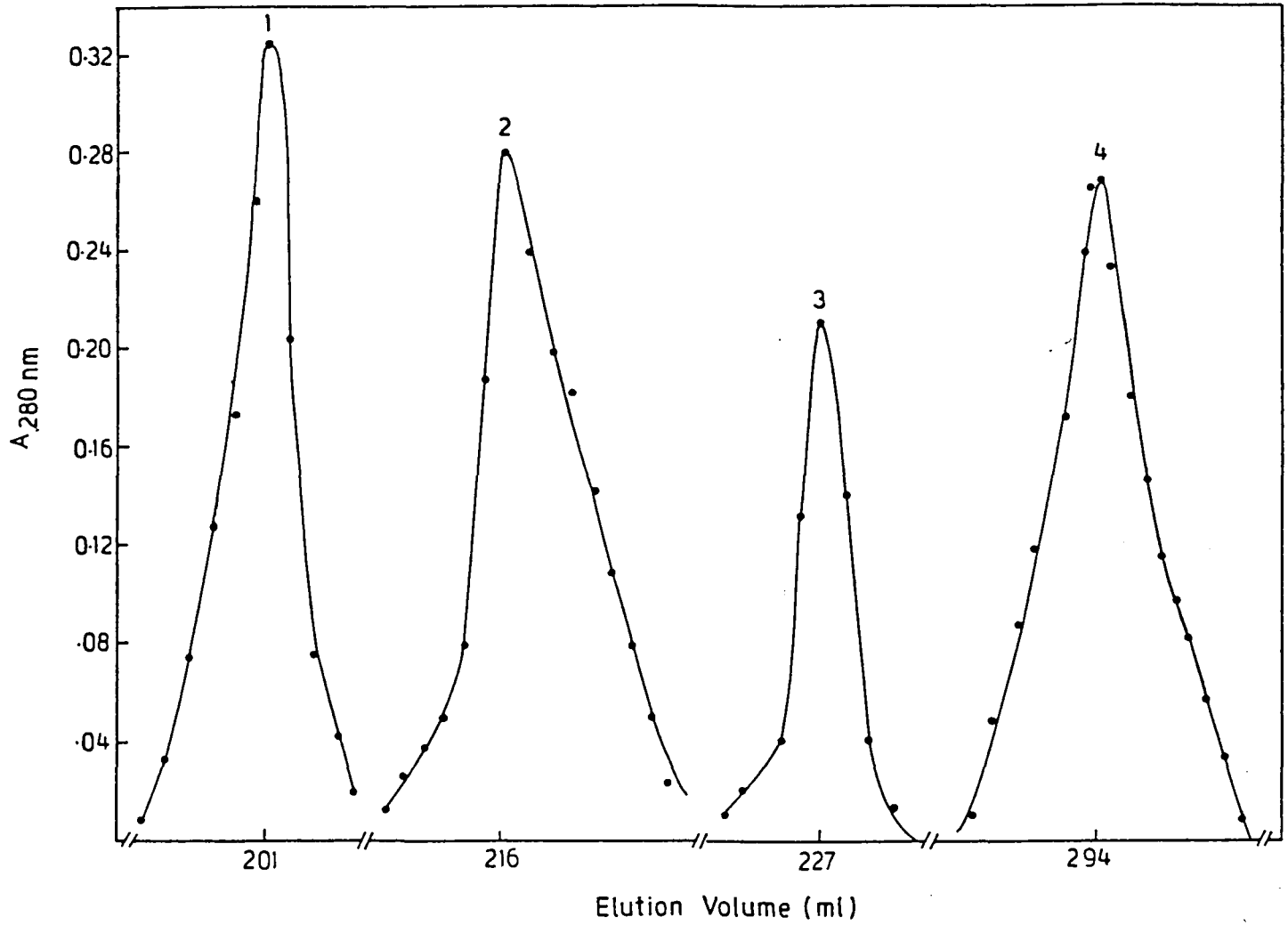


Fig. 5. Gel filtration chromatography of various marker proteins on a Sephacryl S-300 HR column. The markers used were (1) thyroglobulin, 669 kDa; (2) ferritin,. 480 kDa; (3) human plasma fibronectin, 450 kDa and (4) catalase, 232 kDa. Proteins eluted were monitored at 280 nm and plotted against elution volume. Details of other experimental conditions are mentioned in the Methods section **B. a)**.

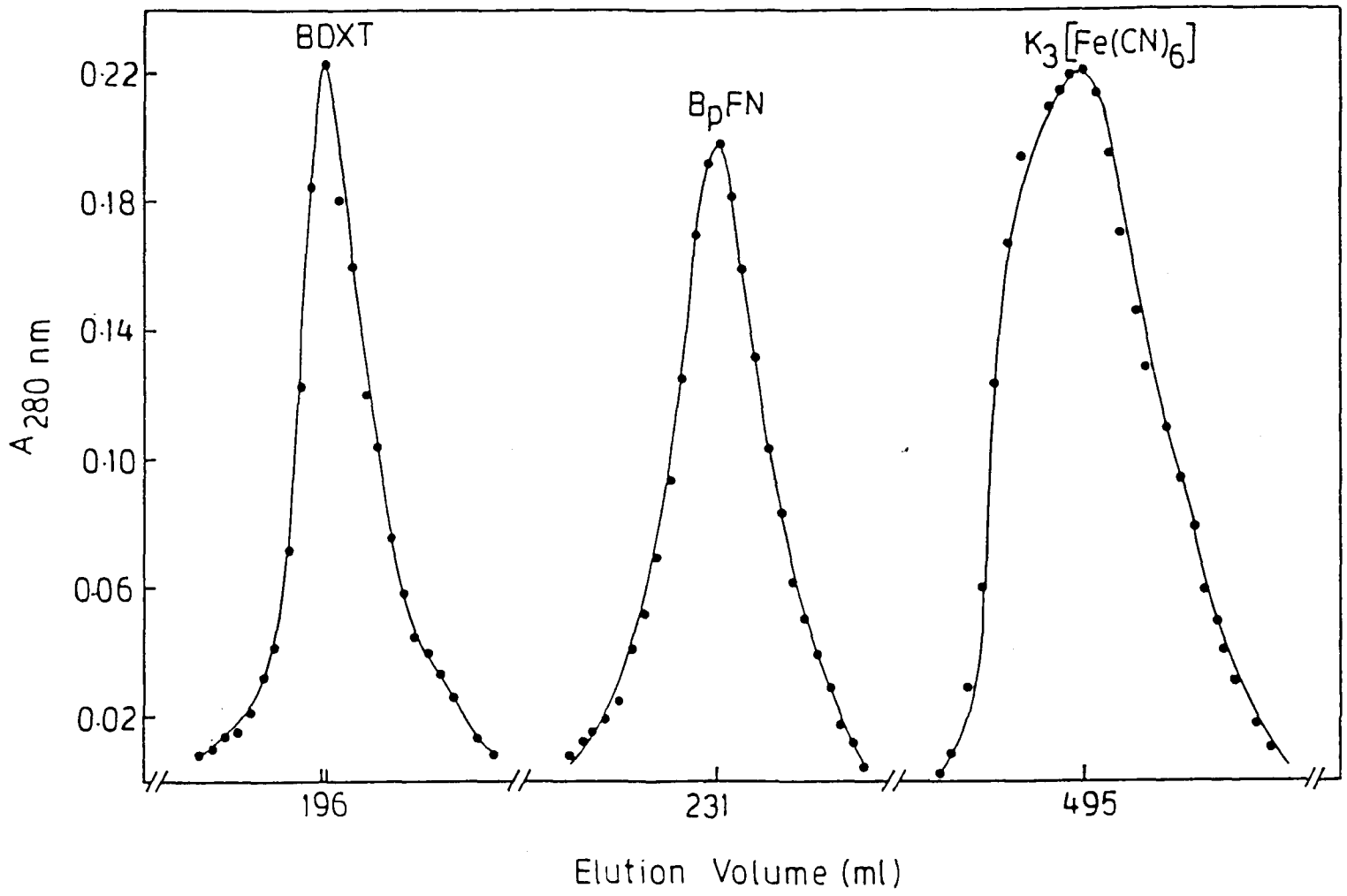


Fig. 6. Gel filtration chromatography of Blue Dextran 2000 (BDXT), buffalo plasma fibronectin (BpFN) and potassium ferricyanide $K_3[Fe(CN)_6]$ through a calibrated Sephacryl S-300 HR column. Experimental details are as described in the legend to Fig. 5.

passing Blue Dextran 2000 through the column. The elution volume, V_p of $K_3[Fe(CN)_6]$ was 495 ml.

The total volume, V_t of the column, computed using the relation $V_t=\pi r^2 l$, where r is the radius and l, the length of the column, was 509.43 ml. The elution volume, V_e of different marker proteins as well as buffalo pFN were determined from their elution profiles. The distribution coefficient, K_d and the available distribution coefficient, K_{av} were calculated with the help of Andrews (1970) and Porath (1963) equations:

$$K_d = (V_e-V_o)/V_i$$

$$K_{av} = (V_e-V_o)/(V_t-V_o)$$

where V_t and V_i are the total and inner volumes of the column respectively. The values of K_d and K_{av} were obtained by theoretical treatment of analytical gel filtration data.

The average molecular weight of buffalo pFN as determined by the plot of V_e/V_o vs log M was found to be 442 kDa whereas the plot of $M^{1/3}$ vs $Kd^{1/3}$ gave an average molecular weight of 422 kDa for this protein. The value of K_d for buffalo pFN was 0.117 and K_{av}, 0.112 (Table I and II and Fig 7 and 8).

ii). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis :

Molecular weight of buffalo pFN was also determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 6% resolving gel with a 3.5% stacking gel (Fig 9). Using the relative mobilities, R_m of different standard molecular weight marker proteins, a plot of R_m against their corresponding log of molecular weights, gave a straight line. SDS-PAGE showed two closely-spaced bands at 216.2 kDa and 208.9 kDa approximately (Fig 11 and Table III). This was further confirmed by SDS-PAGE phastgel electrophoresis on a 4-15% gradient gel using Pharmacia Phast System (Fig 10). Two bands were obtained for

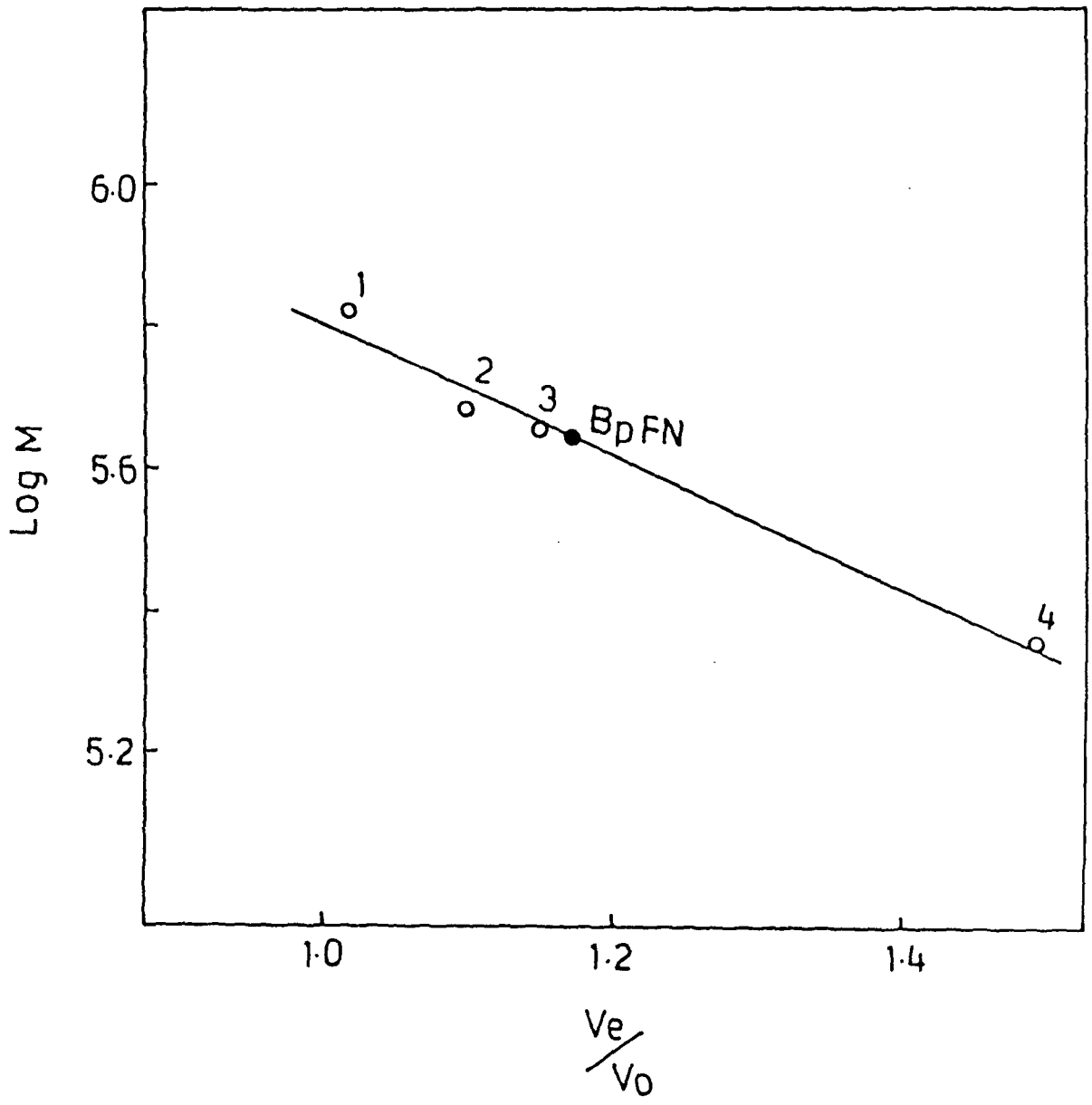


Fig. 7. Plot of elution volume/void volume (V_e/V_o) versus logarithm of molecular weight (log M). The marker proteins (1-4) are the same as given in the legend to Fig.5. The straight line was drawn by the method of least squares which fits the equation : Log M = 6.716 - $0.910V_e/V_o$.

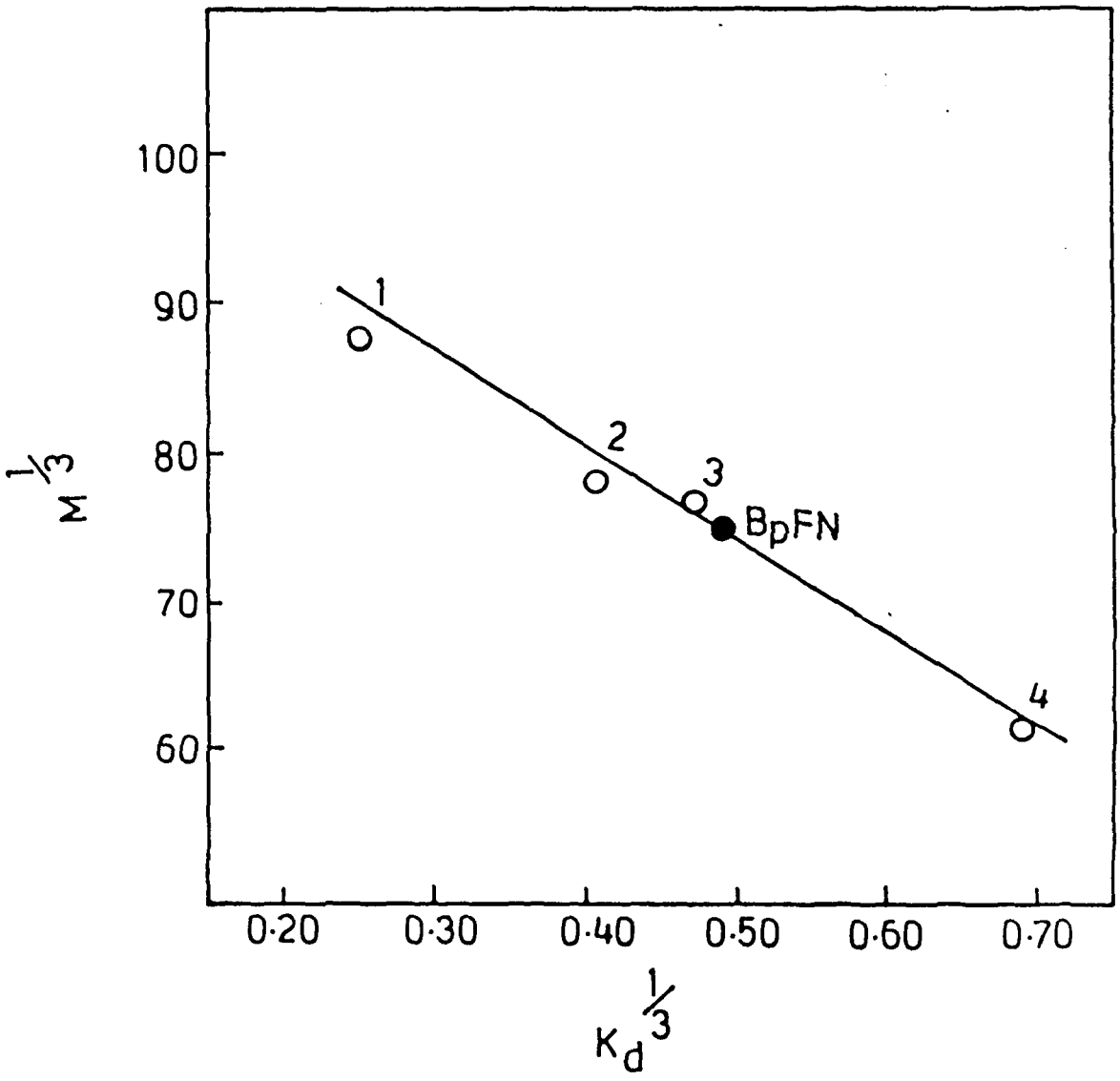


Fig. 8. Porath plot for the determination of molecular weight of buffalo plasma fibronectin (BpFN). The marker proteins (1-4) are same as described in the legend to Fig.5. The linear plot obtained by the method of least squares fits the equation $M^{1/3} = 103.092 - 59.605\ K_d^{1/3}$.

Table I

Hydrodynamic parameters of standard marker proteins and buffalo plasma fibronectin determined from analytical gel filtration

Protein	V_e/V_0	K_d	$K_d^{1/3}$	K_{av}	$(-\log K_{av})^{1/2}$	$\text{erfc}^{-1} K_d$
Thyroglobulin	1.025	0.016	0.255	0.016	1.340	1.647
Ferritin	1.102	0.066	0.405	0.064	1.093	1.297
Human pFN	1.158	0.103	0.469	0.099	1.002	1.144
Catalase	1.500	0.327	0.689	0.313	0.710	0.689
Buffalo pFN	1.178	0.117	0.489	0.112	0.975	1.102

Table II

Molecular weight and Stokes radii of standard marker proteins and buffalo plasma fibronectin		
Protein	Molecular weight [a] (kDa)	Stokes radius (A^0)
Thyroglobulin	669	85
Ferritin	480	70
Human pFN	450 [b]	
Catalase	232	52
Buffalo pFN	442 [c]	64.8 [c,d]
		66.3 [c,e]

a: Andrews, 1970
b: Khan, 1990b
c: Determined in this study
d: Laurent and Killander, 1964
e: Ackers, 1967

Fig. 9. SDS-PAGE (6%) of purified buffalo plasma fibronectin (BpFN). Positions of various standard molecular weight marker proteins (β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; BSA, 66 kDa; catalase, 58 kDa; ovalbumin 45 kDa) are depicted at the left. Lane 1 shows the position of human plasma fibronectin, 225 kDa and lane 2 represents buffalo plasma fibronectin.

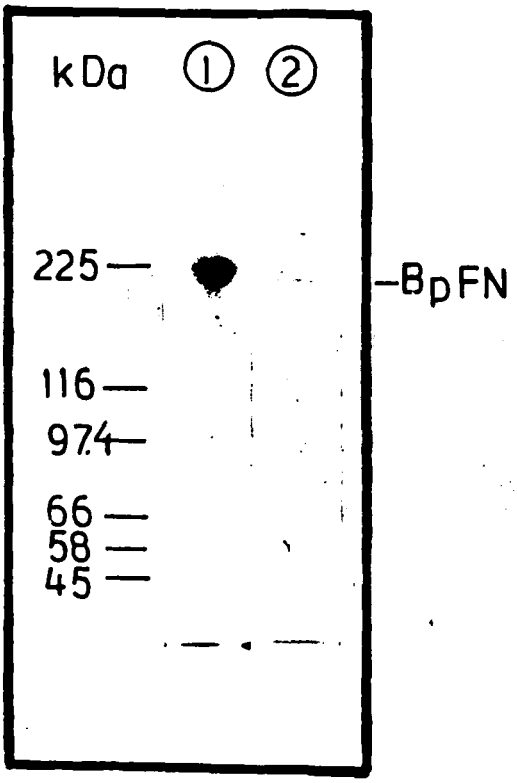

kDa
①
②
225
116
97.4
66
58
45
BpFN

Fig.10. SDS-PAGE of buffalo plasma fibronectin (BpFN) with PhastGel (4-15) gradient. Samples from left to right : Pharmacia Biotech high molecular weight calibration proteins (myosin, 212 kDa; α-2 macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin 76 kDa; glutamic dehydrogenase, 53 kDa), lane 1 human plasma fibronectin 225 kDa and lane 2 buffalo plasma fibronectin.

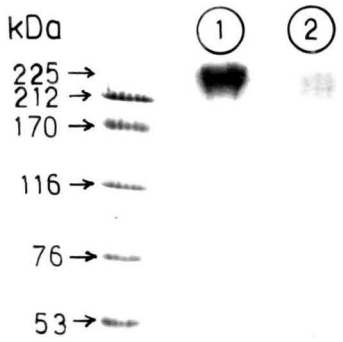

kDa
1
2
225
212
170
116
76
53

Table III

Molecular weight (M) and relative mobility (R_m) values of standard marker proteins and buffalo plasma fibronectin on SDS-PAGE

Protein	Molecular weight (kDa)	log M	R_m
Human pFN	225	5.35	0.182
β–Galactosidase	116	5.06	0.472
Phosphorylase b	97.4	4.99	0.568
BSA	66	4.82	0.781
Catalase	58	4.76	0.836
Ovalbumin	45	4.65	0.910
Buffalo pFN			
Subunit I	216.2	—	0.180
Subunit II	208.9	—	0.200

buffalo pFN and the molecular weights as determined from the plot of R_m vs log M for standard molecular weight marker proteins, were found to be 214 kDa and 204 kDa for the two subunits respectively (Fig 12 and Table IV).

The molecular weights determined both by gel filtration and SDS-PAGE was thus comparable to the values (subunit molecular weight 215-220 kDa and 400-450 kDa for the native protein), obtained for plasma FN reported earlier from other sources.

Lower values of molecular weights for the subunits obtained by SDS-PAGE, compared to a higher weight obtained by gel filtration for the native buffalo pFN indicate that the protein is hydrated under native conditions.

b). Determination of Stokes radius:

Stokes radius of buffalo pFN was determined from the data obtained by analytical gel filtration, according to the method of Laurent and Killander (1964) and Ackers (1967). The results are shown in Fig 13 and 14 and tabulated in Table II. The values obtained were 64.8Å and 66.3Å, respectively.

c). Optical properties:

UV absorption and fluorescence spectra of buffalo pFN was measured in 10 mM tris-HCl pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF/0.02% NaN_3. Two peaks were obtained in the spectrophotometric spectrum of buffalo pFN. The relevant peaks were between 240-245 nm and 277-278 nm

The fluorimetric emission spectrum gave a maxima at 315 nm when excited at 280 nm and 338 nm when excited at 300 nm. The excitation spectrum gave a maxima at 320 nm when emitted both at 315 nm and 338 nm.

The specific excitation coefficient ($E^{1\%}_{1cm}$) determined for the protein was 14.02. Human pFN has a specific extinction of 12.8. The isoionic pH of BpFN was found to be 6.12. This agreed well

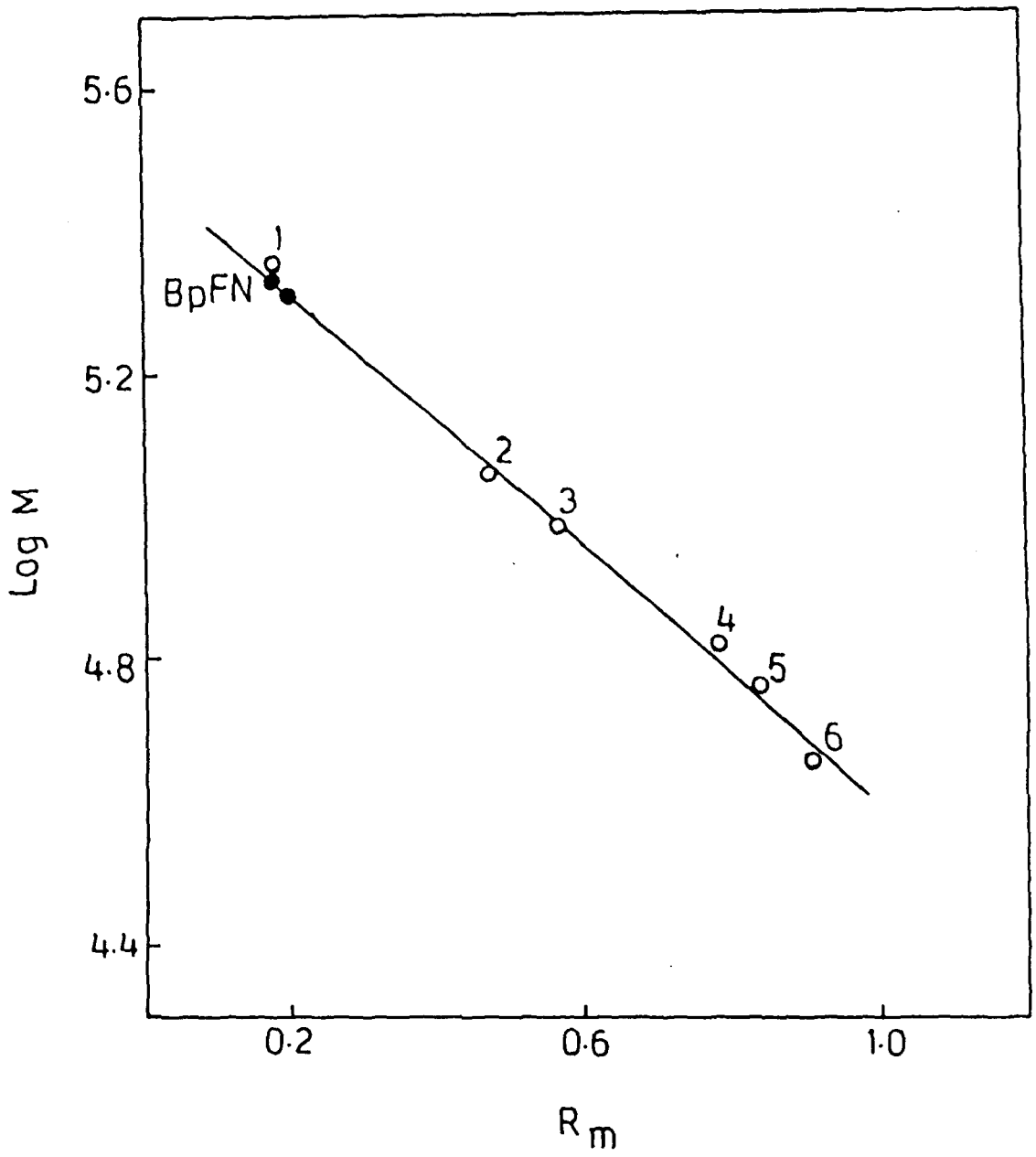


Fig 11. Determination of subunit molecular weight of buffalo plasma fibronectin (BpFN) using relative mobilities (R_m) on SDS-PAGE (6%). The marker proteins (1-6) are the same as described in the legend to Fig.9. The straight line was plotted according to the method of least squares which follows the equation log $M = 0.874R_m - 5.483$.

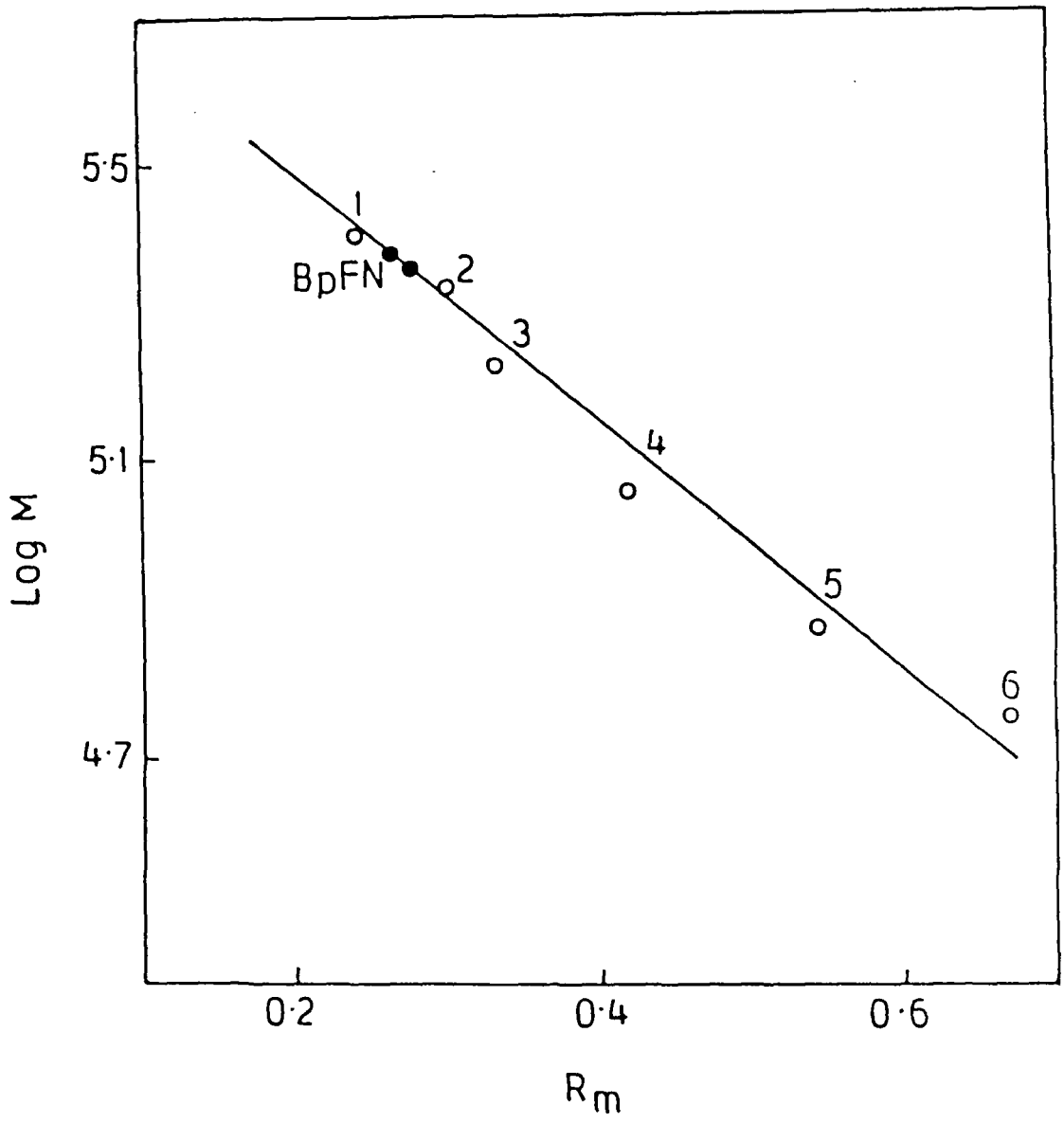


Fig.12. Determination of subunit molecular weight of buffalo plasma fibronectin using relative mobilities (R_m) on SDS-PAGE (PhastGel gradient 4-15). The marker proteins from 1-6 are the same as described in the legend to Fig.10. The straight line was plotted according to the method of least squares which follows the equation log M = 5.756- 1.576R_m.

Table IV

Molecular weight (M) and relative mobility (R_m) values of standard marker proteins and buffalo plasma fibronectin on SDS-PAGE (PhastGel gradient 4-15)

Protein	Molecular weight (kDa)	log M	R_m
Human pFN	225	5.35	0.242
Myosin	212	5.33	0.303
α_2-Macroglobulin	170	5.23	0.333
β-Galactosidase	116	5.06	0.424
Transferrin	76	4.88	0.667
Glutamic dehydrogenese	53	4.72	0.667
Buffalo pFN			
Subunit I	214	—	0.273
Subunit II	204	—	0.288

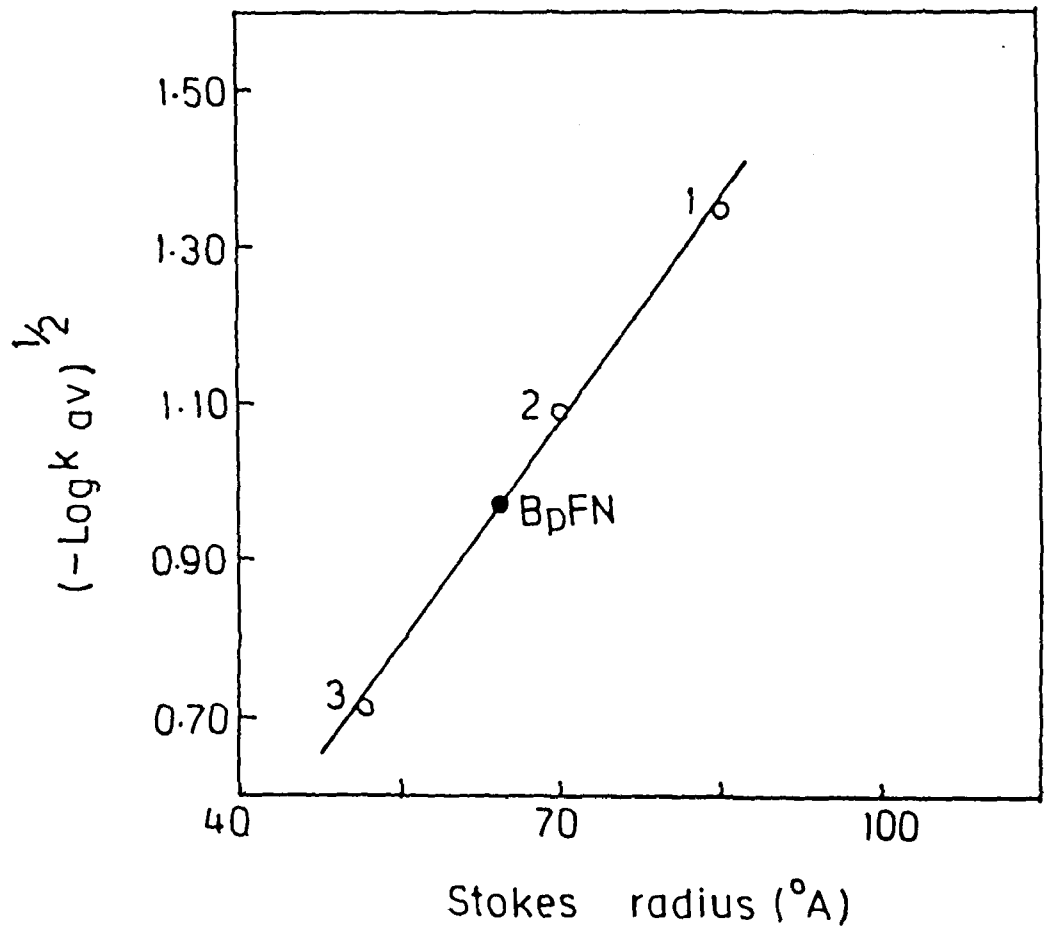


Fig.13. Determination of Stokes radius of buffalo plasma fibronectin according to the method of Laurent and Killander (1964). The marker proteins are (1) thyroglobulin; (2) ferritin and (3) catalase whose respective Stokes radii are listed in Table II. The straight line was drawn by the method of least squares which fits the equation, $(-\log K_{av})^{1/2} = 0.0192r - 0.273$, where $K_{a/v}$ represents available distribution coefficient and r, the relative mobility.

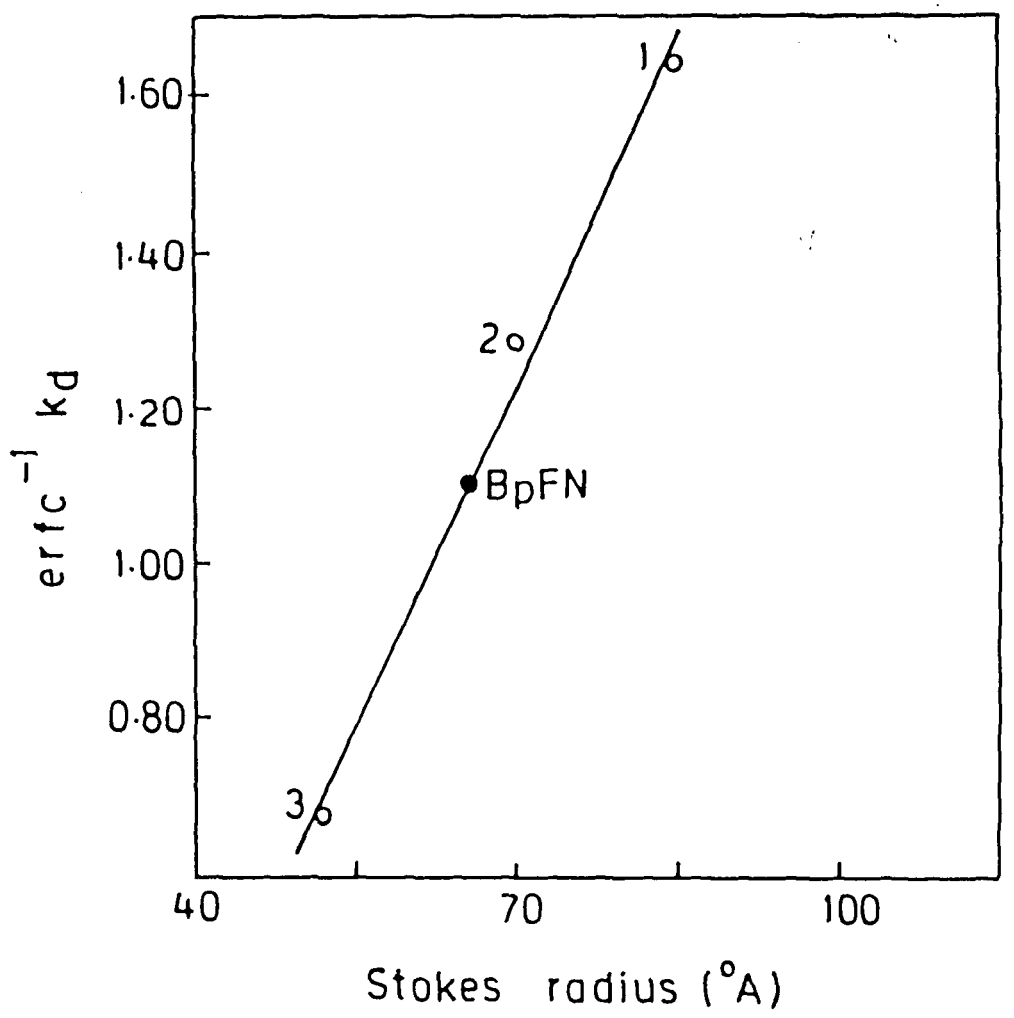


Fig. 14. Determination of Stokes radius of buffalo plasma *fibronectin* according to the method of Ackers (1967). The marker proteins are the same as described in the legend to Fig.13. The *straight line* was drawn according to the method of least squares which fits the equation $(\text{erfc}^{-1}K_d) = 0.0294r - 0.817$.

the values of 5.5-6.3 reported in other sources.

d). End group analyses:

The amino-terminal amino acid residue of buffalo pFN was identified by using dansylation method. Table V shows the R_f values of standard dansylated amino acids in four different solvent systems. The R_f value for the dansyl derivative of the amino-terminal residue of the protein was found to correspond with the value obtained for the standard dansylated derivative of alanine in the same solvent system (Table VI). These results therefore showed that the amino-terminal amino acid residue of buffalo pFN was alanine.

The carboxyl-terminal amino acid residue of buffalo pFN was identified using carboxypeptidase method. The TLC analysis of buffalo pFN on enzymatic digestion with carboxypeptidase showed only one released amino acid . A comparison of the observed R_f values with those obtained for the standard dansylated amino acids showed this amino acid residue as phenylalanine.

Hence, the amino-terminal amino acid residue of buffalo pFN differs from that of human and bovine pFN , both of which have blocked amino-terminal ends (pyrrolidone carboxylic acid and pyroglutamate residues respectively).

e). Determination of free sulfhydryl groups:

The free sulfhydryl content of buffalo pFN was determined essentially by the method of Ellman. Buffalo pFN was found to contain 1.97 free sulfhydryl groups per one subunit of the protein, under denaturing conditions with 6.3 M urea. The sulfhydryl groups of intact pFN were not accessible to titration in the absence of the denaturant. This result is quite consistent with the value reported for human pFN.

Table V

Relative front (R_f) of dansylated amino acids on polyamide TLC Plates				
	Solvent Systems			
Dansylated Amino acids	Formic acid 1.5%	Benzene: Acetic acid (9:1 v/v)	Ethylacetate: Acetic acid: Methanol (20:1:1 v/v)	0.05 M Sod. Phosphate buffer in 25% ethanol
Alanine	0.604	0.667	0.957	0.744
Arginine	–	0.578	0.468	0.558
Asparagine	0.596	0.767	0.658	–
Aspartic acid	0.312	0.233	0.786	0.953
Glutamic acid	0.532	0.333	0.689	0.930
Glutamine	0.830	0.519	–	0.837
Glycine	0.560	0.467	0.956	0.720
Isoleucine	0.257	0.953	0.977	–
Leucine	0.234	0.689	0.844	–
Methionine	0.245	0.756	0.933	0.698
Phenylalanine	0.206	0.800	0.933	0.511
Proline	0.415	0.933	0.544	–
Serine	0.768	0.256	0.041	0.837
Threonine	0.766	0.222	0.796	0.790
Tryptophan	0.128	0.422	0.889	0.395
Tyrosine	0.522	0.200	0.916	0.395
Valine	0.389	0.711	0.936	0.651

Table VI

Dansylated amino acid	Solvent Systems				
	Formic acid 1.5%	Benzene: Acetic acid (9:1 v/v)	Ethylacetate: Acetic acid: Methanol (20:1:1)	0.05 M Sod. phosphate buffer in 25% ethanol	Result
NH_2-terminal	0.628	0.644	0.956	--	Ala
COOH-terminal	--	0.790	0.927	0.500	Phe

f). Determination of the total carbohydrate content:

The total carbohydrate content was determined for buffalo pFN . It was found to contain 4.9% carbohydrate as expressed in terms of D-glucose equivalent values obtained from the standard glucose curve (Fig 15). The carbohydrate content reported from various sources was in the range 4.4-5.7%.

g). Determination of the number of tryptophan residues:

Tryptophan estimation was done according to the procedure detailed by Spies and Chambers (1949). The amount of tryptophan in buffalo pFN was found to be 73.08 moles/mole of protein as was determined from a standard curve of tryptophan (Fig. 16).

h). Amino acid analysis:

Amino acid composition of buffalo pFN was determined using high performance liquid chromatography (HPLC), on a Li^+ column. The results are summarized in Table VII. As given in the Table, the amino acid composition of the protein showed close similarities with pFN from other sources like human and bovine, except Gly, Met and Leu whose contents were significantly lower in buffalo pFN.

i). Immunological studies:

Polyclonal antibodies were raised against buffalo pFN in rabbits. Precipitin line was obtained by Ouchterlony double diffusion, when buffalo pFN antiserum reacted against purified buffalo pFN (Fig 17). Cross-reactivity of anti-buffalo pFN antibody was observed with human pFN, goat pFN and goat cFN as indicated in Fig 18.

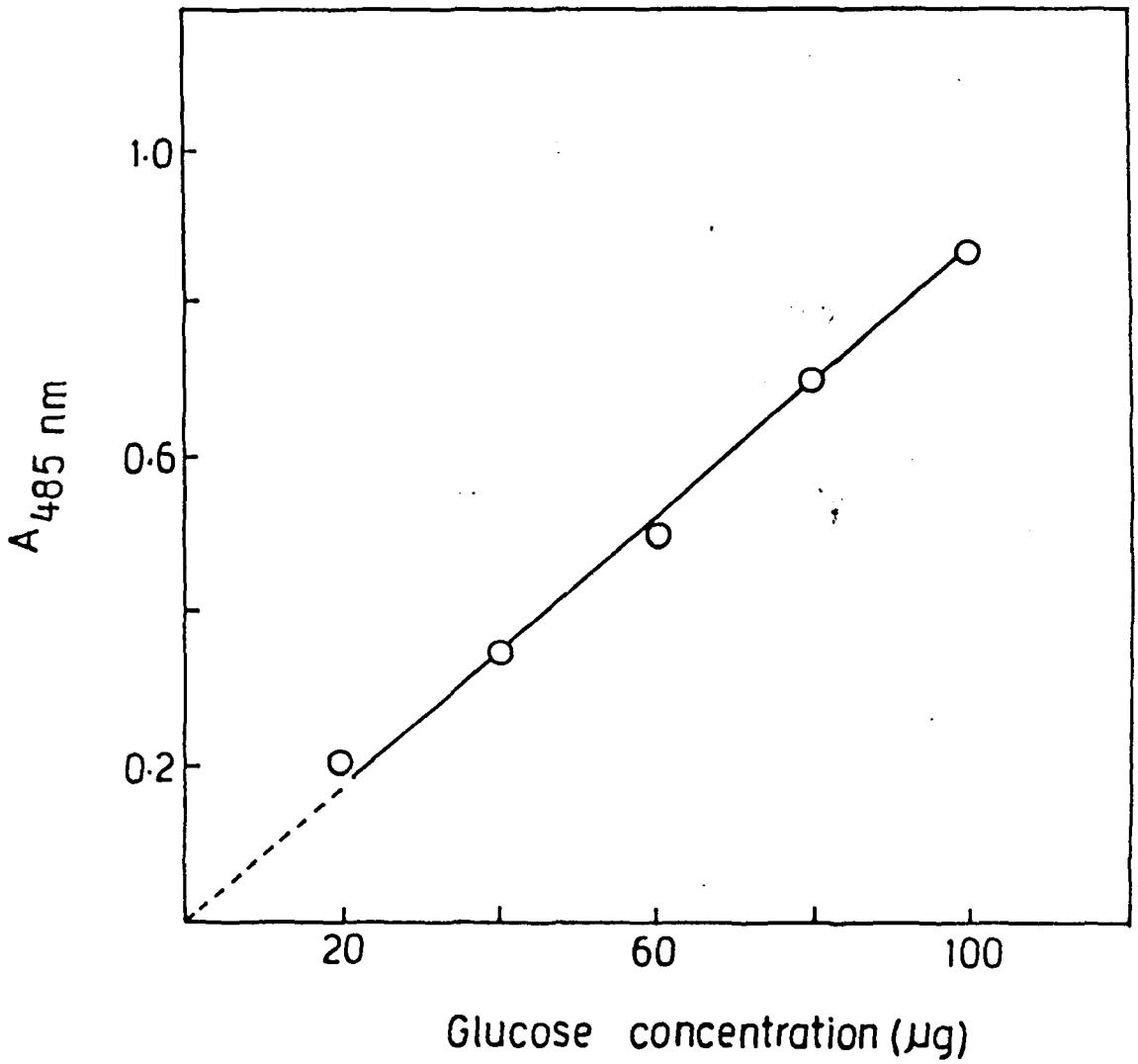


Fig. 15. Standard curve of D- glucose for the estimation of total carbohydrate content. Colour developed was measured at 485 nm and plotted against concentrations of glucose. Details of procedures are described in Methods section B. f).

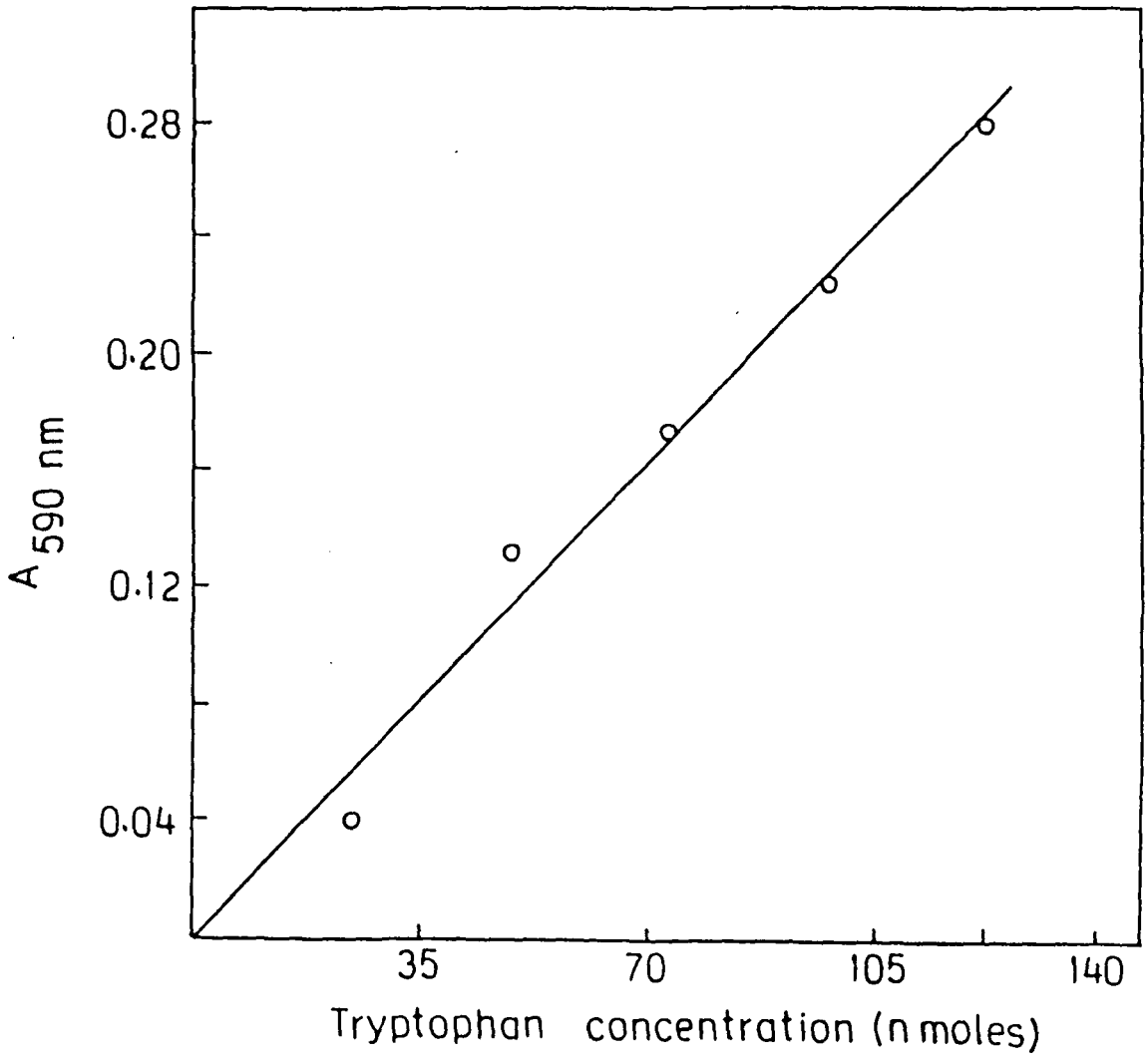


Fig. 16. Standard curve of tryptophan for the estimation of the number of tryptophan residues. Colour developed was measured at 590 nm and plotted against respective concentrations of tryptophan. details of other procedures are given in the Methods section **B. g)**.

Table VII

Amino acid composition of buffalo plasma fibronectin (pFN) determined by HPLC

Amino acids Mole %	Human pFN [a]	Bovine pFN [b]	Buffalo pFN [c]
Cys	2.6	3.3	ND
Asx	9.3	8.4	8.3
Thr	9.7	9.7	8.7
Ser	6.8	7.3	7.8
Glx	11.6	12.1	10.4
Pro	7.6	8.5	8.9
Gly	8.0	8.0	5.0
Ala	4.3	4.3	ND
Val	8.1	9.0	6.8
Met	1.1	1.1	0.1
Ile	4.4	4.4	5.1
Leu	5.7	5.7	1.5
Tyr	4.5	4.1	3.3
Phe	2.7	2.1	2.1
Lys	3.7	3.7	2.1
His	2.1	1.9	1.9
Arg	5.2	5.0	6.1
Trp	2.8	1.3	1.9 [d]

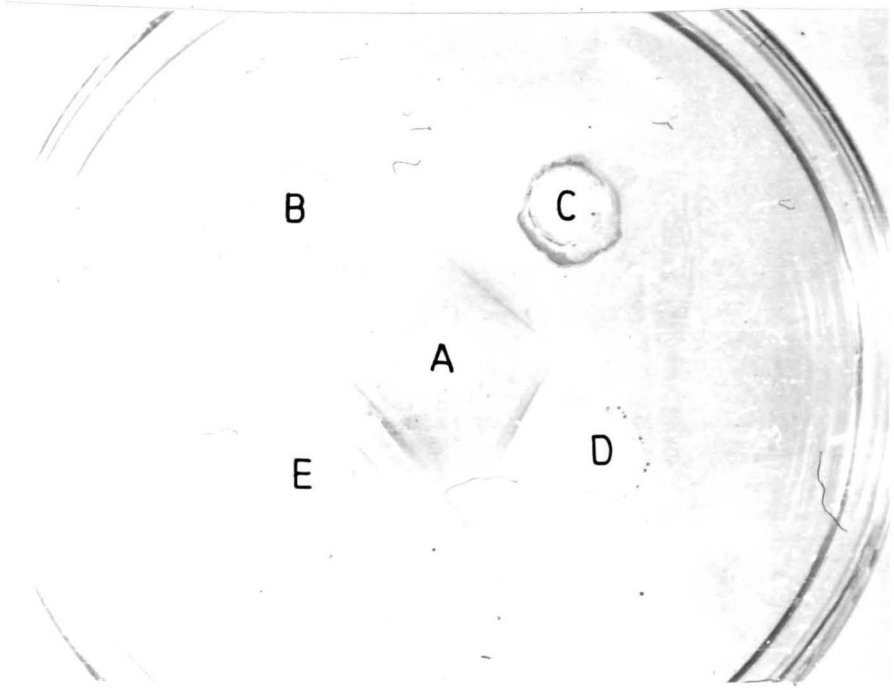
a: Mosesson *et al* ., 1975
b: Skorstengaard *et al* ., 1982
c: Determined in this study
d: Determined seperately
ND: Not determined

Fig. 17. Ouchterlony double immunodiffusion of anti-buffalo plasma fibronectin (Anti- FN) antiserum against purified buffalo plasma fibronectin (FN). Precipitin line is visible in the middle. Experimental details are given in the Methods section **B. i).**

FN

ANTI-FN

Fig 18. Ouchterlony double immunodiffusion of anti-buffalo plasma fibronectin antiserum against plasma fibronectin and cellular fibronectin. About 150 µl of antiserum was taken in the central well (A) and the same amount of purified goat cellular fibronectin (B), goat plasma fibronectin, (C) human plasma fibronectin (D) and buffalo plasma fibronectin (E) was applied in the specified wells .


B
C
A
D
E

j). Thermal unfolding of buffalo plasma fibronectin:

The effect of increasing temperature on the intrinsic fluorescence of buffalo pFN is represented graphically in Fig 19. The results showed that when temperature was increased every 3°C, the intrinsic fluorescence intensity of the protein gradually decreased and then underwent a small sigmoidal transition with a mid-point between 61°-62°C. The transition was irreversible. Upon cooling to 25°C, the fluorescence returned to a value, 79% higher than the original. Human pFN has also been reported to undergo a similar irreversible transition with a mid-point centered at around 63.5°C.

k). Solvent perturbation studies:

The effect of various perturbants (DMSO, PEG 200, and PEG 400) on the intrinsic fluorescence of buffalo pFN was studied, as a function of temperature. The results obtained are shown in Fig 20 A, B, C and D.

DMSO (MW 62.07) is a small perturbant and therefore can easily enter the protein molecule. This easy access, at 20°C caused perturbation by quenching the intrinsic fluorescence of buffalo pFN, as the perturbant concentration gradually increased to 25%. At higher temperatures (37°C and 47°C) the same extent of perturbation was achieved even at a lower DMSO concentration of 20%. At still higher temperature (65°C), only about 15% perturbant concentration was required for the same degree of decrease in intrinsic fluorescence intensity.

PEG 400 (MW 380-420) is a large perturbant and is unable to enter the interior of the protein molecule at temperatures of 20° and 37°C. At higher temperatures (47° and 65°C), buffalo pFN undergoes a conformational change and the buried fluorophores become available to PEG 400 for perturbation. Therefore, PEG 400 caused a decrease in fluorescence intensity of buffalo pFN at 65°C (Fig 20 D).

PEG 200 (MW 190-210), being a moderate size perturbant

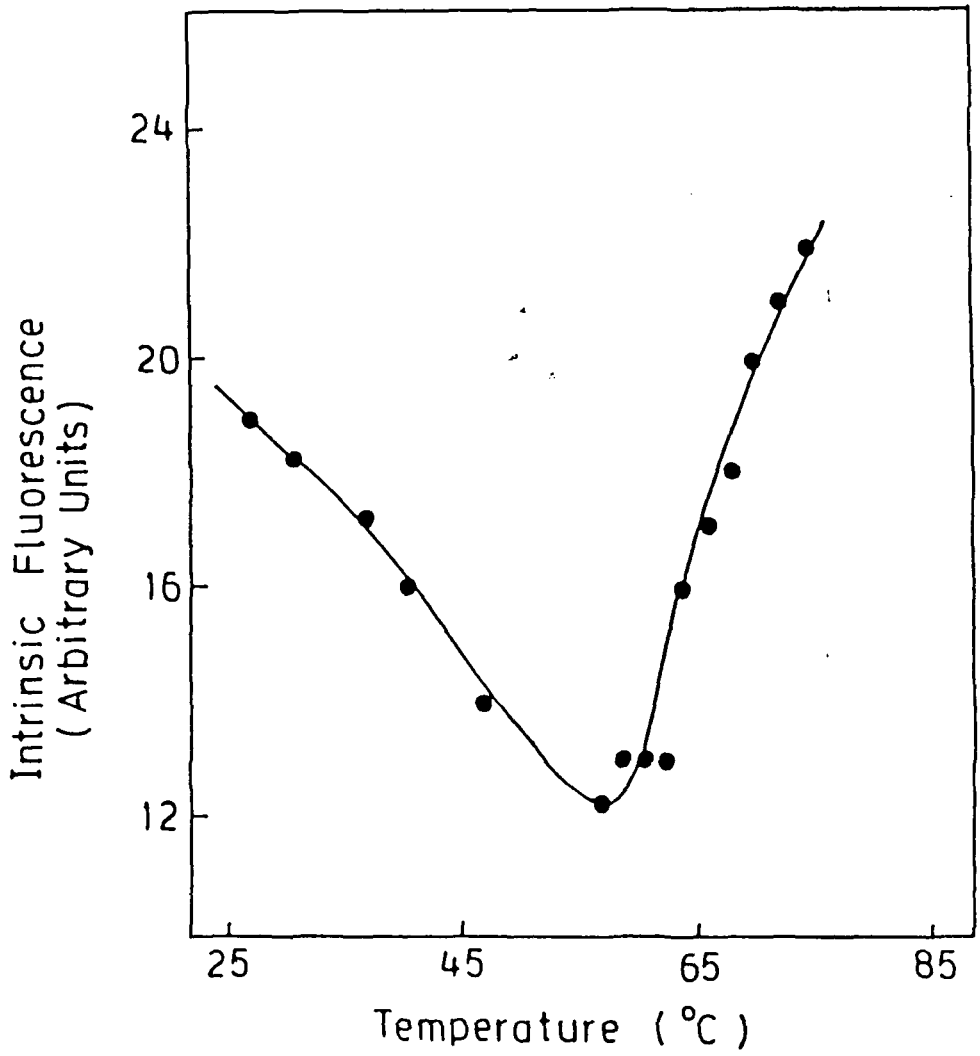


Fig. 19. Effect of temperature on the intrinsic fluorescence of buffalo plasma fibronectin.Intrinsic fluorescence was monitored every 3°C temperature. The excitation wavelength was 280 nm.

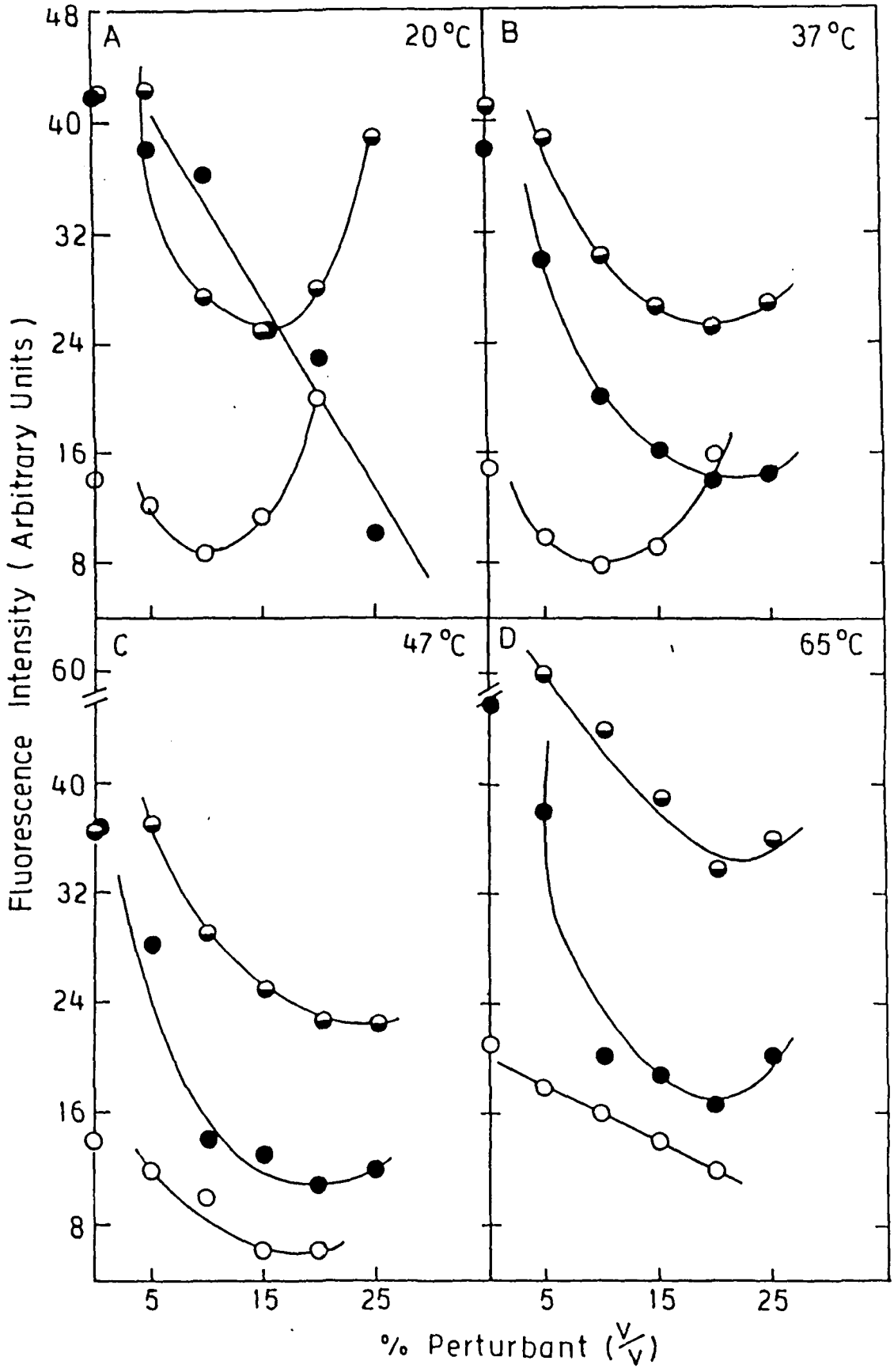


Fig. 20. Effect of perturbants, DMSO (●), PEG 200 (◒) and PEG 400 (O) on the intrinsic fluorescence of buffalo plasma fibronectin at temperatures (A) 20°C (B) 37°C (C) 47°C and (D) 65°C. In each case, 0.3 ml protein sample (2 mg/ml), was taken and the excitation wavelength was 280 nm. Details of the procedure are described in the Methods section B. j).

generated the response of perturbation, falling in between DMSO and PEG 400.

I). Heparin-binding property of buffalo plasma fibronectin:

i) Effect of temperature :

The effect of temperature on the dissociation of buffalo pFN, upon binding to heparin-agarose affinity gel was studied. It was found that even a high temperature of upto 50°C was not sufficient to cause elution of buffalo pFN. However, when the ionic strength was increased to 0.5 M NaCl from 0.15 M NaCl, buffalo pFN was released from heparin-agarose gel, even at 24°C (Table VIII).

ii) Effect of pH :

The effect of pH on the elution of buffalo pFN, upon binding to heparin-agarose gel was studied. A variation in pH between pH 6-9 caused no significant dissociation of buffalo pFN from heparin. Dissociation was, however, achieved at pH 7.4, when the ionic strength was increased to 0.5 M NaCl from 0.15 M NaCl (Table IX).

iii). Effect of ionic strength :

The effect of ionic strength on the elution of buffalo pFN , upon binding to heparin-agarose affinity gel was studied at 25°C, pH 7.4. The elution profile is depicted in Fig 21. Maximal elution of buffalo pFN was found to occur at an ionic strength of 0.65 M NaCl, when a gradient of 0.15-1.0 M NaCl was applied.

Table VIII

Effect of temperature on dissociation of buffalo plasma fibronectin from heparin-agarose gel	
Amount of BpFN bound: 480 μg	
Temperature (^{0}C)	BpFN released (μg)
24	14
26	16
28	40
30	33
32	48
35	52
37	24
40	24
42	30
45	36
50	35
24 + 0.5 M NaCl	400

Table IX

Effect of pH on dissociation of buffalo plasma fibronectin from heparin-agarose gel	
Amount of BpFN bound: 380 µg	
pH	BpFN released (µg)
6.0	32
6.5	37
7.0	31
7.5	37
8.0	41
8.5	37
9.0	32
7.0 + 0.5 M NaCl	202

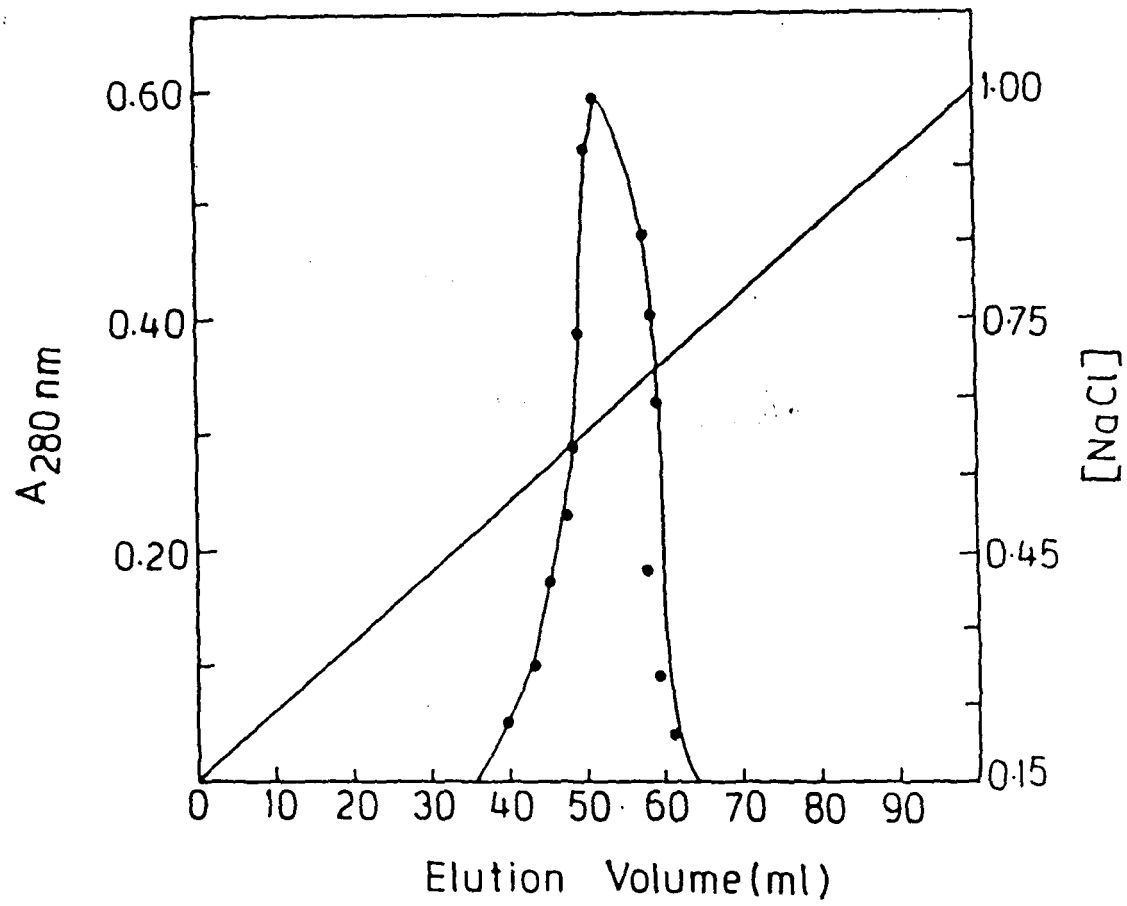


Fig. 21. Effect of ionic strength on the elution of buffalo plasma fibronectin from heparin-agarose affinity gel. Protein sample, (2 mg/ml) in 10 mM tris-HCl, pH 7.4 was loaded onto the column and was eluted with a linear gradient of 0.15 - 1 M NaCl in the same buffer. Other experimental details are given in the Methods section **B. k)**. *iii)*.

DISCUSSION

Fibronectins (FNs) are adhesive glycoproteins, found in all vertebrates. In the soluble form, it is present in the plasma as well as in other body fluids (Hynes, 1990). It also exists in an insoluble form on the surface of a variety of cells, as a component of the extracellular matrix, in basement membranes and loose connective tissues (Olden and Yamada, 1977; Yamada and Olden, 1978). Cellular and plasma FNs share common structural organization and immunological identity (McDonald and Kelley, 1980) and have similar though not identical, biological activities, solubility properties and peptide composition (Garcia-Pardo *et al.*, 1983). Both forms are large dimeric molecules, composed of similar disulfide-linked subunits of approximately 215-220 kDa each (Homandberg and Erickson, 1986). An analysis of proteolytic digests of FN reveals that each subunit is folded into a series of domains, joined by flexible polypeptide sequences (Wagner and Hynes, 1980). The biological activities of FN are diverse, which include wound healing, platelet adhesion, clot formation, cell-cell and cell-extracellular matrix interactions. These functions are related to its capacity to bind extracellular macromolecules like collagen, fibrin, proteoglycans and cell-surface receptors (Hynes, 1990). Although much information is now available on FN, its secondary and tertiary structures are as yet, unclear. In an attempt to further elucidate the above properties and contribute to the existing information, the present work was undertaken to systematically analyze FN from an hitherto unstudied source, buffalo plasma.

In the present study, modifications were incorporated in the method of Hayashi and Yamada (1982) to isolate and purify plasma FN from buffalo blood. Blood was anticoagulated with glucose-citrate buffer, pH 6.1, instead of heparin, which, apart from affecting the interaction of pFN with gelatin, also precipitates FN (Hynes, 1990). EDTA, at a concentration of 0.2 M was also added to the anticoagulant, before collecting the blood,

as this was found to keep the blood in an uncoagulated form for a longer period. After collection of blood, freshly prepared PMSF (0.2 M) was added to prevent pFN from being degraded by proteases like trypsin, thrombin, plasmin and others (Hynes, 1990).

The gelatin-agarose affinity column was equilibrated with 75 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF, instead of 10 mM tris-HCl, pH 7.4/0.15 M NaCl/5 mM EDTA/2 mM PMSF. The yield of purified buffalo pFN was comparatively low, as significant amounts were lost due to the formation of large gelatinous precipitate in the plasma, stored at low temperatures. pFN was also lost due to the formation of a stringy precipitate if stored beyond 36-48 hours, especially at low temperatures. The formation of the precipitate could, however be avoided to a large extent, if the purified protein was stored at 25°C. Previous reports have also described the formation of FN filaments, resembling *in vivo* fibrils that were produced *in vitro* by prolonged incubation at low temperatures (Homandberg and Erickson, 1986). Inspite of such precautions, some amount of aggregation was always present (observed as turbidity in the protein solution) and therefore, the protein solutions always had to be filtered, prior to commencement of an experiment. Although, the columns (sepharose CL-6B, gelatin-agarose, heparin-agarose) were run at 25°C and not under cold conditions, repacking was often necessary as the gels tended to clump after passing a few batches of plasma. The columns were also required to be washed with 1M NaCl to keep them in running condition (Hynes, 1990).

The purity of pFN, obtained after affinity chromatography on heparin-agarose was checked by PAGE which yielded a single band, both upon Coomassie Brilliant Blue staining as well as silver staining. This showed that the protein was relatively pure and homogeneous. SDS-PAGE of buffalo pFN in a homogeneous 6% gel showed that it consists of two subunits, which migrated as closely-spaced doublet. This is in agreement with previous studies wherein human pFN has also been shown to be a dimer

(Click and Balian,1985; McCarthy *et al.*, 1988). The molecular weight of the two subunits, determined by SDS-PAGE on a 6% homogeneous gel as well as on a Pharmacia PhastGel gradient (4-15%) gel were 216.2 and 208.9 kDa and 214 and 204 kDa, respectively. The molecular weight of BpFN, determined by analytical gel filtration, yielded a value of 442 kDa. The molecular weights, determined both by analytical gel filtration as well as SDS-PAGE are in agreement with the earlier reported values of 400-450 kDa for the native protein and 215-250 kDa for each of the subunits (Chen et al., 1977; Hynes, 1990). The molecular weight, determined by gel filtration was found to be comparatively higher than that determined by SDS-PAGE. This overestimation could be due to the enhanced tendency of the native protein for hydration as pFNs are having about 5% carbohydrate (Tanford, 1961). The Stokes radius of BpFN was found to have a value of 65.52Å. However human pFN has been reported to have a Stokes radius of 100Å (Rocco *et al.*, 1987). The results on end group analysis showed alanine to be the amino-terminal and phenylalanine to be the carboxyl-terminal amino acid residues. The amino-terminal residue in human and bovine pFN is blocked. It is pyrrolidone carboxylic acid in human pFN (Garcia-Pardo *et al*, 1983) and pyroglutamate in bovine pFN (Petersen *et al*, 1983). In contrast, buffalo pFN has alanine as amino-terminal residue, which is not blocked.

After denaturation with urea, 1.97 free sulfhydryl groups per subunit of BpFN could be titrated. However, the sulfhydryl groups could not be titrated in the absence of a denaturant. This result is quite consistent with the value (1.6 per subunit) for human pFN (Smith *et al.*, 1982). The amino acid composition of BpFN showed close similarities with pFN from human and bovine sources except for glycine, methionine and leucine which were significantly lower in BpFN.

The UV absorption maxima of BpFN had two peaks, one between 240-245 nm and the other between 277-278 nm. The specific extinction coefficient, $E^{1\%}_{1cm}$ of the protein was determined to be 14.02. This value is higher than the value

reported for human pFN (Mosesson and Umfleet, 1970). The fluorescence spectra of BpFN with an excitation at 280 nm and 300 nm is characteristic of proteins containing tryptophan residues (Teale, 1960). In proteins containing all three aromatic amino acid residues, the observed emission is due mainly to tryptophan, and the fluorescence from phenylalanines is hardly ever observed. Proteins containing both tyrosine and tryptophan do not show an emission band due to tyrosine (Brandt and Witholt, 1967).

The isoionic pH of BpFN was found to be 6.12 which is consistent with the values 5.5-6.3 for pFN reported from other sources (Tamkun and Hynes, 1983; Paul and Hynes, 1984).This indicates an acidic nature of the protein. Carbohydrate content of BpFN was found to be 4.9% which is well within the range of 4.4-5.7% carbohydrate in pFN from other sources (Hynes, 1990). The estimation of tryptophan content in BpFN yielded a value of 73.08 moles of tryptophan per mole of BpFN. The number of residues of tryptophan per subunit of protein, reported from bovine pFN is 38. Therefore, the value obtained for BpFN tallies well with this report (Hynes, 1990).

BpFN was found to be antigenic as polyclonal antibodies were produced in rabbits which gave a precipitin line when anti-BpFN antiserum cross-reacted with BpFN (Fig 17). Anti-BpFN antiserum was specific not only for BpFN but also for human pFN, goat pFN and goat cFN (Fig 18). This shows that there is not much evolutionary change in the epitopes between species as well as tissues, exhibiting greater conservation of immunological property in this extracellular protein.

When BpFN was subjected to thermal denaturation with temperature being increased at a rate of 3°C, the intrinsic fluorescence of fibronectin gradually decreased and then underwent an irreversible denaturation transition between 61°-62°C (Fig 19), similar to that observed for human pFN (Ingham *et al.*, 1984). Upon cooling to the original temperature, the fluorescence intensity value was 79% higher than the original, in contrast to a value of 37% for human pFN. This increase might

be due to the fact that the protein solution became turbid at a temperature of 70°C and above. This turbidity might be a consequence of aggregation of this protein. Wallace *et al.*, (1982) have reported that pFN does undergo rapid heat-induced aggregation. The smooth decrease in the intrinsic fluorescence intensity with increasing temperature below the transition (the descending limb in Fig 19) indicates that BpFN, while unfolding undergoes a conformational change. The tryptophan residues, exposed in the native protein, are probably camouflaged by the unfolding of the protein, resulting in a quenching of the fluorescence (Ingham *et al.*, 1984).

No significant shift in λ_{max} was observed, either upon heating or cooling. The wavelength maxima was centered around 315 nm, which is said to lie on the shorter wavelength side. This suggests that the intrinsic fluorescence of BpFN originates from tryptophans in non-polar environments (Ingham *et al.*, 1984). Tryptophan residues which are located in the interior of proteins and shielded from solvents have short wavelength maxima (between 308-330 nm). Thus, no shift in the wavelength maxima to longer wavelengths was observed(as is consistent with complete unfolding of the protein).This indicates that some amount of tertiary structure persists and that aggregation shields some of the tryptophan residues from the solvent (Ingham *et al.*, 1984).

The solvent perturbation studies are applied to the location and changes in the location of chromophoric/fluorophoric amino acid residues in proteins. The spectra of tyrosine and tryptophan residues located on the surface of proteins are usually perturbed by changes in the physical properties of the solvent such as polarity (changes in dielectric constant), polarizability (changes in refractive index), solvent-solute interactions (hydrogen-bonding) and the charge state of the immediate environment of chromophoric/ fluorophoric residues of proteins. On the other hand, chromophores/fluorophores, deeply buried in the interior folds of the protein are shielded from the perturbing effect of the

solvent, since they do not come in contact with it and so their spectra are not effected. Mild and/or inert substances, which do not alter the conformation of the protein but produce measurable shifts in the spectra of the protein are employed as perturbants in varying concentrations (5-25%). Sucrose, glycerol, ethylene glycol, polyethylene glycol and dimethylsulfoxide are suitable purturbants for solvent perturbation studies (Herskovits, 1967).

The solvent perturbation technique was employed to the study of BpFN, using three different perturbants, DMSO, PEG 200 and PEG 400 at four different temperatures (20°,37°,47° and 65°C) (Fig 20). At 20°C, the extent of perturbation increases (as measured by decrease in fluorescence intensity),with the increase in DMSO concentration upto 25% (Fig 20A). At 37°C (Fig 20B) and 47°C (Fig 20C) the same extent of perturbation was achieved at 20% DMSO concentration, whereas at 65°C (Fig 20D), only 15% DMSO concentration was required to achieve similar perturbation. Since perturbation effect is a diffusion-governed concentration-dependent process, reduced concentration of DMSO, required to achieve the same level of perturbation at higher temperature would mean that BpFN acquires a more extended conformation that facilitated perturbation at higher temperatures. Presuming that moderately high temperatures of upto 47°C would not cause significant loss in secondary structure of the protein (Ingham *et al.*, 1984), this change in the conformation of BpFN may be attributed to a separation of its domains.

The effect PEG 400 had on BpFN further corroborated this hypothesis. PEG 400 is a large and bulky perturbant that is unlikely to reach the fluorophores located in the protein interior, at or near physiological temperatures. At higher temperatures, however, BpFN acquires an extended conformation, where buried fluorophores become available for perturbation, even with a large perturbant like PEG 400.

Deviation from linearity of perturbation curves with PEG 400 (Fig 20A) at lower temperatures could be due to the

"denaturation effect" of the perturbant which causes a structural alteration in BpFN at higher perturbation concentrations (20% and above). This alteration is accompanied by an increase in fluorescence intensity resulting in an inverted bell-shaped curve (Fig 20A). Since BpFN is proposed to exist in an extended conformation at higher temperatures, PEG 400 would act only as a perturbant at these temperatures. the curves produced by such an effect would be linear (Fig 20D). PEG 200, being intermediate in size, had an effect, partially shared by each of the two perturbants, DMSO and PEG 400.

Binding studies with heparin-agarose have shown that even high temperature of upto 50°C and a wide pH range of 6-9 were not sufficient to cause dissociation of BpFN from heparin-agarose gel and therefore BpFN retains its heparin-binding property at higher temperatures and a.wide range of pHs. More stringent conditions like high ionic strength (0.5 M NaCl) are required for dissociation of BpFN from heparin. This is in agreement with the fact that although BpFN acquires an extended conformation at high temperatures or at extreme pH conditions, its secondary structure remains essentially unaltered. Structural transformations under these conditions remain largely restricted to the separation of its domains.

Maximal elution of BpFN from heparin-agarose affinity gel was found to occur at 0.65 M NaCl in contrast to 0.5 M NaCl required for elution of human pFN. This could mean that there is stronger association of the heparin-binding regions of BpFN to heparin. The pI of these regions is basic (Hynes, 1990) in human pFN. The amount of basic amino acid residues in the heparin-binding regions of BpFN might be higher than that of human pFN. Although pH changes also involve ionic interactions, the dissociation, the dissociation of BpFN from heparin-agarose gel might be dependent both on the charge as well as mass of ions. In light of this observation, the low yield of BpFN as mentioned earlier might have been due to the use of 0.5 M NaCl for elution in heparin-agarose affinity chromatography.

Thus, BpFN shares most of its physico-chemical properties

with its counterparts from other sources. However, it does differ in certain aspects like extinction coefficient, amino-terminal end and a higher ionic strength requirement for elution from heparin-agarose affinity column. The protein which is not completely globular in nature, acquires an even more extended conformation at higher temperatures. These structural alterations, especially at moderately high temperatures, do not involve major structural change but are sufficient to make the protein more flexible, such that some molecules/ions are able to reach the protein interior with greater ease. These findings support the postulation that multiple structural forms of FN, obtained under different conditions might play important roles in various FN-mediated physiological processes(Khan, 1990a) and provide suitable adaptation to cells and organs for better cell-cell interaction and other associated processes in organisms.

SUMMARY

The study of fibronectins as a distinct class of proteins, started in the middle of this century when it was found that this protein co-precipitated with fibrinogen in the cold. The term, "fibronectin" (Latin: fibra-fiber, nectene-to connect or link), however, was adopted much later. Purification and characterization proved that fibronectin was a distinct entity, different from fibrinogen and other blood proteins. A protein, similar to fibronectin was also found to exist on the cell-surface of cultured fibroblasts. By late 1970s, to the general acceptance of all workers, fibronectins were classified as adhesive glycoproteins of high molecular weight. They exist in two forms:

1) Plasma fibronectin is the soluble form and is present, predominantly in the plasma.

2) The other form is insoluble and is called cell-surface fibronectin. This form is associated with basement membrane of connective tissues as well as a component of the extracellular matrix.

Both forms of FN share certain structural and physical properties. The proteins are made up of non-identical, but similar subunits of nearly equal sizes, joined at their carboxyl-terminal ends by two disulfide bonds. Each subunit is folded into discrete globular functional domains. These domains possess specific binding sites for biological macromolecules. Because of fibronectins' ability to bind these macromolecules, this protein mediates a large number of diverse and multiple roles, participating in cell adhesion, cellular morphology and spreading, cytoskeletal organization, wound healing, clot formation and phagocytosis.

Fibronectins have been studied from a large number of sources, viz., bovine, human, chicken, hamster and mice. Despite the wide diversity of sources, the overall structural and functional properties of this protein appear to be similar. Nevertheless, a few differences, specially in solubility have been observed. The secondary and tertiary structures of this protein

and the structure-function relationship have not yet been fully revealed.

The endeavour of this study was to isolate, purify and characterize fibronectin from buffalo plasma. The standardized isolation and purification process used was modified at certain steps to overcome a few difficulties encountered, especially with regard to storage of the protein. However, inspite of the modifications incorporated, the yield of plasma fibronectin was poor, and the tendency of the protein to aggregate could not be abolished altogether.

Purified buffalo plasma fibronectin (BpFN) yielded a single band on PAGE, both upon Coomassie Brilliant Blue staining as well as silver staining. Molecular weight of BpFN, determined by gel filtration was 442 kDa. In SDS-PAGE (homogeneous), BpFN migrated as a closely spaced doublet, the subunits corresponding to molecular weights of 216.2 kDa and 208.9 kDa, respectively. Two subunits were also obtained by SDS-PAGE in a PhastGel gradient 4-15, whose molecular weights were found to be 214 kDa and 204 kDa, respectively. The Stokes radius of the protein was calculated by two different plots and the values obtained were 64.8Å and 66.3Å, respectively. Although the molecular weight of the native protein tallied closely with that reported from other sources, the values of Stokes radius were significantly different from that of human pFN.

The amino-terminal end of BpFN was found to be alanine and the carboxyl-terminal end was phenylalanine for both the subunits. Human and bovine pFN have a blocked amino-terminal residue. The protein was found to contain 1.97 free sulfhydryl groups per subunit of BpFN and the sulfhydryl groups were not accessible to titration in absence of the denaturant. There were 73.08 moles of tryptophan residues per mole of BpFN. Carbohydrate content of the protein was 4.9%, a value lying well within the range of those reported from various sources.

The amino acid composition of BpFN showed close similarities with human and bovine pFN, except that the

contents of Gly, Met and Leu, were significantly lower. The protein had an isoionic pH of 6.12, showing that the protein is acidic in nature and this value is consistent with the values of pH range 5.5-6.3, reported from different sources. Two peaks were obtained in the UV absorption spectrum of BpFN, the first between 240-245 nm and the second between 277-278 nm. The fluorescence emission maxima was at 315 nm when excited at 280 nm and at 338 nm when excited at 300 nm. The fluorescence excitation maxima was centered at 320 nm when emitted both at 315 nm and 338 nm. The fluorescence spectrum of BpFN is characteristic of proteins containing tryptophan residues. The specific extinction coefficient ($E^{1\%}_{1cm}$) of the protein was 14 .02, higher than that reported for human pFN.

Polyclonal antibodies were produced when purified BpFN was injected in rabbits and a precipitin line was obtained upon cross-reaction of anti-BpFN antiserum with BpFN. Precipitin lines were also obtained when anti-BpFN antiserum cross-reacted with goat cFN, goat pFN and human pFN, showing that anti-BpFN antiserum obtained in this study shares immunological properties with FNs from other sources. Heating BpFN resulted in a decrease in the intensity of the protein's intrinsic fluorescence upto a temperature of 61°C, after which it underwent an irreversible transition with a mid-point between 61-62°C. Human pFN has also been observed to undergo a similar irreversible transition with a mid-point at around 63.5°C.

The effects of perturbants, DMSO, PEG 200 and PEG 400 on the intrinsic fluorescence of BpFN were studied at four different temperatures, 20°, 37°, 47° and 65°C. DMSO, at 20°C causes perturbation by progressive quenching of intrinsic fluorescence of the protein, because, being a small molecule, it is able to enter the interior of BpFN. At higher temperatures, the same extent of perturbation is achieved at lower DMSO concentrations. PEG 400, a large perturbant is unable to enter the protein interior at 20°C and 37°C. However, at 47°C, BpFN slowly unfolds and undergoes a conformational change at 65°C, with the result that the

intrinsic fluorescence of BpFN is quenched at these temperatures. PEG 200 is a perturbant, intermediate in size between DMSO and PEG 400. Thus, it generates an effect of perturbation also intermediate between the effects of DMSO and PEG 400. These responses suggest that, the conformational change that BpFN undergoes is restricted to a separation of its domains without significant loss in secondary structure.

A temperature of upto 50°C and a pH range of 6-9 caused no significant dissociation of BpFN from heparin-agarose affinity gel. BpFN, however, dissociated from heparin-agarose gel when the ionic strength was increased to 0.5 M NaCl at pH 7.4, indicating that the protein retains its heparin-binding property at high temperatures and between pH 6-9. Maximal elution of BpFN from heparin-agarose gel was found to occur at 0.65 M NaCl, in contrast to 0.5 M NaCl required for elution of human pFN. This might be indicative of the presence of a larger number of basic amino acid residues within the heparin-binding domains of the protein.

Thus BpFN shares most of its physico-chemical properties with its counterparts from human and bovine sources, although differing in certain aspects like extinction coefficient, amino-terminal end and a higher ionic strength requirement for elution from heparin-agarose affinity column. The findings support the postulation that multiple structural forms of FN, obtained under different conditions might play important roles in various FN-mediated physiological processes and provide suitable adaptation to cells and organs for better cell-cell interaction and other associated processes in organisms.

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