PURIFICATION AND PROPERTIES OF BUFFALO (*Bubalus bubalis*) PLASMA FIBRONECTIN

BY
NANDINI CHOUDHURY

THESIS
SUBMITTED IN FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

TO

NORTH-EASTERN HILL UNIVERSITY
SHILLONG – 793022
INDIA
1997

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.

NEHU LIBRARY
Acc No... 103657
Acc By...
Date... 14-8-07
Class
Sub.H...
Ent... by ...
Tran...