Characterization of a 59 kDa gelatin-binding fragment of buffalo plasma fibronectin

Nizamuddin Ahmed* and Naganath Swamy
Division of Biochemistry, Indian Veterinary Research Institute, Izatnagar 243 122 (UP), India

Received 12 February 2001; revised and accepted 14 June 2001

Limited proteolysis of buffalo plasma fibronectin (FN) by thermolysin yielded four gelatin-binding fragments of which, the major 59 kDa fragment, GBF1, was isolated by gelatin-Sepharose and heparin-Sepharose affinity columns. GBF1 appeared during early phase of thermolysin digestion and remained intact even after 4 hr of digestion. GBF1 may be similar to 56 kDa gelatin-binding fragment of FNs from human and hamster plasma. But, it is more resistant to thermolysin cleavage. The fragment binds to heparin with low affinity. On the basis of the structure of human plasma FN, the modular structure of GBF1 may be given as: 6Fn1 5Fn2 4Fn3 3Fn1 2Fn1 1Fn2 1Fn3. Biophysical properties of GBF1 suggest an expanded native conformation. The interaction of the fragment with gelatin is pH-dependent and independent of NaCl concentration.

FN, a multidomain glycoprotein, is found on the surface of cells, in the extracellular matrix and in plasma and other body fluids. Each of its two similar polypeptide chains is comprised of three types of modules, Fn1, Fn2 and Fn3. Various modules have been organized to form functional domains in FN such as cell-, gelatin- and heparin-binding domains. These domains can be isolated by limited proteolysis of FN with thermolysin and other proteases.

The interaction of FN to collagen is important in the formation of extracellular matrix. The gelatin-binding domain (42 kDa) of human plasma FN has affinity for human erythrocyte transglutaminase and associated with substratum-dependent stimulation of fibroblast migration in collagen matrices. Recent studies suggest that cell-surface transglutaminase promotes FN assembly via interaction with the gelatin-binding domain of FN. A 56 kDa gelatin-binding domain/fragment of FN from human and hamster plasma is released during early phase of thermolysin digestion. The module 1Fn3, a structural component of 56 kDa gelatin-binding fragment, of human plasma FN binds to heparin and DNA and is also involved in FN matrix assembly. But, the major gelatin-binding fragment of 42 kDa is produced by 4 hr digestion of FN with thermolysin. This fragment of human plasma FN has been studied by several workers. Although, buffalo plasma FN has been characterized, no information is available on the gelatin-binding domain of this protein. The present communication reports the identification of the major gelatin-binding fragment of buffalo plasma FN and compares its properties with gelatin-binding fragments/domains of plasma FN from other species.

Materials and Methods

Materials

Sodium phosphate (mono- and dibasic), calcium chloride, glycine, acetic acid (glacial) and methanol were procured from Merck, India. Tris and urea (molecular biology grade), acrylamide (3 x crystallized), ammonium persulfate and SDS were purchased from Sigma, USA. Nitrocellulose membrane (Schleicher and Schuell, Germany), BSA, chymotrypsin, horse radish peroxidase conjugate; PMSF, phenyl methyl sulfonyl fluoride; STI, soybean trypsin inhibitor.

Abbreviations used—e-ACA, e-Amino-n-caproic acid; B-HCl, benzamidine hydrochloride; BSA, bovine serum albumin, DAB, 3, 3' diamino benzic acid; FN, fibronectin; FnX, type X module of fibronectin; GBFs, gelatin-binding fragments of buffalo plasma fibronectin derived by thermolysin digestion; HRPC, horse radish peroxidase conjugate; PMSF, phenyl methyl sulfonyl fluoride; STI, soybean trypsin inhibitor.
procured from Bangalore Genei, India. Sepharose 4B, Sephadex G-75 and gel filtration markers were supplied by Pharmacia Biotech, Hong Kong. Rabbit anti-human plasma FN was a kind gift from Prof D F Mosher, University of Wisconsin, Madison, USA. All other chemicals used in this study were of analytical grade and procured from standard commercial sources.

**Methods**

**Isolation of buffalo plasma FN**

Buffalo plasma was treated with STI (0.1 mg/ml), B-HCI (10 mM), e-ACA (100 mM), EDTA (10 mM), PMSF (2 mM) and sodium azide (0.025%). The treated plasma was subjected to precipitation by BaCl2 at 4°C for removal of vitamin K-dependent coagulation proteins and subsequent addition of (NH4)2SO4 to remove excess Barium2. FN was isolated from Sepharose 4B-filtered plasma by gelatin-Sepharose and heparin-Sepharose columns as described earlier23,24. All isolation procedures were carried out at room temperature.

**Isolation of GBF1**

Purified buffalo plasma FN (1 mg/ml) in 25 mM Tris-HCl buffer, pH 7.6, containing 50 mM NaCl, 0.5 mM EDTA and 2.5 mM CaCl2 was digested with thermolysin (5 µg/ml) at 22°C for 4 hr as described by Zardi et al. The digest was fractionated on gelatin-Sepharose and heparin-Sepharose columns as described earlier23,24. All isolation procedures were carried out at room temperature.

**SDS-PAGE and Western blotting**

SDS-PAGE of FN and the fragments was performed on 5-15% or 4-18% gradient gel according to Laemmli25. Gels were stained with Coomassie Blue. The fragments were subjected to Western blot analysis using semi-dry blotter as described by Towbin et al.26. GBFs were electroblotted onto nitrocellulose membrane and then probed with rabbit anti-human plasma FN at (1:250) dilution followed by goat anti-rabbit IgG (secondary antibody) conjugated to horseradish peroxidase. The blot was developed with DAB reagent.

**Results and Discussion**

**FN Preparation**

The homogeneity of FN preparation isolated from buffalo plasma was established by SDS-PAGE. Reducing SDS-PAGE yielded a doublet (characteristic of plasma FN), representing two subunits with mol. mass of 239 kDa and 225 kDa, respectively. The FN preparation promoted cell adhesion and spreading of Madin Durbey Bovine Kidney (MDBK) cells on polystyrene wells (results not shown).

**Digestion of FN by thermolysin**

Thermolysin digest of FN at various time intervals (30 min-4 hr) was analysed by reducing SDS-PAGE (Fig. 1). As evident from the figure, the digestion was consistent between 90 min-4 hr (lanes 7-12). Thermolysin digestion FN for 4 hr yielded 11 major fragments of mol. mass in the range of 10-200 kDa (results not shown), similar to that had been reported in the case of FNs of human and hamster plasma13,14.

Fractionation of thermolysin digest on gelatin-Sepharose column yielded four gelatin-binding fragments of 40-60 kDa (Fig. 2, lane 2). Analysis of heparin-bound fragments revealed four fragments in high mol. mass range and other two fragments of mol. mass of 60 kDa and 29 kDa (Fig. 2, lane 4).

![Fig. 1—Time course of thermolysin digestion of buffalo plasma FN](image-url)
AHMED & SWAMY: GELATIN-BINDING FRAGMENT OF BUFFALO PLASMA FIBRONECTIN

115

1 2 3 4 M kDa

97.4

68.0

43.0

29.0

20.0

14.3

Fig. 2—SDS-PAGE analysis of thermolysin digest of FN fractionated on gelatin- and heparin-Sepharose columns (Buffalo plasma FN was digested with thermolysin as described in legend to Fig. 1 and fractionated on gelatin- and heparin-Sepharose columns followed by reducing SDS-PAGE on 4-18% gradient gel. Gel was stained with Coomassie Blue. Lanes 1 and 2, unbound and bound fragments to gelatin column, respectively; lanes 3 and 4, unbound and bound fragments to heparin column, respectively; lane M, mol. mass markers—phosphorylase b, 97.4 kDa; BSA, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; STI, 20 kDa; lysozyme, 14.3 kDa)

Fig. 3—Analysis of GBFs by SDS-PAGE on 5-15% gradient gel under reducing and non-reducing conditions (Gel was stained with Coomassie Blue. Lane M, mol. mass markers as mentioned in legend to Fig. 2; lanes 1 and 4, GBF1-GBF4; lanes 2 and 5, GBF2-GBF4; lanes 3 and 6, GBF1; lanes M and 1-3 with 2% mercaptoethanol and lanes 4-6 without mercaptoethanol)

Gelatin-binding fragments (GBFs)

Migration on SDS-gels

Migration of GBFs (GBF1, GBF2, GBF3 and GBF4, designated in order of their mobilities on the SDS-gel) under reducing and non-reducing conditions are shown in Fig. 3. Each GBF migrated as a single band on SDS-gels under reducing and non-reducing conditions suggesting that these fragments do not have inter-chain disulfide cross-linkages and each fragment is made up of a single polypeptide chain. These results are in agreement with earlier reports for gelatin-binding fragments of human plasma FN. The mobility of GBFs on the gel, under non-reducing conditions, is more, because unreduced polypeptides have compact structures as compared to their extended conformation under reducing conditions.

Molecular mass

Molecular mass of GBF1, GBF2, GBF3 and GBF4 (determined by SDS-PAGE) was found to be 59 kDa, 48 kDa, 45 kDa and 41 kDa, respectively (Fig. 4). A gelatin-binding fragment of 56 kDa is generated after 1 hr thermolysin digestion of human and hamster plasma FNs. However, the major gelatin-binding fragment, of around 40 kDa, was produced after 4 hr thermolysin digestion of human plasma FN. Borsi et al. and Zardi et al. reported gelatin-binding fragments of 30 kDa and 40 kDa by digestion of human plasma FN with thermolysin. A 45 kDa gelatin-binding fragment had been isolated by digestion of bovine plasma FN by plasmin and chymotrypsin.
Our studies suggest that molecular size of GBFs is significantly different from gelatin-binding fragments of plasma FNs from other species. 

**Immunological cross-reactivity**

As analyzed by Western blotting (Fig. 5), GBF2-GBF4 did not cross-react with anti-human plasma FN antibody and hence epitopes, required for cross-reactivity were absent in these fragments. Only the high mol. mass fragment (GBF1) cross-reacted with the antibody. The specific epitope is, probably, present in the extra structural region of GBF1, which is absent in other fragments. It is also likely that the required regions may be buried inside the folded structures of GBF2-GBF4 and hence are not available for immunological reaction.

**Studies with GBF1**

Our results clearly suggest that GBF1 appears during early phase of thermolysin digestion and remains intact even after 4 hr of digestion (Fig. 1). GBF1 seems to be somewhat resistant to thermolysin cleavage as compared to the 56 kDa gelatin-binding fragment of FN, from human and hamster plasma, which is degraded by thermolysin after 1 hr of digestion.13,13 The difference in thermolysin-sensitivity of GBF1 may be because of the amino acid sequence of GBF1.

---

**GBF1**

---

Fig. 5—Western blot of GBFs derived from thermolysin digestion of buffalo plasma FN [GBF1-GBF4 were subjected to reducing SDS-PAGE and transferred to nitrocellulose membrane. The blot was developed using anti-human FN as described under 'Methods'. No band was observed for GBF2-GBF4]

buffalo plasma FN. The carbohydrates of FN present in gelatin-binding domain appear to be responsible for the resistance to proteolysis of this domain.29 Also, deglycosylation severely affects the stability of gelatin-binding fragment of human plasma FN.15 The content and/or composition of carbohydrate moieties attached to the polypeptide chain of GBF1 possibly make it less susceptible to thermolysin degradation.

The relative concentrations of GBF1-GBF4 determined by gel scanning (of protein bands of lane 1 in Fig. 4; data not shown) is in the ratio of 56:18:13:13, respectively. The concentration of GBF1 produced by chymotrypsin digestion was also significantly higher (results not shown). Thus, GBF1 is the main gelatin-binding fragment produced by limited proteolysis of buffalo plasma FN by thermolysin or chymotrypsin.

**Isolation**

We used a simple procedure to isolate GBF1 from thermolysin digest of FN using gelatin- and heparin-Sepharose affinity columns. The preparation was homogeneous on SDS-gel (Fig. 4, lane 2). Others5,5 had used hydroxyapatite column to isolate gelatin-binding fragments from thermolysin digest of human plasma FN. GBF1 can also be isolated from chymotrypsin digest of FN using similar chromatography procedures.

**Binding with heparin**

GBF1 binds to heparin with weak affinity. It binds to heparin-Sepharose only at a very low ionic strength.
Table 1—Gel filtration parameters of marker proteins and GBFI

<table>
<thead>
<tr>
<th>Proteins</th>
<th>(V_0)</th>
<th>(V_d/V_0)</th>
<th>(K_{eq}=V_c/V_d)</th>
<th>(K_{d}=V_c/V_t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>165</td>
<td>1.154</td>
<td>0.084</td>
<td>0.089</td>
</tr>
<tr>
<td>Ovalbumin chymotrypsinogen A</td>
<td>186</td>
<td>1.301</td>
<td>0.164</td>
<td>0.173</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>267</td>
<td>1.867</td>
<td>0.473</td>
<td>0.500</td>
</tr>
<tr>
<td>GBFI</td>
<td>165</td>
<td>1.154</td>
<td>0.084</td>
<td>0.089</td>
</tr>
</tbody>
</table>

\(V_0\), elution volume of proteins; \(V_0\), \(V_t\), and \(V_c\) represent void volume, bed volume and internal volume of the column, respectively.

Table 2—Biophysical properties of GBFI

<table>
<thead>
<tr>
<th>Properties</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass</td>
<td>63 kDa</td>
</tr>
<tr>
<td>Stokes radius</td>
<td>36.0 Å</td>
</tr>
<tr>
<td>Intrinsic viscosity [(\eta)]</td>
<td>4.88 mlp/g</td>
</tr>
<tr>
<td>Diffusion coefficient ((D_{20w}))</td>
<td>6.1x10^-7 cm²/sec</td>
</tr>
</tbody>
</table>

Fig. 6—Effect of pH on the binding of GBFI with gelatin [GBFI (1.1 mg) in each buffer passed on the column (bed volume, 2 ml) equilibrated with the same buffer and eluted with 4 M urea in 50 mM Tris buffer, pH 7.6 containing 10 mM EDTA and 1 mM PMSF].

The structure of GBFI seems to be similar to that of 56 kDa gelatin-binding fragment of human plasma FN with various concentrations of NaCl and (ii), at different pH using gelatin-Sepharose affinity column. NaCl upto 1 M concentration did not affect the binding of the fragment with gelatin (data not shown). The effect of pH on binding of GBFI with gelatin has been shown in Fig. 6. At pH 6.0, the binding was 50% whereas at pH 9.0, it was only 13% compared to the binding at physiological pH. Hence, the interaction of the fragment with gelatin is independent of ionic strength or salt concentration. But, it is severely affected by the pH of the media. pH-dependent ionization of the groups in the fragment and/or gelatin may be responsible for such observation. Earlier report also showed that 3-5 M NaCl or KCl did not interfere with the interaction of FN with gelatin. Vuento et al. reported that the essential charged amino acids of human plasma FN were involved in the binding of FN with gelatin. Reversible unfolding studies of 42 kDa fragment of human plasma FN suggested the involvement of some lysine residues in the interaction. However, chemical modification of this fragment by chloramine-T suggested the involvement of methionine residues of FN in gelatin binding.

Interaction with gelatin

Interaction of GBFI with gelatin was studied: (i), with various concentrations of NaCl and (ii), at different pH using gelatin-Sepharose affinity column. NaCl upto 1 M concentration did not affect the binding of the fragment with gelatin (data not shown). The effect of pH on binding of GBFI with gelatin has been shown in Fig. 6. At pH 6.0, the binding was 50% whereas at pH 9.0, it was only 13% compared to the binding at physiological pH. Hence, the interaction of the fragment with gelatin is independent of ionic strength or salt concentration. But, it is severely affected by the pH of the media. pH-dependent ionization of the groups in the fragment and/or gelatin may be responsible for such observation. Earlier report also showed that 3-5 M NaCl or KCl did not interfere with the interaction of FN with gelatin. Vuento et al. reported that the essential charged amino acids of human plasma FN were involved in the binding of FN with gelatin. Reversible unfolding studies of 42 kDa fragment of human plasma FN suggested the involvement of some lysine residues in the interaction. However, chemical modification of this fragment by chloramine-T suggested the involvement of methionine residues of FN in gelatin binding.

Structure

The structure of GBFI seems to be similar to that of 56 kDa gelatin-binding fragment of human plasma FN with various concentrations of NaCl and (ii), at different pH using gelatin-Sepharose affinity column. NaCl upto 1 M concentration did not affect the binding of the fragment with gelatin (data not shown). The effect of pH on binding of GBFI with gelatin has been shown in Fig. 6. At pH 6.0, the binding was 50% whereas at pH 9.0, it was only 13% compared to the binding at physiological pH. Hence, the interaction of the fragment with gelatin is independent of ionic strength or salt concentration. But, it is severely affected by the pH of the media. pH-dependent ionization of the groups in the fragment and/or gelatin may be responsible for such observation. Earlier report also showed that 3-5 M NaCl or KCl did not interfere with the interaction of FN with gelatin. Vuento et al. reported that the essential charged amino acids of human plasma FN were involved in the binding of FN with gelatin. Reversible unfolding studies of 42 kDa fragment of human plasma FN suggested the involvement of some lysine residues in the interaction. However, chemical modification of this fragment by chloramine-T suggested the involvement of methionine residues of FN in gelatin binding.
FN. After 1 hr thermolysin digestion of human plasma FN or its 56 kDa fragment, the 42 kDa gelatin-binding fragment is generated. The release of 42 kDa fragment of buffalo plasma FN is restricted because GBF1 is more resistant to thermolysin cleavage as compared to the 56 kDa fragment. However, digestion of buffalo plasma FN beyond 4 hr may yield the 42 kDa fragment in significant amount. The presence of FN3, the module binding to DNA and heparin, in 56 kDa fragment of human plasma FN gives an extra mass of 14 kDa to this fragment. The thermolysin cleavage site in the 56 kDa fragment to generate 42 kDa fragment, probably, lies in the structural region between FN1 and FN3. By the knowledge of the primary structure of this region and thermolysin specificity, one can pinpoint the cleavage site. Assuming that buffalo plasma FN has a structure similar to that of human plasma FN, the modular structure of GBF1 can be speculated as: FN1 FN2 FN3 FN4 FN1 FN1 FN1 FN3.

Although buffalo plasma FN has similar physicochemical properties to that of human plasma FN, its domain structure may differ due to its different primary structure around gelatin-binding domain and other regions. Further, structural and functional studies of GBF1 are required for understanding the role of this fragment in important physiological processes like assembly of FN in extracellular matrix and signal transduction.

References
34. Laurent K C & Killander J (1964) J Chronatogr 14, 317-330