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Research paper

Fibronectin-binding peptides. I. Isolation and characterization of two unique fibronectin-binding peptides from gelatin

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Abstract

Gelatin binds to fibronectin with a high affinity although the fibronectin-binding components have not been located. Fibronectin plays an important role in tumor cell metastasis and gelatin may have a profound effect on the metastatic process. In this study, fractionated acid-washed gelatin was cleaved with trypsin and resultant peptides fractionated by fibronectin-Sepharose affinity chromatography. After further purification using size exclusion HPLC and then reverse-phase HPLC, two unique peptides were obtained and sequenced. The binding affinities of these two peptides to fibronectin were evaluated by an ELISA method developed during this study and compared with the gelatin. Both possessed significantly higher binding affinities to fibronectin than gelatin alone. © 1998 Elsevier Science B.V.

Keywords: Gelatin; Fibronectin-binding peptide; Binding affinity

1. Introduction

The propensity of tumor cells to spread from their sites of origin to another part of the body, i.e. to invade and metastasize, is actually the principal cause of death from cancer and is one of the key reasons why conventional regimens for cancer treatment fail. Currently only about half of all diagnosed cancer patients survive at least 5 years when treated with conventional regimes based on surgery, chemotherapy, and radiotherapy; the treatment failure groups are generally victims of the incomplete eradication of their metastases. If the formation of metastases could be successfully treated or even prevented, then the incidence of death from cancer should be dramatically reduced.

The metastatic process is a complex cascade of events with, initially, groups of cells breaking free from the primary tumor and invading adjacent stromal tissues. These cells then penetrate vascular or lymphatic vessels for transportation to their final resting sites. Most cells are killed in the circulatory system by hemodynamic forces and by cells of the immune system. It has been estimated that 10^5 to 10^7 cells are shed from a primary mouse tumor per day but only a small percentage of these go on to form metastases [1,2]. Circulating tumor cells adhere both to each other and to blood cells such as platelets. The embolus formation caused by these adhesions favors the lodgment of tumor cells in capillary beds and serves to protect them against immune surveillance. Although the actual arrest of tumor cells in the target organ may be facilitated by non-specific trapping of emboli, it is likely that metastasizing cells recognize specific adhesive proteins in the endothelial layer, especially in the subendothelial extracellular matrix. These adhesin molecules may also support the subsequent migration of the tumor cells during the invasion of the subendothelial connective tissue of the target organ [3,4]. One of the major adhesions that has an in vivo distribution consistent with a possible role in these key processes is fibronectin.

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Fibronectin is a large glycoprotein composed of two similar, but not identical, disulfide-linked subunits, each of which has a molecular mass of 250–280 kDa. It is a normal constituent of plasma and is widely deposited in connective tissue, blood vessel walls and basement membranes. The presence of fibronectin in and around tumors [5–9], together with its ability to support the adhesion of a variety of normal and transformed cells, suggests that fibronectin may play a role in certain adhesive aspects of target organ colonization by metastatic cells.

When the complexity of the metastatic process is considered, coupled with the fact that the malignant cell must successfully accomplish each component step, suggests many different ways to intervene therapeutically in the metastatic cascade. It is also conceivable that if a single critical step could be blocked by, for example, specifically and completely blocking the interaction of tumor cells with a crucial extracellular matrix molecule such as fibronectin, then the whole metastatic process would fail.

A number of different approaches have been used to test the ability of anti-adhesive peptides to interfere with metastatic colonization [10–13]. The pentapeptide GRGDS, representing the principal recognition site in the central cell-binding domain of fibronectin, has been evaluated in an experimental metastasis model system with a dose-dependent inhibition of colonization being observed [14]. Activity of this small peptide was found not to be due to its toxicity, but correlated with its anti-adhesive activity. Unfortunately, the low specific activity and instability are pharmaceutical problems that inhibit further development.

Savagner et al. [15] demonstrated that invasion of lymphoid precursor cells across basement membrane could be inhibited by a number of anti-fibronectin antibodies. The strategy here would be to look for other peptides with higher specific activity and couple them onto an optimal adjuvant, not only to improve their activity, but also their stability. Subsequently both polymeric and cyclic peptides have been shown to be more potent inhibitors of both adhesion and experimental metastasis [16].

Gelatin binds to fibronectin with high affinity [17–22]. By specifically binding to fibronectin, gelatin should also interfere with the binding of cells to fibronectin surfaces [23]. Gelatin is a complicated and presumably variable mixture of macromolecules with a wide range of molecular weights, and taking into account the observation of severe side effects apparently resulting from decreasing plasma fibronectin levels after infusion of gelatin-based plasma substitutes in man [24–30], the clinical use of gelatin per se has been limited.

In the present study, gelatin was cleaved with trypsin and fibronectin-binding peptides isolated by fibronectin-Sepharose affinity chromatography. After further purification with size-exclusion HPLC and reverse-phase HPLC, two peptides were sequenced. Their binding affinities to fibronectin were evaluated by an ELISA method developed in this study and compared with that of the gelatin. The two peptides were both found to possess significantly higher binding affinities to fibronectin than the starting gelatin alone.

2. Materials and methods

2.1. Materials

Materials were obtained from Sigma Chemical, St. Louis, MO, Fisher Scientific, Fair Lawn, NY, or as noted.

Peptides I and II were synthesized by Genosys Biotechnologies (Woodlands, TX) at a purity of better than 98%.

2.2. Purification of a commercial gelatin

Bovine skin gelatin (lime cured, type B, Bloom strength 225 g, Sigma), dissolved in a Tris buffer (0.02 M Tris + 0.025 M KCl) was applied to the Sephadex G-200 (superfine, Pharmacia Fine Chemicals, Uppsala, Sweden) column (50 × 1.5 cm i.d.) and eluted with the Tris buffer (flow rate 3 ml/h). Each 2-ml fraction was collected with a Bio-Rad Model 2110 Fraction Collector (Richmond, CA). The absorbance of the fractions were monitored at UV 215 nm (Beckman DU-65 Spectrophotometer, Beckman Instruments, Fullerton, CA).

2.3. Fractionation of the fibronectin-binding domain of gelatin

2.3.1. Preparation of the affinity matrix

CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden), 1.36 g, was completely swollen in 1 mM HCl and washed extensively with about 400 ml 1 mM HCl. Well drained, the swollen gel was washed with 100 ml of coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3), and finally equilibrated with 8 ml of the coupling buffer. Human plasma fibronectin (Gibco BRL Life Technologies, Grand Island, NY) was covalently coupled to ~4 ml of the swollen CNBr-activated Sepharose 4B at 3.33 mg/ml in the coupling buffer. The coupling process was carried out on a Hematology/Chemistry Mixer (Mixer Model 346, Fisher Scientific, Fair Lawn, NY) at refrigerator conditions for 20 h. After the coupling was completed, the matrix was again washed extensively with the coupling buffer (120 ml), and blocked with 15 ml blocking buffer (0.2 M Tris-HCl containing 0.2 M glycine at pH 8) at 4°C for 2 h on the Hematology/Chemistry Mixer. The blocked matrix was washed alternatively three times with the coupling buffer and acetate buffer (0.1 M acetate buffer (pH 4.0) + 0.5 M NaCl). The matrix was finally equilibrated with 0.01 M phosphate buffer (PB) and loaded onto a Poly-Prep Chromatography column (4 × 0.8 cm i.d., Bio-Rad, Hercules, CA) before use. After each use, the fibronectin-Sepharose was re-equilibrated by washing with 4 M urea, distilled

water and finally 0.01 M PB. The concentration of fibronectin before and after the coupling was measured using the μBCA Protein Assay Reagent with an albumin standard (Pierce, Rockford, IL), following the manufacturers' instructions.

2.3.2. Fractionation of fibronectin-binding fragments of gelatin after trypsin digestion

The selected gelatin fraction purified by Sephadex G-200 (above) was digested with TPCK-trypsin (Sigma, St. Louis, MO) (25:1, w/w) for 90 min at 37°C in a digestion buffer (0.1 M NH_4HCO_3, 0.1 mM $CaCl_2$, pH 8.0). The digested mixture was applied to the fibronectin-Sepharose column, prepared above, at 5 mg protein/ml of swollen gel. Unbound fragments were removed by washing the column with the digestion buffer and the fragments that bound with high affinity were eluted with 0.5 M Tris-HCl (pH 7.2) buffer containing 8 M urea. These fragment-containing fractions were fast-desalted by passing through a Sephadex G-25 M column (PD-10 column, Pharmacia Biotech, Uppsala, Sweden) and stored at 4°C before further purification.

2.4. Size exclusion HPLC analysis of the fibronectin-binding fragments-containing fractions

2.4.1. Instrument

Waters high-performance liquid chromatography (HPLC) system (Waters, Millipore, Bedford MA), including Waters 501 HPLC pump, Waters U6K injector, Waters 486 Tunable Absorbance Detector, Waters Pump Control Module and Millennium™ 2010 software, version 2.0.

2.4.2. Samples

Purified gelatin by Sephadex G-200, digested mixture and fibronectin-binding fragment(s)-containing fraction.

2.4.3. Method

Samples, 10 μl, were loaded onto the column (Bio-Sil SEC-125 column, 300 × 7.8 mm i.d., Bio-Rad) and eluted with mobile phase (0.05 M NaH_2PO_4 + 0.05 M Na_2HPO_4 + 0.15 M NaCl, pH 6.8) at a flow rate of 1.0 ml/min. The detector wavelength was set at 215 nm. Peaks with various molecular weights were estimated by calibration with Gel Filtration Molecular Weight Standards (Bio-Rad). Peaks in the fibronectin-binding fragment(s)-containing fraction were manually collected and the same peak from each run pooled and concentrated using a Savant Speed-Vac concentrator (Farmingdale, NY).

2.5. Further purification and subsequent sequencing

The concentrated fragment solutions were further purified by reverse-phase HPLC (Waters system, as described above) on the Macrosphere 300 C18 71 column (250 × 4.6 mm i.d., Alltech, Deerfield, IL) eluted with 0.1% trifluoroacetic acid (TFA) in acetonitrile/water (25:75, v/v). The fractions thus obtained were pooled and

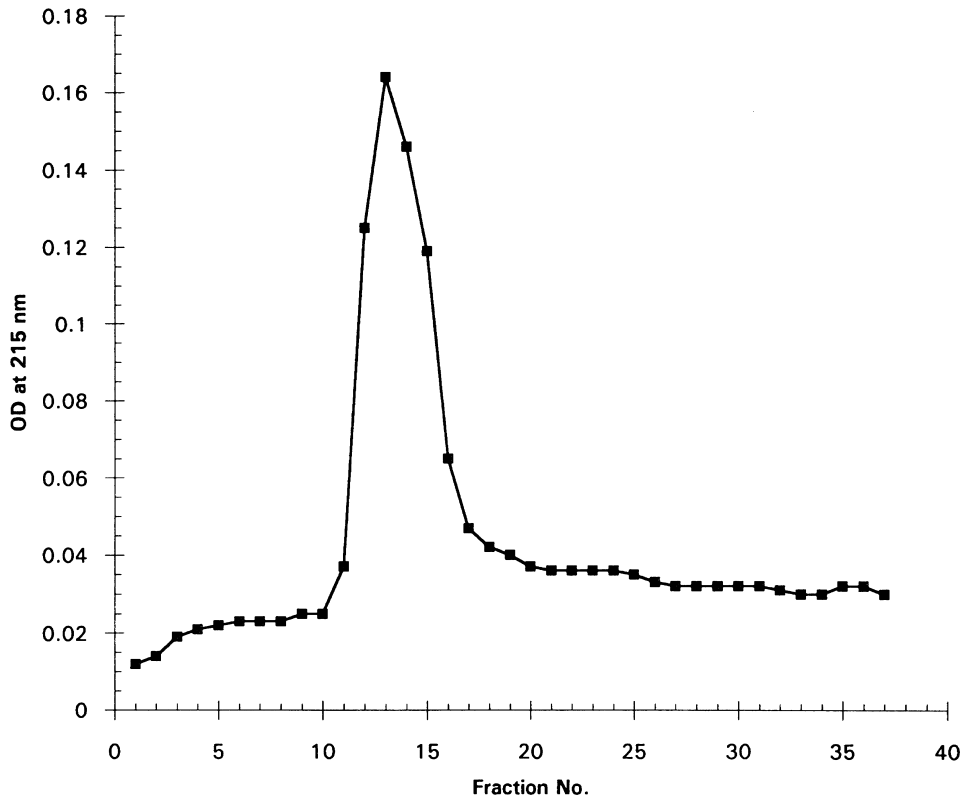

Fig. 1. Elution profile of limed bovine skin gelatin (type B, Bloom strength 225 g) on Sephadex G-200.

concentrated prior to estimating by use of the μBCA Protein Assay Reagent (Pierce). Sequences of the fragments were identified using the Applied Biosystems Model 477A Protein Sequencer.

2.6. Evaluation of the binding affinities of the fibronectin-binding peptides to fibronectin by an enzyme-linked immunosorbent assay (ELISA)

Microtiter wells (96-well vinyl EIA/RIA plates, Gibco) were first coated with gelatin or each fractionated peptide and incubated at 4°C for 2 days. After the removal of the supernatants, the wells were washed with PBS-T (0.01 M phosphate buffer saline, pH 7.4 (PBS) supplemented with 0.05% Tween-20 and 0.02% sodium azide). The wells were then blocked with blocking buffer (0.25% bovine serum albumin (BSA)) for 1 h at 37°C. After addition of serial dilutions of fibronectin at 37°C for 2 h, anti-fibronectin antiserum (Rabbit, Calbiochem, San Diego, CA) in PBS-T (1:1000) was added to the wells and incubated at 37°C for 1.5 h. The wells were then further incubated with goat-anti-rabbit IgG (H + L) alkaline phosphatase conjugate (Bio-Rad) diluted 1000 times with conjugate buffer (0.05 M Tris + 1% BSA + 0.02% sodium Azide, pH 8.0) at 37°C for 30 min. The plates were incubated with the substrate buffer (0.05 M glycine + 1.5 mM magnesium chloride, pH 10.5) and substrate (*p*-nitrophenyl phosphate disodium, Sigma) for 15 min at 37°C. The enzymatic reaction was terminated by the addition of 1 M NaOH and absorbance at 410 nm measured using the Dynatech plate reader (MR 300, Dynatech Laboratories, Chantilly, VA). The wells coated with coating buffer served as controls. The negative control was provided by replacing the anti-fibronectin (human plasma) antiserum (rabbit) with normal rabbit serum. The inhibition experiment was conducted by pre-incubating a concentration of fibronectin (10 μg/ml) with various amounts of gelatin.

The binding data were analyzed by nonlinear regression using GraphPad Prism™, version 2.01 (San Diego, CA). Data were fitted into both one-site binding and two-site binding models and compared. The best fit model was selected by the program and the binding constants calculated.

$$\text{One-site binding}: \quad A = A_{\max} \times [\mathrm{L}] / (K_{\mathrm{d}} + [\mathrm{L}]) \tag{1}$$

$$\text{Two-site binding}: \quad A = A_{\max 1} \times [\mathrm{L}] / (K_{\mathrm{d}1} + [\mathrm{L}]) + B_{\max 2} \times [\mathrm{L}] / (K_{\mathrm{d}2} + [\mathrm{L}]) \tag{2}$$

where A is the absorbance at 410 nm, $A_{\max}$ is the maximum absorbance at saturation, [L] is the molar concentration of the free fibronectin, and K_{d} is the dissociation constant.

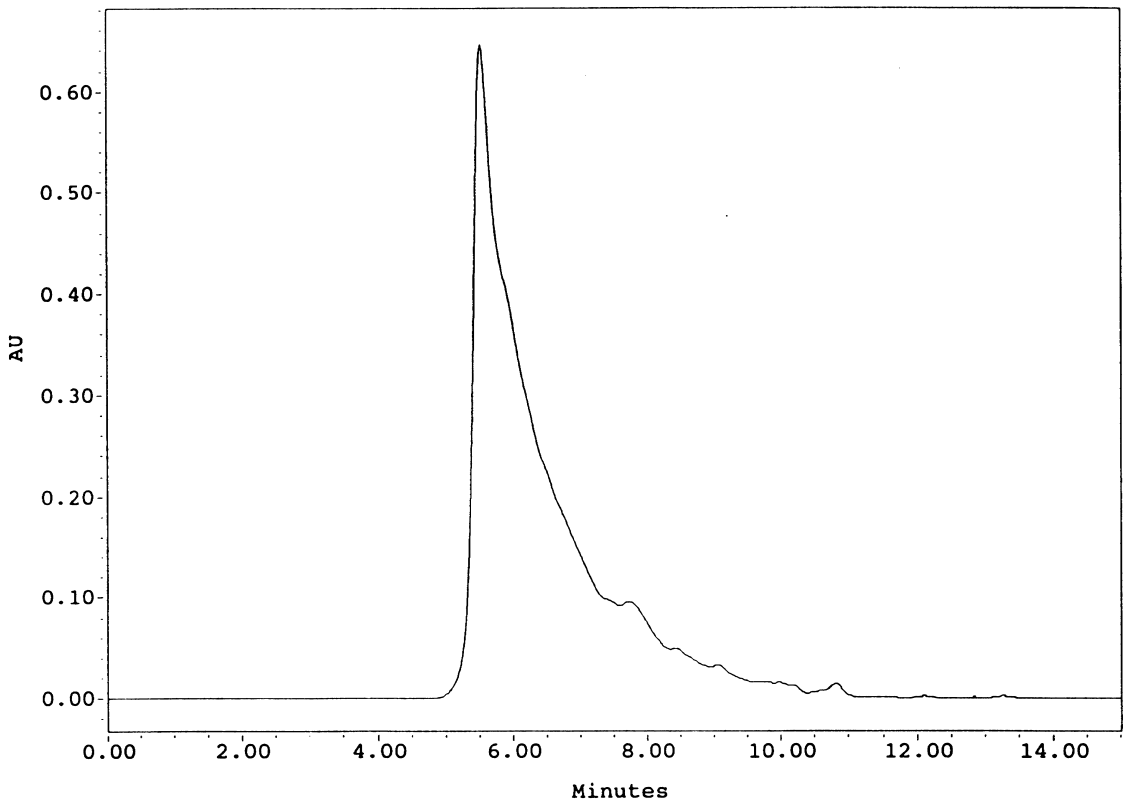

Fig. 2. Size exclusion HPLC profile of the purified, limed bovine skin gelatin (type B, Bloom strength 225 g). Column: Bio-Sil SEC-125 (300 × 7.8 mm i.d.). Sample: gelatin purified by Sephadex G-200, 20 μl. Mobile phase: 0.05 M NaH_2PO_4 + 0.05 M Na_2HPO_4 + 0.15 M NaCl, pH 6.8. Flow rate: 1 ml/min. Detector: UV, 215 nm.

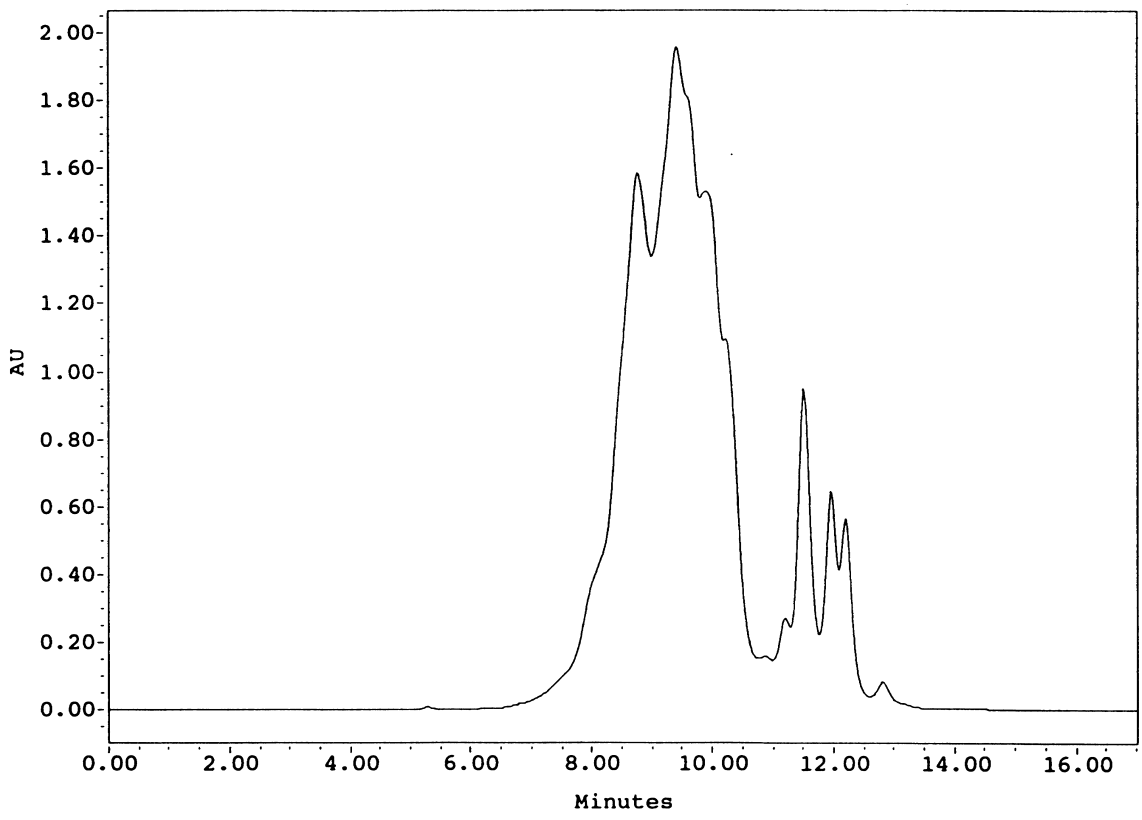

Fig. 3. Size exclusion HPLC profile of the trypsinized gelatin. Column: Bio-Sil SEC-125 (300 × 7.8 mm i.d.). Sample: gelatin/trypsin (25:1, w/w) at 37°C for 90 min in digestion buffer (0.1 M NH_4HCO_3, 0.1 mM $CaCl_2$, pH 8.0), 10 μl. Mobile phase: 0.05 M NaH_2PO_4 + 0.05 M Na_2HPO_4 + 0.15 M NaCl, pH 6.8. Flow rate: 1 ml/min. Detector: UV, 215 nm.

3. Results and discussion

The gelatin used here exhibited only a single peak on Sephadex G-200 (Fig. 1), indicating that this commercial material could be considered relatively homogeneous at the chromatographic level. The fractions within this major peak were collected to conduct further experiments.

Trypsin digests proteins only when they are in their denatured form. It catalyses the hydrolysis of lysyl and arginyl bonds, except where the following residue is a proline. The mode of action of trypsin on gelatin is believed to involve attack of the terminal peptides, leading to the breaking of intra- and intermolecular cross-links and leaving the helical region intact [31]. Size-exclusion HPLC profiles of the gelatin and the cleaved gelatin mixture were constructed (Figs. 2 and 3). It is apparent that the gelatin had been cleaved into a series of smaller molecular fragments ranging from approximately 1.0 to 10.0 kDa after trypsin digestion, while the initial starting material contained materials with molecular sizes ranging from 33.7 to 413.4 kDa. The wide range of molecular weight of gelatin components is readily explained by the method of commercial preparation from various forms of collagen. Single-chain species of approximately 100 kDa and two-chain species of approximately 200 kDa are the main components. A disordered form of tropocollagen, itself having a molecular weight of approximately 300 kDa, may also be found [31]. The results here show that the gelatin contains all types of denatured forms and confirms the complex constitution.

Sixteen types of collagen have been identified [32]. However, the collagen type known for the longest time and most intensively investigated is collagen type I which represents the main constituent of skin, tendon, and the proteinaceous matter of bone [31]. The molecule consists of two α1(I)-chains and one α2(I)-chain [33,34]. The whole amino acid sequence of the α1 chain on the triple helical region from calf skin collagen has been published [35,36]. Sixteen and nine residues, respectively, within the non-triple helical regions at the N-terminus of the α1(I)- and the α2-chain have been characterized for collagens from calf (Fig. 4). The sequence of the non-triple helical regions at the C-terminal is known only for the α1(I)-chain of the calf (Fig. 5) and the sequence within the non-triple helical region

α1(I)

pGlu-Leu-Ser-Tyr-Gly-Tyr-Asp-Glu-Lys-Ser-Thr-Gly- Ile-Ser-Val-Pro-

α2(I)

pGlu-Phe- Asp-Ala-Lys-Gly-Gly- - Gly-Pro-

Fig. 4. Amino acid sequences of the non-triple helical N-terminal regions of α1(I)- and α2(I)-chains from calf [33,35,36].

α1(I)

Ser-Gly-Gly-Tyr-Asp-Leu-Ser-Phe-Leu-Pro-Gln-Pro-Pro-Gln-Gln-Glx-Lys-Ala-His-Asp-Gly-Gly-

Arg-Tyr-Tyr

α2(I)

Ser-Gly-Gly-Tyr-Glu-Phe-

Fig. 5. Amino acid sequences of the non-triple helical C-terminal regions of the α1(I)- and α2(I)-chains from calf [33].

at the C-terminal of the α2-chain apparently has not been elucidated, with only the sequence of the first six residues being known (Fig. 5) [35,37,38].

Fibronectin-binding site(s) on collagen have not been studied systematically and were reviewed by Vartio [39] based, in turn, on the work by Dessau et al. [40]. The major interaction site of fibronectin with native type I collagen is at the site associated with the cleavage of collagen by vertebrate collagenase, in the region of residues 757–791 of the α1(I) collagen chain or parallel regions in the α2(I) chain. The sequence between residues 772 and 785 lacks proline and hydroxyproline, and the resulting local uncoiling of the collagen helix may expose the active fibronectin-binding site. Adelmann-Grill [41] found that most of the activity resided in the α1(I) chain, and more specifically in the cyanogen bromide-derived peptide α1(I)-CB7 which consisted of residues 552–822 of the α1(I) chain. Interpreting the results of their own work, Dessau et al. [40] described the location of the reactive sites in collagen α-chain as two overlapping peptides, residues 552–819 and 643–932, which showed equal fibronectin-binding activity. It appears likely that the most active binding region of the α1(I)-chain is located between residues 643 and 819 of the sequence [33]. It is possible, however, that other portions in the α-chain also participate in the reaction. For example, distinct activity has also been found on peptide α1(I)-CB8, which occupies positions 124–402 in the α1(I)-chain [33].

Deductions about the active binding sites on gelatin for fibronectin above were mainly obtained by limited cleavage of collagen to obtain certain segments initially and then using these as inhibitors against the binding reaction between gelatin and fibronectin. The fragments possessing

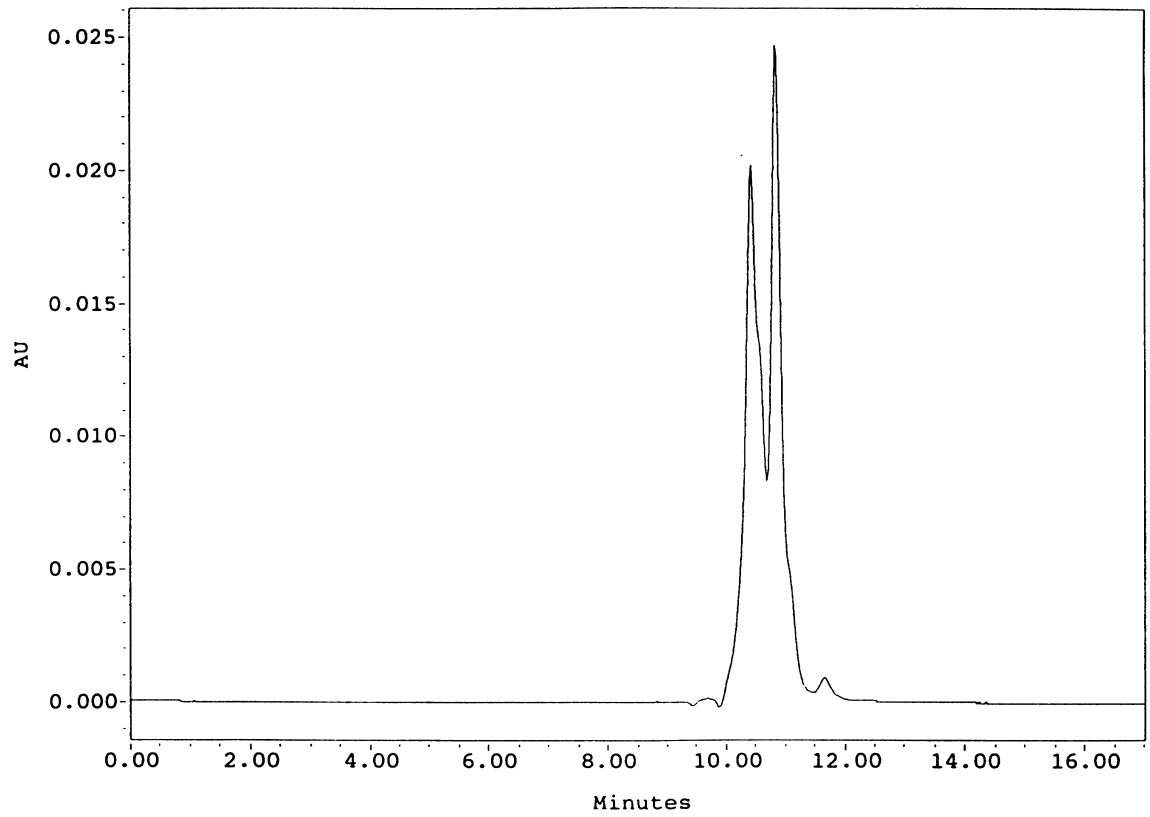

Fig. 6. Size exclusion HPLC profile of fibronectin-binding fragments fractionated by fibronectin-Sepharose affinity chromatography. Column: Bio-Sil SEC-125 (300 × 7.8 mm i.d.). Sample size: 10 μl. Mobile phase: 0.05 M NaH_2PO_4 + 0.05 M Na_2HPO_4 + 0.15 M NaCl, pH 6.8. Flow rate: 1 ml/min. Detector: UV, 215 nm.

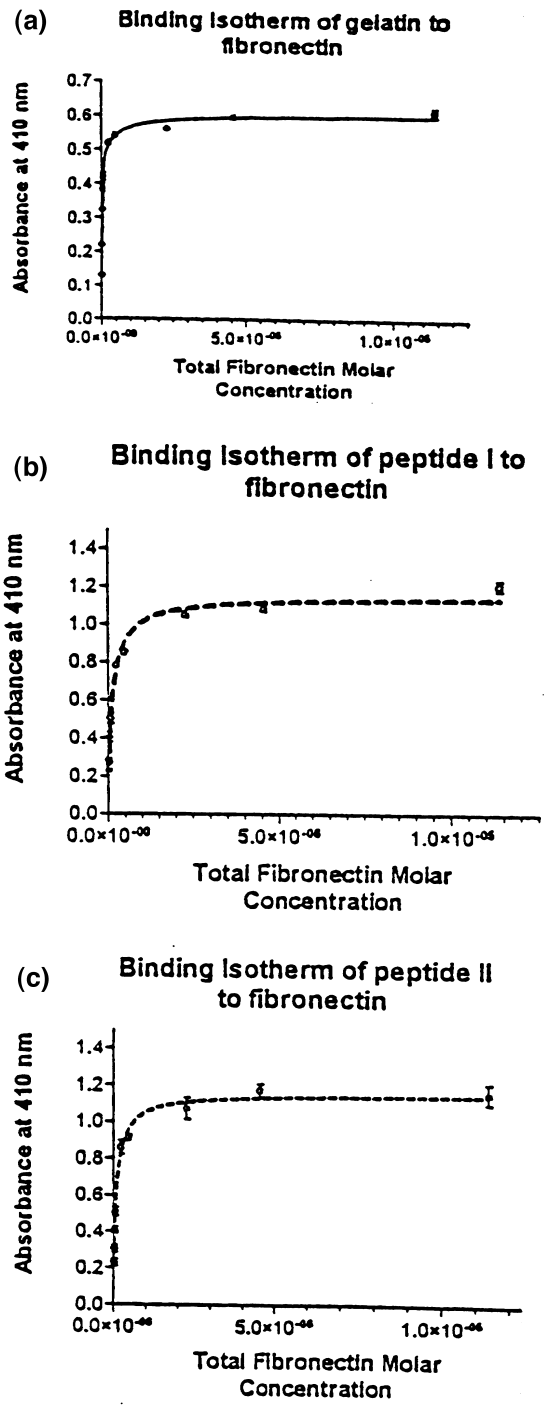

Fig. 7. Binding to fibronectin with increasing fibronectin concentration: (a) gelatin, (b) Peptide I and (c) Peptide II ($n = 3$).

the highest inhibiting activity were considered to contain the active binding site(s). In this present study, however, the fibronectin-binding segments were fractionated by fibronectin-liganded affinity chromatography, a procedure that has not been previously employed for this purpose. Although affinity chromatography has been applied quite extensively in the field of modern biotechnology, this appears to be the first time that the fibronectin-binding site(s) of gelatin (denatured collagen) have been located by this technology.

Two fragments with high binding affinity were separated from the gelatin-digested mixture (Fig. 6) and are summarized in Table 1. Further purification by reverse-phase HPLC and concentration brought the two fragments to concentrations of 91.43 μg/ml for the larger fragment and 50.28 μg/ml for the smaller fragment.

The two fragments were later sequenced as: Thr-Leu-Gln-Pro-Val-Tyr-Glu-Tyr-Met-(X)-Gly-Val (here Thr-Leu-Gln-Pro-Val-Tyr-Glu-Tyr-Met-(Val)-Gly-Val (Peptide I) and Thr-Gly-Leu-Pro-Val-Gly-Val-Gly-Tyr-Val-Val-Thr-Val-Leu-Thr (Peptide II)). These two fibronectin-binding peptides appear to reside in the non-triple helical region of collagen since they do not exhibit the amino acid motif of Gly-Xaa-Yaa, which is common to the whole sequence for the triple helical regions. The portion of the binding site(s) obtained in this study is therefore different from those described above, i.e. within the residue range of 552–932 on the α1(I) chain. This suggests that another two new fibronectin-binding sites from gelatin have been uncovered in this present work.

In addition, Peptides I and II do not show homology with either of the non-triple helical peptides at the N- or C-terminus of the α1(I) chain (Figs. 4 and 5), which might also suggest that they could be from other portions of collagen, where the sequences have not been completely elucidated, such as the non-triple helical peptides of the α2(I) chain.

ELISA methodology is known for its sensitivity, specificity, accuracy, reproducibility and requirement for small quantities of sample [42–44]. Although ELISA has been used to quantitate fibronectin concentration in the plasma [45–47], it has not been previously used to measure the binding affinity between fibronectin and its bound components. Determined by this ELISA method, the binding of gelatin and the two fibronectin-binding peptides to fibronectin exhibited the typical binding saturation curves with an increasing concentration of fibronectin (Fig. 7). The negative control, obtained by replacing the anti-fibronectin (human plasma) antiserum (rabbit) with normal rabbit serum, did not result in any subsequent absorbance reading, indicating that there was no binding between normal rabbit serum and human plasma fibronectin. Accordingly, color development in the assay was totally based on binding between human plasma fibronectin on wells and the anti-fibronectin (human plasma) antiserum (rabbit). Moreover, the binding of fibronectin to the plastic-bound gelatin could be inhibited in a dose-dependent fashion by pre-incubating the fibronectin solutions with gelatin (Fig. 8), which further indicated that the ELISA was solely based on the specific interaction between fibronectin and gelatin. By combining binding data measured by ELISA with advanced non-linear regression analysis, binding models and binding constants could be obtained accurately.

The binding of gelatin and fibronectin-binding peptides to

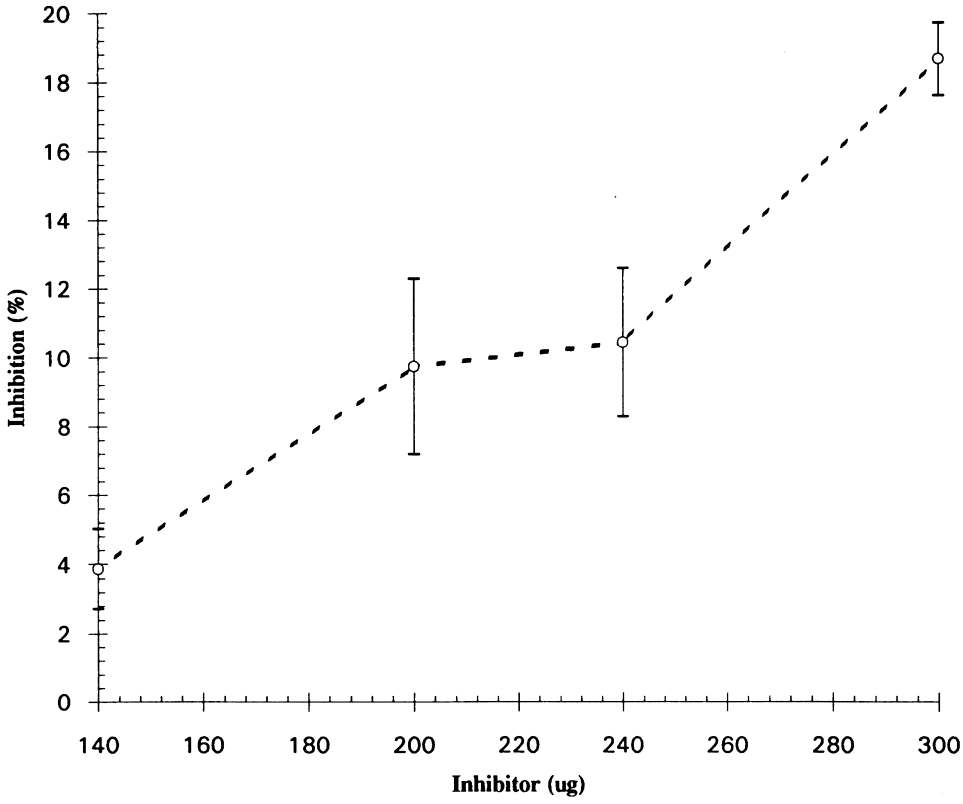

Fig. 8. Inhibition of the binding of fibronectin to plastic-bound gelatin by gelatin in the ELISA. Bar = standard error ($n = 3$).

fibronectin fitted a two-site binding model (Table 2). The binding constant of gelatin at the higher affinity site ($K_{d1} = 5.38 \times 10^{-9}$ M) showed values similar to those in the literature which had been obtained by different methodologies [17–21], further suggesting the reliability of the method.

The two fibronectin-binding peptides fractionated from gelatin all possessed significant higher binding affinities to fibronectin than gelatin alone ($t = 5.616$, df = 4, $P < 0.01$ for Peptide I; $t = 4.476$, df = 4, $P < 0.01$ for Peptide II). However, the binding affinity to fibronectin between Peptide I and Peptide II did not show any significant difference ($t = 0.08956$, df = 4, $P > 0.1$). In conclusion, these two unique peptides, apparently derived from the non-helical region of the gelatin structure, represent hitherto unknown fibronectin-binding regions. Both peptides possessed signif-

Table 1
Molecular sizes and their relative fractions estimated by size-exclusion HPLC, together with the size of digested fractions

Digest mixture			Gelatin			Fibronectin-binding fragments		
RT (min)	kDa[a]	%[b]	RT (min)	kDa	%	RT (min)	kDa	%
5.27	443.30	0.26	5.53	326.90	98.47			
8.73	7.72	26.64	7.80	22.96	1.16			
9.40	3.51	29.05						
9.99	1.77	5.16						
10.29	1.25	5.80				10.42	1.07	51.82
10.88	0.62	0.15	10.81	0.68	0.37	10.85	0.65	48.18
11.18	0.44	1.16						
11.50	0.30	19.17						
11.94	0.18	6.09						
12.20	0.13	5.46						
12.81	0.06	1.06						

RT, retention time.
[a]kDa was estimated according to calibration curve of the molecular weight standards.
[b]Percent fraction was based on percentage of each peak area.

Table 2

Non-linear regression result of binding of gelatin and fibronectin-binding peptides to fibronectin, determined by the ELISA and analyzed by GraphPad Prism™, version 2.01

Sample	K_{d1} (M)	K_{d2} (M)	R^2
Gelatin	5.382×10^{-9}	3.385×10^{-7}	0.9839
Peptide I	6.681×10^{-10}	1.851×10^{-7}	0.9804
Peptide II	7.709×10^{-10}	1.267×10^{-7}	0.9785

icantly higher binding affinities to fibronectin than their parent gelatin.

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