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Separation of fibronectin from a plasma gelatinase using immobilized metal affinity chromatography

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Conventional preparations of plasma fibronectin are known to contain a co-purifying gelatinase [1986, J. Biol. Chem. 261, 4363–4366], but so far useful methods to remove the protease have not been available. In this study a number of different methods were tested in order to achieve separation of the two proteins. Immobilized metal affinity chromatography was found to be efficient for this purpose, and a convenient procedure to separate the two proteins under nondenaturing conditions on chelating Sepharose charged with Co^{2+}, Ni^{2+}, or Zn^{2+} is described. An alternative method employing pH gradient elution of an Fe^{3+} gel also resolved fibronectin from the gelatinase. The Fe^{3+} gel bound both proteins at pH 6.0 but not at pH 7.4, suggesting that the two proteins were phosphorylated. The described procedures will now allow studies of the functions of fibronectin in the absence of the contaminating protease.

Fibronectin; Gelatinase; Immobilised metal affinity chromatography; Zymography; Chromatofocussing

1. INTRODUCTION

Fibronectin is a major cell adhesion protein of extracellular matrices and body fluids which is involved in a variety of biological processes including cell migration, thrombus formation, and wound healing (for reviews see [1–3]). It is commonly purified from blood plasma by affinity chromatography on gelatin–Sepharose [4]. However, a plasma protease with an unreduced M_r of 72 kDa contaminates such fibronectin preparations due to its ability to bind gelatin with similar affinity as fibronectin [5]. The enzyme is mainly obtained in a latent form but may be activated by proteases, autoactivation, or by other mechanisms [5], similarly to other known collagenases and gelatinases. After activation, the enzyme degrades nonhelical collagens [5]. In the absence of appropriate methods to separate fibronectin from the gelatinase, essentially all experiments with fibronectin have thus been performed in the presence of this potential source of artifacts. Indeed, some cell biological activities that have been ascribed to fibronectin or to fibronectin fragments may have been due to this enzyme [6–8].

The gelatinase is a metal-dependent enzyme and its activity can be inhibited by the addition of EDTA [5]. However, this approach to control the enzyme activity cannot be used in biological contexts since EDTA has numerous effects on cells, including inactivation of fibronectin receptors (integrins). In this report we provide means to solving this problem. Convenient methods for purification of fibronectin from gelatinase under non-denaturing conditions by immobilized metal affinity chromatography (IMAC) are described.

Abbreviations: IMAC, immobilized metal affinity chromatography; PBE, polybuffer exchanger; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Fibronectin was isolated from human plasma as described [4]. The following reagents were purchased: Tris, gelatin, EDTA and MES from Sigma; acrylamide and bisacrylamide from Bio Rad; metal salts and imidazole from Merck; HiTrap Chelating columns (1 ml), Polybuffer and Polybuffer exchanger gel (PBE) from Pharmacia.

2.2. *Chromatofocusing*

The PBE 94 column (0.9 × 10 cm) was equilibrated with 25 mM imidazole, pH 7.4 and after applying the fibronectin preparation (3 mg) in the same buffer, elution with the polybuffer was started (dilution 1:8, pH 4.0). The collected fractions (2 ml) were analyzed by absorbance at 280 nm and by zymography.

2.3. *Metal affinity chromatography*

Metal chelating gels were used in prepacked 1 ml HiTrap Chelating columns. After washing with five bed vols. of deionized water, the columns were charged by applying 3 vols. of 100 mM $CuSo_4 \cdot 5\ H_2O$, $ZnCl_2$, $FeCl_3 \cdot 6\ H_2O$, $CaCl_2 \cdot 2\ H_2O$, $Ni(NO_3)_2 \cdot 6\ H_2O$ and $CoCl_2 \cdot 6\ H_2O$, respectively. The excess metal was washed out with 5 vols. of deionized water, followed by equilibration with 5 vols. of 50 mM Tris, pH 7.4, containing 1 M NaCl or 0.5 M K_2SO_4 unless otherwise indicated. A sample of the fibronectin preparation (2 mg in 2 ml equilibration

buffer) was applied to each column, and after further washing with five column vols. of equilibration buffer, the retained proteins together with complexed metal ions were eluted with 100 mM EDTA. The flow rate was 60 ml/h. Fractions (1 ml) were collected and analyzed by absorbance at 280 nm and by zymography. Maximal fibronectin binding capacity of the gels was determined by applying the sample (1 mg/ml; 60 ml/h) until fibronectin was detected in the effluent.

2.4. *Electrophoresis and zymography*

SDS-PAGE was performed according to the method of Blobel and Dobberstein [9]. Molecular weight marker proteins were from Sigma. Zymography was carried out in polyacrylamide gels containing 2 mg/ml gelatin as described [5]. Briefly, after the electrophoresis the gels were washed with 1% Triton X-100, 50 mM Tris, pH 7.4, for 2 h and then incubated in 50 mM $CaCl_2$, 1 μM $ZnCl_2$, 50 mM Tris, pH 7.4, at 37°C for 15 h and stained with Coomassie brilliant blue R-250 in order to visualize the non-degraded gelatin.

3. RESULTS

After unsuccessful attempts to separate fibronectin from plasma gelatinase by ion exchange chromatography, heparin–Sepharose affinity chromatography and differential precipitation with NH_4SO_4 or PEG, we tried chromatofocusing. When the standard procedure recommended by the manufacturer for the pH range 7–4 (pI of fibronectin is 5.8) was employed, gelatinase activity co-eluted with fibronectin (results not shown). By increasing the NaCl concentration in the polybuffer, it was found that at 200 mM NaCl most of the gelatinase was eluted with the first fractions of the starting buffer, separated from the following fibronectin peak (not shown). However, at this salt concentration the pH gradient in the column was distorted, and the fibronectin was trailing slowly off the column between pH 6.0–6.5. Thus, the result indicates, that the two proteins were associated with each other at low ionic strength, but that the higher salt conditions were not suitable for purification of fibronectin by this technique.

Because of the above mentioned problems, we turned our attention toward finding conditions to separate the two proteins at high ionic strength by immobilized metal affinity chromatography (IMAC). HiTrap Chelating prepacked columns (Pharmacia) were charged with Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Ca^{2+} and Fe^{3+} ions, respectively, as described in section 2. In the first set of experiments the binding of fibronectin and gelatinase to different metal ions were compared in the presence of either 1 M NaCl or 0.5 M K_2SO_4. Fibronectin was found to bind to Cu^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+} in both buffers, whereas the gelatinase was retained on columns charged with Cu^{2+}, Zn^{2+} and Ni^{2+} in the presence of 0.5 M K_2SO_4 but only to the Cu^{2+}-gel in the presence of 1 M NaCl. Examples of the analysis data obtained are shown in Fig. 1 and the results are summarized in Table I. In all cases more than 90% of the fibronectin applied to the columns was present either in the flow-through fraction or in the EDTA-eluted fractions. Judging from the zymography assay, the binding of the gelatinase to the columns occurred in a similar 'all-or-non' fashion. Neither of the proteins bound to columns which had not been charged with metal ions.

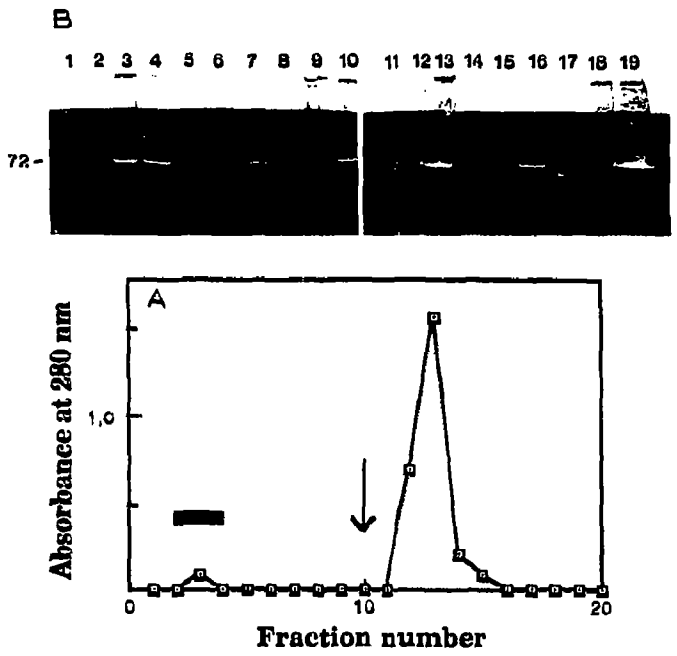

Fig. 1. Chromatography of fibronectin and gelatinase on HiTrap Chelating columns charged with different metal ions. Samples of isolated fibronectin (2 mg) containing gelatinase were chromatographed on the columns as described in Section 2. In (A) the absorbance at 280 nm for the chromatography on the Zn^{2+} column in buffer containing 1 M NaCl is shown. The arrow indicates the start of the elution with the 100 mM EDTA solution. Fractions containing gelatinase activity are indicated by the bar (■). In (B) the gelatinase activity is shown of the starting material (lane 19) and in samples from chromatographies in the presence of 1 M NaCl on columns charged with Cu^{2+} (lanes 1–3), Co^{2+} (lanes 4–6), Ni^{2+} (lanes 7–9), Ca^{2+} (lanes 10–12), Fe^{3+} (lanes 13–15) and Zn^{2+} (lanes 16–18). Samples from the flow-through fractions (lanes 1,4,7,10,13,16), the wash fractions (lanes 2,5,8,11,14,17) and the EDTA-eluted fractions (lanes 3,6,9,12,15,18) were analyzed on the zymography gel as described in Section 2. Note that in addition to the white gelatinase bands, fibronectin is visible as dark bands at the top of the gel in some lanes.

When the conditions for chromatography on the Fe^{3+} column was changed to 50 mM MES, pH 6.0, 1 M NaCl as equilibration buffer, both proteins were retained on the gel. By applying a pH gradient as shown in Fig. 2, the peak gelatinase activity was found in fractions at pH 6.6, while fibronectin was eluted at pH 6.8. Attempts to elute the proteins separately from the Cu^{2+} column with an imidazole gradient (1 mM–1,000 mM in the presence of 1 M NaCl) failed. Fibronectin and gelatinase were eluted together at an imidazole concentration of 60–70 mM (results not shown).

To evaluate the capacity of the columns charged with different metal ions, a solution of fibronectin was passed through the respective gel. The amount of protein adsorbed to the gel until elevated levels of A_{280} in the eluent were detected, was taken as the binding capacity of the gel. Values around 20 mg of fibronectin

bound per ml of gel were obtained for the tested metal ions (Table II).

4. DISCUSSION

This study was undertaken in order to find a method for separating fibronectin under non-denaturing conditions from a previously identified 72 kDa gelatinase [5]. IMAC was found to be suited for this purpose. The high ionic strength at which the chromatography is performed in order to reduce non-specific electrostatic binding to the gel, also eliminated interactions between fibronectin and gelatinase. IMAC is based on the affinity between proteins and heavy-metal ions, usually chelated to iminodiacetate-substituted agarose. Fractionation of proteins depends primarily on the relative content of imidazole residues (histidines) on the surface of the proteins, but also tryptophane, cysteine and other residues probably contribute to the interactions [10]. Several reviews describing properties and applications of the technique have been published [11–13]. For our purpose we tested six ions and found conditions for separation of the investigated proteins with four of them: Zn^{2+}, Ni^{2+}, Co^{2+} and Fe^{3+}. Cu^{2+} bound both proteins with similar affinity, while neither of the proteins bound to the Ca^{2+} gel.

The interactions of the two proteins with Fe^{3+} differed from those with Zn^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+}. While the latter ions bound the proteins at neutral pH but not at pH 6.0 (not shown), Fe^{3+} exhibited the reversed pH dependence. At pH 6.0 the histidine residues are protonated and cannot participate in chelation of metal ions, but phosphoryl groups have been found to bind Fe^{3+} preferentially at this pH [13]. Thus, our results suggest that the interactions were due to the presence of phosphate groups on the protein surface [13]. Different degrees of phosphorylation may explain the different retention on the gel during pH gradient elution. In line with this interpretation, metabolically $^{32}PO_4$-labeled fibronectin has been isolated from fibroblast cultures [14,15]. However, from those studies it was not clear if all fibronectin molecules or only a subfraction were labeled. In bovine plasma fibronectin, one serine residue close to the C-terminus was identified to carry phosphate in approximately 50% of the subunits [16]. Whether one such subunit is present in each fibronectin dimer, and if there are additional phosphorylated sites in plasma fibronectin, is not known. Phosphorylated gelatinases or collagenases have so far not been reported. Our results indicate that it would be worthwhile to investigate these issues further.

The procedures described in this report will allow rapid separation of fibronectin from plasma gelatinase under non-denaturing conditions. The possibility to use four different ions for this purpose makes the method adaptable to different situations. The availability of protease-free fibronectin is an obvious pre-requisite for the adequate elucidation of its biological roles, and the possibility of obtaining such preparations may result in re-evaluation of some of the suggested activities of fibronectin.

Table I

Binding of fibronectin and gelatinase to different immobilized metal ions

	Metal ions					
	Cu^{2+}	Zn^{2+}	Ni^{2+}	Co^{2+}	Ca^{2+}	Fe^{3+}
0.5 M K_2SO_4						
Fibronectin	+	+	+	+	–	–
Gelatinase	+	+	+	–	–	–
1 M NaCl						
Fibronectin	+	+	+	+	–	–
Gelatinase	+	–	–	–	–	–

Samples of isolated fibronectin containing gelatinase in equilibration buffer (0.5 M K_2SO_4 or 1 M NaCl in 50 mM Tris pH 7.4) were chromatographed as described in Section 2, on columns charged with the indicated metal ions. The collected fractions were analyzed as shown in Fig. 1A and B. (+) and (–) denote binding and non-binding, respectively, of the proteins to the column.

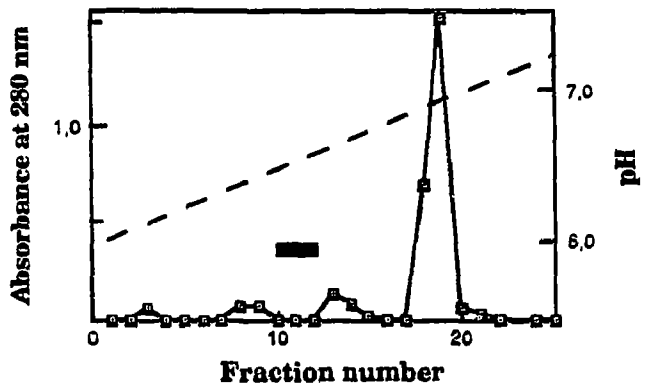

Fig. 2. IMAC of fibronectin and gelatinase on Fe^{3+} column. Isolated fibronectin containing gelatinase was applied to an Fe^{3+} column, equilibrated in 50 mM MES, pH 6.0, 1 M NaCl. The column was eluted with a pH gradient from pH 6.0 to pH 7.4 (dashed line). Fractions of 1 ml were collected and analyzed for fibronectin and gelatinase (zymography). Fractions containing gelatinase activity are indicated by the bar (■).

Table II

Fibronectin binding capacity of gels charged with different metal ions

	Metal ions				
	Cu^{2+}	Zn^{2+}	Ni^{2+}	Co^{2+}	Fe^{3+}
Fibronectin (mg/ml gel)	22	16	25	20	20

The equilibration buffers used were 50 mM Tris, 1 M NaCl, pH 7.4, for the columns charged with Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}, and 50 mM MES, 1 M NaCl, pH 6.0, for the Fe^{3+} column.

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