Characterization of cryptic human fibronectin proteases

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I. Introduction

Fibronectin (FN) is a multifunctional plasma and extracellular matrix glycoprotein, which is secreted as a dimer of two similar but not identical polypeptide chains of approximately 220-250 kDa connected by two disulfide bonds near the C-terminus of the protein [1, 2] (Fig.1).

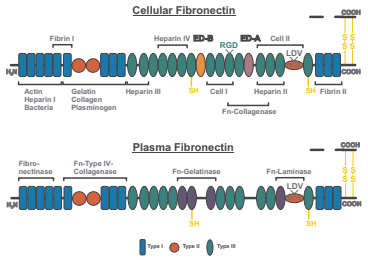


Fig. 1. Schematic domain structure of cellular and plasma fibronectin. Fibronectins are composed of three types of internal repeating modules designated type I, II, and III. The binding domains of fibronectin are indicated at the cFN molecule. The localization of FN-proteases is indicated by black brackets at the pFN molecule.

The binding properties shown in Fig.1 indicate that FN is implicated in a variety of cellular properties including cell adhesion, wound healing, cytoskeletal organisation, migration, differentiation, phagocytosis, hemostasis, thrombosis, and pathogenesis of diseases including cancer and bacterial infection [1-4]. In contrast to these properties other investigations demonstrated that FN fragments may perform quite different biological functions than the intact molecule. Several studies described various proteolytic activities of pFN fragments. There are four latent proteases in human pFN designated as fibronectinase (Ser-enzyme), FN-gelatinase (Asp-enzyme), FN-lamininase (Asp-enzyme) and FN-type IV-collagenase (metallo-enzyme) [5-9] (Fig.2).

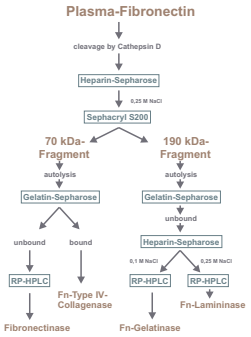


Fig. 2. Isolation and purification of the four cryptic fibronectin proteases after cathepsin D digestion of human plasma fibronectin.

II. Results

Expression and purification of FnColA and FnColB

Recombinant FN-type IV-collagenases A and B were obtained from *E. coli* [BL21(DE3)] transformed with parts of the full length cDNA of pFN cloned into pET 11a-vector. After induction with IPTG the expression reached a maximum after 4 h. Molecular weights of FnColA (35 kDa) and FnColB (28 kDa) were determined by SDS-PAGE. For identification we determined the N-termini by automated Edman sequencing (V^{293}VQPQPHP) as the N-terminus of the collagen binding domain of pFN.

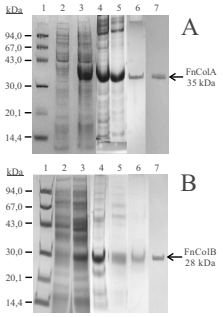


Fig. 3. SDS-PAGE analysis of the expression and purification of recombinant FnColA (A) and FnColB (B). The homogeneity is documented after silver staining. Lane 1: LMW protein marker; lane 2: expression of the recombinant proteins before induction with IPTG; lane 3: expression 4 h after induction with IPTG; lane 4: solution of inclusion bodies; lane 5: FN-collagenases eluted from Q-Sepharose; lane 6: homogenous FN-collagenases after gelfiltration on Sephacryl S-100; lane 7: immunoblot analysis of purified FnColA (A) and FnColB (B) with monoclonal antibody IST-10 (Chemicon).

Catalytic characteristics

After folding of the recombinant proteins with an oxido shuffling system we observed an autocatalytic degradation as shown by Keil-Dlouha et al. [7]. Zymography was used to define which of the resulting fragments exhibited catalytic activity against potential substrates. As shown in Fig. 4, gelatin, a- and b-casein were substrates for the recombinant FN-type IV-collagenase. From FnColA and FnColB several catalytic active fragments of 20, 24, 40, 43 and 66 kDa were observed. Fragments with lower molecular weight than the cloned proteins were probably generated by autodigestion, whereas the higher molecular weight forms could be the result of oligomerization. Further investigations also revealed digestion of type II- and type IV-collagen and the synthetic Mca-peptide.

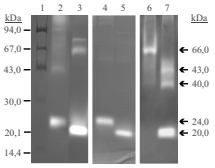


Fig. 4. Gelatin zymogram of FnColA and FnColB folding reactions stained with Coomassie Blue. Lane 1: LMW protein marker; lanes 2+3: folding reactions of FnColA led to two active low molecular weight fragments with either M, 24 kDa or 20 kDa and several high molecular weight fragments; lanes 4-7: folding reactions of FnColB led to the same active fragments as described for FnCol A. Arrows show most significant active fragments. The same results were observed with a- and b-casein.

The obtained enzymes were not stable, underwent further autodigestion, and lost proteolytic activity. We observed the same carboxy terminal degradation in both recombinant FnColA and FnColB enzymes. Figure 5 shows a possible model for the processing of FN-type IV collagenases A and B leading to the active 20 kDa and 24 kDa fragments.

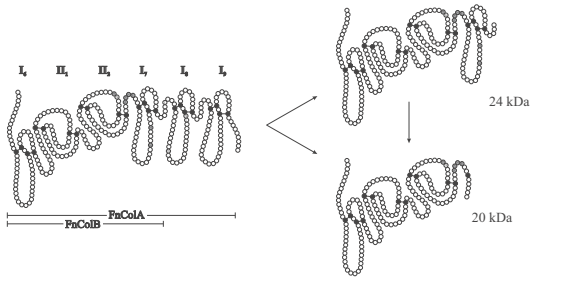
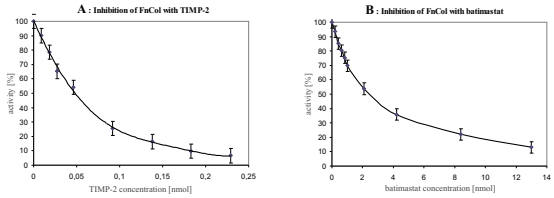


Fig. 5. Model of recombinant FN-type IV collagenases A and B and its processing leading to active 20 kDa and 24 kDa fragments. Type I and type II modules are indicated by I_x and II_y. Shaded amino acids show the zinc binding motif -AAHE- and further histidine residues with possibility of zinc binding. The figure also shows three possible Asn residues for glycosilation.

The cleavage specificity against the insulin b-chain was investigated by Edman sequencing of the insulin fragments obtained after digestion with recombinant and isolated Fn-type IV-collagenase, isolated FN-gelatinase, FN-lamininase, and fibronectinase. We found the same cleavage sites for both the recombinant and the isolated form but there was one additional cleavage site at residues Phe^{25}-Tyr^{26} with the recombinant FN-type IV-collagenase (Fig. 6).

Fig. 6. Cleavage specificity of fibronectin proteases against insulin b chain. No cleavage sites were found for fibronectinase.

Inhibition studies demonstrated that EDTA and batimastat were effective inhibitors of the FN-collagenases. In contrast to the protein fragment isolated from the digest of plasma fibronectin, bovine TIMP-2 and the N-terminal inhibitory domain of human TIMP-2 (TIMP-2) also inhibited the catalytic activity of the recombinant enzymes. The studies show TIMP-2 as a better inhibitor for recombinant FN-type IV-collagenase than batimastat.

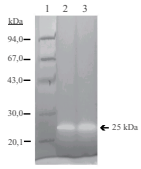


Expression, purification and characterization of fibronectinase

The recombinant fibronectinase was expressed in a transformed *E. coli* [BL21(DE3)] strain. The protein was purified using DEAE-Sepharose as an anion exchanger and Sephacryl S-100 as a matrix for gelfiltration chromatography. For identification we determined the N-terminus by automated Edman sequencing (R^4QAQQMVQPQS) as the N-terminus of the whole fibronectin molecule. Furthermore Western-Blot analysis with a monoclonal antibody against the Hepain I binding domain of fibronectin was used for identification.

The folding procedure of the denatured inclusion body protein was performed with an oxido shuffling system and the recombinant fibronectinase was activated under Ca^{2+}-incubation. As a substrate for the recombinant fibronectinase we identified fibronectin isolated from human plasma (Fig. 7).

Fig. 7. Fibronectin zymogram of recombinant Fnase stained with Coomassie Blue. Lane 1: LMW protein marker; lanes 2+3: folding reactions of Fnase led to an active fragment with M, 25 kDa.



	Enzyme	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [mM^{-1} s^{-1}]
Ac-Ile-Glu-Gly-Lys-pNA	Fibro-nectinase	1.4 ±0.5	33.4 ±0.7	23.86 ±0.45
Bz-Ile-Glu-Gly-Arg-pNA	Fibro-nectinase	1.2 ±0.5	46.7 ±0.8	38.92 ±0.64

Fig. 8. Kinetics of hydrolysis of p-nitroaniline-substrates by isolated fibronectinase

III. References

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