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Affinity purification and enzymatic cleavage of inter-alpha inhibitor proteins using antibody and elastase immobilized on CIM monolithic disks

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Abstract

Epoxy-activated monolithic CIM disks seem to be excellent supports for immobilization of protein ligands. The potential use of enzymes, immobilized on monolithic disks for rapid preparative cleavage proteins in solution was investigated. Digestion of complex plasma proteins was demonstrated by using inter-alpha inhibitors with elastase, immobilized on epoxy-activated CIM disks. Recently, a monoclonal antibody against human inter-alpha inhibitor proteins (MAb 69.31) was developed. MAb 69.31 blocks the inhibitory activity of inter-alpha inhibitor proteins to serine proteases. These results suggest that the epitope defined by this antibody is located within or proximal to the active site of the inhibitor molecule. This antibody, immobilized on monolithic disk, was used for very rapid isolation of inter-alpha proteins. The isolated complex protein was used for enzymatic digestion and isolation of cleavage products, especially from inter-alpha inhibitor light chain to elucidate precisely the target sequence for MAb 69.31 by N-terminal amino acid sequencing. Bovine pancreatic elastase immobilized on monolithic disk cleaves inter-alpha inhibitor protein complex into small fragments which are still reactive with MAb 69.31. One of these proteolytic fragments was isolated and partially sequenced. It could be shown that this sequence is located at the beginning of two proteinase inhibitor domains of the inter-alpha inhibitor light chain (bikunin). Elastase immobilized on monolithic disk offers a simple and rapid method for preparative isolation of protease cleavage fragments. The immobilized enzyme is stable and still active after repeated runs. A partial or complete digestion can be achieved by varying the flow rate.

Keywords: Inter-alpha inhibitors; Bikunin; Preparative cleavage; Enzyme immobilization; Monolithic chromatographic supports; Epitope mapping

1. Introduction

Proteolytic digestion plays a key role in protein analysis and identification. The enzyme trypsin is most commonly used and the protein digestion can be performed in solution or in-gel after separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Further analysis of the cleavage products can be facilitated by peptide mass mapping or tandem mass spectrometry (MS–MS)-based peptide sequencing [1].

Monolithic supports such as disks, rods and tube shaped columns are characterized by an almost complete lack of diffusion resistance during mass transfer. They, therefore, represent ideal supports for immobilization of enzymes and fast conversion of substrates [2,3]. Recent developments have shown that the enzymes immobilized on the monoliths can be used for analytical as well as preparative purposes [4,5].

For proteolytic digestion, trypsin immobilized on monolithic supports has been used most often [2,3]. It could be demonstrated, by use on trypsin immobilized on monoliths, that the enzymatic conversion depends to a large extent on the flow rate. Additionally, the fact that denaturation of the high-molecular-mass substrates before proteolytic digestion

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by adding SDS and reducing agent beta-mercaptoethanol improves its conversion, suggests that steric effects, i.e. accessibility to the cleavage site in the protein, play an important role in the interaction with the enzyme [3]. Recent progress and development of monoliths toward further miniaturization makes them also suitable for use as supports in microfluidic devices for immobilization of proteolytic enzymes in the field of proteomics. Enzymatic microreactors in capillaries and on microfluidic chips prepared by immobilizing trypsin on porous polymer monoliths have been reported [6,7].

The inter-alpha inhibitor protein (IaIp) family is comprised of complex plasma proteins that consist of a combination of multiple polypeptide chains (light and heavy chains) covalently linked by a chondroitin sulphate chain. The major forms found in human plasma in relatively high concentration (between 0.6 and 1.2 g/L) are inter-alpha inhibitor (IaI) which consists of two heavy chains (H1 and H2) and one light chain (also termed as 'bikunin') and pre-alpha inhibitor (PaI) which consists of one heavy chain (H3) and one light chain [8,9]. Bikunin contains an N-linked oligosaccharide and bears additionally a chondroitin sulphate chain that is esterified via an internal unsulphated *N*-acetyl galactosamine residue with the C-terminal amino acid of the heavy chain(s) of both inter- and pre-alpha inhibitor. The uncomplexed light chain (bikunin) is known to inhibit several serine proteases, such as trypsin, human leukocyte elastase (HLE), plasmin, Factor Xa and cathepsin G. IaIp have have been implicated to play a role in inflammation/sepsis, tumor invasion and formation of metastasis [10–12,14]. The role of the light chain as well as the heavy chains in inhibition of tumor growth and metastasis has also been discussed [15,16].

The monoclonal antibody (MAb) 69.31 directed towards human IaIp has been developed and partially characterized in our laboratory [10]. In this report, the affinity purification of IaIp using MAb 69.31 immobilized on monolithic CIM disk is described. Affinity purified IaIp were subsequently cleaved by elastase, also immobilized on monolithic disks. It could be shown that MAb 69.31 is directed against bikunin, the active part of the inhibitor molecules.

2. Materials and methods

2.1. Chemicals

All fine chemicals purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of human IaIp

For isolation of pure IaIp, partially purified IaIp mixture was used. These proteins were isolated by size-exclusion chromatography as a side fraction from solvent/detergent and heat-treated, clotting factor VIII (fVIII) preparation (Octanate, Octapharma Pharmazeutika, Vienna, Austria) as described elsewhere [17].

2.3. Electrophoretic methods

SDS–PAGE was performed according to Laemmli [18]. The protein samples were separated on 12.5% Tris, etc. Glycine gel. The electrophoresis was performed using the Mini Protean II electrophoresis system (Bio-Rad, Hercules, CA, USA).

2.4. Trypsin inhibition assay

The ability of IaIp to inhibit trypsin was measured spectrophotometrically by using the synthetic substrate *p*-toluenesulphonyl-L-arginine methyl ester (TAME). The hydrolysis of the substrate by trypsin in the presence of the inhibitors was measured on BioTek microplate reader at 410 nm wavelength.

2.5. Immunochemical methods

A competitive enzyme-linked immnosorbent method (ELISA) using MAb 69.31 to measure IaIp concentration in biological mixtures has been developed in our laboratory and has been described elsewhere [9]. Western blot analysis was performed by using the same monoclonal antibody or polyclonal antibodies R20 against IaIp [10]. After SDS–PAGE, proteins were transferred onto nitrocellulose membrane using a semi-dry TransBlot apparatus (Bio-Rad) according to the manufacturer's instruction. Membranes were blocked with 5% non-fat dry milk in wash buffer [phosphate-buffered saline (PBS) + 0.1% Tween 20)] and primary antibody was incubated at 37 °C for 1 h. After extensive washing, horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Zymed, San Fransisco, CA, USA) was added. The positive reactivity was visualized by using metal-enhanced DAB (Pierce) or chemiluminescence substrate SuperSignal (Pierce, Rockford, IL, USA).

2.6. Immobilization of monoclonal antibody and elastase on CIM disks

For antibody immobilization, 2 mg of purified monoclonal antibody 69.31, was used. The antibody was diluted in the immobilization buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5) at a concentration of 0.5 mg/mL and immobilized to epoxy-activated CIM monolithic disk with 16 mm diameter, 3 mm thickness and volume of 0.36 mL (BIASeparations, Ljubljana, Slovenia). The immobilization procedure was performed according to the manufacturer's protocol. The blocking buffer was 0.2 M Tris–HCl, pH 8.5. The ligand density was estimated about 2 mg antibody/CIM disk. The binding capacity of the epoxy CIM disk is 3–5 mg of IgG per disk.

For enzyme immobilization, 5 mg purified elastase (Sigma) dissolved in the immobilization buffer was used. Further procedure was identical as for antibody immobilization. The ligand density after immobilization was 1.5–2.0 mg elastase/CIM disk.

2.7. Chromatography

All chromatographic separations were performed on Bio-Rad BioLogic Workstation. For immunoaffinity chromatography, CIM disk with immobilized monoclonal antibody 69.31 was applied. For IaIp isolation, 5 mL of fVIII concentrate in PBS, pH 7.4, with a concentration of 100 international units (IU)/mL was applied. The bound IaIp were eluted with 0.1 M citric acid, pH 2.4 and were immediately neutralized by adding 1 M NaOH. The flow rate during sample application and elution was 5 mL/min.

For enzymatic cleavage, 0.5 mg of purified human plasma derived IaIp in 1 mL PBS, pH 7.4, were pumped through a disk with immobilized elastase with a flow rate ranging from 0.1 to 2.0 mL/min. For the system cleaning, 0.1 M citric acid, pH 2.4, was used.

2.8. Protein identification

Mass spectrometric and N-terminal amino acid sequence analysis was performed at the Core Laboratory for Proteomic Mass Spectrometry at University of Massachussets Medical School, Worcester Foundation Campus, Shrewsbury, MA, USA.

3. Results and discussion

3.1. Protein isolation and identification

The monoclonal antibody 69.31 was generated by using a subtractive immunization protocol against the components of human plasma derived fVIII preparations. Immunoaffinity chromatography on immobilized MAb 69.31 CIM disk of human plasma fVIII concentrate resulted in two protein bands with apparent molecular masses in SDS–PAGE of ca. 250,000 and 125,000, respectively (Fig. 1A–C). Partial N-terminal amino acid sequence analysis of the 250,000 band identified peptide sequences belong to inter-alpha inhibitor heavy chain H2 precursor and light chain (bikunin) (Fig. 2). Both polypeptide chains are characteristic components of inter-alpha inhibitor (IaI) [1,2]. Using the same methods, inter-alpha inhibitor heavy chain precursor H3 and bikunin were identified in the 125,000 protein band (Fig. 2). Both polypeptide chains are components of pre-alpha inhibitor (PaI) [1,2].

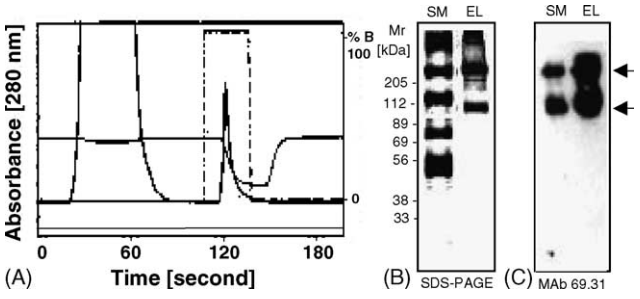


Fig. 1. Affinity purification of IaIp from fVIII concentrate Octanate. Five hundred IU fVIII were solved in 5 mL PBS, pH 7.4 and applied to the column with MAb 69.31, immobilized on epoxy-activated CIM disk. Flow rate during application, washing and elution was 5 mL/min, pressure 0.1 MPA. Buffer A: PBS, pH 7.4, buffer B (elution buffer): 0.1 M citric acid, pH 2.4 (A). SDS–PAGE analysis of the starting material (SM) and eluted fraction (EL) of the immunoaffinity on immobilized MAb 69.31 CIM disk (B). Western blot analysis with MAb 69.31 detected both IaI with Mr ca. 250,000 and PaI with ca. 125,000 (arrows) in the starting material and eluted fraction (C).

3.2. Inhibitory activity of isolated iaIp

The ability of IaIp to inhibit trypsin hydrolysis of synthetic substrate TAME is shown in Fig. 3. Trypsin alone showed a typical activity curve. Affinity purified IaIp inhibited trypsin activity in a concentration-dependent manner (partial inhibition at 5 μg and complete inhibition at 10 μg). The addition of purified MAb 69.31 abolished the inhibitory activity of IaIp to trypsin. This effect was specific since the addition of an unrelated antibody (MAb 69.20 against fVIII) did not block the inhibitory activity of IaIp and the addition of MAb 69.31 alone to trypsin did not have any significant effects (Fig. 3). It was determined previously that MAb 69.31 was reactive with the light chain (bikunin) as demonstrated by Western blot analysis of IaIp treated with hyaluronidase, an enzyme that cleaves the glycoaminoglycan chain linking the heavy and the light chain of the IaI and PaI complex [9]. The results of the trypsin inhibition assay suggest that the epitope of MAb 69.31 is located at or proximal to the active site of bikunin. Moreover, in in vitro three-dimensional cell invasion assay, purified human IaIp specifically and significantly inhibit the migration of invasive cancer cells (Lim et al, manuscript in preparation). The inhibitory activity of IaIp in this assay was abolished by the addition of purified MAb 69.31, suggesting that the epitope defined by this monoclonal antibody plays important role in cancer cell invasion. Precise mapping of the epitope is therefore desirable for further study of the anti-invasive effects of IaIp.

3.3. Enzymatic digestion

In order to isolate the proteolytic digest of IaIp in solution, bovine pancreatic elastase was immobilized on epoxy-activated CIM disk. Affinity purified human plasma derived IaIp (see Fig. 1) was used as a starting material. Elastase immobilized on CIM disk cleaved human plasma derived IaIp complex into small fragments (Fig. 4A). The digested fragments produced by elastase were smaller compared to the cleavage products after hyaluronidase treatment [10]. The size of proteolytic fragments was also dependent of the flow rate. The proteolytic digestion at flow rates between 0.25 and 2.0 mL/min was incomplete and residual complexed forms of IaI and PaI were still detectable by Western blot with MAb 69.31 and rabbit polyclonal antibody against human IaIp (R20) (Fig. 4B and C). The rabbit antibody R20 is reactive against heavy chains as well as light chain of IaI and PaI,

Fig. 2. Identification of isolated IaIp by N-terminal amino acid sequence. In the upper band with the apparent molecular mass of 250,000, bikunin and heavy chain H2 (HC-2) could be identified as the components of inter-alpha inhibitor (IaI). The polypeptides identified in the lower band (125,000) were bikunin and heavy chain H3 (HC-3) as the components of Pre-alpha inhibitor (PaI).

whereas MAb 69.31 is reactive specifically against the light chain (bikunin).

This result agrees with earlier findings with trypsin, immobilized on monolithic supports. Also in this case, when high-molecular mass substrates were used, the degree of proteolytic digestion was dependent on the flow rate [3,13]. The elastase CIM disk was stable and still active after repeated runs and cleaning with 0.1 M citric acid, pH 2.4. Even after a 6 months storage in PBS, no significant activity loss of the immobilized enzyme disk was observed, suggesting possible long term use of the immobilized CIM disks.

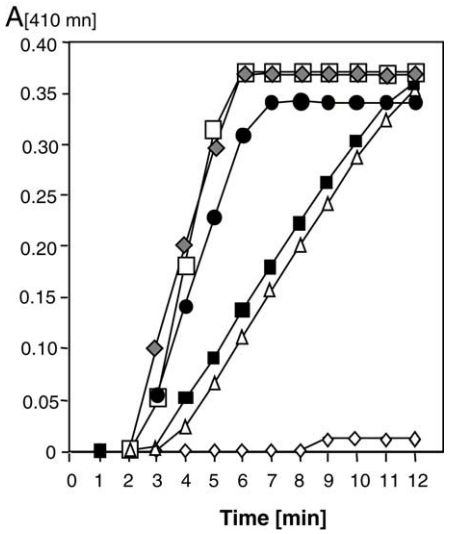


Fig. 3. Inhibitory activity of IaIp to the serine protease trypsin. The ability of IaIp to inhibit trypsin hydrolysis of *p*-toluenesulphonyl-L-arginine methyl ester (TAME) was measured spectrophotometrically. Trypsin alone showed a typical activity curve (□). Affinity purified IaIp inhibited trypsin activity in concentration dependent manner [partial inhibition at 5 μg (■) and complete inhibition at 10 μg (◊)]. The addition of purified MAb 69.31 abolished the inhibitory activity of IaIp to trypsin (●). This effect was specific since the addition of an unrelated antibody (MAb 69.20, against clotting factor VIII) did not block the inhibitory activity of IaIp (△) and the addition of MAb 69.31 alone to trypsin did not have any significant effects (♦).

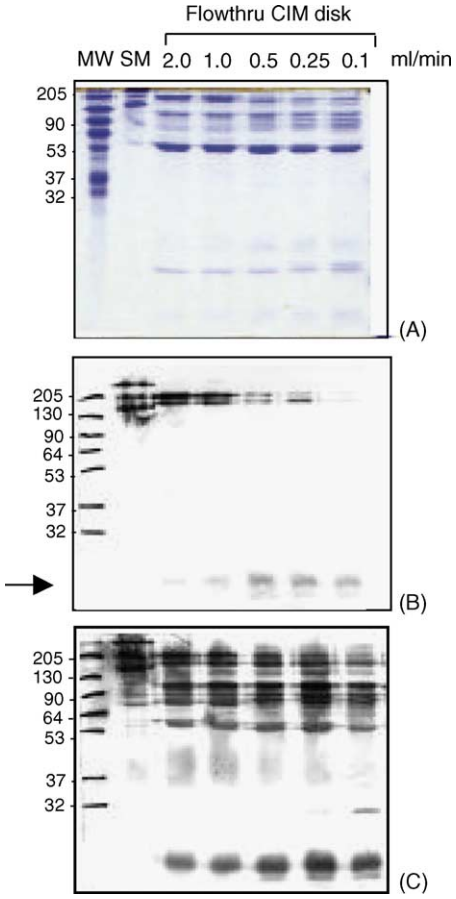
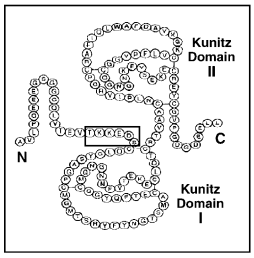


Fig. 4. Enzymatic digestion of IaIp on immobilized elastase CIM disk. The starting material (SM), human plasma derived IaIp was applied to the immobilized elastase CIM disk at various flow rates (from 0.1 to 2 mL/min). The digested IaIp fragments were separated on SDS–PAGE and stained with coomassie blue (A), or transferred onto nitrocellulose for Western blot analysis using monoclonal antibody 69.31 (B) or polyclonal antibodies R-20 against human IaIp (C). The rabbit antibody R20 is reactive against heavy chains as well as light chain of IaI and PaI, whereas MAb 69.31 is reactive specifically against the light chain (bikunin) with an apparent molecular mass of ca. 25,000 (B, arrow). Mw: molecular mass, values are $\times 10^3$.

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    kecreycgvp  gdgdeellrf  sn
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Fig. 5. Amino acid sequence of human bikunin (the light chain of IaIp, SWISSPROT accession P02760). The N-terminal sequence analysis revealed the first six amino acids (225–230; TKKEDS) of the proteolytic fragment of bikunin that is still reactive vith MAb 69.31 (boxed).

As shown in Fig. 4B, the smallest proteolytic fragment was still reactive with MAb 69.31 in Western blot assay. The target sequence of this protein fragment was obtained by N-terminal analysis using Edman degradation. The results revealed the first six amino acid residues (225–230; TKKEDS) of the proteolytic fragment of the light chain that is still reactive with the monoclonal antibody (Fig. 5). This six amino acid sequence is located at the beginning of two proteinase inhibitor domains of the Kunitz type (Domains I and II) and will be used as a starting point for the synthesis of overlapping peptide arrays to determine precisely the epitope defined by MAb 69.31.

One of the advantages in using monolithic disks is the possibility to combine multiple disks with different properties in a series in one housing. A combination of bioconversion with immuno-affinity disks would be possible to permit a rapid isolation of specific proteolytic digests in a single step. In this setting, purified IaIp can be applied to the conjoint liquid chromatographic disks with the immobilized Elastase CIM disk and MAb 69.31-immobilized affinity disk. The proteolytic fragments will be captured specifically by the antibody in the second disk and can be eluted subsequently.

4. Conclusion

Monoclonal antibody 69.31 immobilized on epoxy-activated CIM disk enabled a rapid and simple method for isolation of corresponding antigens, a mixture of IaIp. Immobilized enzyme CIM disk offers a rapid method for preparative isolation of protease cleavage fragments.

The smallest cleavage fragment of the light chain of IaIp complex was achieved by use of immobilized elastase CIM disk. Edman degradation of the fragment provided the primary amino acid sequence needed for designing peptide arrays to elucidate precisely the epitope of the MAb 69.31.

The immobilized enzyme on the CIM disk is stable and still active after repeated runs. A partial or complete digestion can be achieved by varying the flow rate.

Acknowledgement

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