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Research paper

Three low molecular weight cysteine proteinase inhibitors of human seminal fluid: Purification and enzyme kinetic properties

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ABSTRACT

The cystatins form a superfamily of structurally related proteins with highly conserved structural folds. They are all potent, reversible, competitive inhibitors of cysteine proteinases (CPs). Proteins from this group present differences in proteinase inhibition despite their high level of structural similarities. In this study, three cysteine proteinase inhibitors (CPIs) of low molecular weight were isolated from human seminal fluid (HSF) by affinity chromatography on carboxymethyl (CM)-papain–Sepharose column, purified using various chromatographic procedures and checked for purity on sodium-dodecyl PAGE (SDS-PAGE). Matrix-assisted laser desorption-ionization-time-of flight-mass spectrometry (MALDI-TOF-MS) identified these proteins as cystatin 9, cystatin SN, and SAP-1 (an N-terminal truncated form of cystatin S). All three CPIs suppressed the activity of papain potentially and showed remarkable heat stability. Interestingly SAP-1 also inhibits the activity of trypsin, chymotrypsin, pepsin, and PSA (prostate specific antigen) and acts as a cross-class protease inhibitor in *in vitro* studies. Using Surface Plasmon Resonance, we have also observed that SAP-1 shows a significant binding with all these proteases. These studies suggest that SAP-1 is a cross-class inhibitor that may regulate activity of various classes of proteases within the reproductive systems. To our knowledge, this is the first report about purification of CPIs from HSF; the identification of such proteins could provide better insights into the physiological processes and offer intimation for further research.

1. Introduction

Cysteine proteases are accountable for a lot of biological processes occurring in human body [1]. The main physiological role of CPs is intracellular catabolism of peptides and proteins [2], malfunctioning has been implicated in the development and progression of many diseases [3–6]. The activity of CPs is regulated by their specific natural protein inhibitors called cystatins. Cystatins have been isolated and characterized from different human tissues and body fluids [7–14]. Their inhibitory profiles, as well as their affinities for target enzymes, vary with different CPs. Appropriate steadiness between free CPs and their complexes with inhibitors is critical for the regulation of the proteolytic activity under normal physiological conditions [15–18]. Based on the molecule complexity, cystatins have been categorized into three families [19,20]: Family 1 cystatins (stefins) are found mainly intracellularly and have molecular weights of 12 kDa, family 2 cystatins (S, SA, SN, C) are essentially found extracellularly and have a molecular weight of 14 kDa, and family 3 cystatins are the high molecular weight kininogens. All of the characterized cystatins exhibit sequence homologies.

The presence of the different cystatins and their exact functions in the male accessory sex glands is largely still unknown. Cystatin A has been demonstrated to be present in the basal cells, in all cases of benign prostatic hyperplasias (BPH), low-grade prostatic intraepithelial neoplasias (PIN), and high-grade PIN and can aid in the diagnosis of prostatic adenocarcinoma [21]. Cystatin C is highly expressed and widely distributed throughout the male genital tract, suggesting that cystatin C is an important regulator for normal and pathological proteolysis in the male reproductive system [22]. Moreover, the presence of cystatin-related epididymal spermatogenic (CRES) proteins in the sperm acrosome, suggests their role in sperm maturation and fertilization [23]. Seminal

Abbreviations: CM, carboxymethyl; CPs, cysteine proteinases; CPIs, cysteine proteinase inhibitors; CRES, cystatin-related epididymal spermatogenic; HSF, human seminal fluid; LMW, low molecular weight; PCI, protein C inhibitor; PSA, prostate specific antigen.

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plasma also contains cystatin S, D, and B in relatively high amounts; however their roles remain to be established. CPIs are the dominant inhibitors in seminal plasma [13,22] therefore, may also have some important, although as yet unknown, roles in fertilization. Further studies are needed to explore their role with special attention to fertility and other functions in semen. In the present study, we have purified and identified three LMW cystatins of HSF using various chromatographic steps in a sequential manner and subsequent characterization of these proteins may promote our knowledge of biochemical mechanisms involved in human fertilization in future. We have also determined the enzymatic properties of the inhibitors to be able to assess their potential capacity as physiologically important inhibitors of CPs of human reproductive tract and/or in HSF.

2. Material and methods

2.1. Sample collection

Freshly ejaculated normal human semen was collected and pooled from the Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi. Ethical permission was taken from the same institute for the study (Ref. No.IESC/T-154/2010). Proteases (Papain, trypsin, chymotrypsin, pepsin, and proteinase K), hemoglobin, and trichloroacetic acid were purchased from Sigma–Aldrich (St Louis, MO, USA). Molecular weight marker (Unstained Protein Markers) was from Fermentas. All other chemical and reagents were of analytical grade and obtained from a local supplier.

2.2. Isolation and purification of LMW cystatins

To obtain seminal plasma, 20 ml of semen was centrifuged at 1300 × *g* for 15 min at 4 °C. The seminal plasma was further clarified by centrifugation at 10,000 × *g* for 15 min. The supernatant was then dialyzed against 50 mM sodium phosphate buffer (pH 6.5, containing 0.2 M NaCl, 2 mM EDTA, and 10 mM sodium azide) and was chromatographed at 4 °C on CM-papain–Sepharose column, prepared as described in Ref. [24]. After thorough washing with the buffer, proteins with affinity for carboxymethylated papain were eluted with 0.2 M trisodium phosphate, pH 12, containing NaCl, EDTA, and sodium azide, as in the binding buffer above. Eluted sample was neutralized with 2 M sodium phosphate buffer, pH 6.0, and concentrated at 4 °C using ultrafiltration (Millipore, Billerica, MA). The concentrated eluate from affinity column was further applied on DEAE–Sephacel (10 × 2.6 cm) column using 50 mM sodium phosphate, pH 6.0. After extensive washing, bound proteins were eluted with linear gradient of NaCl (0–0.5 M) in same buffer (Fig. 1A).

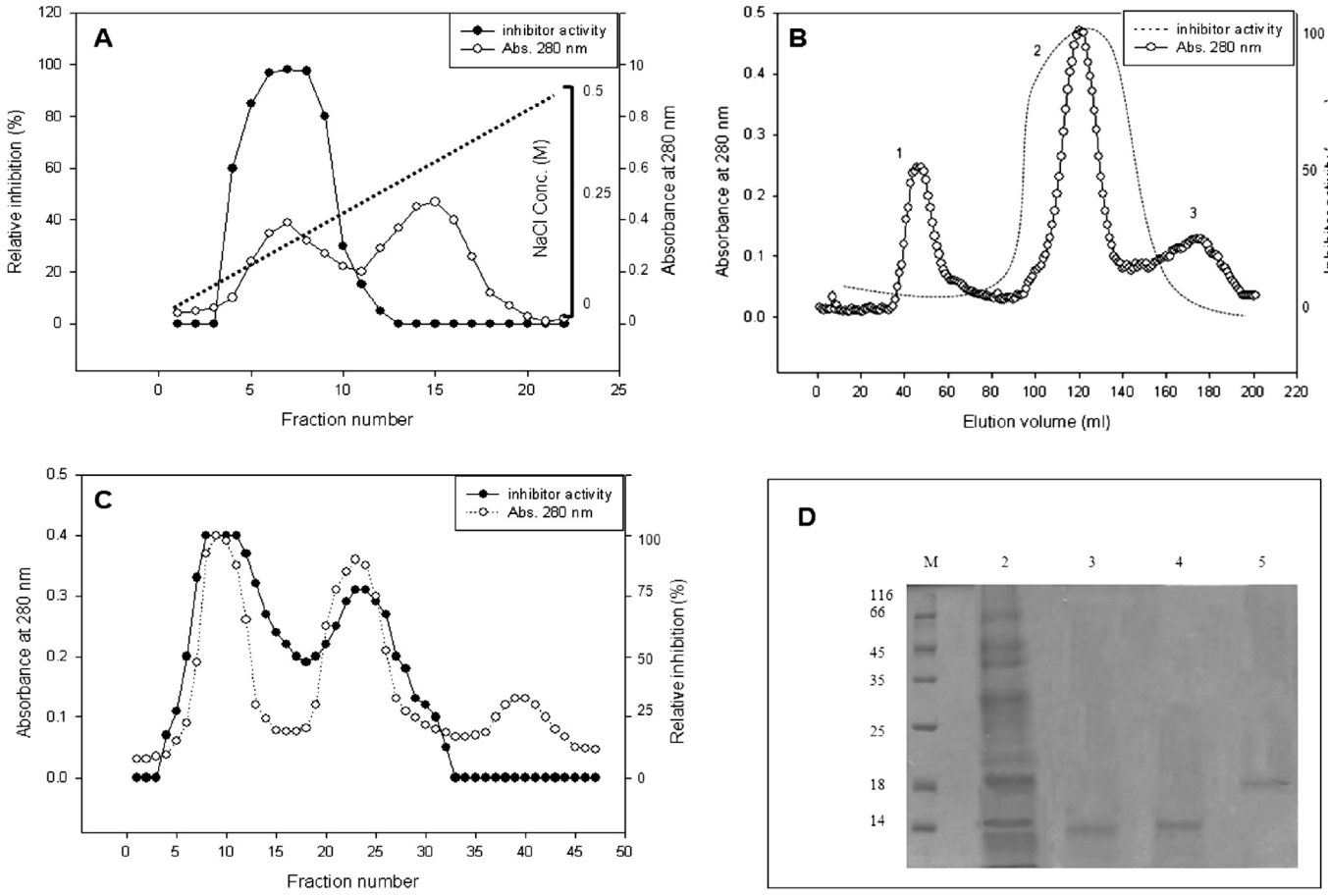

Fig. 1. Purification of HSF cystatins using affinity, ion exchange and gel filtration chromatography. (A) Elution profile on anion exchanger DEAE–Sephacel. The fraction with affinity for carboxymethylated papain was applied to DEAE–Sephacel column in 50 mM sodium phosphate, pH 6.0. Elution was carried out with a linear gradient of 0–0.5 M NaCl. (B) Purification of SAP-1 on Sephadex G-50. The fraction (peak 2) containing inhibitory activity was pooled and concentrated. (C) Purification of cystatin SN and cystatin 9 on strong-anion exchanger column (Resource Q). The flow-through fractions of the DEAE–Sephacel column was used as starting material. Cystatin SN was eluted at 0.05 M NaCl and cystatin 9 at 0.1 M (w/v) NaCl. (D) SDS-PAGE pattern of purified cystatins of HSF under reducing condition and stained with Coomassie blue. Lane 1, molecular weight markers (116 kDa: β-galactosidase b, 66 kDa: Bovine Serum Albumin, 45 kDa: Ovalbumin, 35 kDa: Lactate dehydrogenase, 25 kDa: REase Bsp 981, 18.8 kDa: β-Lactoglobulin and Lysozyme: 14.4 kDa); Lane 2, affinity eluent of CM-papain–Sepharose; Lane 3, SAP-1; Lane 4, cystatin SN; Lane 5, cystatin 9.