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Purification and characterization of CRISP-3 from human seminal plasma and its real-time binding kinetics with PSP94

Jenifer H. Anklesaria [a], Rushika R. Pandya [a], Bhakti R. Pathak [a], Smita D. Mahale [a,b,*]

[a] Division of Structural Biology, India
[b] ICMR Biomedical Informatics Centre, National Institute for Research in Reproductive Health (Indian Council of Medical Research), Jehangir Merwanji Street, Parel, Mumbai, 400 012, India

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ABSTRACT

Cysteine-rich secretory proteins (CRISPs) have been postulated to have a role in male reproduction and prostate pathophysiology. Of the mammalian CRISPs, CRISP-3 levels in particular have been shown to be upregulated in prostate cancer. Efforts have been made to obtain highly pure CRISP-3 for gaining structure-function information of this protein. However, well characterized and highly pure protein is not available yet. CRISPs from snake venom have been purified using prostate secretory protein of 94 amino acids (PSP94) has been reported earlier. In the present study, CRISP-3 was purified to homogeneity from human seminal plasma using human PSP94-immnobilized affinity column. The molecular mass of the purified protein was determined by SDS-PAGE followed by immunoblotting and found to be ~26 kDa and ~28 kDa. The purity was further verified using MALDI-TOF MS analysis, where two peaks at m/z 25509 and 27715 were obtained. The lower molecular weight peak corresponds to the calculated molecular mass of CRISP-3 (~26 kDa); whereas the higher molecular weight peak was confirmed to be the glycosylated form (~28 kDa) from the deglycosylation experiment. Binding of PSP94 in increasing concentrations to purified CRISP-3 immobilized chip was further validated using surface plasmon resonance. The kinetics data suggested that purified CRISP-3 binds specifically and with high affinity to PSP94. In conclusion, a homogeneous preparation of highly pure CRISP-3 protein is obtained from human seminal plasma.

1. Introduction

The cysteine-rich secretory protein (CRISP) family of proteins are characterized by 16 highly conserved cysteine residues and mainly found in mammals and reptiles [1]. CRISP proteins comprise of a C-terminal CRISP domain, which includes an ion-channel regulatory (ICR) region and a hinge region. The hinge region links the ICR region to the N-terminal CAP (Cysteine-rich secretory proteins, Antigen 5) domain [2]. Three CRISPs have been reported in humans, namely, CRISP-1, CRISP-2 and CRISP-3, according to their tissue distribution [1]. CRISP-1 expression is restricted to the epididymis [3,4], while CRISP-2 is expressed mainly in the testis [5,6]. As for CRISP-3, the transcripts are predominantly found in the salivary gland, pancreas and prostate, and at a lower level in the epididymis, ovary, thymus and colon [1].

Cysteine-rich secretory protein 3 (CRISP-3), previously referred to as specific granule protein of 28 kDa (SGP28) is a constituent of the blood plasma and exocrine secretions, such as saliva, sweat and seminal plasma [7,8]. It is a glycoprotein first isolated from human neutrophils [9]. The occurrence of CRISP-3 in neutrophil granules and exocrine secretions, and its sequence similarity to plant pathogenesis-related proteins involved in host defense is suggestive of a role in innate immunity. It has also been shown to play a role in prostate cancer; wherein its levels are up-regulated [10,11]. In humans, CRISP-3 has been shown to interact with prostate secretory protein of 94 amino acids (PSP94) in seminal plasma [12] and α1B-glycoprotein in blood plasma [13]. It is postulated that the ion channel regulatory activity of CRISP-3 might be inhibited by its binding partners [12–14]. Additionally, it has been reported that PSP94-CRISP interactions are not limited to humans [15,16] and could be of functional relevance to all mammalian CRISPs.

Previous studies from our lab have successfully demonstrated the purification of PSP94 from human seminal plasma [17] and CRISP-3 was identified as one of the proteins interacting with PSP94 apart from prostatic acid phosphatase (PAP) [18]. Although the binding of CRISP-3 to PSP94 has been previously reported, the

* Corresponding author at: Division of Structural Biology and ICMR Biomedical Informatics Centre, National Institute for Research in Reproductive Health (Indian Council of Medical Research), Jehangir Merwanji Street, Parel, Mumbai-400 012, India.
E-mail address: smitamahale@hotmail.com (S.D. Mahale).

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function of either protein has not been clearly defined. Investigating the function of CRISP-3 in the past has been constrained by difficulty in obtaining the pure protein. Several attempts have been made to purify human CRISP-3 from neutrophil extracts [9,12] or as a recombinant C-terminally truncated form of CRISP-3 (rCRISP-3Δ) protein expressed in bacterial cells [7]. Recently, Volpert et al. reported the expression and purification of native CRISP-3 in HEK 293 cells [19]. However, these methods had their limitations and well characterized, highly pure human CRISP-3 for structure/function analysis is yet not available. As a result, we attempted purification of native CRISP-3 from human seminal plasma to a high degree of purity.

In this paper, we utilized the property of CRISP-3 interacting with PSP94 to purify and characterize native CRISP-3 from human seminal plasma by affinity chromatography. The affinity purified CRISP-3 was further validated for its binding kinetics with PSP94 using surface plasmon resonance analysis.

2. Materials and methods

2.1. Samples

This study was approved by the Institutional Ethics Committee for Clinical Studies (No.: 200/2011). Freshly ejaculated semen from healthy volunteers was collected according to World Health Organization recommendations [20] and allowed to liquefy for 1 h at room temperature (RT). The semen samples were then centrifuged for 20 min at 500*g* at 26 °C to separate sperm from seminal plasma. The seminal plasma was further centrifuged at 15,000*g* for 20 min and the supernatant with protease inhibitor cocktail (Roche, Penzberg, Germany) was stored at −20 °C until use.

2.2. Fractionation of human seminal plasma proteins

Fractionation of human seminal plasma proteins was carried out as described earlier [17]. In brief, human seminal plasma was subjected to ammonium sulphate precipitation followed by hydrophobic interaction chromatography (HIC) using Phenyl Sepharose 6 Fast Flow (high substitution) beads (Amersham Biosciences, Uppsala, Sweden). All samples were filtered through a 0.22 μm filter (Millipore, Darmstadt, Germany). The bound fraction following elution was further separated by reverse phase high performance liquid chromatography (RP-HPLC) using a 250 × 21.5 mm Hi-Pore 318 C-18 (BioRad Laboratories, Hercules, CA, USA) preparative column. The separation was carried out over a period of 3 h using a linear gradient of 0%–70% Acetonitrile in 0.1% Trifluoroacetic acid (TFA)/H_2O. Fractions of 4 mL at a flow rate of 2 mL/min were collected and subsequently concentrated. PSP94 and CRISP-3 containing fractions were further identified by immunoblotting.

2.3. Polyacrylamide gel electrophoresis and immunoblotting

Fraction I (10 ng), fraction II and III (10 μg each) were resolved on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, followed by transfer onto a nitrocellulose membrane. After blocking with 5% non-fat dry milk (NFDM) in TBS-T buffer (20 mM Tris Buffered Saline, pH 7.4 containing 0.1% Tween 20) overnight at 4 °C, the blot was incubated with goat anti-human CRISP-3 antibody (R&D systems Inc., Minneapolis, USA) and affinity-purified rabbit anti-human PSP94 antibody raised in the laboratory [17]. The blot was further incubated with horseradish-peroxidase conjugated polyclonal rabbit anti-goat (Bangalore Genei, Bangalore, India) and goat anti-rabbit (Bangalore Genei, Bangalore, India) secondary antibody respectively. All incubations were in 1:2000 dilution with TBS-T containing 1% NFDM for 1 h at room temperature (RT). Finally, the blot was developed using enhanced chemiluminescence (ECL Plus, GE Healthcare, Buckinghamshire, UK).

2.4. Purification of CRISP-3 using affinity chromatography

The CRISP-3 containing fraction (fraction II) was subjected to affinity chromatography using PSP94 immobilized column. Briefly, PSP94 affinity column was prepared by coupling native human PSP94 (~8 mg), previously purified in our laboratory [17], to a 1 mL HiTrap NHS-activated HP column (GE Healthcare BioSciences AB, Uppsala, Sweden) according to the manufacturer's protocol. The coupling efficiency was calculated as the difference in concentrations of PSP94 in the coupling solution and in the wash solution after coupling. To this, fraction II (~2 mg protein) from RP-HPLC was applied. The unbound proteins were collected by thoroughly washing the column with 50 mM sodium phosphate buffer pH 7.4 containing 0.1 M NaCl followed by the same buffer containing 0.5 M NaCl. Finally, the bound proteins were eluted in 0.2 M glycine pH 2.0. Fractions of 1 mL were collected at a flow rate of 0.25 mL/min. The absorbance of the eluate was continuously monitored at 280 nm with AKTA explorer system (Amersham Biosciences, Uppsala, Sweden) and the part corresponding to the peak of absorbance was collected, neutralized with 2 mL of 50 mM sodium phosphate buffer pH 7.4 containing 0.5 M NaCl. The affinity purified fraction (eluate) was concentrated to a fixed volume by ultrafiltration using a 3 kDa molecular weight cut-off membrane (Merck Millipore, Darmstadt, Germany). Protein concentration was determined using modified Folin Lowry's method [21]. Bovine serum albumin was used as a standard.

2.5. Characterization of affinity purified CRISP-3

The eluate post affinity chromatography was characterized by SDS-PAGE followed by silver staining and western blot analysis using anti-human CRISP-3 antibody. In order to confirm that the eluate contained only CRISP-3 and not CRISP-2 known to be present on the sperm, it was further subjected to immunoblotting as described earlier using mouse anti-CRISP-2 antibody (R&D systems Inc., Minneapolis, USA) and goat anti-mouse secondary antibody (Santacruz Biotech Inc., CA, USA).

2.6. Analysis of CRISP-3 at each purification stage

In a separate experiment, fractions at each stage of purification were resolved on two SDS-PAGE gels and subjected to silver staining and immunoblotting. Purity of CRISP-3 at each stage was determined using densitometric analysis of the total protein from the silver stained gel and immunoreactive bands of CRISP-3 from western blot as compared with recombinant human CRISP-3 (rhCRISP-3) as a standard (R&D systems Inc., Minneapolis, USA) [19]. The ImageQuantTL Software from GE Healthcare (Buckinghamshire, UK) was used for densitometric analysis.

2.7. Molecular mass analysis of affinity purified CRISP-3

In order to assess the purity and molecular mass of the affinity purified protein, matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis was performed (outsourced to Proteomics facility, ACTREC, Mumbai). The eluate was suspended in 2 μL of 50% ACN and 0.1% TFA and spotted onto sample plate with matrix solution containing 5 mg/mL sinapinic acid in ACN and 0.1% TFA.