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β-Microseminoprotein binds CRISP-3 in human seminal plasma

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Abstract

β -Microseminoprotein (MSP) and cysteine-rich secretory protein 3 (CRISP-3) are abundant constituents of human seminal plasma. Immunoprecipitation and gel filtration of seminal plasma proteins combined with examination of the proteins in their pure form showed that MSP and CRISP-3 form stable, non-covalent complexes. CRISP-3 binds MSP with very high affinity, as evidenced by surface plasmon resonance. Due to far higher abundance of MSP in prostatic fluid, it manifests large overcapacity for CRISP-3 binding. Structural similarity with an MSP-binding protein from blood plasma suggests that CRISP-3 binds MSP through its aminoterminal SCP-domain.

Keywords

Cysteine-rich secretory protein; β-Microseminoprotein; PSP94; Seminal plasma; Prostate cancer; Protein complex; SCP-domain; Mass spectrometry; Surface plasmon resonance

Human cysteine-rich secretory protein 3 (CRISP-3; also known as SGP28) was first isolated from neutrophilic granulocytes, but it is also a constituent of blood plasma and exocrine secretions, such as saliva and seminal plasma [1]. It belongs to a family of CRISPs found in mammals and reptiles, which is characterized by 16 highly conserved cysteine residues of a total of 220–230 amino acids and 35–85% identity in the primary structure [2–4]. Recently, the crystal structure of a snake venom CRISP was elucidated [5]. This confirmed the previously proposed two-domain structure of CRISPs with a large N-terminal SCP domain (after sperm coating protein, an alternative name for murine CRISP-1) and a smaller, compact C-terminal domain, separated by a hinge-region [3,5].

A novel protein with an N-terminal SCP domain has been identified in human blood plasma and is called PSP94-binding protein (PSPBP) as it binds to β -microseminoprotein (MSP), also known as prostate secretory protein of 94 amino acids (PSP94) [6]. MSP is one of the three predominant proteins secreted by the prostate gland, but it is also present in other body fluids, e.g., tracheobronchial fluid. In general, MSP-synthesis is associated with tissue producing

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mucous secretions [7,8]. It is yet unknown whether MSP binds to PSPBP through the SCP domain or via the large C-terminal part of the molecule [6]. One way to answer this question is to investigate whether MSP binds other proteins with an SCP domain, and the abundance of both MSP and CRISP-3 in human seminal plasma prompted us to test the hypothesis that these proteins form high-affinity complexes.

Materials and methods

Materials

Human MSP was purified from seminal plasma with the method described for the purification of pig MSP [9]. Human CRISP-3 was purified from granulocytes [10]. Specific antisera were raised in rabbits against recombinant C-terminally truncated human CRISP-3 and native human MSP [1,11]. Semen was collected from healthy volunteers and allowed to liquefy for 1 h at room temperature (RT), followed by centrifugation to remove spermatozoa and was subsequently stored at −20 °C until use.

Gel filtration

Proteins were separated according to their molecular size by gel filtration on a Superose 12 HR 10/30 column using Äkta FPLC (Amersham Biosciences, Uppsala, Sweden). Samples were diluted in PBS (pH 7.4) and incubated at 37 ° C for 15 min followed by centrifugation. Samples of 200 μl were applied to the column, which was equilibrated in PBS. Fractions of 0.25 ml were collected at a flow rate of 0.25 ml/min. The column was calibrated using the same conditions with a combination of high and low molecular weight markers (aldolase (158 kDa), bovine serum albumin (dimer 134 kDa, monomer 67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa)). Elution volumes were determined from the chromatograms and a calibration curve was constructed using linear regression.

Quantification of proteins

The concentration of pure samples was determined using a spectrophotometer and the calculated molar extinction coefficients at 280 nm for CRISP-3 (55,580 M^{-1} cm^{-1}) and MSP (17,100 M^{-1} cm^{-1}) [12]. The concentration of CRISP-3 in seminal plasma was measured by enzyme-linked immunosorbent assay [1] and MSP with a modification of the competitive immunoassay for human MSP [11].

Immunoprecipitation of seminal plasma proteins

The immunoglobulin G (IgG) fraction of anti-CRISP-3 and anti-MSP antisera was isolated on HiTrap protein A columns, immobilized on CNBr-activated Sepharose, and packed into separate columns according to the instructions by the manufacturer (Amersham Biosciences). Normal rabbit IgG (DAKO (X0936), Glostrup, Denmark) was immobilized on a separate column to serve as a control. Seminal plasma was centrifuged 15,000 rpm for 5 min, diluted 10-fold in buffer (50 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, 0.5 M NaCl, and 0.5% Triton X-100), and supplemented with 1 mM phenylmethylsulfonyl fluoride. Samples of 2 ml were applied to the control column and the flow-through was applied to either the anti-MSP or anti-CRISP-3 column. Columns were eluted with 0.2 M glycine–HCl (pH 2.5) after extensive washing with buffer. Eluted proteins were neutralized with 2 M Tris–HCl (pH 9.0) and concentrated using microcon 10 microconcentrators (Amicon, Beverly, MA).

SDS–PAGE and immunoblotting

Precipitated proteins were boiled in reducing sample buffer and subjected to SDS–PAGE in 12% separating and 3% stacking gels. Immunoblotting was performed as described [10]. The

IgG fraction of anti-CRISP-3 or anti-MSP antiserum was used as primary antibody (IgG concentration 3 μg/ml). Blots were developed with DAB/metal concentrate and stable peroxide substrate buffer (Pierce, Rockford, IL).

Mass spectrometry

Co-precipitated proteins from the anti-CRISP-3 column were identified by MALDI-TOF mass spectrometry of tryptic digests [10]. The peptide molecular mass fingerprints were used for a search of the SWISS-PROT and TrEMBL databases using the ''PeptIdent'' tool on the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics. An aliquot of the protein digest was introduced into a Q-Tof-2 tandem mass spectrometer (Micromass, Manchester, UK) using the nanospray interface and analyzed in MS mode as well as in MSMS mode to obtain specific sequence information.

Amino acid sequencing

Proteins from peak fractions corresponding to the MSP–CRISP-3 complexes in gel filtrations were desalted and concentrated on a ProBlott PVDF membrane using a ProSpin centrifugal cartridge (Applied Biosystems). The PVDF membrane was punched out, washed in 500 μl of 20% methanol, and transferred to the blot cartridge of the ''Procise'' protein sequencer (model 494A, Perkin-Elmer ABD, Foster City, CA), where it was analyzed using essentially the preprogrammed ''Blot'' cycles [10]. As a control of the yield of individual amino acids, β -lactoglobulin was analyzed by the same procedure.

Surface plasmon resonance analysis of binding kinetics

Kinetic measurements of the interaction between MSP and CRISP-3 were performed by real-time interaction analysis using a BIAcore 2000 instrument (Biacore, Uppsala, Sweden). Flow cells of CM5 sensor chips were activated with 20 μl of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 0.05 M *N*-hydroxysulfosuccinimide at a flow rate of 5 μl/min. Ligands, at 20 μg/ml in 10 mM Na-acetate, pH 3.8, were injected to reach 2000 resonance units, whereafter unreacted groups were blocked with 20 μl of 1 M ethanolamine, pH 8.5. A negative control consisted of an activated and blocked flow cell to which no ligand was added. The analytes, diluted to various concentrations in 10 mM Hepes, pH 7.4, 0.15 M NaCl, 5 mM EDTA, and 0.005% Tween 20, were injected for 100 s during the association phase at a constant flow rate of 30 μl/min. The dissociation was subsequently followed for 1200 s at the same flow rate. To regenerate the surface, a 10 μl bolus of 2 M NaCl in 10 mM HCl was used to remove bound analyte. Sensograms were corrected by subtraction of the signal from the negative control surface and used to calculate the rate and affinity constants with the BiaEvaluation 3.0 software (Biacore, Uppsala, Sweden).

Results

Formation and stoichiometry of MSP–CRISP-3 complex

Human MSP and CRISP-3 were incubated in a physiologic buffer and separated by size exclusion chromatography (Fig. 1A). CRISP-3 eluted as a double peak as we used a mixture of glycosylated and unglycosylated protein (shown in inset) with an apparent molecular mass of the glycosylated form estimated (from the calibration curve) to be 24 kDa, which is close to the expected monomeric size [1]. MSP eluted as a single peak corresponding to 21 kDa, suggesting that MSP either forms a homodimer or has an extended conformation under native conditions, since the theoretical mass of MSP is 10.7 kDa [7]. Following co-incubation of equimolar amounts of MSP and CRISP-3, the formation of complexes with a 1:1 stoichiometry was indicated as the two proteins eluted together in a single peak approximately 1 ml before the free proteins corresponding to a mass of 38 kDa. A fixed amount of CRISP-3 was then

incubated with either double or half the amount of MSP. Again the formation of 1:1 stoichiometric complexes was indicated (Fig. 1B), and the excess of MSP or CRISP-3 eluted according to their free form.

The quantitative relation between CRISP-3 and MSP was further evaluated by N-terminal protein sequence analysis of the complex. Proteins in the peak fraction from the gel filtrations with equimolar amounts of CRISP-3 and MSP (Fig. 1A, dashed line), and with double amount of MSP (Fig. 1B, dotted line) were examined. The first 15 residues were analyzed and two parallel sequences were identified, corresponding to the N-termini of CRISP-3 and MSP. Extrapolation of the combined repetitive yields to ‘‘residue 0’’ resulted in estimated initial yields of MSP and CRISP-3 of 20.0 and 12.4 pmol, respectively, in the sample from gel filtration with equimolar amounts of the proteins applied to the Superose column. The sample from the gel filtration with an excess of MSP gave initial yields of 35.8 and 18.8 pmol, respectively. Thus in both cases, an MSP–CRISP-3 ratio closer to 2 was found (1.6 and 1.9, respectively), suggesting a 2:1 stoichiometry. Due to a very different amino acid composition of the N-terminal sequences of MSP and CRISP-3 (SCYFIPNEG VPGDST and NEDKDPAFTALLTTQ), the ratios between the yield of individual amino acids from the two sequences were compared with the ratio between the yield of the same amino acids in a control protein (N-terminal sequence: LIVTQTMKGLDIQKVA). Six pairs of amino acids were chosen for evaluation, e.g., the ratios between the yield of residue number 5 from both MSP and CRISP-3 (I and D) were divided by the ratio between the yield of residue number 12 and 11 (I and D) in the control protein. This estimation resulted in an average MSP–CRISP-3 ratio of 2.0 (range 1.4–2.7) in the sample with the highest initial yields, i.e., from the gel filtration with an excess of MSP.

Identification of MSP–CRISP-3 complex in seminal plasma

Proteins were immunoprecipitated from seminal plasma with anti-CRISP-3, anti-MSP, and control IgG, respectively, and analyzed by SDS–PAGE and immuno-blotting (Fig. 2). The glycosylated and unglycosylated forms of CRISP-3, corresponding to bands of 29 and 27 kDa, were precipitated with anti-CRISP-3 IgG. A co-precipitated protein of 16 kDa was likely MSP (lane 2). The same protein band was also precipitated with the anti-MSP IgG (lane 3) and reacted with these antibodies by immunoblotting (lanes 8–9). Although CRISP-3 was barely visible in the Coomassie-stained anti-MSP precipitate, the 2 bands were clearly detected in immunoblotting (lane 6).

The co-precipitated proteins from the anti-CRISP-3 column were analyzed by mass spectrometry of tryptic peptides of the bands excised from the gel. In the 16 kDa band, a molecular mass corresponding to the N-terminal tryptic peptide of MSP was detected (observed protonated molecular mass 1798.89 Da, theoretical 1798.81 Da) by MALDI-TOF MS. The identity of this mass was verified by Q-TOF tandem mass spectrometry. No other MSP-derived fragments were detected by MALDI-TOF MS, which could be expected since the theoretical tryptic fragments are either too small or too large, or are devoid of basic amino acids for positive ionization. By Q-TOF tandem mass spectrometry, however, two other molecular masses were observed, corresponding to two different C-terminal fragments (KTCSVSEWII and TCSVSEWII), although with a lower score.

The 33 kDa band observed in all 3 precipitates (lanes 1–3) showed no reaction with anti-CRISP-3 or anti-MSP antibodies in immunoblotting (lanes 4–9). It was identified as prostate specific antigen (PSA) by mass spectrometry and its co-precipitation may be a result of unspecific binding to IgG or the Sepharose itself. The smeared bands around 16 kDa also contained several masses corresponding to tryptic fragments of semenogelins (holoproteins of 52–76 kDa), the major substrates of PSA [7]. In addition, the 16 kDa prolactin-inducible protein was identified, possibly present due to its ability to bind IgGs [13,14].

To further study the complex between CRISP-3 and MSP, seminal plasma proteins were separated by gel filtration (Fig. 3A) and specific immunoassays were used to measure CRISP-3 and MSP in each individual fraction (Fig. 4A). The majority of MSP was found in the same fractions as when the pure protein was examined alone (Fig. 1A). A minor proportion was found in the fractions with CRISP-3, corresponding to the complex. This was expected, as the concentration of MSP in seminal plasma (600–900 mg/L) is >20-fold higher than the concentration of CRISP-3 (3–30 mg/L) [7,15].

To evaluate the capacity of CRISP-3 binding, a small sample of seminal plasma was supplemented with a slight molar excess of pure CRISP-3, i.e., a final concentration of 2 mg CRISP-3 per milliliter seminal plasma, and evaluated by gel filtration. Compared to the gel filtration of normal seminal plasma, a new peak exactly corresponding to the complex formed with the pure proteins was observed in the UV-chromatogram (Fig. 3B). Immunoassay analyses of fractions revealed that all of MSP eluted according to the size of the complex, whereas a minor proportion of CRISP-3 eluted corresponding to its unbound form (Fig. 4B).

Kinetics of the MSP–CRISP-3 complex formation

We already knew that CRISP-3 is not covalently bound to other seminal plasma proteins, as separation of the proteins by SDS–PAGE followed by immunoblotting for CRISP-3 resulted in 2 bands corresponding to the expected sizes, also under non-reducing conditions [1]. The gel filtration experiments described above show that the MSP–CRISP-3 complex is stable and forms rapidly. The complex in seminal plasma did not dissociate in the buffer used for immunoprecipitation despite its high content of salt and detergent. The apparently high affinity between the proteins was further examined in detail by continuous monitoring of binding and dissociation by surface plasmon resonance technology. The affinity was evaluated separately for the 2 forms of CRISP-3 found in seminal plasma. The sensograms (Fig. 5) show that both glycosylated and unglycosylated CRISP-3 have very high affinity to MSP as indicated by very low dissociation rates. Global evaluation, using the 1:1 Langmuir model of binding, yielded kinetic binding parameters of the two isoforms (i.e., glycosylated and unglycosylated CRISP-3) that were indistinguishable (Table 1), indicating that the binding is unaffected by the glycosylation. The very low dissociation constants also suggest that the interaction between CRISP-3 and MSP is highly specific.

Discussion

We have recently studied CRISP-3 in the human male reproductive tract, and found mRNA and protein expression throughout the secretory epithelium from the epididymis to the prostate gland [15]. In that work, it was erroneously assumed that CRISP-3 is present in a non-complexed form in seminal plasma as gel filtration suggested a size close to its monomeric form. However, the experiments in this work reveal that this is unlikely. Instead, CRISP-3 in human seminal plasma is bound to MSP in a high-affinity complex. In contrast, almost all MSP in seminal plasma is present in a free, non-complexed form due to the manyfold higher concentration of MSP than of CRISP-3.

We were unable to define the exact stoichiometry of the MSP–CRISP-3 complex, but most likely one CRISP-3 molecule interacts directly with one MSP-molecule. As concluded in a previous report [16], the behavior of MSP in gel filtration (Fig. 1A) and non-reducing SDS–PAGE (shown in supplementary material) indicates that MSP exists as a homodimer. It is thus possible that one CRISP-3 molecule binds one MSP-dimer.

The fact that human MSP can bind both CRISP-3 and PSPBP, which are only structurally related in the N-terminal SCP domain, indicates that MSP binds both proteins through this domain. This is in agreement with the results shown here that binding is similar to glycosylated

and unglycosylated CRISP-3, since the glycosylation site is located at the C-terminus of CRISP-3 [1]. The limited sequence identity (35%) between the SCP domains of CRISP-3 and PSPBP suggests that MSP is likely to bind other proteins with an SCP domain, e.g., CRISP-1, which is present in seminal plasma in humans and rodents [2,3,17]. Moreover, the low degree of conservation of the primary structure of SCP domains suggests that formation of complexes is primarily dependent on the overall three-dimensional structure of the interacting molecules.

As the function of both MSP and CRISP-3 remains largely unknown, the function of the complex is not evident. The C-terminal domain of CRISP-3 has structural similarity to potassium-channel inhibitors [5] and several reptile CRISPs can inhibit different ion-channels [4]. Recently, CRISP-1 in rats was shown to inhibit capacitation of spermatozoa and thus prevent premature sperm activation [17]. MSP is not mixed with spermatozoa before ejaculation as it is exclusively secreted by the prostate gland in the human male reproductive tract [7,18]. This implies that CRISP-3 in the epididymal fluid may be available to interact with spermatozoa and epithelium, but that any excess of CRISP-3 will be bound in complex with MSP shortly after ejaculatory mixing with the prostatic fluid. We therefore suggest that one function of MSP is to complex-bind CRISP-3 and thereby inhibit an activity of CRISP-3, which is undesired after ejaculation.

We have recently proposed a similar function of α_1B-glycoprotein, which binds CRISP-3 in blood plasma [10]. Although both MSP and CRISP-3 also are present in blood plasma, we did not detect MSP–CRISP-3 complexes in this fluid. This is probably due to the much lower concentration of MSP (5–20 μg/L) compared to CRISP-3 (2–10 mg/L) and the fact that each protein has another more suitable binding partner in blood [6,10].

In prostate cancer, expression of MSP often decreases with higher tumor grade [19,20], whereas expression of CRISP-3 follows the reverse pattern with high expression in high-grade tumors [21,22], unpublished observations. A recent report showing that MSP reduces tumor growth in a prostate cancer rat model [23] suggests that the acquired imbalance in MSP–CRISP-3 expression may play a role in the pathophysiology of human prostate cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.bbrc.2005.05.139.

Abbreviations

CRISP
: cysteine-rich secretory protein

MSP
: β -microseminoprotein

PSA
: prostate specific antigen

PSP94
: prostate secretory protein of 94 amino acids

PSPBP
: PSP94-binding protein

SCP
: sperm coating protein

SGP28
: specific granule protein of 28 kDa

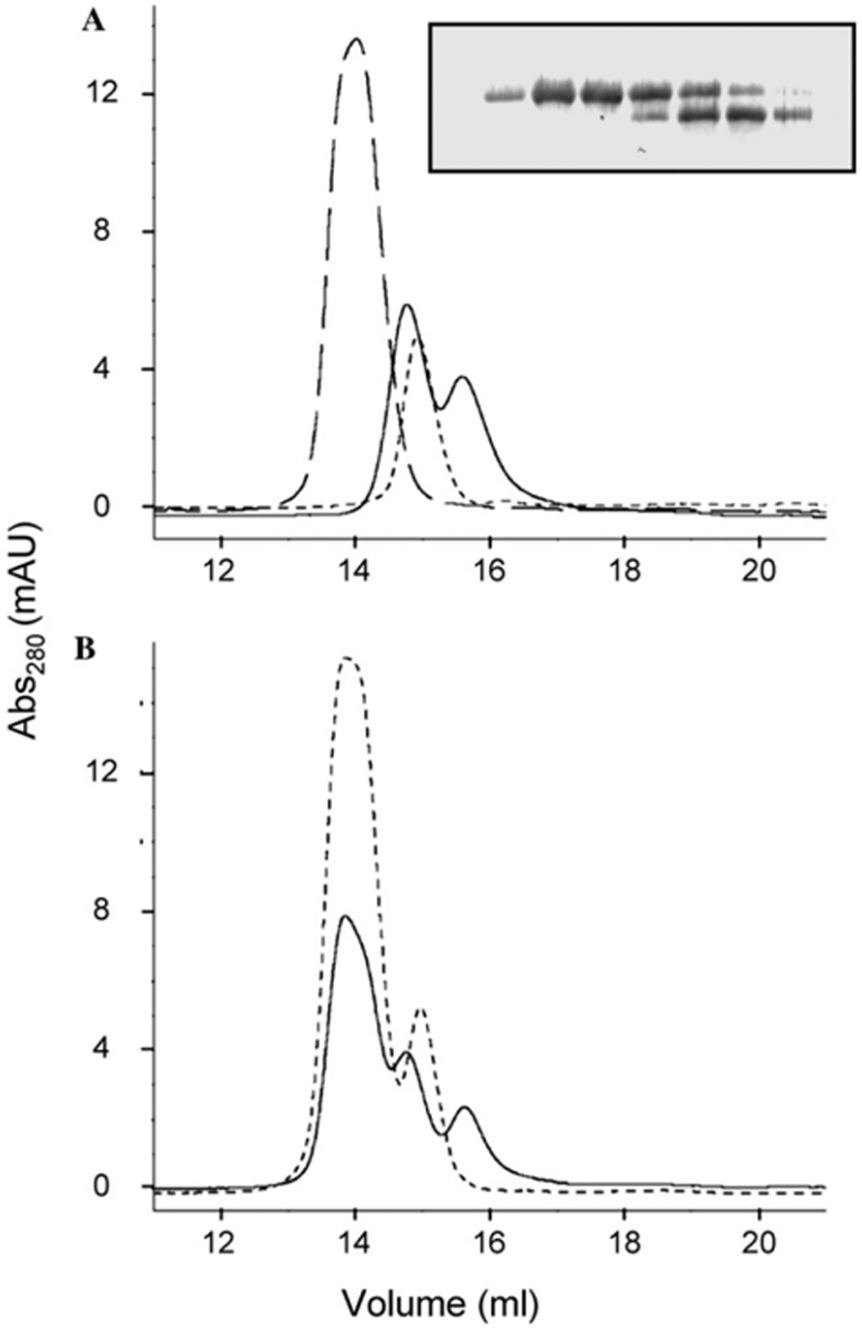

Fig 1.

Overlaid UV-chromatograms from gel filtration of pure CRISP-3 and MSP. (A) CRISP-3 alone (—), MSP alone (· · ·), or MSP and CRISP-3 (– – –) in equimolar concentration. (B) Same amount of CRISP-3 together with half (—) or double molar amount (· · ·) of MSP. Inset: Immunoblotting of fractions corresponding to the CRISP-3 double peak.

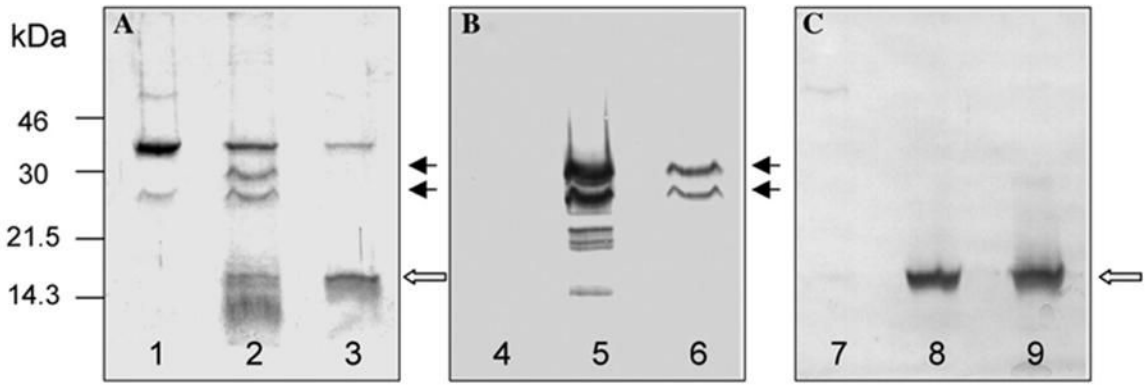

Fig 2.

Immunoprecipitation of proteins from seminal plasma. (A) Twelve percent gel stained in Coomassie blue; immunoblotting with anti-CRISP-3 IgG (B); and anti-MSP IgG (C). Precipitate from control column (lanes 1, 4, and 7), from anti-CRISP-3 column (lanes 2, 5, and 8), and from anti-MSP column (lanes 3, 6, and 9). Small arrows indicate the position of CRISP-3 holoprotein. Long arrows indicate the position of MSP.

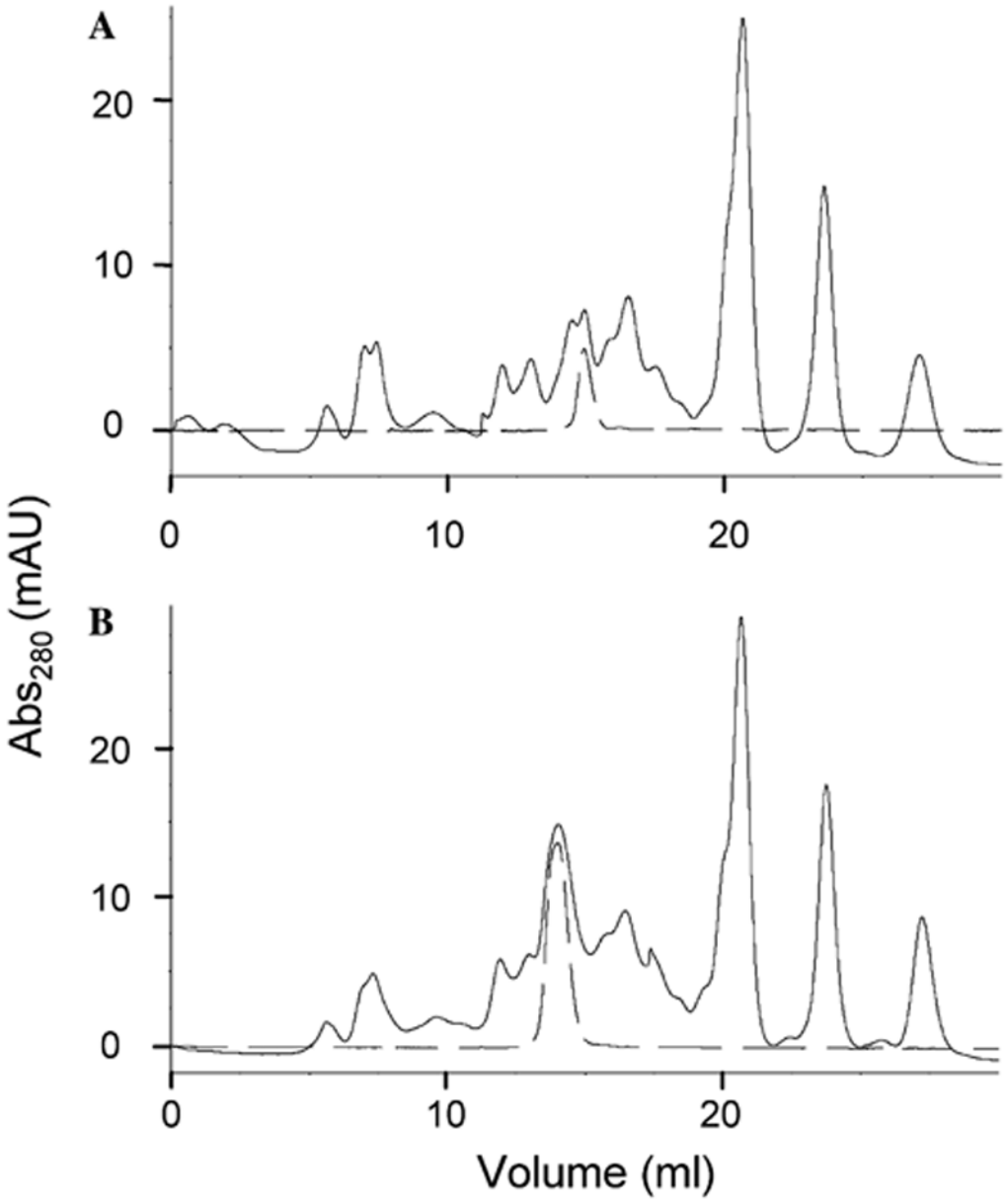

Fig 3.

Gel filtration of seminal plasma. (A) A sample of 5 μl seminal plasma and 195 μl PBS was applied. The UV-chromatogram (—) is compared with the gel filtration of pure MSP (– – –) from Fig. 1A. (B) A sample of 5 μl seminal plasma supplemented with 10 μg CRISP-3 in 195 μl PBS was applied. The UV-chromatogram (—) is compared with the gel filtration of pure MSP-CRISP-3 complex (– – –) from Fig. 1A.

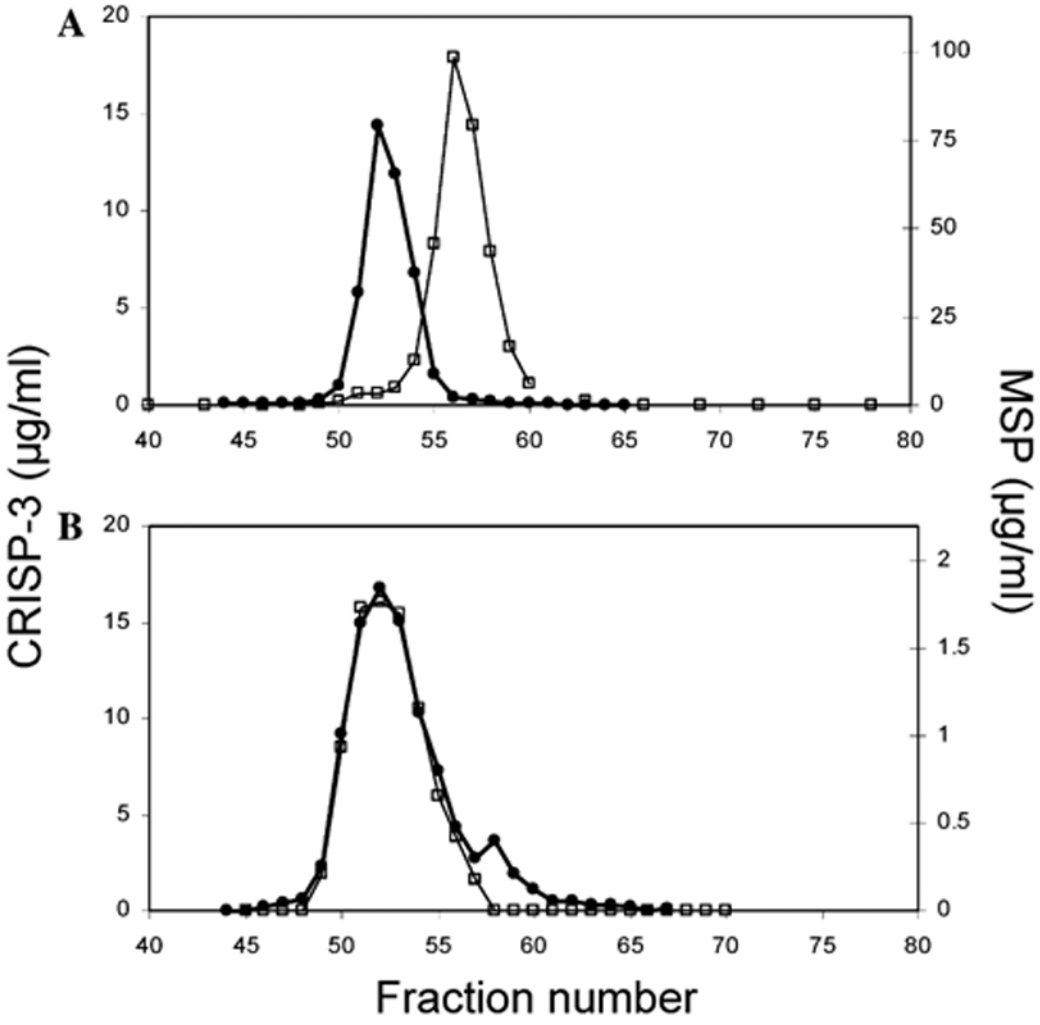

Fig 4.

CRISP-3 and MSP in fractions from gel filtration of seminal plasma. (A) A sample of 100 µl seminal plasma and 100 µl PBS was applied. (B) A sample of 5 µl seminal plasma supplemented with 10 µg CRISP-3 in 195 µl PBS was applied. The concentration of CRISP-3 (●) and MSP (□) was measured in relevant fractions.

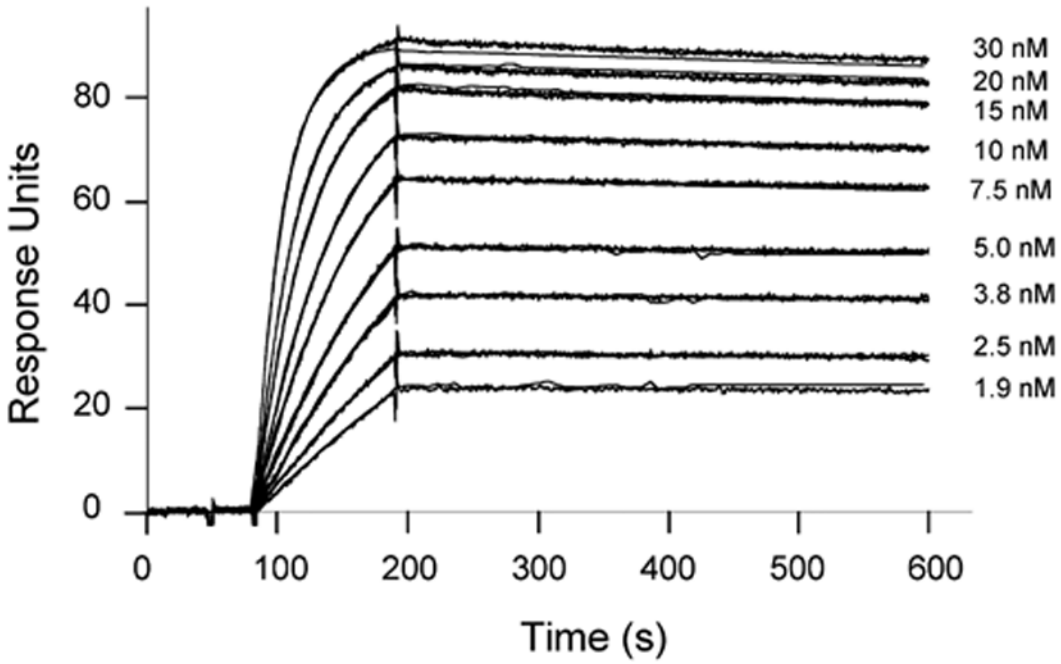

Fig 5.

Surface plasmon resonance analysis of the binding of MSP to immobilized CRISP-3. Sensograms were obtained from injections of MSP at different concentrations ranging from 1.9 to 30 nM. The sensograms and fitted curves from the experiments with unglycosylated CRISP-3 are shown.

Table 1

Surface plasmon resonance analysis of the binding of MSP to immobilized CRISP-3

Ligand	k_a (1/Ms)	k_d (1/s)	K_D (M)
Unglycosylated CRISP-3	1.45×10^6	9.11×10^{-5}	6.28×10^{-11} ($3.86–8.71 \times 10^{-11}$)
Glycosylated CRISP-3	1.13×10^6	7.65×10^{-5}	6.77×10^{-11} ($2.61–10.92 \times 10^{-11}$)

The kinetics of the interaction between CRISP-3 and MSP was evaluated with a Biacore 2000 instrument. The ligand CRISP-3 was immobilized on a surface that was challenged with different concentrations of MSP, and the association and dissociation rates (k_a and k_d) were measured. The equilibrium dissociation constants (K_D) are given with a 95% confidence interval within brackets.