Radioimmunoassay of β-Microseminoprotein, a Prostatic-Secreted Protein Present in Sera of Both Men and Women

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We describe a simple radioimmunoassay of β-microseminoprotein, one of the three most abundant secretory proteins of the prostate gland. The detection limit of the assay is 1 µg/L, and its precision, expressed as the total coefficient of variation, is <10% for values between 10 and 150 µg/L. Using this assay, we found that β-microseminoprotein immunoreactivity was present in sera from both sexes at about the same concentration. The protein detected had the same molecular size on gel chromatography as the protein isolated from seminal plasma, and dilution curves for the sera paralleled that for the pure protein. The findings suggest that β-microseminoprotein is present in serum of healthy subjects of both sexes and that it originates in tissue other than the prostate gland. The range of the serum concentration was 0–10.6 µg/L (median 4.1) for 51 healthy adult women and 1.1–14.7 µg/L (median 6.2) for 35 healthy adult men not older than 40 years. In males with prostatic cancer the concentration in serum was highly variable and often greatly increased. The concentration of β-microseminoprotein was correlated with that of creatinine in serum, suggesting that the protein is eliminated—at least partly—from the circulation by glomerular filtration. Little of the protein was present in the urine of women. In urine from men the concentration was high and variable, probably because of local contribution from the prostate gland to the urethral urine.

Additional Keyphrases: fertility · gonadotropins · β-inhibin · prostatic cancer · reference values · renal function · seminal plasma · urine

The three most abundant proteins in the secretions produced by the human prostate gland are prostatic acid phosphatase, prostate-specific antigen, and β-microseminoprotein (1). All three proteins are present in seminal plasma at about 1 g/L each (1–4). β-Microseminoprotein, which has an apparent molecular mass of 14 to 16 kDa on sodium dodecyl sulfate (SDS) electrophoresis (1, 5, 6), has been called β-inhibin (7), β-microseminoprotein (β-MSP) (8), and prostatic secretory protein of 94 amino acids (PSP94) (5) by various authors.

The amino acid sequence of β-microseminoprotein has been determined, both by protein sequencing methodology (6, 8, 9) and by complementary DNA sequencing (10). A 94-amino acid peptide containing 10 cysteine residues and five pairs of adjacent basic amino acids, and lacking carbohydrate, β-microseminoprotein shows little structural similarity to other proteins.

The biological function of β-microseminoprotein is still unknown. This protein was originally isolated from semen as a factor inhibiting the pituitary release of follitropin (follicle-stimulating hormone) (5), but this activity has now been questioned (11, 12) and several authors have argued against the name inhibin (13).

The potential of β-microseminoprotein as a marker for prostatic disease (14–16) and also a general interest in its biological function have prompted us to develop an assay for this protein. Here we describe the properties of our radioimmunoassay of β-microseminoprotein and report the results of measurements of this protein in serum and urine of adult humans of both sexes.

Materials and Methods

Materials

The purification of β-microseminoprotein from seminal plasma has been described previously (1). The product obtained was homogeneous, with an apparent molecular mass of 16 kDa as judged by SDS/polyacrylamide gel electrophoresis, and its amino acid composition and NH₂-terminal sequence were in agreement with published data (1, 5–10). We dissolved the purified protein in 50 mmol/L Tris HCl buffer, pH 7.4, containing NaCl, 0.15 mol/L (Tris saline), and stored this stock solution in aliquots at −20°C. The concentration of β-microseminoprotein in the stock solution was determined by quantitative amino acid analysis.

Antiserum to β-microseminoprotein was obtained by immunizing New Zealand White rabbits with the purified protein. The antigen (0.1 mg per animal) was injected subcutaneously on 10 different sites on the back of each animal. Freund's complete adjuvant was used the first time and Freund's incomplete adjuvant for repeated injections, done at monthly intervals. Antibody titers were checked regularly, and the antiserum from the best-responding animal was used. The antiserum was monospecific when tested by immunoelectrophoresis against normal seminal plasma. The single precipitation line obtained corresponded to the position for purified β-microseminoprotein, but no precipitation line was obtained with blood serum. Anti-rabbit IgG antiserum was raised in goats by standard methods (17). A sheep anti-rabbit immunoglobulin coupled to a solid phase (Pharmacia Decanting Suspension III) was obtained from Pharmacia, Uppsala, Sweden. Purified mouse IgG was kindly provided by Dr. Anders Grubb, Department of Clinical Chemistry, Malmö General Hospital, Malmö, Sweden. Bovine serum albumin (Cohn Fraction V) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, U.K.; human prolactin from Hormone Laboratory, Aker Hospital, Oslo, Norway; polyethylene glycol (mean M, 6,000) from Kebo AB, Stockholm, Sweden; Chloramine T from Merck AG, Darmstadt, F.R.G.; Na₁₂⁵¹ (17.4 kCi/g) from NEN Products, Boston, MA; and porcine insulin from Novo AS, Copenhagen, Denmark. Blue Dextran 2000, molecular-size marker proteins for electrophoresis, Sephadex G-25 superfine, and Superscan 12 HR10/30.
column were from Pharmacia. Water was purified by an Elga Spectrum R.O.1 system (The Elga Group, Buckinghamshire, U.K.). All other chemicals used were of analytical grade.

Procedures

**General methods.** SDS/polyacrylamide slab-gel electrophoresis was performed in 100 to 150 g/L gradient gels (18) and immunoelectrophoresis was carried out with standard methodology (19). For amino acid analysis we used a Beckman 6300 automatic amino acid analyzer after hydrolysis of an aliquot of β-microsemionoprotein stock solution in an evacuated borosilicate glass tube for 24 h at 110 °C in 6 mol/L HCl, with norleucine added as internal standard.

**Gel chromatography.** Gel chromatography, as a test of the homogeneity of labeled β-microsemionoprotein and for estimating the molecular size of immunoreacting material, was performed at room temperature (approximately 20 °C) on a 10 × 310 mm column of Superose 12 HR 10/30. The elution buffer—sodium barbital, 75 mmol/L, pH 8.6, containing 1.0 g of bovine serum albumin and 0.05 g of sodium azide per liter—was delivered with a 2150 HPLC-pump (LKB, Bromma, Sweden) at a flow rate of 0.7 mL/min. The column was calibrated with Blue Dextran 2000 (average molecular mass 2.0 MDa), mouse IgG (150 kDa), bovine serum albumin (68 kDa), human prolactin (23 kDa), and porcine insulin (6.0 kDa). The effluent was collected in 15-drop fractions and the volume of each fraction was determined by weighing. We monitored the fractions by radioactivity measurements when testing tracer homogeneity and by radioimmunoassay in the size-estimation experiments.

**Radioiodination.** The iodination was performed at room temperature. To a 11 × 55 mm glass tube with a small magnetic stirrer we added 5 μL of Na<sup>125</sup>I (18.5 MBq) and 50 μL of purified β-microsemionoprotein (35 μg) in Tris-saline. The reaction was started by adding 10 μL of Chloramine T trihydrate dissolved in water (2.5 g/L) and stopped after 30 s by adding 10 μL of sodium metabisulfite (5 g/L) and 30 μL of 75 mmol/L sodium barbital buffer, pH 8.6, containing 50 mg of sodium azide per liter. The iodinated protein was separated from unreacted iodide by chromatography at 20 °C on a 1.0 × 10 cm column of Sephadex G-25 (superfine), equilibrated and eluted with 75 mmol/L sodium barbital buffer, pH 8.6, containing 50 mg of sodium azide per liter. The flow rate was 0.7 mL/min and the eluate was collected in tubes containing 0.5 mL of the elution buffer supplemented with 20 g of bovine serum albumin per liter.

**Radioimmunoassay procedure.** The assay buffer was 75 mmol/L sodium barbital, pH 8.6, containing 2.5 g each of disodium EDTA and bovine serum albumin per liter. We used antisemir diluted 4000-fold in the assay buffer. Serum from nonimmunized rabbits was included to give a concentration of rabbit serum in the working antisemir solution corresponding to a 160-fold dilution.

Standards were prepared from the stock solution of purified β-microsemionoprotein by dilution in assay buffer to give concentrations ranging from 0 to 350 μg/L. These were stored in aliquots at −70 °C.

We performed the assay by mixing, in 11 × 55 mm polystyrene tubes, 100 μL of standard or sample, 200 μL of diluted antisemir, and 200 μL of <sup>125</sup>I-labeled β-microsemionoprotein (diluted in assay buffer to give about 40 000 counts/min per 200 μL). Nonspecific binding was deter-

mined by excluding the antisemir (i.e., using only serum from nonimmunized rabbits). After the mixture was incubated at room temperature for 3 h, 100 μL of a pooled specimen of serum from normal humans, all with β-microsemionoprotein <5 μg/L, was added to the standard tubes and to sample tubes with diluted serum or non-serum fluids. We added 100 μL of assay buffer to the sample tubes with undiluted serum. Immediately afterwards, we precipitated immunoglobulins by adding 500 μL of a goat anti-rabbit IgG antisemir diluted 40-fold in assay buffer containing 50 g of polyethylene glycol per liter. Alternatively, we precipitated the bound tracer with 0.5 mL of a suspension of solid-phase coupled sheep anti-rabbit immunoglobulin (Pharmacia Decanting Suspension III). After letting the samples stand for 1 h at room temperature, we centrifuged them at 2000 × g and 4 °C for 15 min. The supernates were discarded and the radioactivity in the sediments was measured with 70% efficiency in an LKB-Wallac 1277 GammaMaster 10-channel gamma counter (LKB, Bromma, Sweden).

Samples were run in duplicate and results were read from a curve fitted to the counts obtained with the standards (each point in triplicate) by using a smoothed, cubic spline function with the standard errors of the means as weighting factors (20).

**Assay evaluation.** The sensitivity of the assay was defined as the reading on the standard curve at the mean counts for the zero-concentration standard minus three standard deviations of the counts.

We determined the intra-assay coefficient of variation (CV) at three different levels by analyzing three different samples 20 times in order to assay run, 10 in the beginning of the series and 10 at the end. The intra-assay CV was also estimated from the replicate values of samples (covering the range of values between 5 and 175 μg/L) in 20 consecutive assays.

We estimated the total CV (intra-assay + interassay) by assaying three different serum pools in 10 consecutive assays. Aliquots of each sample were kept at −20 °C, so that repeated thawing was avoided. Each result was the mean of duplicate values. During the experimental period two different tracer preparations were used.

We tested analytical recovery by adding three different amounts of purified β-microsemionoprotein from the standard stock solution to one serum sample from a woman with a low concentration of β-microsemionoprotein (5.5 μg/L) and two samples from men, one with a low concentration (9.0 μg/L) and the other with a high (73.8 μg/L) concentration. The amounts added were calculated to increase the concentration with 47.8, 87.5, and 175 μg/L, respectively.

**Assay of serum antibodies to β-microsemionoprotein.** This was performed by an immunosorbert radioassay according to Ericsson et al. (21), β-microsemionoprotein labeled as described above being used as tracer.

**Serum creatinine.** This was determined in the routine clinical chemistry laboratory with an alkaline picric acid reagent method (22). The normal reference intervals were 60–100 μmol/L and 80–115 μmol/L for adult women and men, respectively.

**Gonadotropins.** Follitropin and lutropin (luteinizing hormone) were measured by radioimmunoassay (23).

Subjects and Collection of Samples

The reference population consisted of 51 healthy women.
(ages 20–56 years, 10 older than 40) and 43 healthy men (ages 22–59 years, eight older than 40). Samples were also obtained from 28 patients (ages 56 to 98 years, median 74) with prostatic cancer. All patients had tumors in stages T3 or T4 of the Tumor, Node, and Metastasis System of staging prostate cancers, which includes five stages of increasing severity (T0 to T4) (24).

Blood was collected by venipuncture. We obtained serum by centrifugation at 2000 \( \times g \) for 20 min after allowing the blood to clot for 60 min at room temperature or overnight at 4 °C. To obtain plasma, we sampled blood with EDTA as anticoagulant. The serum and plasma samples were stored at −20 °C.

Urine was collected without additives during 24 h from 24 healthy women (ages 20–56 years) and six healthy men (ages 20–40 years). The volume was measured and an aliquot was stored at −20 °C until analyzed.

Seminal plasma from healthy men was obtained as described previously (I). It was stored at −20 °C.

Serum samples for correlation of serum creatinine with \( \beta \)-microsemionoprotein were selected from samples referred to the clinical chemistry laboratory for routine tests. They were selected to give a suitable distribution of serum creatinine values but with regard to the sex of the patient, clinical data, or results of other blood tests. There were 46 samples from females (ages 14–92 years, median 65) and 55 from males (ages 15–97 years, median 74).

**Results**

**\( \beta \)-Microsemionoprotein Assay**

*Labeling of \( \beta \)-Microsemionoprotein.* With the labeling procedure used, between 65% and 85% of the radioactivity (five labeling experiments) was incorporated into the material eluting in the void volume of the G-25 column. Analysis of this material by gel chromatography on a Superose 12 column, which has a higher resolving power than the G-25 column, showed that more than 95% of the radioactivity was eluted in a peak corresponding to the elution position of pure \( \beta \)-microsemionoprotein (determined in a previous run on the same column). The specific radioactivity of the labeled \( \beta \)-microsemionoprotein was 420 GBq/g. The amount of tracer used in the assay procedure (approximately 40 000 counts/min) corresponded to about 2 ng of labeled \( \beta \)-microsemionoprotein per tube.

*Antiserum.* Incubation of the labeled \( \beta \)-microsemionoprotein with various dilutions of the antiserum, followed by precipitation of immunoglobulins with second antibody, showed a suitable dilution of the antiserum in the incubation mixture to be 10 000-fold (Figure 1), i.e., a 4000-fold dilution in the working antiserum solution. With antiserum in excess—i.e., a dilution <2500-fold—more than 90% of the tracer was precipitated (Figure 1). When no antiserum was included (nonspecific binding), only a few percent of the tracer was precipitated (Figure 1). An equally low nonspecific binding was obtained when standards or samples (more than 100 representative samples tested) were included, making correction for nonspecific binding in the assay procedure unnecessary.

*Standard.* The standards we used in our procedure were prepared in buffer containing bovine serum albumin. Initially we found that the precipitation with second antibody was more reproducible with serum samples than with standards. This phenomenon was probably caused by components (e.g., plasma proteins) present in serum samples, but not in the buffer used for the standards, that made the precipitates firmer and more adhesive. The ideal approach would be to prepare the standard in a serum free from \( \beta \)-microsemionoprotein, but we found it difficult to find such sera. Therefore, we adopted a procedure in which 100 μL of pooled serum with a low concentration of \( \beta \)-microsemionoprotein was added to the standard tubes immediately before the precipitation step. To make the volumes equal in standards and samples, the same volume of buffer was added to the serum sample tubes.

*Separating system.* In the assay procedure adopted for routine use we separated bound and free tracer by polyethylene glycol-reinforced second-antibody precipitation. Separation with solid-phase-coupled anti-rabbit immunoglobulin (Pharmacia Decanting Suspension III) gave identical results (not shown) but was not used because of the higher reagent cost.

*Assay performance.* A typical standard curve is shown in Figure 2 together with dilution curves for three different samples. The sensitivity of the assay was 1 μg/L. Estimates of the intra-assay CV obtained with three different samples in a single run were 3.3% (for a sample with a mean concentration of 10.7 μg/L), 2.3% (sample mean 81.7 μg/L), and 2.4% (sample mean 159 μg/L), respectively. Estimation from sample duplicates in 20 different assay runs gave 3.0%. The estimates of the total CV (i.e., intra-assay plus inter-assay) obtained for three different pooled sera in 10 consecutive assays were 6.3% (at the mean concentration of 9.8 μg/L), 5.8% (at 80.2 μg/L), and 4.3% (at 156 μg/L), respectively. Analytical recovery of added purified \( \beta \)-microsemionoprotein varied between 94% and 116% in the nine recovery experiments described under Materials and Methods. There was no systematic difference in recovery between the three test sera used for these experiments.

*Sampling.* Values did not differ significantly between plasma and serum (Student's paired two-tailed t-test) according to the results with plasma and serum samples collected simultaneously from 30 subjects.
Stability of $\beta$-microsemimoprotein in stored serum samples. Thirty-six serum samples (five from healthy men, five from healthy women, and 26 from men with prostatic disease) that had been assayed six months previously and kept at $-20^\circ$C in the meantime were re-assayed. The values obtained on the first occasion ranged from 5 to 74 $\mu$g/L. The mean difference between the second and the first value was plus 4.9%, with a standard deviation of 12.9%.

$\beta$-Microsemimoprotein in Serum and Urine

$\beta$-Microsemimoprotein was detected not only in sera from men but also in sera from women and in urine. In Figure 2 are shown the dilution curves for the immunoreactivity in serum from a woman and serum from a man, and in seminal plasma, together with the standard curve obtained with purified $\beta$-microsemimoprotein. The curves are all parallel. The immunoreactivity in serum was also investigated by gel chromatography, which separates molecules according to molecular size. The results obtained with serum from a normal man, a normal woman, and a patient with prostatic cancer are shown in Figure 3, which, for comparison, also shows the chromatograms obtained with seminal plasma and purified $\beta$-microsemimoprotein. In all cases except for the prostatic-cancer patient, the immunoreactivity was eluted as a single peak at a position and with a width indistinguishable from that of pure $\beta$-microsemimoprotein. According to the calibration of the column with molecular-mass markers, the elution position corresponded to a molecular mass of about 16 kDa. In the patient with prostatic cancer the main immunoreactivity eluted as in the other samples but an extra minor immunoreactive component was seen eluting before the main component (Figure 3). The molecular mass of the extra component was estimated to exceed 150 kDa. Possibly the two other serum samples contained a similar component, but with a concentration close to the detection limit of the assay (Figure 3).

Reference values. In the reference population, sera from males tended to have a higher concentration of $\beta$-microsemimoprotein than sera from females, but the difference was not great (Figure 4). If subjects older than 40 years were excluded, the difference between males and females was even less pronounced (Figure 4). The range was 0.0-10.6 $\mu$g/L (median 4.1) for the women and 1.1-14.7 $\mu$g/L (median 6.2) for the men not older than 40 years. There was no correlation in the reference population between age and the concentration of $\beta$-microsemimoprotein in serum, either for women alone ($r = 0.079$), men alone ($r = 0.224$), or for all subjects together ($r = 0.154$).

The concentration of $\beta$-microsemimoprotein was measured in urine from 24 healthy women. Values were below the detection limit (1 $\mu$g/L) in 17 of the subjects, and the
highest value measured was 7.7 μg/L. In urines from men the concentration was much higher and variable. For six individuals the range was 12.2 to 223 μg/L (median 81.1 μg/L).

Prostatic-cancer patients. The concentration of β-microsemionprotein in sera from 28 patients with prostatic cancer varied greatly. The range was 0 to 486 μg/L (median 14.3 μg/L). Fourteen of the patients had values exceeding the upper reference limit and four had values below the lower limit. Patients with more severe disease (stage T4) tended to have higher values than patients in stage T3, a difference that was statistically significant (P = 0.003) according to the Mann–Whitney U-test.

Antibodies to β-Microsemionprotein in Serum

Serum from 10 healthy women, 10 healthy men (all <40 years old), and 28 patients with prostatic cancer were tested for the presence of circulating antibodies to β-microsemionprotein. None contained detectable antibodies.

β-Microsemionprotein and Serum Creatinine

The relation between the serum concentrations of creatinine and β-microsemionprotein is shown in Figure 5 for 46 women and 55 men. The low positive correlation between the two variables (r = 0.49) is nevertheless statistically significant (P < 0.001).

β-Microsemionprotein and Gonadotropins

Follitropin and lutropin were measured in the serum samples of the reference subjects. There was no significant correlation between the concentrations of any of the gonadotropins on the one hand and β-microsemionprotein on the other, either in women (n = 51) or men (n = 43).

Discussion

β-Microsemionprotein is present in seminal plasma in a relatively high concentration. According to evidence obtained with histochemical techniques (1, 14, 25), northern blot analysis (10), and observations in patients lacking seminal vesicles (1) the β-microsemionprotein in seminal plasma originates from the prostate gland. Not unexpectedly, therefore, β-microsemionprotein may appear in serum of men with prostatic disease, as demonstrated with previously developed assays for this protein (5, 26).

Our results with the sensitive assay described in this work show that immunoreactive β-microsemionprotein is present in serum of both men and women and at about the same concentration. Does this immunoreactivity represent the same protein that has been isolated from seminal plasma? Our belief that this is so is supported by two of our findings: First, the immunoreactivity in serum—both from men and women—displayed a dilution curve that paralleled that of the assay standard curve, the latter prepared with β-microsemionprotein purified from seminal plasma. Second, fractionation of the serum immunoreactivity according to molecular size showed that almost all of it represents a homogeneous component with the same molecular size as β-microsemionprotein. Although only isolation and analysis of the immunoreactive component could provide an ultimate proof, the evidence strongly suggests identity of the immunoreactivity present in sera of women with β-microsemionprotein.

The nature of the minor immunoreactive component with a larger molecular size than β-microsemionprotein present in the sample from the patient with prostatic cancer—and perhaps also in sera of healthy subjects—is unclear. The component could be an aggregated form of β-microsemionprotein or perhaps β-microsemionprotein in complex with a high-molecular-mass binder in the patient's plasma.

The concentration of β-microsemionprotein in sera of women, when measured with our assay, is almost the same as in healthy men not older than 40 years, an age below which benign prostatic hyperplasia or prostatic cancer is rare. Although the mean value for men is statistically higher than for women, the overlap is large, and one may conclude that, for healthy subjects, the concentration is essentially the same in both sexes. This finding suggests that the prostate gland is not an important source for
serum 3-microseminoprotein in healthy men, and that blood of men and women have a common source for this protein.

In men with prostatic cancer the concentration of 3-microseminoprotein in serum was often markedly increased, similar to what has been observed with other prostate-specific markers, such as acid phosphatase and prostate-specific antigen. This increase most probably represents a direct release of 3-microseminoprotein into blood from the diseased prostate gland, and it may well be that the high values occasionally seen in subjectively healthy men older than 40 years also represent contributions from the prostate gland.

The release of 3-microseminoprotein into the circulation as a consequence of a disease process affecting the tissue producing this protein might stimulate the production of antibodies directed against 3-microseminoprotein. If present, such antibodies would interfere with our assay and probably cause falsely increased values. Our failure to detect antibodies in any of our group of patients with prostatic disease (many with high values for 3-microseminoprotein in their blood) suggests that this does not happen frequently.

Although it appears to be somewhat larger in SDS electrophoresis and gel chromatography (16 kDa), 3-microseminoprotein is a small protein of 94 amino acids with a calculated molecular mass of about 11 kDa ([1, 5, 6, 8–10]). Proteins of this size are usually more or less freely filtered in the renal glomeruli. We therefore measured 3-microseminoprotein in urine from females and from males as well. In females the concentration was low, more than half of the patients having values below the detection limit of our assay. Men had much higher and more variable concentrations of 3-microseminoprotein in their urine, and occasionally very high values were encountered. The low values in women suggest that 3-microseminoprotein, if filtered in the glomeruli, is effectively reabsorbed by the tubule cells and that the urine finally is almost free of the protein. The high values seen for urine from men thus probably represent a local contribution from the prostate gland to the urethral urine.

Filtering of 3-microseminoprotein in the renal glomeruli and subsequent reabsorption in the tubuli might be a major catabolic route for this protein. One would then expect a correlation between the concentrations in blood of 3-microseminoprotein and a marker of glomerular filtration rate, such as creatinine. Our measurements of 3-microseminoprotein in a number of patients with various serum creatinine concentrations showed a statistically significant positive correlation between these two variables, giving support to the hypothesis that at least part of the catabolism of 3-microseminoprotein takes place by glomerular filtration. If the input of 3-microseminoprotein into the circulation varied little in these patients and glomerular filtration was responsible for the elimination of the protein, one would have expected a correlation higher than that observed. But many of the patients in this study had severe diseases besides their renal insufficiency, and it is not unlikely that many of the high values for 3-microseminoprotein seen in these patients were partly caused by an increased input of 3-microseminoprotein into the circulation as a consequence of a disease affecting an organ synthesizing 3-microseminoprotein, e.g., the prostate gland in the men.

The function of 3-microseminoprotein is still unknown.

Earlier proposals ([7] that 3-microseminoprotein has inhibitory activity–i.e., inhibition of release of follitropin from the pituitary–have been questioned ([11, 12]). This is consistent with our finding of a zero correlation between 3-microseminoprotein and follitropin.

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