

Proteins of Human Semen

I. Two-Dimensional Mapping of Human Seminal Fluid

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The proteins in human seminal plasma were mapped by high-resolution two-dimensional electrophoresis (ISO-DALT and BASO-DALT systems). When analyzed under dissociating conditions, samples from normal fertile males revealed a pattern of over 200 proteins, ranging in mass from 10 000 to 100 000 daltons. Comparison of the mapped proteins from these males and those who had undergone vasectomy allowed us to identify one series of glycoproteins as missing from the semen from vasectomized individuals. Glycoproteins isolated by affinity chromatography with use of concanavalin A were also mapped. Some of the protein spots were identified either by co-electrophoresis with purified proteins or by the electrophoretic transfer of proteins to nitrocellulose sheets and subsequent detection by immunological procedures. The proteins identified include a number of serum proteins as well as prostatic acid phosphatase and creatine kinase. Proteolytic events shown to occur during the liquefaction of semen that occurs early after collection indicate the importance of carefully controlled collection and preparation methods for clinical evaluation of seminal plasma. Ethylenediaminetetraacetic acid and phenylmethylsulfonyl fluoride inhibit this proteolysis.

Additional Keyphrases: ISO-DALT and BASO-DALT systems • electrophoresis, polyacrylamide gel • "mapping" of proteins • glycoproteins • proteins • enzymes • chromatography, affinity • immunological identification procedures • effects of vasectomy

Human semen contains many distinct protein components, which are important in the functioning and survival of spermatozoa and which reflect the functioning of the male accessory glands. Earlier analyses of human semen have been concerned primarily with the qualitative evaluation of sperm morphology and vitality, and with numbers of sperm, particularly as related to fertility capacity (1-4) and in the diagnosis of disease (5, 6). The sperm-free, nondialyzable fraction of human semen has a complex composition (7) and enzymology (6, 8-11), and shows genetic polymorphisms (12-14). These characteristics are useful in clinical studies of age-related changes (15), correlations between disease and infertility (16), diagnosis of disease (10), and the physiology of reproduction (17, 18), as well as in forensic science (12, 19).

With two-dimensional electrophoresis techniques one can resolve complex mixtures of proteins such as occur in seminal plasma on the basis of two separate, nonrelated properties of each polypeptide. The position of each polypeptide on the map is determined by separation on the basis of charge in the first dimension, followed by separation based upon relative molecular mass in the second dimension. This technique, utilizing both the ISO-DALT (20, 21) and BASO-DALT (22)

systems has been used to map other complex mixtures of proteins from human tissues (23-25).

This paper presents the initial two-dimensional mapping of seminal plasma, with emphasis on the differences observed between normal and vasectomized males, identifies a number of the mapped proteins, describes the major glycoproteins binding to concanavalin A (Con A),¹ and presents initial studies on the proteolytic degradation of the proteins in seminal plasma. Mapping and identification of the protein components of seminal plasma are basic to further investigations aimed at (a) the early diagnosis of disease, including prostatic cancer; (b) exploration of possible relationships of some protein components to male infertility; (c) the identification of the proteins secreted by individual accessory glands; and (d) the analysis of some genetic polymorphic proteins.

Materials and Methods

Materials

Antibodies to human proteins were purchased from DAKO (via Accurate Chemical and Scientific Corp., Westbury, NY 11590) and prostatic acid phosphatase (EC 3.1.3.2) from JBL Biochemical Co., San Luis Obispo, CA 93410. Creatine kinase (EC 2.7.3.2; BB isoenzyme, from human brain) was a gift from Dr. Peter Jackson, Postgraduate Medical School, Cambridge, England. Lactoferrin purified from human milk was a gift from Dr. Anatoly Bezkorovainy, Rush Presbyterian-St. Luke's Hospital, Chicago, IL 60612. Ampholytes for ISO-DALT separations were from LKB, Bromma, Sweden. BASO-DALT separations were made with ampholytes from Serva Fine Biochemicals, Inc., Garden City Park, NY 11040. Nitrocellulose paper, type BA85, was from Schleicher and Schuell, Keene, NH 03431.

Methods

Preparation of seminal plasma. Semen samples were obtained from healthy volunteers (five normal and three vasectomized) after at least 36 h of sexual abstinence. All volunteers had at one time proven fertile by fathering at least one child. The samples were not allowed to liquefy, but were immediately diluted with one-half volume of a dilution buffer containing, per liter, 123 mmol of NaCl, 5 mmol of KCl, 1 mmol of MgSO₄, and 37 mmol of Tris · HCl, pH 8.0, as described by Pulkkinen et al. (26). Except in proteolysis experiments, the buffer was modified to contain 1 mmol of ethylenediaminetetraacetic acid (EDTA) per liter, and phenylmethylsulfonyl fluoride was added to the sample to a final concentration of 2.5 mmol/L from a stock solution of 100 mmol/L in ethanol, to prevent protease activity. The sample was then chilled to 4 °C, vortex-mixed to ensure complete mixing, and centrifuged at high speed (25 000 × g, 5 min) to pellet particulate material. The supernatant fluid was re-

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¹ Nonstandard abbreviations used: Con A, concanavalin A; EDTA, ethylenediaminetetraacetic acid; and Tris, tris(hydroxymethyl)methylamine; ISO-DALT and BASO-DALT, names given to gel electrophoresis systems used in the Molecular Anatomy Program. Abbreviations used in Figures are explained in the legends.

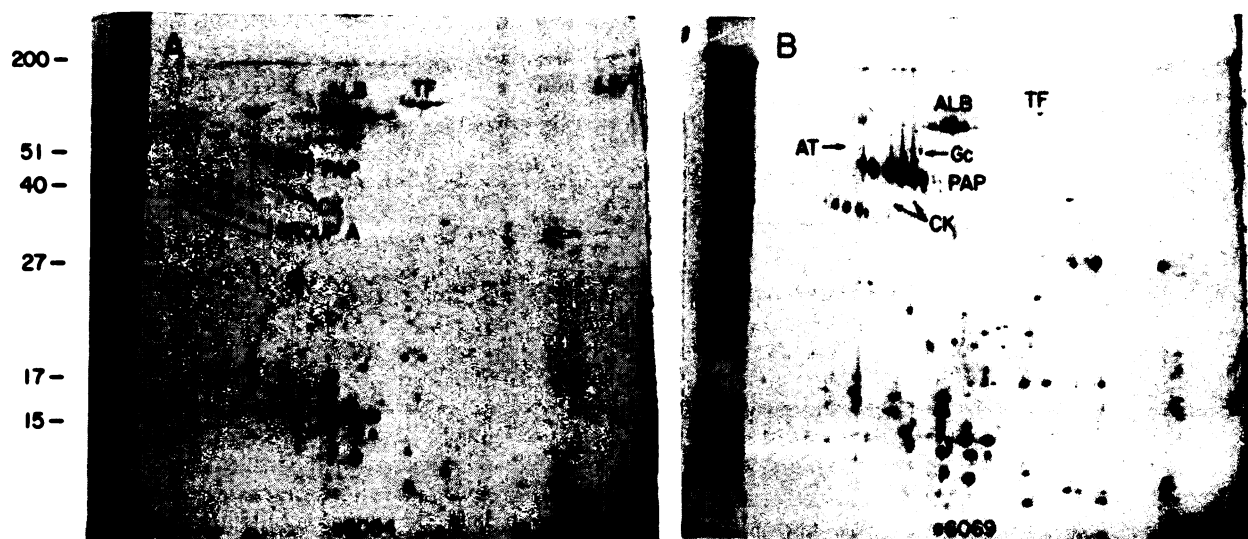


Fig. 1. Two-dimensional ISO-DALT electrophoretic patterns of seminal plasma of a normal human male (A) and a vasectomized male (B)

The gel is oriented with the acidic side to the left. Abbreviations: LF, lactoferrin; TF, transferrin; ALB, albumin; AT, α_1 -antitrypsin; PAP, prostatic acid phosphatase; Gc, Gc globulin; CK, creatine kinase. Spots labeled Group A are discussed in the text. Molecular-mass standards are rat heart muscle (29). Numbers on left are molecular mass in thousands of daltons

moved and made to contain, per liter, 9 mol of urea, 20 g of dithioerythritol, and 40 mL of LKB Ampholine, pH 3.5–10. Samples were stored in 0.2-mL aliquots at -80°C .

Preparation of purified proteins. Lactoferrin, prostatic acid phosphatase and creatine kinase were each solubilized to a final concentration of 5 g/L in a solution containing, per liter, 9 mol of urea, 20 g of dithioerythritol, 50 mL of LKB Ampholines (pH 3.5–10), and 2 g of sodium dodecyl sulfate.

Isolation of Con A binding proteins. Semen samples were diluted with 10 volumes of dilution buffer, phenylmethylsulfonyl fluoride was added to a final concentration of 2.5 mmol/L, and the samples were centrifuged at high speed. The diluted plasma was dialyzed overnight at 4°C against 100 volumes of 0.1 mmol/L EDTA, then lyophilized.

Con A-Sepharose 4B was packed into a 100×10 mm column and washed extensively with an equilibration buffer containing, per liter, 200 mmol of NaCl, 100 mmol of Tris-HCl (pH 7.4), 1 mL of Triton X-100 surfactant, and 1 mmol each of CaCl_2 , MgCl_2 , and MnCl_2 . Lyophilized seminal plasma (50 mg) was solubilized in 3 mL of the equilibration buffer and applied to the column. Eluted material was monitored for protein by ultraviolet absorbance at 280 nm. The detergent-solubilized material was passed through the column twice, to ensure maximum binding. Material that remained bound to the column was subsequently eluted with equilibration buffer modified to contain 100 mmol of α -D-methylglucopyranoside per liter. Material obtained from the column was dialyzed overnight against 1 mmol/L EDTA, then lyophilized. For electrophoretic analysis the lyophilized powder was solubilized to give a final concentration of 80 g/L in the solution used for the solubilization of the purified proteins.

Proteolytic degradation of seminal plasma. Pooled semen samples were divided into two equal portions. To one sample, one-fourth volume of dilution buffer, modified to contain 5 mmol of EDTA and 2.5 mmol of phenylmethylsulfonyl fluoride per liter, was added. The other sample was diluted with one-fourth volume of a 150 mmol/L solution of NaCl. The samples were kept at room temperature, and at specific time intervals aliquots were removed and centrifuged for 2 min on a Microfuge (Beckman Instruments, Inc., Fullerton, CA 92634), and the supernates were treated as described above for seminal plasma.

Two-dimensional electrophoresis. Seminal plasma proteins and purified proteins were separated electrophoretically, with use of the ISO-DALT system (20, 21) to resolve acidic and neutral proteins and the BASO-DALT system (22) to resolve neutral and basic proteins.

Focusing in the first-dimension ISO gels was done in 40 g/L polyacrylamide containing, per liter, 2.4 g of *N,N*-methylenebisacrylamide as cross-linker, 9 mol of urea, 50 mL of LKB ampholytes (80% 3.5–10 and 20% 5–8) and 20 mL of Nonidet P40 (Particle Data Labs., Elmhurst, IL 60126) for 10 000 volt-hours. We separated proteins in the first dimension with the BASO system exactly as described by Willard et al. (22).

The second-dimension molecular-mass separations were in slab gels consisting of a linear polyacrylamide gradient (100–200 g/L, and containing 8 g of cross-linker per liter). The electrophoretic buffer was a Tris-glycine-sodium dodecyl sulfate solution previously described (24).

Spot identification. Protein subunits were identified immunologically after electrophoretic transfer of the pattern from the second-dimension slab gel to type BA85 nitrocellulose paper, essentially as described by Towbin et al. (27). The transferred protein patterns were treated with monospecific antisera and the precipitated antigen-antibody complex was reacted with horseradish-peroxidase-conjugated IgG vs the first antibody. The complex was then made visible by treatment with 3',3'-diaminobenzidine tetrahydrochloride. A detailed description of the transfer and detection process used here will be published elsewhere (manuscript in preparation).

The spots identified as creatine kinase and lactoferrin were identified by co-electrophoresis of purified protein. Prostatic acid phosphatase was identified both by the immunological procedure described and by co-electrophoresis of purified enzyme.

Results

Figure 1 shows the two-dimensional ISO-DALT electrophoretic map of human seminal plasma. The plasma from normal fertile individuals, when analyzed by this method, contained over 100 polypeptides, ranging in molecular mass from 10 000 to 100 000 daltons. Many of the larger forms demonstrated a high degree of charge heterogeneity, indicative

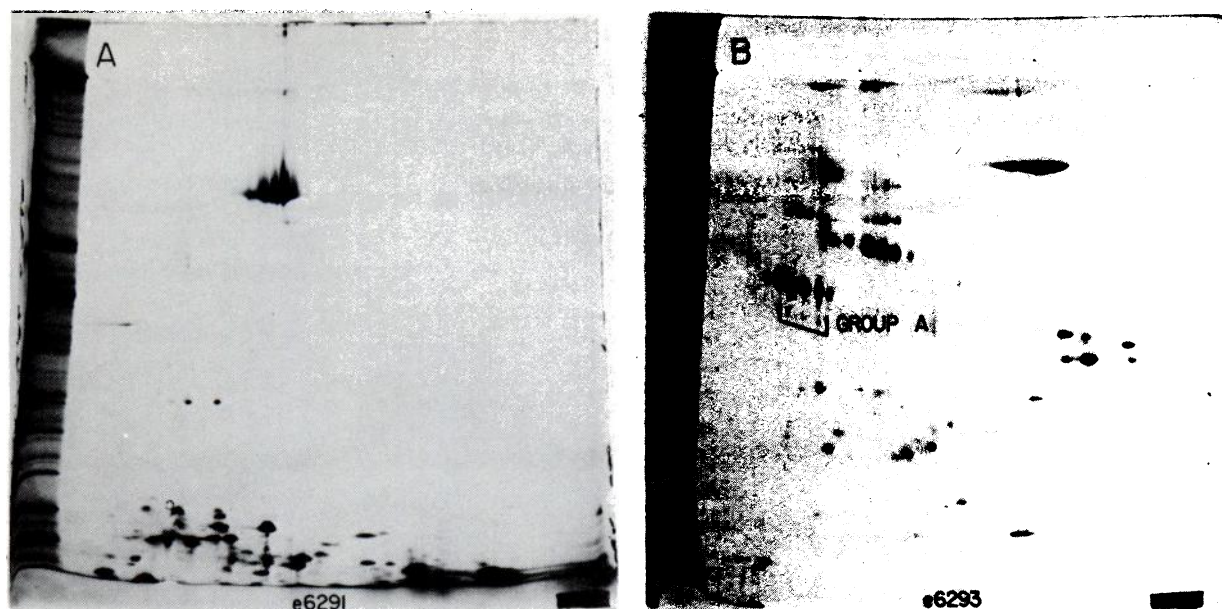


Fig. 2. Fractions of normal human seminal plasma proteins separated by use of concanavalin A

Seminal plasma was reconstituted to approximate the original concentration after Con A fractionation procedures; 12 μ L of sample applied to each gel. (A) Seminal plasma proteins not bound to Con A. (B) Seminal plasma proteins bound to Con A and eluted with α -D-methylglucopyranoside. Molecular-mass standards and gel orientations as in Figure 1

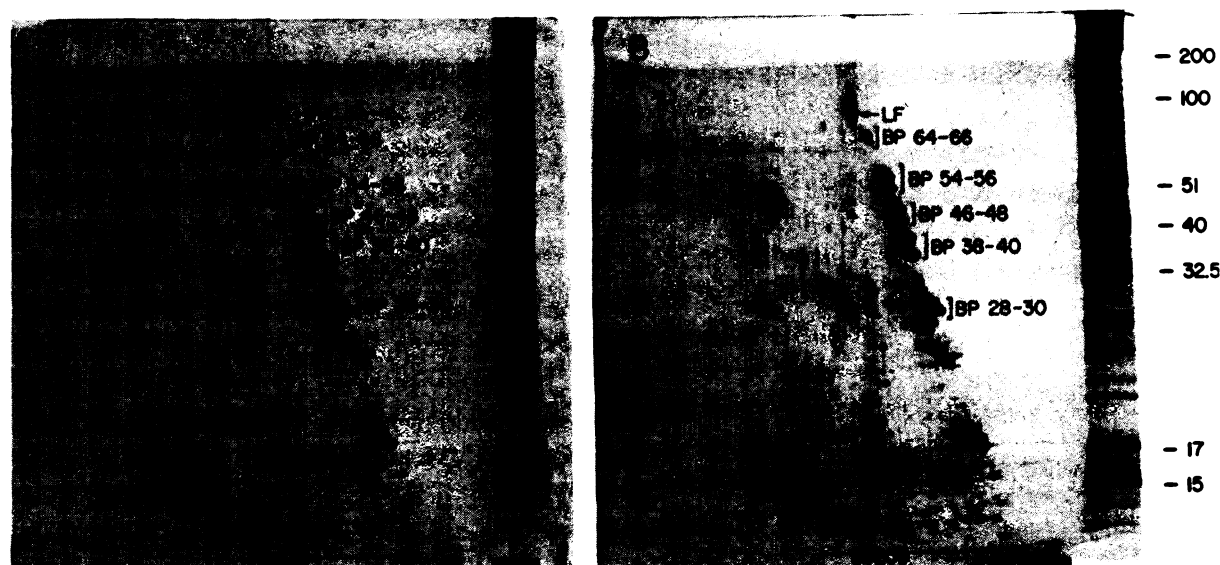


Fig. 3. Two-dimensional BASO-DALT electrophoretic patterns of seminal plasma of a normal human male (A) and a vasectomized male (B), after electrophoresis in the first dimension for 3600 volt-hours

Lactoferrin (LF) is identified for reference. Labeling of spots is explained in text. Molecular-mass standards and orientation of the gels as in Figure 1. Numbers on right are molecular mass in thousands of daltons

of a glycoprotein nature. Most of the protein in these samples (based upon staining intensity) is found in the molecular mass region below, 30 000 daltons. We believe that most of the smaller polypeptides are probably not cleavage products of higher-molecular-mass components, because of (a) the effects of added protease inhibitors at the time of sample collection and (b) the close resemblance between the pattern of some of the larger polypeptides and that previously observed in human blood serum samples (24). Of the proteins identified in Figure 1, some are known components of serum: albumin, transferrin, α_1 -antitrypsin, and Gc globulin. Identification of these proteins was based on their reaction with antibodies to human serum proteins after the transfer to nitrocellulose paper. Other proteins identified in these patterns include prostatic acid phosphatase and creatine kinase. All are present in seminal

plasma preparations of both normal and vasectomized males.

The electrophoretic patterns obtained with seminal plasma of normal and vasectomized individuals are very similar, with one notable exception (Figure 1). The seminal plasma of normal individuals contained a series of spots with mass about 30 000 daltons at the acid end of the gel. These spots were missing from all vasectomy samples tested and therefore may represent a protein of testicular origin. This group of polypeptides, labeled as Group A in Figure 1A, did not react with any antisera tested, nor did they appear to co-migrate with proteins known to be present in human serum. Their functional identification remains to be determined. The only other difference noted between the seminal plasma of normal and vasectomized males was that in many of the samples tested

there were quantitative differences in some protein spots (based upon visual interpretation of staining intensity), as best illustrated by the proteins identified as transferrin and prostatic acid phosphatase.

When preparations of seminal plasma solubilized with Triton X-100 are applied to immobilized Con A, a total of about 87 to 90% of the material can be recovered from the column with the procedures described. Of this, about 80% is not bound to the column but is eluted with the equilibration buffer. The remaining 20% is eluted when a 0.1 mol/L solution of α -D-methylglucopyranoside is added to the buffer. Figure 2 shows the two-dimensional ISO-DALT patterns of seminal plasma after this treatment. Albumin and most of the components of lower relative molecular mass are not bound to the column and are removed with the equilibration buffer (Figure 2A), while most of the high-mass material (>30 000 daltons) is bound to the column and eluted in the presence of the hapten sugar, as would be expected for molecules containing carbohydrate residues (Figure 2B). Included in the material binding to Con A was the series of spots designated Group A, found in normal samples but not in those from vasectomized individuals.

When the seminal plasma was analyzed by BASO-DALT electrophoresis, a substantial amount of protein was present that was not resolved by isoelectric focusing to equilibrium. The patterns obtained for seminal plasma from normal and vasectomized males were extremely similar (Figure 3). However, one unexpected result was the pattern exhibited by some of the fastest migrating (most basic) polypeptides. They appear to separate into groups of two in the molecular-mass dimension, with the lighter forms of the first four migrating fastest. There are five groups, labeled BP 28–30, BP 38–40, BP 46–48, BP 54–56, and BP 64–66 in Figure 3. The letters refer to the basic protein nature of each group while the numbers refer to the approximate molecular mass (in thousands of daltons) of the components in each group. As will be discussed below, these proteins may be involved in the process of liquefaction.

A significant change was observed in the protein map of seminal plasma when the sample was allowed to liquefy at room temperature in the absence of proteolysis inhibitors. The changes in the BP 28–30 through BP 64–66 proteins are probably associated with the process of liquefaction, because addition of the chelating agent EDTA has been shown to inhibit the collagenase-like peptidase that is thought (9) to be active during the first phase of liquefaction. As seen in Figure 4A–E, if proteolysis is inhibited by adding phenylmethylsulfonyl fluoride and EDTA, the high-molecular-mass proteins resolved by the BASO-DALT system (BP 28–30 through BP 64–66) remain relatively constant in amount for 30 min after collection. However, if phenylmethylsulfonyl fluoride and EDTA are not added, and proteolysis takes place, these proteins begin to disappear almost immediately and are completely lost within 30 min (F–J). It is not known whether both phenylmethylsulfonyl fluoride and EDTA are necessary to prevent the observed proteolysis. Pieces of the proteins that disappear from the pattern if proteolysis is permitted have not been located on the gels; no concomitant increases in the density of any remaining spots have been observed, nor have any new spots appeared. The BASO-DALT technique is a nonequilibrium electrophoretic system; therefore it cannot be used to determine whether the pieces removed are of an acidic or basic nature. It is uncertain whether this series of basic proteins represents products of one parent protein in which the progress of degradation is halted by the added inhibitors or whether many unrelated proteins are involved. This can only be determined when quantitative analyses of the protein spots can be accurately made.

Three additional series of spots—labeled 1, 2, and 3 in

Figure 4—also reflect the presence or absence of added inhibitors. In the sample containing proteolytic inhibitors (A–E), the rate of disappearance for Series 1 and 2 is slow but progressive, while Series 3 remains stable. In the sample containing no proteolytic inhibitors (F–J), all three series of spots begin to disappear immediately and are lost to the sample within about 15 min. The functions of all the proteins that are seen to be changing with time in these samples remain to be determined. We analyzed a similar series of gels, using seminal plasma from a vasectomized individual, with results (data not shown) identical to those described above, indicating that sperm is not involved in these degradation processes. ISO-DALT electrophoresis of similar semen samples (of normal and vasectomized males) revealed no noticeable changes in the proteins observed by that technique.

Prostatic acid phosphatase is one of the most extensively studied enzymes found in seminal plasma. Figure 5 shows the ISO-DALT patterns obtained with purified enzyme after staining with Coomassie Brilliant Blue R-250 (A) and seminal plasma stained immunologically after transfer of the ISO-DALT pattern to nitrocellulose paper (B). The charge heterogeneity found in this enzyme is apparent in both samples. Nine charge species are identifiable by the immunological staining technique. The median molecular mass of prostatic acid phosphatase subunits analyzed under these conditions is about 48 000 daltons.

Discussion

The advent of two-dimensional electrophoretic techniques makes practical the analysis and description of a large fraction of the protein components found in human tissues and fluids. Historically, the clinical analysis of human semen has primarily been concerned with such characteristics as volume, sperm density, morphology, and motility, as well as the activity of certain enzymes. As valuable as these characteristics are, much remains to be discovered. Previous one-dimensional sodium dodecyl sulfate electrophoretic analyses of human semen have resolved up to 40 polypeptide bands, and albumin, lactoferrin, and acid phosphatase have been identified (19). Isoelectric focusing has been used primarily to determine the presence and amount of certain enzymes (11, 14). The results reported here show the complexity of the protein moieties of human seminal plasma and demonstrate the usefulness of the current high-resolution electrophoretic systems. This paper also makes apparent that the rapid rate of proteolytic degradation makes highly controlled sample-collection and sample-preparation techniques essential for clinical evaluations of seminal plasma.

The two-dimensional patterns obtained with a combination of the ISO- and BASO-DALT systems show 200–300 proteins or protein subunits resolved with a high degree of reproducibility from very small samples. However, relatively few of these many proteins have been identified. The fact that many of the components are present in relatively small quantities makes the functional identification of these proteins difficult. We propose to approach this problem by using small-scale preparative techniques for enzyme isolation, followed by co-electrophoresis with seminal plasma, and by using highly purified commercially available antibodies and the very sensitive techniques of immunological staining.

With two-dimensional techniques now available for multiple sample analysis, many aspects of the biochemistry of seminal plasma can now be studied and differences between clinically normal and abnormal samples examined in detail. Correlation of genetic polymorphisms and alterations in concentration of individual proteins with disease should provide useful indications of the functions of the many proteins visible in the two-dimensional map of seminal plasma. Accurate information about the quantity of each protein will

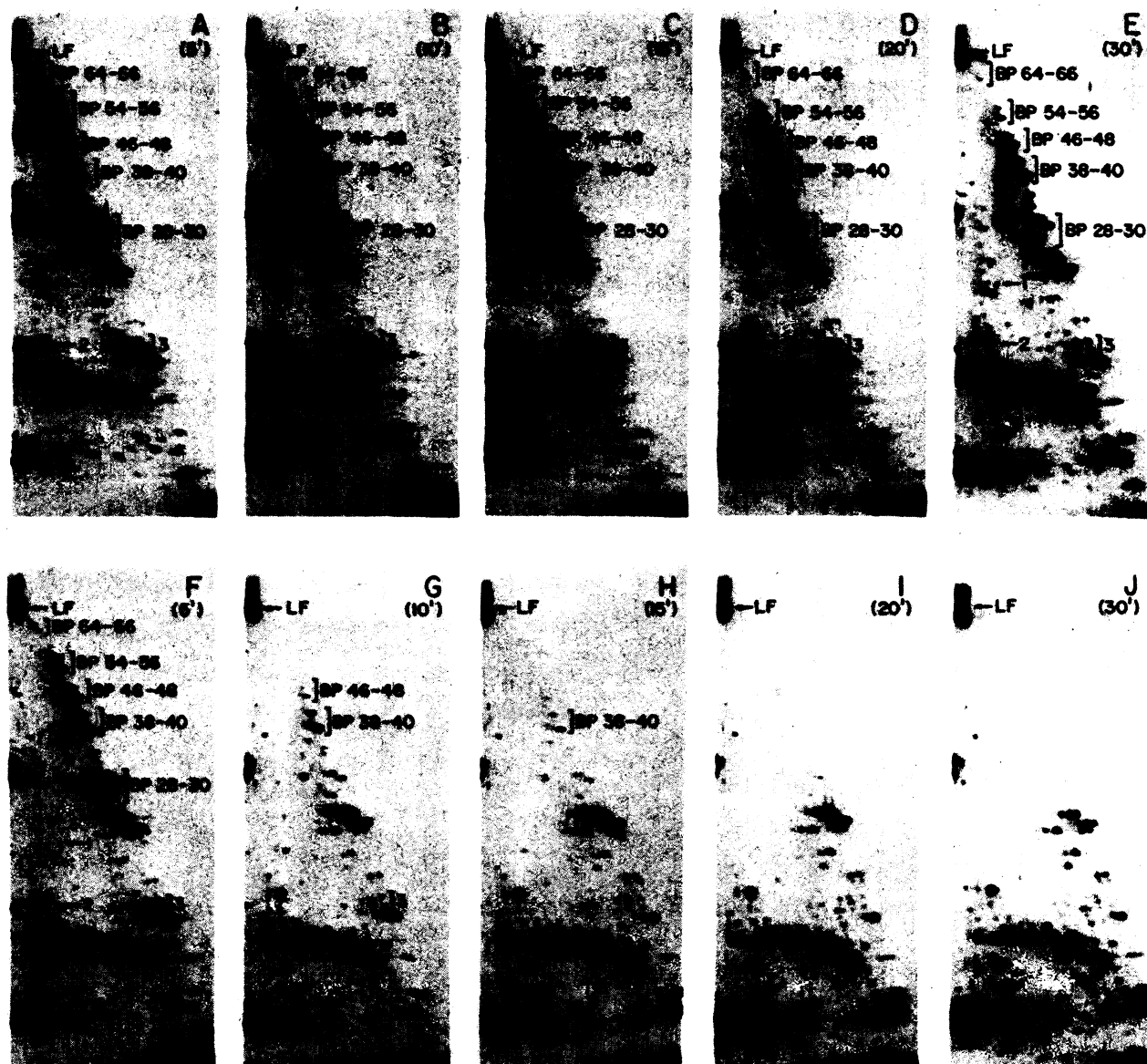


Fig. 4. Effect of combined phenylmethylsulfonyl fluoride and EDTA on the proteolytic degradation of basic proteins of normal human seminal plasma

Portions of BASO-DALT patterns of seminal plasma containing both phenylmethylsulfonyl fluoride and EDTA (A-E) or no proteolytic inhibitors (F-J). At specific time intervals after collection, proteolysis was stopped by the addition of urea and dithioerythritol, as described in *Methods*. Time intervals are: A and F, 5 min; B and G, 10 min; C and H, 15 min; D and I, 20 min; and E and J, 30 min. Abbreviations and gel orientation as in Figure 1

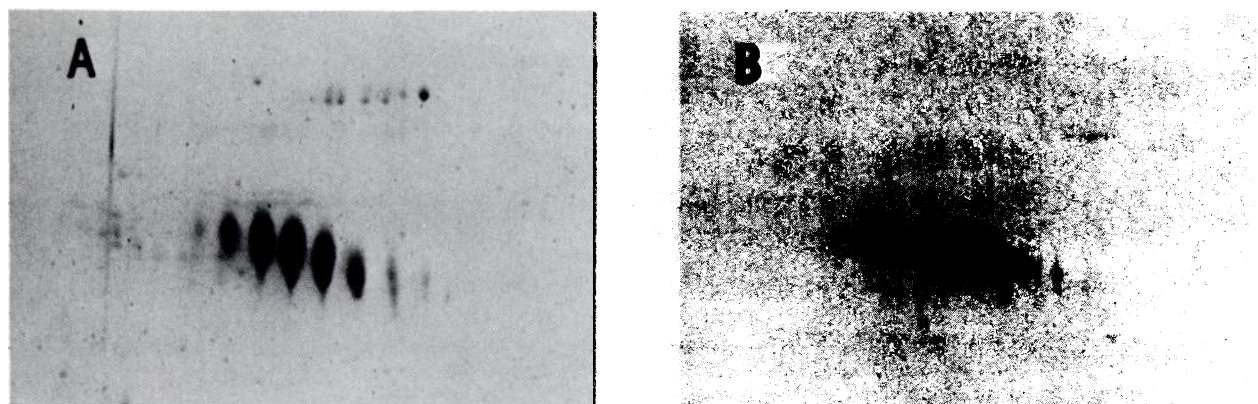


Fig. 5. Charge heterogeneity of purified human prostatic acid phosphatase after ISO-DALT electrophoretic separation

(A) Ten micrograms of enzyme stained with Coomassie Brilliant Blue R-250; (B) enzyme after transfer of the two-dimensional pattern of seminal plasma to nitrocellulose paper and immunological staining as described in *Methods*

help to provide a method for choosing the most useful markers for diagnosis of disease. To this end, methods for quantitating mapped spots are now under development in our laboratory. We are also developing internal charge standard markers for pI measurement (28) and high-resolution sodium dodecyl sulfate molecular-mass markers (29), which will provide a reproducible coordinate system to be incorporated in each gel. The following paper in this series of studies will present two-dimensional maps of human sperm proteins.

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