TECHNICAL NOTE

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The Simultaneous Identification of Seminal Acid Phosphatase and Phosphoglucomutase by Starch Gel Electrophoresis

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ABSTRACT: Although elevated acid phosphatase (AP) activity in vaginal fluid is a consistent indicator for semen, differentiation between vaginal AP and seminal AP provides a more meaningful result. Detection of seminal AP in mixtures of vaginal AP, feces, and blood is accomplished by starch gel electrophoresis, employing the substrate thymolphthalein monophosphate as a selective visualization agent. Genetic phosphoglucomutase isoenzymes are simultaneously separated by this method and allow differentiation in some semen/vaginal fluid mixtures.

KEY WORDS: pathology and biology, phosphatases, phosphoglucomutase, electrophoresis

The positive identification of aspermic semen deposits has long burdened the forensic analyst. Approaches using the high concentration of enzymatic seminal acid phosphatase (orthophosphoric monoester phosphohydrolase, AP) have been considered; however, the specific origin of any enzymatic activity is not established by traditional methods. This problem is compounded when the seminal deposit is mixed with vaginal secretions, saliva, or feces, since these materials themselves may be sources of AP.

Recent attempts to differentiate acid phosphatases have relied on factors of electrophoretic mobility and enzyme-substrate specificity. Separation has been accomplished on starch and polyacrylamide gels differentiating seminal from a number of other acid phosphates. By combining and adapting the methods of Refs *1* to *4*, a system has been developed to simultaneously resolve seminal AP and phosphoglucomutase (PGM) types with increased AP enzyme-substrate specificity.

Experimental Procedure

The buffering reagents and Connaught brand hydrolyzed starch used in this study were supplied by Fisher Scientific Co. (Fairlawn, N.J.). Visualization agents were purchased

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from Sigma Chemical Co. (St. Louis, Mo.); exceptions were 4-methylumbelliferyl phosphate (ICN Pharmaceuticals, Inc., Plainview, N.Y.) and thymolphthalein monophosphate, disodium salt (J. T. Baker Co., Phillipsburg, N.J.).

Preparation of Buffers and Gels

Culliford's bridge buffer is prepared by adjusting a solution of 0.1*M* tris(hydroxymethyl) aminomethane (Tris), 0.1*M* maleic acid, 0.01*M* ethylenediaminetetraacetic acid (EDTA), and 0.01*M* magnesium chloride in distilled water to a pH of 7.4 with sodium hydroxide. A 1:15 dilution of this tank buffer is used for preparing a 1-mm 10% starch gel. Preparation of these plates and use of this general electrophoretic technique is adequately described elsewhere [*1*].

Preparation of Samples and Electrophoresis

Standards consisting of cloth strips impregnated with an aqueous extract of human prostate [*2*], or whole human semen, were dried in a vacuum desiccator and stored frozen at −20°C. Vaginal and anal swabs were air-dried and frozen. Whole blood was deposited on cotton cloth and air-dried. Unknowns sampled were provided by a local planned parenthood clinic.

Samples of approximately 10 by 1 mm were cut from the standards and stained unknowns and were moistened on a microscope slide with the minimum of gel buffer. These threads were inserted in the gel, at 1-cm intervals, about 2.5 cm from the cathode wick. Electrophoresis was carried out on 20- by 20-cm plates at 150 V (12.5 V/cm) for 5 h employing a cooling plate circulating 1°C water.

Development

The PGM agar overlay visualization method of Culliford (or one of its modifications [*5*]) may be used. This system consists of a solution of 35 mg glucose-1-phosphate (containing 1% glucose-1,6-diphosphate), 20 mg magnesium chloride, 1 mg nicotinamide-adenine dinucleotide phosphate (NADP), 1 mg 3-(4,5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide (MTT), 1 mg phenazine methosulfate, and 1 unit glucose-6-phosphate dehydrogenase in 10 ml of 2% agar solution at 56°C.

The AP visualization was accomplished by overlay of a filter paper sheet saturated with a solution of 1 mg 4-methylumbelliferyl phosphate (MUP) and 5 mg thymolphthalein monophosphate, disodium salt (TMP) in 10 ml of 0.1*M* citrate buffer of pH 4.5. When ultraviolet visualization indicated sufficient substrate consumption (fluorescent areas develop) the mixed substrate developer was removed and the seminal AP regions were visualized by overlaying a second filter paper saturated with 0.1*N* sodium hydroxide; this rapidly converted the reaction product thymolphthalein into its visible (blue) chromogenic form.

Upon completion of the run, the plate was divided in half by placing glass barriers in the starch gel. The agar PGM overlay was poured over the lower portion of the plate while the upper half was developed with the MUP-TMP reagent. Incubation was at room temperature. Visualization of fluorescent zones in the upper overlay was followed by sodium hydroxide quenching. The upper portion of the plate was photographed and the plate was then incubated at 37°C for complete PGM development (see Fig. 1).

Results

Figures 1 and 2 demonstrate that this modified PGM starch gel electrophoresis procedure clearly differentiates seminal AP from vaginal and (most) fecal APs. While PGM

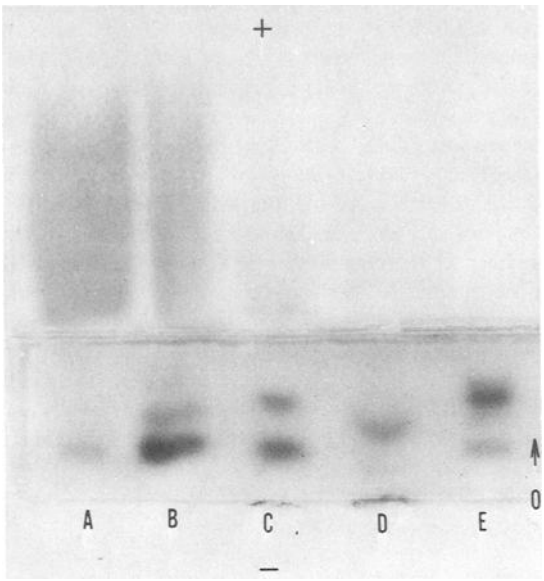

FIG. 1—*The visualization of acid phosphatases and PGM isoenzymes:* (left to right) *semen, extracted prostate, vaginal, fecal, and blood; PGM groups* (left to right) *2-1, 2-1, 1-1, nonspecific, and 1-1.*

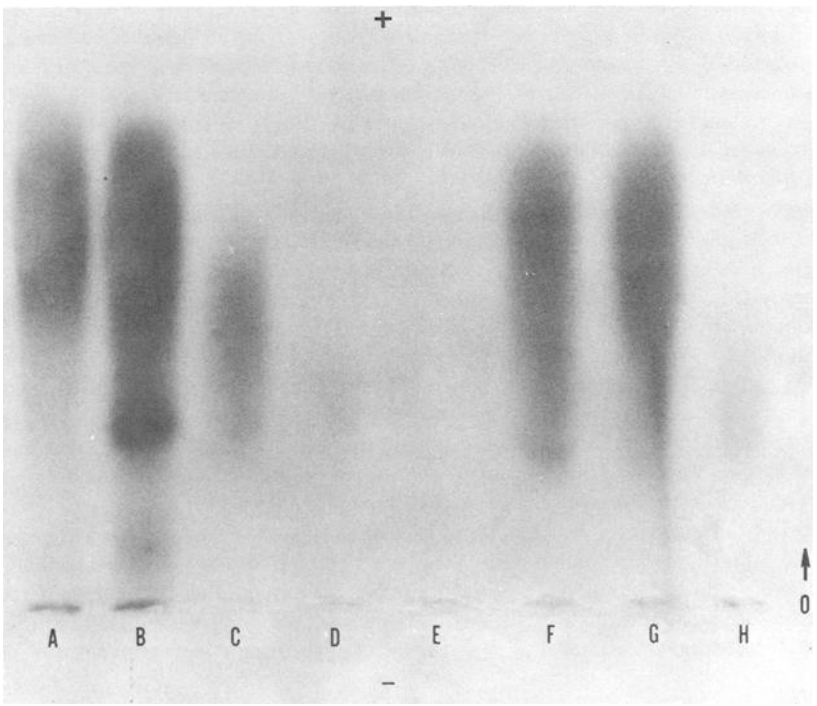

FIG. 2—*The visualization of acid phosphatases using MUP-TMP:* (left to right) *semen, extracted prostate, vaginal, fecal, blood, vaginal plus seminal, fecal plus seminal, and fecal.*

types are not as widely separated in a 5-h run at 150 V as they are during an overnight electrophoretic procedure, the bands are still clearly recognizable. The rapid migration of seminal AP in this buffer system precludes a longer migration path to further resolve PGM zones. In all cases tested seminal AP migration exceeded that of vaginal AP by about 3 cm. When whole semen was diluted with saline, to produce the same AP activity as that for vaginal swabs, the electrophoretic migration difference between these enzymes remained the same. Although seminal AP can be clearly differentiated from vaginal AP, it is not usually possible to positively identify vaginal AP in a mixed stain. While fecal samples may react under ultraviolet light, certain samples also develop a weak blue band; however, this is usually below the seminal AP region. Hemoglobin migrates below the origin in this system and offers no interference in cases of bloody vaginal or anal swabs.

Although use of cellulose acetate in a Gelman Sepratek apparatus,[3] employing the same buffer system run at 240 V for 30 min, gave good resolution and migration for prostate, vaginal, and seminal AP, the activity of the vaginal AP samples was low. After 30 min contact with an agar gel containing the TMP reagent the separated vaginal region was barely visible.

Discussion

Because of a favorable population frequency distribution PGM isoenzyme types are frequently determined in case work involving blood and semen. Since electrophoretic starch gel grouping of heterogeneous PGM enzymes can potentially eliminate suspects involved in sexual assault cases, and since fecal stains appear to display only nonspecific PGM activity, a simultaneous separation of seminal AP from other APs with concurrent PGM identification makes starch gel an attractive alternative for the identification of semen, even on anal swabs.

Using polyacrylamide gel electrophoresis, Lam et al [*6*] have separated 21 different human tissue APs into at least two zones of activity, varying in mobility or band pattern from semen and prostate. Additionally, Adams and Wraxall [*3*] have differentiated vaginal and seminal AP by electrophoretic mobility by employing an acrylamide gel. These authors, as well as Stolorow et al [*7*], have noted difficulty in differentiating seminal AP from some fecal APs when using MUP or α-naphthyl phosphate as visualization substrates for this method.

Starch gel electrophoresis by Smith and Whitby [*2*] has separated prostatic AP into 20 bands in a discontinuous citrate buffer system. That electrophoretic pattern was interpreted as a single enzyme protein with increasing numbers of sialic acid residues. Those authors noted that electrophoresis on starch, in almost any buffer of about pH 6, resolves prostatic AP into two zones.

A number of variations of the polyamide system of Adams and Wraxall [*3*] resolved prostatic AP into two zones. It was found, however, that starch gel gave similar resolution when the same barbiturate buffer system was employed. Moreover, use of the standard PGM starch separation system gave clear differentiation of seminal, vaginal, and most fecal APs (see Fig. 2). This convenient separation method justifies an increased use of electrophoresis for PGM grouping in cases of rape [*8*].

A variety of substrates for APs have been investigated; among these are α-naphthyl phosphate [*9–12*], *p*-nitrophenyl phosphate [*13*], phenyl phosphate [*14*], MUP, and TMP. Roy et al [*4*] have demonstrated by comparative hydrolysis rates that the substrate thymolphthalein monophosphate is the least affected by the nonprostatic APs. Pragay et al [*15*] also noted the effectiveness of this reagent in analysis of evidence involved in cases of rape.

[3]Gelman Instrument Co., Ann Arbor, Mich.

Greater differentiation between APs can be accomplished by a mixed substrate method incorporating TMP and MUP. After the nonspecific fluorescent zones (corresponding to various APs) are viewed under ultraviolet light, the enzymes are quenched with base and the blue bands of thymolphthalein visualized. Since this reagent forms its own chromophore no diazo dye coupling is necessary. The comparative specificity of seminal AP for TMP preferentially visualizes the seminal zones an immediate blue; weaker vaginal AP (migrating below seminal AP) completely develops within the first minute.

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