TECHNICAL NOTE

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A New Enzymatic Method for the Demonstration of Spermine in Human Seminal Stains

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ABSTRACT: A new, simple method for the identification of seminal stains based on the reaction of bovine plasma amine oxidase (spermine oxidase) with spermine is described. The key reaction is the production of hydrogen peroxide by the oxidase action on spermine; the hydrogen peroxide oxidizes 2′,7′-dichlorofluorescin to produce a light green color by which the presence of seminal fluid can be identified. Some data are presented on the stability and specificity of the present method.

KEY WORDS: pathology and biology, semen, chemical analysis

Many methods have been developed to identify seminal fluid and stains. As an absolute proof, spermatozoa are looked for microscopically, but their absence is not always indicative of the absence of semen: aspermatic and old seminal stains give negative results. In these cases, chemical methods are required for the identification of semen. It is well known that the concentration of spermine in human semen is higher than in most animal body fluids and tissues [*1,2*]. On the basis of this unique distribution, Barberio [*3*] and Puranen [*4*] developed classical crystallographic methods for the identification of semen. More recently, methods for the identification of seminal spermine by paper and thin-layer chromatography were also reported [*5–8*]. The use of seminal spermine as an indicator of human semen has an advantage over the use of enzyme proteins such as acid phosphatase [*9*] in that this amine is fairly resistant to heat or putrefaction. Very recently, bovine plasma amine oxidase (spermine oxidase) [*10*], which oxidizes spermine quite actively, has become commercially available, and an extremely sensitive photometric assay for hydrogen peroxide was also

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developed [*11*]. These have enabled us to devise a qualitative analysis for spermine in seminal stains that is much simpler than the previous ones [*3–8*]. The details of the procedure and its suitability for application to forensic science examination are presented in this paper.

Materials and Methods

Chemicals

Bovine plasma amine oxidase was obtained from Worthington Biochemical Corp., Freehold, N.J.; horseradish peroxidase (Type II) from Sigma Chemical Company, St. Louis, Mo.; 2′,7′-dichlorofluorescin diacetate from Eastman Kodak Co., Rochester, N.Y.; and spermine tetrahydrochloride, semicarbazide hydrochloride, and aminoguanidine carbonate from Nakarai Chemicals, Ltd., Kyoto. Other common chemicals used were of the highest purity commercially available.

Stains

Human semen was collected from volunteers by masturbation. Various human body fluids including semen were dropped or smeared on filter paper (Toyo Roshi No. 2, Tokyo). The paper was allowed to dry at room temperature for a few hours and cut into small pieces (2 by 5 mm). Seminal stains kept at room temperature for periods of one week to 20 years in our laboratory were also used.

Preparation of Reagents

Buffer solution: 1.2 g (final concentration, 0.1*M*) of tris(hydroxymethyl)aminomethane, 0.62 g (final concentration, 0.1*M*) of boric acid, and 3.7 mg (final concentration, 0.1 m*M*) of disodium ethylenediaminetetraacetate (EDTA) were dissolved in 100 ml of distilled water and the pH was adjusted to 7.0 by adding 1*N* hydrochloric acid solution. The borate was added to enhance the reaction; EDTA was used to protect the enzymes from various ions in the stains.

Reagent I: 2 mg of horseradish peroxidase and 2 mg of bovine plasma amine oxidase (22 IU/mg) were dissolved in 10 ml of the buffer solution.

Reagent II: 1 mg of 2′,7′-dichlorofluorescin diacetate was dissolved in 3 ml of 0.1*N* sodium hydroxide to hydrolyze the acetyl ester, and the pH was adjusted to 7.0 by adding 0.3*N* hydrochloric acid. The volume of the solution was made to 10 ml with the buffer solution. Since 2′,7′-dichlorofluorescin is sensitive to autoxidation, it should be prepared just before use and kept cold in the dark.

Standard Procedure

Various conditions of the assay were tested and the following procedure is recommended as a standard assay. A small piece of the stained material was placed in the bottom of a small test tube. Two drops of Reagent I and two drops of Reagent II were added to the stain, which was then incubated in the dark at 37°C for 5 to 10 min in a water bath. As a blank test, unstained filter paper of the same size was subjected to the assay. After incubation, a light green color developed that was observed by the naked eye.

Results

In order to confirm that the color reaction is due to polyamine present in the seminal stain, we subtracted bovine plasma amine oxidase from the complete assay mixture. This

resulted in no reaction. Furthermore, the addition of semicarbazide (final concentration, 3.6 m*M*) and aminoguanidine (final concentration, 2.9 m*M*), the inhibitors of amine oxidases, to the reaction mixture suppressed the reaction almost completely, showing that the reaction is catalyzed by the amine oxidase.

To check the sensitivity of the present method, human semen was diluted stepwise, dropped on filter paper, and dried to make small seminal stains, which were then treated by the standard procedure. As a result, all samples were positive up to an eightfold dilution. From the authentic samples of spermine, it was estimated that the seminal strains of the eightfold dilution contained about 1 nmole of spermine.

Seminal stains left at room temperature for various periods were subjected to the present method. As shown in Table 1, all the samples were positive up to 5 years; only one of ten samples was negative after 11 to 20 years of aging. These results confirmed that spermine in the stain is stable for a long period.

The results on various human body fluids are presented in Table 2. The color could be detected only in semen and in two samples of urine. However, the color in the former was much more intense than in the latter. The two positive samples might be due to the contamination of urine with semen.

Since seminal fluid may coexist with blood, vaginal fluid, or rectal fluid in actual cases, we tested the effects of these materials on the present reaction by adding their stains to the reaction mixtures. No interference of the materials with the reaction was observed.

Discussion

A new method for the demonstration of spermine in human seminal stains is based on the bovine plasma amine oxidase-dependent production of hydrogen peroxide [*10*]. In this

TABLE 1—*Polyamine test on human seminal stains of various ages.*

Age of Stain	Tested, *n*	Positive, *n*	Negative, *n*
1 day to 1 week	15	15	0
2 weeks to 1 month	9	9	0
2 to 6 months	10	10	0
7 months to 1 year	10	10	0
2 years	10	10	0
3 to 5 years	3	3	0
6 to 10 years	3	2	1
11 to 20 years	10	9	1

TABLE 2—*Polyamine test on the stains of various human body fluids.*

Human Body Fluid	Tested, *n*	Positive, *n*	Negative, *n*
Semen	54	54	0
Blood	11	0	11
Serum	24	0	24
Saliva	11	0	11
Nasal discharge	7	0	7
Tears	4	0	4
Breast milk	7	0	7
Sweat	8	0	8
Vaginal fluid	24	0	24
Urine	28	2	26
Feces	4	0	4

reaction, the hydrogen peroxide-dependent oxidation of 2',7'-dichlorofluorescin to 2',7'-dichlorofluorescein, in the presence of horseradish peroxidase, produces a light green color [*11*].

Although both spermine and spermidine are active substrates for bovine plasma amine oxidase, the reaction of the enzyme with spermine is twice as active as that with spermidine [*10*]. In addition, the concentration of spermine in human semen is much higher than that of spermidine [*2*]. It is therefore concluded that the present reaction with human seminal stains is almost exclusively due to spermine.

Seminal spermine is useful for the identification of semen because it is fairly resistant to heat and putrefaction. In addition, the amine is suitable for the examination of seminal stains excreted by vasectomized individuals because seminal spermine is derived not from the testis but largely from the ventral prostate [*1,12*].

Our present method is sensitive enough to detect as little as 1 nmole of spermine present in the stain. This is due to the extremely high molar extinction coefficient (91 000 $M^{-1}cm^{-1}$) of 2',7'-dichlorofluorescein, the product of the reaction, and to the unique stoichiometry that one mole of hydrogen peroxide generates 5.3 moles of 2',7'-dichlorofluorescein [*11*]. In addition, our method is much simpler and quicker than other methods reported so far [*3–8*]: more than 100 samples can be treated within 30 min. Therefore, the method seems suitable as a preliminary test for the identification of human semen in medicolegal practice.

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