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A New Enzymatic Method for Quantitation of Spermine in Human Semen

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Summary. A new enzymatic method for the quantitation of spermine in human semen, based on the specific reaction of barley seedling polyamine oxidase with spermine, is described. Small amounts of human semen were incubated with the polyamine oxidase; hydrogen peroxide formed in the oxidase reaction was measured photometrically by coupling 4-aminoantipyrine with N-ethyl-N-(3-methylphenyl)-N′-acetylethylenediamine in the presence of peroxidase. The detection limit of spermine of this method was about 10 nmol per tube. The mean level of spermine in human semen was 2.41 μmol/ml; the levels in vaginal fluid, saliva, serum, and urine were below the detectable limit by this procedure.

Key words: Spermine, enzymatic assay – Identification of human semen, enzymatic method

Zusammenfassung. Es wird eine neue enzymatische Methode zur quantitativen Bestimmung von Spermin im menschlichen Samen beschrieben, die auf einer spezifischen Reaktion von Polyamin-Oxidase aus Gerstensämlingen mit Spermin beruht. Geringe Mengen menschlichen Samens wurden mit der Polyamin-Oxidase inkubiert und das durch die Oxidase-Reaktion gebildete Wasserstoffperoxid photometrisch nach Kupplung von 4-Aminoantipyrin mit N-Äthyl-N-(3-methylphenyl)-N′-acetylethylendiamin in Gegenwart von Peroxidase gemessen. Die Nachweisgrenze von Spermin in der Probe liegt bei etwa 10 nmol. Die mittlere Konzentration von Spermin im menschlichen Samen beträgt 2.41 μmol/ml; die Werte für Vaginalflüssigkeit, Speichel, Serum und Urin liegen unter der Nachweisgrenze der Methode.

Schlüsselwörter: Spermin, enzymatische Bestimmung – Spermauntersuchung, enzymatisch – Spurenuntersuchung, Sperminnachweis

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The concentration of spermine in human semen is much higher than in most mammalian body fluids and tissues [1, 2]. On the basis of this unique distribution, Barberio [3] and Puranen [4] developed classical crystal methods for the identification of human semen. Some qualitative methods for the identification of seminal spermine by paper and thin-layer chromatography were also reported [5–8]. Recently, we reported a qualitative enzymatic method for the demonstration of spermine in human seminal stains [9]. In the present paper, we have established a detailed procedure of a new simple and specific method for the quantitation of spermine in human semen, since quantitative analyses often give more accurate information for the identification of semen.

Materials and Methods

Chemicals

4-Aminoantipyrine was obtained from Wako Pure Chemical Industries, Ltd., Osaka (Japan); horseradish peroxidase (type II) and spermine-4 HCl from Sigma Chemical Co., St. Louis, MO (USA); N-ethyl-N-(3-methylphenyl)-N′-acetylethylenediamine (EMAE)[1] was a product of Kyowa Medicus, Co., Ltd., Tokyo (Japan). Other chemicals used were of the highest purity commercially available.

Human Samples

Human semen was collected from volunteers by masturbation. Vaginal fluids were collected at the Dept. of Obstetrics and Gynecology at the Nagoya University Hospital, Nagoya (Japan). Microscopic examinations of spermatozoa and the acid phosphatase test were performed to check the contamination of the samples by semen. Saliva, serum, and urine were obtained from healthy subjects of either sex. All samples were kept frozen at − 20° C until analyzed.

Preparation of Barley Seedling Polyamine Oxidase[2]

Barleys (seeds of *Hordeum vulgare*), obtained from a local farm, were germinated on a moist vermiculite at about 20° C for 2–3 weeks in the dark. After this period, the seedlings were excised and washed in tap water. One hundred fifty grams of the material was homogenized with cooling in 300 ml of 0.5 *M* NaCl solution in a Waring blender, and filtered with gauze. The resulting juice was brought to 45% saturation with ammonium sulfate followed by centrifugation (18,000 × *g*, 15 min). The supernatant fraction was then brought to 65% saturation with the same salt, and centrifuged (18,000 × *g*, 15 min). The sediment was dissolved in 10 vol. of 0.01 *M* sodium phosphate buffer (pH 6.5), dialyzed against 50 vol. (with three changes) of the same buffer solution with stirring for 24 h, and finally lyophilized. The lyophilized enzyme revealed its activity of 1.45 μmol/mg dry weight/60 min when 1.0 mM spermine was used as substrate, and was stable for more than 3 months at − 20° C.

Results

Substrate Specificity of Barley Seedling Polyamine Oxidase

To check specificity of the present method, a number of amines and amino acids were tested for their oxidation by polyamine oxidase from barley seedlings. As shown in Table 1, this enzyme was highly specific for spermine.

1 EMAE will be kindly provided, upon request, by Kyowa Medicus Co., Ltd., 1-6-1 Ote-machi, Chiyoda-ku, Tokyo, Japan

2 Although the purification procedure of polyamine oxidase from barley seedlings is very simple, the lyophilized enzyme will be provided by Dr. T. Matsumoto upon request

Table 1. Substrate specificity of polyamine oxidase from barley seedlings[a]

Substrate (final, 1.0 mM)	Optical density
Spermine	0.609
Spermidine	0.004
Cadaverine	0
Putrescine	0
1,2-Ethylenediamine	0
1,3-Propanediamine	0
1,6-Hexamethylenediamine	0
1,7-Diaminoheptane	0
Agmatine	0
Ethylamine	0
n-Propylamine	0
n-Butylamine	0
Octopamine	0
Serotonin	0
Tryptamine	0
Benzylamine	0
β-Phenylethylamine	0
Tyramine	0
Synephrine	0
L-Alanine	0
L-Tryptophan	0
L-Glycine	0
L-Serine	0
L-Aspartic acid	0
L-Arginine	0
L-Histidine	0

[a] The incubation mixture (final, 1.82 ml) contained 0.3 ml of 0.5 *M* sodium citrate buffer (pH 4.5), 0.3 ml of peroxidase solution (0.2 mg/ml), 0.3 ml of 4-aminoantipyrine solution (1 mg/ml), 0.3 ml of EMAE solution (0.5 mg/ml), 0.3 ml of polyamine oxidase solution (0.2 mg/ml), 0.3 ml of water and 20 µl of each substrate (final, 1.0 mM). After incubation at 37° C for 60 min, optical density was measured at 555 nm

Effect of Incubation Time

The assay mixtures (final volume, 1.82 ml), containing 120 nmol spermine and 0.3 mg of polyamine oxidase, were incubated at 37° C for various periods. As illustrated in Fig. 1, the optical density almost reached its maximal level after only 5 min of incubation; it decreased only slightly from 15 to 60 min. Therefore, 15 min of incubation was adopted to secure complete consumption of spermine in assay mixtures. The optical density was found to be stable for several hours at room temperature.

Calibration Curve

The relationship between the optical density resulted from the enzyme reaction and the amounts of spermine is presented in Fig. 2. Satisfactory linearity was obtained

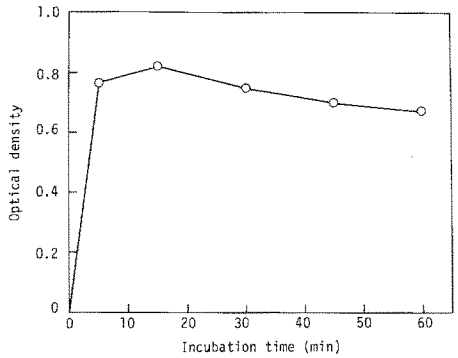

Fig. 1

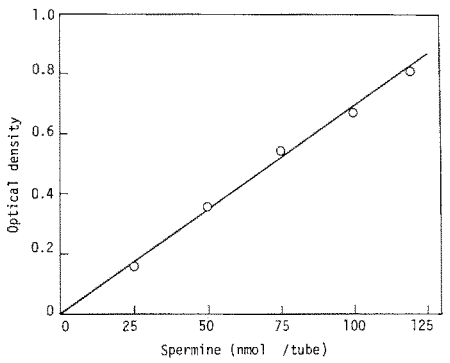

Fig. 2

Fig. 1. Effect of incubation time (37° C) on consumption of spermine by barley seedling polyamine oxidase. The composition of the incubation mixture was the same as specified in Table 1 except that 120 nmol of spermine and 0.3 mg of polyamine oxidase were contained in the incubation mixture (1.82 ml)

Fig. 2. Calibration curve for spermine. Assay procedure is the same as specified in Table 1 except that various amounts of spermine and 0.3 mg of polyamine oxidase were used

Table 2. Effect of contamination by various body fluids on the assay of spermine in human semen[a]

Body fluid added (20 µl)	Optical density at 555 nm	
	No addition	Addition
Vaginal fluid	0.298	0.295
Saliva	0.273	0.276
Serum	0.273	0.278
Urine	0.278	0.256

[a] Twenty microliters of various body fluids were added to the assay mixtures (1.8 ml) prior to the addition of 20 µl of human semen. The values are the means of duplicate determinations

over the concentration range of 25–120 nmol spermine per tube (1.82 ml). The detection limit was about 10 nmol.

Interference by Contaminants

Since seminal fluids often coexist with other body fluids in actual cases, we have tested the effects of vaginal fluid, saliva, serum, and urine on the present reaction by adding these fluids to assay mixtures prior to the addition of seminal fluids as shown in Table 2. Vaginal fluid, saliva, and serum showed no interference. Urine showed slight interference; but this minor interference was fully compensated by taking internal standards.

Table 3. Spermine contents in various human body fluids

Body fluid	Spermine (μmol/ml)
Semen	2.41 ± 1.33 (20)[a]
Vaginal fluid	ND[b] (10)
Saliva	ND (7)
Serum	ND (10)
Urine	ND (10)

[a] The mean ± SD is given; the numbers of determinations are in parentheses
[b] ND: not detected

Recommended Procedure

On the basis of the above data, we adopted the following procedure as a standard assay for spermine in body fluids. The incubation mixture (final, 1.82 ml) contained 0.3 ml of 0.5 *M* sodium citrate buffer (pH 4.5), 0.3 ml of horseradish peroxidase solution (0.2 mg/ml), 0.3 ml of 4-aminoantipyrine solution (1 mg/ml), 0.3 ml of EMAE solution (0.5 mg/ml), 0.3 ml of the polyamine oxidase solution (1 mg/ml), 0.3 ml of water and 20 μl of the body fluid (semen, vaginal fluid, saliva, serum or urine). After incubation at 37° C for 15 min, the optical density of the purple color developed was determined at 555 nm in a cuvette of 1 cm light path. The blank test differed in that 0.3 ml water was added to the mixture in place of the EMAE solution. External standards could be taken for seminal fluid, vaginal fluid, saliva, and serum because of their non interference with the assay. As an external standard, 20 μl (60–120 nmol) of spermine solution (5.2–10.4 mg spermine-4 HCl dissolved in 5 ml water) was added to the mixture in place of the body fluid. When interference in the assay is suspected, internal standards (addition test) should be taken; 20 μl (60 nmol) of spermine solution was added to the mixture together with 20 μl of the body fluid.

Concentration of Spermine in Human Semen

Table 3 shows the determinations of spermine in various body fluids. The high spermine content was confirmed in human semen, while this amine could not be detected in other body fluids by this method.

Discussion

To our knowledge, this is the first report to perform a specific enzymatic assay for spermine in biologic samples. Our present method is applicable to any biologic samples, such as tissue extracts; this line of experiments is now under investigation in our laboratory. Enzymatic assays were tried for spermidine using *Serratia marcescens* cells [10], for combined polyamines (spermine + spermidine) using bovine serum amine oxidase [11], for combined diamines (putrescine + cadaverine)

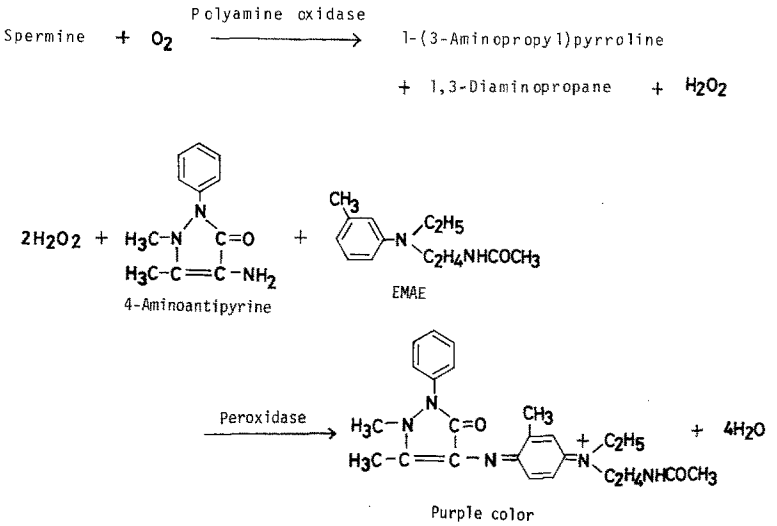

Fig. 3. Reaction mechanism of the present assay

using human placental diamine oxidase [12], and for combined diamines and polyamines using soybean seedling amine oxidase [13].

Our method is based on the unique substrate specificity of barley seedling polyamine oxidase that this enzyme attacks only spermine when the pH of the reaction mixture is 4.5 (Table 1) [14]. Various amine oxidases were reported to be active toward polyamines. The enzymes from bovine serum [15], oat seedlings [16], soybean seedlings [17], and pea seedlings [18] are all active toward both spermine and spermidine. The polyamine oxidase from barley seedlings used in the present assay is the only enzyme, as far as we know, which is specific for spermine.

The properties of barley seedling polyamine oxidase were well studied by Smith et al. [14, 19] although this enzyme has not been highly purified. The crude enzyme preparation only after ammonium sulfate precipitation is sufficient for practical use. The partical purification procedure is very simple; barley is a common material easily obtainable.

We have used, in this method, EMAE as a new coloring reagent [20]. Our method is based on the polyamine oxidase-dependent production of hydrogen peroxide [14]; the hydrogen peroxide-dependent reaction of EMAE with 4-aminoantipyrine in the presence of peroxidase produces a strong purple color [20]. The reaction mechanism is shown in Fig. 3. The present 4-aminoantipyrine-EMAE system is about 2.5-fold as sensitive as the conventional 4-aminoantipyrine-phenol system [21], and is resistant to autoxidation and to interference by bilirubin and proteins [20].

Using this method, we have obtained the spermine level in human semen of 2.41 μmol/ml (Table 3). This value is fairly similar to that (3.04 μmol/ml) of Jänne et al. [2] obtained by paper electrophoresis, supporting the accuracy of our method.

Since the present method consists of the direct addition of small amounts of human semen to assay mixtures, and their incubation for 15 min, it is very simple

and quick; about 100 samples can be treated within 1 h. Therefore, we can recommend our method for the identification of human semen in actual medicolegal practice as well as for pure research purposes.

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