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A Medico-Legal Study on Diamine Oxidase in Human Semen

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The identification of semen is essential in medico-legal practice of sex crimes. For this purpose, the activity of acid phosphatase is widely used.[1] However, although the method with acid phosphatase is highly sensitive, the activity is also present in vaginal fluid and in many kinds of vegetables. In 1948, BERG[2] found the high activity of diamine oxidase (DAO) in human semen using a classical biological method. Since then, no medico-legal report on seminal DAO has appeared. Accordingly, in the present study we investigated the fundamentals on DAO in seminal fluid using a fluorometric assay in order to test if DAO can be used for the identification of semen in medico-legal practice.

Materials and Methods

Materials

Human semen was collected at the Department of Obstetrics and Gynaecology of Nagoya University Hospital. It was frozen immediately after sampling and kept in a deep freezer until analyzed. Vaginal fluid was smeared on filter papers, dried and kept in a freezer until used. The fluid was extracted into a small amount of water just before use. Preceding the estimation, a microscopic examination of spermatozoa and the acid phosphatase test were performed in order to check the contamination of the samples with semen. Normal human serum, serum during pregnancy and saliva were kept in a frozen state until analyzed. Spinach, cabbage, radish, stone-leek, parsley, white potato, sweet potato, cucumber, green pepper, carrot, egg apple, lettuce, celery, mushroom, cauliflower, tomato, lemon, mandarin orange, apple, pear, persimmon and grape were mashed and kept in a freezer until used.

Homovanillic acid, putrescine-2HCl, cadaverine-2HCl, spermidine-3HCl, spermine-4HCl, histamine-$2H_3PO_4$, semicarbazide-HCl, aminoguanidine-H_2CO_3, pyridoxal-5′-phosphate and potassium cyanide were obtained from Nakarai Chemicals, Ltd., Kyoto; horseradish peroxidase from Sigma Chemical Company, St. Louis, Mo.; iodoacetic acid from E. Merck AG, Darmstadt; hydrogen peroxide from Mitsubishi-Gasukagaku, Ltd., Tokyo; radioactive [2,3-^{3}H (N)]-putrescine-2HCl and Omnifluor from New England Nuclear, Boston, Mass.

Assays

DAO activity was measured fluorometrically essentially according to GUILBAULT *et al.*[3] In this method, hydrogen peroxide formed in the DAO reaction was measured fluorometrically by converting homovanillic acid into a highly fluorescent compound in the presence of per-

oxidase. The fluorometric measurements were made in a Shimadzu corrected spectrofluorophotometer RF-502 at 20°C.

In the assay for DAO in urine, the fluorometric measurement was difficult since contaminated fluorescent compounds resulted in quite high blank values. Therefore, we used the radiochemical method of OKUYAMA and KOBAYASHI[4] with a slight modification. The assay mixture (0.6 ml) contained 0.2 ml of 0.1M sodium phosphate buffer (pH 7.8), 0.2 ml of radioactive putrescine solution (final concentration, 0.33 mM) and 0.2 ml of enzyme solution. After incubation at 37°C for 30 min, 200 mg of solid sodium bicarbonate and 5 ml of toluene were added to the mixture, shaken vigorously and centrifuged (3,000 rpm, 5 min). The organic phase was transferred to another centrifuge tube, mixed with 3 ml of water saturated with sodium bicarbonate, shaken vigorously and centrifuged (3,000 rpm, 5 min). One milliliter of the organic phase was subjected to liquid scintillation counting with Aloka LSC-502. This isotopic method showed the sensitivity comparable to that of the fluorometric assay.[3]

Results and Discussion

Conditions for the fluorometric assay

Since no one tried to assay DAO in human semen by the flurometric method of GUILBAULT *et al.*[3] and since the enzymological knowledge on DAO in human semen is rather scanty,[5][6] we investigated the fundamental properties of DAO in human semen in order to establish a detailed fluorescence assay procedure.

Figure 1 shows the effect of pH on the enzyme reaction. The reaction increased up to

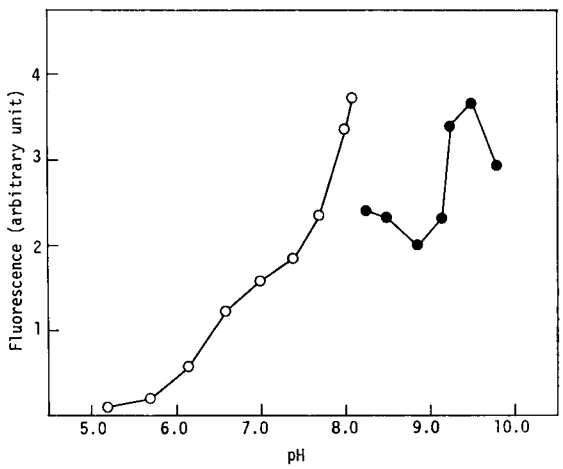

Fig. 1. Effect of pH on the enzyme reaction of seminal DAO-peroxidase system. Sodium phosphate buffer (0.5 M) was used in the pH range 5.0 to 8.5, and sodium borate buffer (0.5 M) in the pH range 8.5 to 10.0. The assay procedure is given in the text.

pH 8.0 with sodium phosphate buffer. A comparable fluorescence was also observed around pH 9.5, but in alkaline pH range blank values were quite high. Therefore, we adjusted the assay mixture to pH 8.0 with sodium phosphate buffer.

Table 1 shows the substrate specificity of human seminal DAO. As can be seen from the

Table 1. Substrate specificity of human seminal DAO

Substrate*	Fluorescence (arbitrary unit)
Putrescine	100
Cadaverine	98.6
Spermidine	15.3
Spermine	7.6
Histamine	1.4

*The concentration of the substrate was 1 mM.

Table 2. Effect of various inhibitors on the enzyme reaction of human seminal DAO

Inhibitor*	Fluorescence (arbitrary unit)
None	100
Semicarbazide	1.2
Aminoguanidine	0.6
Potassium cyanide	6.5
Iodoacetic acid	35.1

*The concentration of the inhibitor was 1 mM. Putrescine (1 mM) was used as substrate.

table, putrescine was oxidized most rapidly. Accordingly, we used putrescine as substrate.

Table 2 shows the effect of some inhibitors on seminal DAO activity. Carbonyl reagents such as semicarbazide and aminoguanidine at the concentration of 1 mM inhibited human seminal DAO almost completely. Thus, we adopted semicarbazide to stop the enzyme reaction. It was confirmed that semicarbazide does not quench the fluorescence developed by the enzyme reaction. Potassium cyanide, a chelator of metals, inhibited DAO appreciably, which is consistent with the observation that DAO is a copper-containing enzyme.[7] Iodoacetic acid also inhibited DAO activity, suggesting that thiol groups of seminal DAO or added peroxidase participate in the enzyme reaction. Effect of pyridoxal-5′-phosphate, which is known to be a cofactor of DAO, was tested. The addition of the cofactor showed strong quenching of the fluorescence. Thus, we did not add this cofactor in our routine assay. It should be recalled that the addition of the cofactor to the assay mixture did not enhance the activity of human seminal DAO.[5]

On the basis of the above data, we adopted the following procedure as a routine assay for human seminal DAO. The assay mixture (0.6 ml) contained 0.1 ml of 0.5 M sodium phosphate buffer (final pH of the assay mixture, 8.0), 0.1 ml of peroxidase solution (1 mg/ml), 0.1 ml of homovanillic acid solution (1 mg/ml), 0.1 ml of putrescine solution (final concentration, 1 mM) and the enzyme solution. The assay mixture was preincubated at 37°C for 10 min before addition of homovanillic acid and putrescine. After incubation at 37°C for 1 h, the enzyme reaction was stopped by addition of 0.1 ml of semicarbazide solution (2 mg/ml). Two milliliters of water were added to the assay mixture and centrifuged at 10,000 rpm for 5 min. The supernatant was decanted into a test tube and the fluorescence intensity of the reaction product was measured with excitation at 315 nm and emission at 425 nm. As a blank test, the assay mixture without putrescine was incubated and mixed with the substrate after addition of semicarbazide. Standard was taken by adding freshly prepared hydrogen peroxide solution (5–10 nmoles) to the assay mixture before incubation. The method is so sensitive that as little as 0.5 nmole of hydrogen peroxide formed in the assay mixture can be determined.

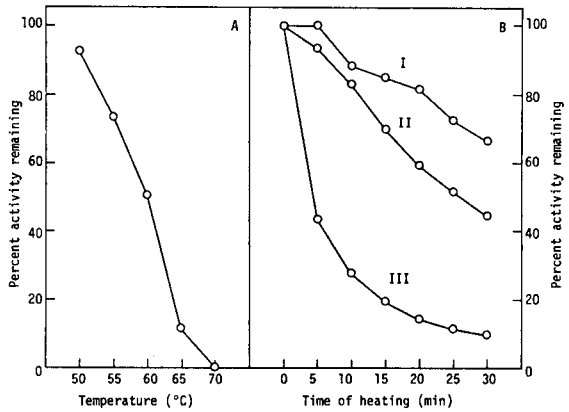

Fig. 2. Heat-stability of human seminal DAO. A: Heat-stability as a function of temperature. The seminal fluid was treated at various temperatures for 30 min. B: Heat-stability as a function of the time of heating. I: 55°C; II: 60°C; III: 65°C.

Effect of heating on human seminal DAO

Since heat-stability is worthwhile to study from the medico-legal point of view, we incubated seminal fluid at various temperatures. The results are illustrated in Fig. 2. As shown in the figure, seminal DAO lost its activity almost completely by heating at 70°C for 30 min. However, after incubation of the enzyme at 60°C for 30 min, an appreciable amount of the activity remained. By incubation at 50°C for 30 min, the enzyme activity was almost unaffected. Accordingly, seminal DAO seems to be fairly heat-stable, which suggests that the human seminal DAO is suitable for medico-legal examination.

DAO activities in various body fluids

DAO activities in various body fluids are summarized in Table3. The activity could be detected only in semen and in the serum of pregnant woman, and was much higher in the former than in the latter. DAO in the semen of azoospermia was as high as that of

Table 3. DAO activities in various body fluids of the human

Sample	Number	DAO activity (nmoles H_2O_2 formed/ml/h)	
		Mean	Standard error
Semen*			
Normospermia	18	148	58
Oligospermia	16	147	34
Azoospermia	4	223	73
Serum			
Normal	10	n.d.***	—
Pregnant	10	9.2	2.8
Urine**	12	n.d.	—
Saliva	7	n.d.	—
Vaginal fluid	7	n.d.	—

*The count of spermatozoa: Normospermia, >40 million/ml; oligospermia, ≦40 million/ml; azoospermia, 0/ml.

**Presence of DAO in urine was tested by the radiochemical method, the procedure of which is given in the text.

***n.d.: Not detected.

normospermia, suggesting that seminal DAO does not originate from testis.

DAO activities in vegetables and fruits:

The presence of DAO was checked in vegetables such as spinach, cabbage, radish, stone-leek, parsley, white potato, sweet potato, cucumber, green pepper, carrot, egg apple, lettuce, celery, mushroom and cauliflower, and in fruits such as tomato, lemon, mandarin orange, apple, pear, persimmon and grape. As a result, the activity was found only in cucumber (18.6 ± 7.6 nmoles H_2O_2 formed/g wet weight/h, mean $\pm$S.E., $n=3$). It should be emphasized that no DAO activity was found in cauliflower which contains a large amount of acid phosphatase[1]).

In the present study, we demonstrated that human seminal fluid contains high DAO activity. In addition, DAO was found not to be ubiquitous: so far as checked in the present study, the activity was found only in human semen, the serum of pregnant woman and cucumber. Seminal fluid revealed much higher activity than the other two. These results suggest the possibility that DAO can be used for the identification of semen in medico-legal practice. The further studies on DAO with human seminal stains are now in progress in our laboratory.

Summary

The fundamental enzymological properties of human seminal diamine oxidase (DAO) were investigated to establish a detailed fluorescence assay procedure for DAO. Using this assay, a high activity of DAO was demonstrated in human seminal fluid. DAO activity was also found in the serum of pregnant woman and cucumber, but not in other body fluids and plants tested. Human seminal fluid revealed much higher activity than the other two. These results suggest the possibility that DAO can be used for the identification of semen in medico-legal practice.

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ヒト精液ジアミン酸化酵素の法医学的研究

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われわれはジアミン酸化酵素（DAO）が精液中に多量に存在することを確認し，本酵素活性を精液の証明の示標として応用することを目的として，いろいろの基礎実験を行つた．DAO の活性測定はペルオキシダーゼを用いる蛍光法を利用した．精液の酵素活性に及ぼす反応液の pH の影響を検討したところ，リン酸緩衝液を用いて pH 8.0で測定するのが適当であることを知つた．各種の基質を用いてその特異性に関してしらべたところ，プトレシンで活性が最大であつた．精液 DAO に及ぼすいろいろの阻害剤の影響をも検討した．DAO の熱安定性をしらべたところ，65℃30分の処理後でもかなりの活性が認められ，50℃30分の処理ではほとんど活性の低下はみられなかつた．それ故精液 DAO は比較的熱に安定であり，法医学的物体検査に適するものと思われる．以上の基礎実験をもとにして DAO の定量法の詳細を設定し，ヒト各種体液中の DAO 活性を測定したところ，精液と妊娠血清中に活性を認めたが他の体液中では活性を認めなかつた．精液中の活性は妊娠血清のそれよりはるかに高かつた．無精子症患者の精液にも高い活性が認められた．さらにいろいろの野菜ならびに果実について DAO 活性の有無をしらべたところ，キューリに低い活性を認めたが他の植物には活性は認められなかつた．以上の結果から，ヒトの精液を検出する手段として DAO 活性を用いることは有用であるものと考えられる．