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Identification of semen in stain by determination of the specific activity of L-tartrate-inhibitable acid phosphatase

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Summary. For identification of semen in stain the specific activity of L-tartrate-inhibitable acid phosphatase (ACP) was determined. With each stain extract, both enzyme activity and protein concentration were determined, and the specific activity (enzyme activity/protein concentration) was calculated. Seminal stains showed a value of 23.8 ± 15.2 (mean ± SD) IU/mg protein, while vaginal fluid stains showed a value of 0.088 ± 0.049 IU/mg protein. Stains of other body fluids did not show any L-tartrate-inhibitable ACP activity. Furthermore, only eight of 30 plant juice stains showed any levels of L-tartrate-inhibitable ACP, although all plants tested showed ACP activity. As the present method enables us to analyze forensic samples quantitatively, it seems to be useful for forensic practice.

Key words: Semen detection, acid phosphatase – L-tartrate inhibitable acid phosphatase – Identification of semen in stain

Zusammenfassung. Zur Identifizierung von Spermaspuren wurde die spezifische Aktivität der durch L-Tartrat hemmbaren sauren Phosphatase (ACP) bestimmt. Für jeden Spurenextrakt wurde die Proteinkonzentration sowie die spezifische Aktivität des Enzyms ermittelt. Spermaspuren wiesen Aktivitätswerte von $23{,}8 \pm 15{,}2$ IU/mg Protein auf, Spuren von Vaginalflüssigkeit hingegen nur von $0{,}088 \pm 0{,}049$ IU/mg Protein. Spuren anderer Körperflüssigkeiten zeigten diese Aktivität nicht. Darüber hinaus konnte nur bei 8 von 30 Spuren von Pflanzensäften Aktivitäten der durch L-Tartrat hemmbaren ACP nachgewiesen werden, obwohl alle untersuchten Pflanzen ACP-Aktivität besaßen. Die Methode scheint für die forensische Praxis geeignet, da sie quantitative Ergebnisse liefert.

Schlüsselwörter: Spermaspuren, Identifizierung – Saure Phosphatase, hemmbar durch L-Tartrat

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Introduction

In cases involving sexual assault, it is very important to identify seminal stains. For that purpose, spermatozoa are investigated microscopically, but their absence is not always indicative of the absence of semen; aspermatic and very old seminal stains usually give negative results. It is well known that human semen shows high activity of acid phosphatase (ACP), and thus simple color tests for ACP are routinely used in many laboratories for identification of semen [1–3]. However, these tests are considered presumptive because some stains of other body fluids or various plants show positive results. In semen, most ACP is prostatic acid phosphatase (PAP), an isoenzyme of ACP which is synthesized by epithelial cells of the prostate. Abdul-Fadl and King [4] reported that L-tartrate inhibits ACP activity of the prostate, while not affecting the ACP of plasma and red cells. In the field of clinical medicine, L-tartrate-inhibitable ACP activity in serum is used as the indicator of the presence of prostatic cancer which secrets PAP into serum. It has been noted by some investigators that low levels of ACP activity are normally present in the vaginal fluids of sexually inactive women [5–7]. This endogenous vaginal ACP should be differentiated from PAP in identification of seminal stains. Sivaram claimed that the inhibition of ACP activity by L-tartrate was characteristic of the seminal ACP [8]. However, some part of this vaginal ACP is inhibited by L-tartrate [6, 7].

In our present study, specific activity (enzyme activity/protein concentration) of L-tartrate-inhibitable ACP was used for the identification of semen in stains. A simple commercial kit was used for determination of enzyme activity level. With each stain extract, enzyme activity and protein concentration were assayed simultaneously, and specific activity was calculated. The usefulness of the specific activity of L-tartrate-inhibitable ACP for identification of seminal stains and seminal contamination in mixed extract from stains is discussed.

Materials and methods

Stains

Human seminal samples were obtained from a fertility clinic in Nagoya University Hospital. Semen stains were made on a piece of filter paper (Whatman no. 2, Maidstone, England), allowed to dry at room temperature, and examined at various intervals after preparation. Other body fluid samples, i.e., blood, breast milk, nasal discharge, saliva, sweat, tears, urine, and vaginal fluid were also obtained from humans, and their stains were prepared as above. Thirty species of vegetables and fruits containing high amounts of ACP [9] were juiced, and the stains were similarly prepared. For aging experiments, seminal stains left at room temperature for up to 4 years under indirect illumination were used.

A piece of the stained filter paper, 7 by 7 mm in area, was soaked in 0.4 ml distilled water in a small test tube, and the stain was extracted for 60 min at room temperature. Supernatant fluid obtained by centrifugation was used as the stain extract. In case of saliva, urine, and some plant stains containing small amounts of protein, an area of filter paper 10 by 10 mm was soaked in 0.4 ml distilled water to get concentrated extract. For experiment using mixed extract from stains of semen and vaginal fluid, the size of the stain and the volume of water for extraction were changed to get proper concentrations.

Measurement of ACP and L-tartrate-inhibitable ACP activity and protein concentration

The enzyme activity of ACP and L-tartrate-inhibitable ACP was assayed by the "Cica PHOS 'acid'" kit (Chugai Pharmaceutical Co., Tokyo, Japan). Of the extract 0.05 ml was added to each of two test tubes containing 0.5 ml substrate (disodium phenylphosphate, 4-amino antipyrine) and 0.5 ml citrate buffer (pH 4.8). One of the test tubes contained 0.02 M of L-tartrate. After 60 min incubation at 37°C, the reaction was stopped by the addition of 1 ml NaOH (0.075 M) containing $K_3[Fe(CN)_6]$. The optical density was measured at 500 nm and enzyme activity was determined using optical densities of phenol standard solutions. Activity of L-tartrate-inhibitable ACP was calculated by subtracting the uninhibited portion from total ACP activity. The protein concentration of stain extract was determined as described by Lowry et al. [10], and specific activity of L-tartrate-inhibitable ACP was calculated for each stain extract.

Indirect hemagglutination inhibition test on mixed extracts

Stain extracts of group A semen were mixed with those of group B vaginal fluid at various protein ratios. Likewise, stain extracts of group B semen were mixed with those of group A vaginal fluid. With each mixed extract, indirect hemagglutination inhibition tests with anti-A, anti-B, and anti-H sera were performed, and specific activity of L-tartrate inhibitable ACP were assayed simultaneously. Human antisera against human anti-A and anti-B were purchased from Ortho Diagnostic Systems Inc. (New Jersey, USA), and *Ulex europaeus* (an anti-H lectin) from EY Laboratories Inc. (San Mateo, Calif., USA). The indirect hemagglutination inhibition test was performed as follows: Each volume of 2-fold serial dilutions of stain extracts was mixed with each volume of antisera adjusted to 1:4 titer against 1% suspensions of test blood cells and incubated at room temperature for 60 min. At the end of incubation, the strength of inhibition was tested with test blood cells mentioned above in a hollowed glass plate.

Results

Mean and SD of the specific activity of L-tartrate-inhibitable ACP determined with five fresh semen samples and their stains stored at room temperature for up to 41 days is shown in Fig. 1. Specific activity of L-tartrate-inhibitable ACP in fresh semen samples was 52.2 ± 21.7 IU/mg protein. When semen samples were dried on paper and then extracted with distilled water the next day, the

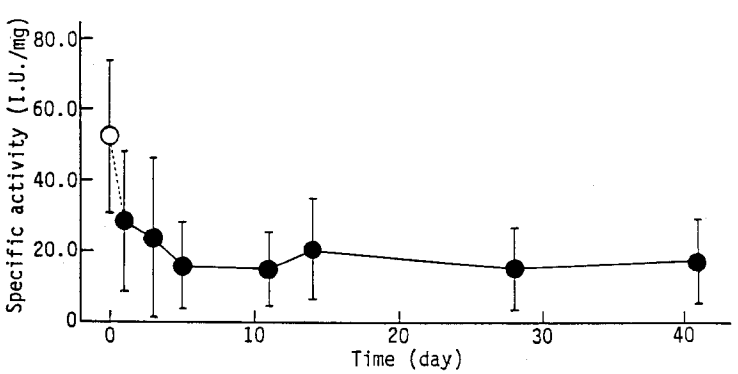

Fig. 1. Specific activity of L-tartrate-inhibitable ACP in fresh semen and seminal stains of various ages. Each point represents mean ± SD of five samples. ○ fresh semen; ● seminal stains

Table 1. Specific activity of L-tartrate-inhibitable acid phosphatase in human seminal stains of various ages

Age of stain	Tested no.	L-tartrate inhibitable ACP (IU/l)	Protein conc. (mg/l)	Specific activity (IU/mg)
1 day to 1 month	20	17000 ± 13800[a]	748 ± 266[a]	23.8 ± 15.2[a]
6 months	6	7310 ± 5280	461 ± 143	19.9 ± 22.8
1 year	6	8870 ± 6620	522 ± 106	18.6 ± 16.0
2 years	6	6520 ± 4700	510 ± 113	12.6 ± 8.3
3 years	5	2810 ± 2220	404 ± 165	6.1 ± 3.8
4 years	6	3440 ± 2750	392 ± 60	8.3 ± 6.1

[a]Mean value ± SD

Table 2. Specific activity of L-tartrate-inhibitable acid phosphatase in stains of various human body fluids

Body fluid	Tested no.	Total ACP (IU/l)	L-tartrate inhibitable ACP (IU/l)	Protein conc. (mg/l)	Specific activity (IU/mg)
Semen	20	18200 ± 14500[a]	17000 ± 13800[a]	748 ± 266[a]	23.8 ± 15.2[a]
Blood	5	21.4 ± 6.2	0	3740 ± 1770	0
Breast milk	5	5.0 ± 0.4	0	233 ± 72	0
Nasal discharge	4	7.2 ± 0.4	0	299 ± 173	0
Saliva[b]	5	2.0 ± 0.1	0	30.0 ± 6.0	0
Sweat	3	4.7	0	35.0	0
Tear	2	2.6	0	78.6	0
Urine[b]	4	1.8 ± 0.2	0	27.4 ± 7.6	0
Vaginal fluid	5	8.1 ± 4.4	6.0 ± 4.0	62.6 ± 21.3	0.088 ± 0.049

Each stain of 7 × 7 mm in area was soaked in 0.4 ml of distilled water and extracted for 60 min at room temperature
[a]Mean value ± SD
[b]The area of stains was 10 × 10 mm

specific activity decreased by about half. However, the specific activity was fairly stable in the case of dried stains; values of 17.1 ± 12.2 (4.8–32.4) were still obtained on the day 41.

Specific activity of L-tartrate-inhibitable ACP determined with seminal stains of various ages is summarized in Table 1. Though the activity decreased gradually with the increase in aging periods, 4-years-old stains still had L-tartrate-inhibitable ACP activity of 8.3 ± 6.1 (0.9–14.7) IU/mg protein. Activity of ACP, L-tartrate-inhibitable ACP and the specific activity of L-tartrate-inhibitable ACP in various body fluids are summarized in Table 2. Though ACP was detectable in all body fluids, L-tartrate-inhibitable ACP was only detectable in stains of semen and vaginal fluid. However, the specific activity of L-tartrate-inhibitable ACP in vaginal fluid stains (0.088 ± 0.049 IU/mg protein) was far lower than that of seminal stains. Specific activity of L-tartrate-inhibitable ACP in various plant stain extracts is summarized in Table 3. Stains of all plants had

Table 3. Specific activity of L-tartrate-inhibitable acid phosphatase in stains of various plant extracts

Plant	Total ACP (IU/l)	L-tartrate inhibitable ACP (IU/l)	Protein conc. (mg/l)	Specific activity (IU/mg)
Asparagus	24.0	0	338	0
Banana	36.7	0	197	0
Broccoli	50.4	7.2	137	0.052
Cabbage	22.3	0	102	0
Carrot	22.3	0	59	0
Cauliflower	40.8	6.2	164	0.038
Celery	9.3	0	49	0
Cucumber[a]	6.7	0	70	0
Egg plant[a]	6.7	0	80	0
Ginger root	26.8	0	318	0
Grape[a]	4.6	0	33	0
Japanese radish	8.6	0	70	0
Leek	16.1	0	80	0
Musk melon	40.5	0	252	0
Onion	15.4	0	254	0
Orange[a]	7.2	0	204	0
Parsley	27.4	0	169	0
Peas with pod	176.0	0	165	0
Pineapple	11.0	0	110	0
Potato	157.8	3.1	309	0.010
Spinach	8.9	0	98	0
Strawberry	7.2	0	147	0
Sweet pepper[a]	6.0	0	97	0
Sweet potato	1060	35.7	329	0.108
Tomato	9.6	0	127	0
Water melon[a]	3.5	0	76	0
Enokidake ($n = 20$) *(Flammulina velutipes)*	61.4 ± 13.7[b]	42.2 ± 10.3[b]	113 ± 20[b]	0.369 ± 0.025[b]
Mushroom ($n = 9$)	170.4 ± 118.0	140.0 ± 103.0	162 ± 55	0.781 ± 0.045
Shiitake[a] ($n = 10$) *(Lentinus edodes)*	50.8 ± 31.5	35.7 ± 24.0	39 ± 11	0.852 ± 0.500
Shimeji ($n = 8$) *(Lyophyllum aggregatum)*	30.9 ± 19.2	18.9 ± 18.2	638 ± 130	0.031 ± 0.032

Each stain of 7 × 7 mm in area was soaked in 0.4 ml distilled water and extracted for 60 min at room temperature

[a] The area of stains was 10 × 10 mm

[b] Mean value ± SD

ACP activity, but those of only eight plants had L-tartrate-inhibitable ACP activity. The specific activity of L-tartrate-inhibitable ACP in such stains were much lower than that of seminal stains.

Results of specific activity of L-tartrate-inhibitable ACP and indirect hemagglutination inhibition tests on mixed stain extract with group A semen and group B vaginal fluid is summarized in Table 4. In the case of semen alone, specific activity was 25.8 IU/mg protein, while that of vaginal fluid alone was 0.18 IU/mg protein. Specific activity in mixed extracts with seminal and vaginal fluid stains and inhibition titer to anti-A and anti-H decreased gradually with an increase in vaginal contents. Inversely, the inhibition titer to anti-B increased with an increase in vaginal contents. Specific activity of L-tartrate-inhibitable ACP and in-

Table 4. Changes in specific activity of L-tartrate-inhibitable acid phosphatase and titer of indirect hemagglutination inhibition test on mixed extract

Semen: Vaginal fluid [(A):(B)]	L-tartrate-inhibitable ACP (IU/l)	Protein (mg/l)	Specific activity (IU/mg)	Inhibition titer (2^{-n})		
				anti-A	anti-B	anti-H
Semen	61420	2380	25.8	7	*N*	8
10:1	46600	2140	21.8	6	2	4
2:1	28200	1800	15.7	5	3	2
1:1	20220	1560	13.0	3	4	1
1:2	13140	1510	8.70	2	4	1
1:10	4776	1320	3.62	0	5	0
1:100	705	1210	0.58	*N*	5	*N*
Vaginal fluid	231	1250	0.18	*N*	6	0

Extract of A seminal stain was mixed that of B vaginal fluid stain at various protein ratios.
N: Sample showed no inhibition titer

Table 5. Changes in specific activity of L-tartrate-inhibitable acid phosphatase and titer of indirect hemagglutination inhibition test on mixed extract

Semen: Vaginal fluid [(B):(A)]	L-tartrate-inhibitable ACP (IU/l)	Protein (mg/l)	Specific activity (IU/mg)	Inhibition titer (2^{-n})		
				anti-A	anti-B	anti-H
Semen	53600	1440	37.2	*N*	8	8
10:1	47820	1480	32.3	2	8	5
2:1	45100	1800	25.1	3	6	2
1:1	45320	2220	20.4	5	5	2
1:2	30840	2680	11.5	6	5	1
1:10	14860	3520	4.22	6	2	0
1:100	2093	3940	0.53	6	*N*	1
Vaginal fluid	196	4120	0.048	6	*N*	0

Extract of B seminal stain was mixed that of A vaginal fluid stain at various protein ratios.
N: Sample showed no inhibition titer

direct hemagglutination inhibition tests on mixed stain extract with group B semen and group A vaginal fluid are summarized in Table 5. Specific activity decreased with a decrease of inhibition titer of semen similar to the results in Table 4.

Discussion

All spot tests for detection of ACP activity have been regarded as supplemental tests for identification of semen, though semen contains far higher amounts of ACP than any other body fluid. The reason is that all of these methods are qualitative in nature, and the amount of semen in stain is usually unknown. Furthermore, human body fluids besides semen and many kinds of plant fluids contain significant amounts of ACP. In the field of clinical medicine, L-tartrate-inhibitable ACP is regarded as PAP, and several kits for determination of activities of PAP and ACP are commercially available. L-tartrate-inhibitable ACP is not absolutely characteristic of semen because considerable amounts of this enzyme have been found on semen-free vaginal swab extracts [6, 7]. Recently, vaginal PAP levels in samples from cases of sexual assault have begun to be assayed directly by radioimmunoassay [11, 12]. However, expensive counting equipment and radioactive probes which constitute a major health hazard are indispensable to these methods. L-tartrate-inhibitable ACP was only detectable in stain extracts of semen, vaginal fluid and eight plants, whereas ACP was detectable in all stain extracts of nine body fluids and 30 kinds of plants that were examined (Tables 2 and 3). Among these, specific activity in seminal stain extract was extremely high. Although L-tartrate-inhibitable ACP activity in semen decreased by half when semen was stained on filter paper and extracted in water the next day, the activity was fairly stable in dried stains (Fig. 1). In stains of various human body fluids other than semen, only vaginal fluid showed detectable L-tartrate-inhibitable ACP activity. Since the mean value of specific activity of L-tartrate-inhibitable ACP in vaginal fluid stains (0.088 IU/mg protein) was far lower than that in seminal stains, semen-free vaginal fluid stain could be easily differentiated from seminal stains. Therefore, the specific activity of L-tartrate-inhibitable ACP gives us a good index to distinguish seminal stain from other body fluids and plant stains. In forensic science practice, semen and vaginal fluid often form mixed stains. From experimentally mixed extracts with seminal and vaginal fluid stain, we found intermediate levels of specific activity of L-tartrate-inhibitable ACP. For example, specific activity in vaginal fluid stain became higher than 3.0 IU/mg protein when the stain was mixed with extract of seminal stain in the ratio of 10:1, while specific activity in vaginal fluid stain was less than 0.2 (Tables 4 and 5). The results suggest that seminal contamination in vaginal fluid in forensic samples can be analyzed quantitatively by using a value of specific activity of L-tartrate-inhibitable ACP activity.

Quantitative analysis of ACP level in the vagina has been reported by several researchers [5–7, 12–14]. Sensabaugh [15] proposed some guidelines for the interpretation of quantitative assay by statistical analysis of endogenous and postcoital ACP level in the vagina. In forensic practice, a vaginal fluid specimen is usually taken using a cotton swab and extracted in a certain amount of liquid,

such as physiologic saline, or sometimes collected as vaginal washing. Therefore, the concentration of ACP may vary from the original, and the exact evaluation of the results is difficult. In our study, we resolved this problem by determination of protein concentration in stain extract and by use of a value of specific activity. Since the value of the specific activity of L-tartrate-inhibitable ACP in stain is not practically influenced by means of extraction or dilution, the results can be analyzed quantitatively even with a mixed stain of semen and vaginal fluid. Furthermore, the method is simple and completed in a short time. For example, it takes only 50 min to measure protein concentration of 20 samples, and even the whole procedure takes only about 3 h to obtain the values of specific activity. Consequently, this method can be used as a confirmative test for identification of seminal stain or seminal contamination and seems to be useful for forensic science practice.

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