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TECHNICAL NOTE

Applicability of ELISA detection of statherin for forensic identification of saliva

Tomoko Akutsu · Ken Watanabe · Yoshihito Fujinami · Koichi Sakurada

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Abstract Statherin is a low molecular-weight phosphoprotein secreted from the parotid gland. Statherin mRNA was previously reported to be a useful marker for mRNA-based saliva identification. In this study, applicability of ELISA detection of statherin for forensic identification of saliva was investigated. The specificity and sensitivity of ELISA for detection of statherin were compared with those of ELISA for α-amylase and the Phadebas® amylase test. Statherin was specifically detected in saliva but not in other body fluids. In addition, statherin was successfully detected in aged saliva stains, mixed body fluids–saliva stains, and simulated casework samples. On the other hand, although ELISA for α-amylase showed higher sensitivity than ELISA for statherin, it was not specific enough to identify saliva. The Phadebas® amylase test also showed positive results in other body fluids that are known to have α-amylase activity; however, it is easy to use for screening forensic casework samples. In conclusion, ELISA for detection of statherin developed in this study could be an effective tool for the forensic identification of saliva because of its specificity for saliva among other body fluids. Forensic casework samples should be tested by ELISA detection or mRNA-based analysis for statherin, depending on the condition of the sample, to supplement presumptive tests for α-amylase, such as the Phadebas® amylase test.

Keywords Forensic science · Saliva identification · Statherin · α-Amylase · ELISA

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T. Akutsu · K. Watanabe · Y. Fujinami · K. Sakurada
National Research Institute of Police Science,
6-3-1, Kashiwanoha,
Kashiwa, Chiba 277-0882, Japan

T. Akutsu (✉)
First Department of Forensic Science, National Research Institute of Police Science,
6-3-1, Kashiwanoha,
Kashiwa, Chiba 277-0882, Japan
e-mail: tomoko@nrips.go.jp

Introduction

Saliva samples, such as those found on the butt of a cigarette smoked by an offender, the lip of a can, or the skin surface of a victim of sexual assault, frequently remain at crime scenes. These forensic samples have been screened with presumptive tests for saliva before DNA analysis. Especially, in the case of sexual assault, identification of saliva from a mixed sample containing semen provides important probative evidence of oral intercourse.

Since the level of α-amylase in saliva is much higher than those in other body fluids, presumptive tests for saliva are generally performed by the detection of α-amylase activity using a starch–iodine assay or the Phadebas® amylase test (Magle Life Science, Lund, Sweden) [1–3]. However, the results of these tests should be interpreted carefully since α-amylase activity is also found in body fluids other than saliva [1, 4, 5]. Recently, an immunochromatographic test, namely, RSID-Saliva (Independent Forensics, Hillside, IL, USA), and a colorimetric test, namely, SALIgAE-saliva test (Abacus Diagnostics, West Hills, CA, USA), have been developed and evaluated for their sensitivity and specificity in the forensic identification of saliva [6]. Although these kits showed higher sensitivity and specificity than the Phadebas® amylase test, positive results were obtained from urine, feces, and some samples

of breast milk. Therefore, it is necessary to develop a more specific marker other than amylase for forensic identification of saliva.

Statherin, a low molecular-weight phosphoprotein comprising 43 amino acids, is secreted from the parotid and submandibular glands [7]. Statherin has been identified as a potent inhibitor of calcium phosphate precipitation [8, 9]. Recently, it was reported that statherin mRNA would be useful as a marker for mRNA-based saliva identification because of its high specificity for saliva [10–13]. Besides, enzyme-linked immunosorbent assay (ELISA) procedures have been developed for identification of body fluids [14–18] as well as ABO blood grouping [19–22] in various forensic samples with an advantage of its applicability for crude samples without purification. Therefore, in this study, the applicability of ELISA detection of statherin polypeptide for forensic identification of saliva was investigated. The specificity and sensitivity of ELISA for detection of statherin were compared with those of ELISA for α-amylase and the Phadebas® amylase test. Simulated casework samples were also tested to verify whether detection of statherin by ELISA could be adopted for identification of saliva in forensic casework samples.

Materials and methods

Reagents

An affinity-purified goat polyclonal antibody raised against the N-terminal peptide of human statherin (anti-statherin) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fractionated rabbit antiserum raised against human α-amylase (anti-α-amylase) and horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG were purchased from Sigma (St. Louis, MO, USA). HRP-conjugated goat anti-rabbit IgG was purchased from Promega (Madison, WI, USA). The Phadebas® amylase test was purchased from Magle Life Science. Other reagents used in this study were of research grade and purchased from Wako Pure Chemical Industries (Osaka, Japan).

Collection of saliva and other body fluids

Saliva samples were collected from 24 volunteers. Semen, vaginal fluid stains, sweat, and urine were also collected from five volunteers by general non-invasive methods. Blood was collected from the brachial vein of five volunteers. The samples were stored at −20°C until use. Vaginal fluid stains, which were obtained by wiping the vaginal mucosa with a sterile cotton swab, were cut into 5× 5-mm squares and extracted with 100 μL of 0.01 M phosphate buffered saline (PBS, pH 7.2). To evaluate the sensitivity of ELISA for detection of statherin and α-amylase, pooled saliva sample was prepared from an equal volume of each saliva sample obtained from five volunteers. Stains of saliva and other body fluids were prepared as follows: 5 μL of samples were spotted onto cotton cloth (approximately 8-mm in diameter) and air-dried at room temperature for 1 week. Saliva stains, which had been stored at 4°C for 1.5–2.5 years, were used as aged saliva stains. A piece of a 1×1-cm square or 5×5-mm square of aged saliva stain was used for ELISA or the Phadebas® amylase test, respectively.

All procedures involving human participants were approved by the Institutional Review Board of the National Research Institute of Police Science.

Mixed stains

To examine the possible interference of body fluids with ELISA for detection of statherin, 5 μL of saliva was mixed with an equal volume of blood, semen, vaginal fluid extract, sweat, or urine. Semen was also mixed with vaginal fluid extract or sweat. Each mixed sample was spotted onto cotton cloth and air-dried at room temperature for 1 week.

Simulated casework samples

The rolling paper and the filter tip of a cigarette butt and the inner surface of a face mask were cut into 1×1-cm squares. The lips of a bottle and a can were wiped with a 1×1-cm square of cotton cloth dampened with distilled water.

ELISA

Saliva, other body fluids, and vaginal fluid extract were diluted from 1:100 to 1:6,400 with 0.05 M bicarbonate buffer (BCB, pH 9.6). Body fluid stains, aged saliva stains, mixed stains, and simulated casework samples were cut into small pieces and extracted with 250 μL of BCB for 1 h. These extracts were diluted from 1:2 to 1:128 with BCB. Aliquots of 50 μL of each diluted sample were added to each well of a 96-well microtiter plate (Sumilon-7296F; Sumitomo Bakelite, Tokyo, Japan) and incubated at 37°C for 1 h. Then, each well was blocked with 200 μL of 1% BSA in PBS at 37°C for 1 h. Anti-statherin or anti-α-amylase was diluted 1:500 with 0.05% Tween 20 in PBS (PBST); 50 μL was added to each well, and incubated at 37°C for 1 h. HRP-conjugated rabbit anti-goat IgG or HRP-conjugated goat anti-rabbit IgG was diluted 1:5,000 with PBST; 50 μL was added to each well, and incubated at 37°C for 1 h. For determination of statherin in stains, HRP-conjugated rabbit anti-goat IgG was diluted 1:1,000. Each well was washed three times with 250 μL of PBST between

each step. A volume of 50 μL of *ortho*-phenylenediamine dissolved at 0.5 mg/mL in McIlvaine buffer (0.1 M citrate–phosphate buffer, pH 5.0) containing 0.06% H_2O_2 was added to each well and incubated at room temperature for 5 min. Color development was stopped by addition of 100 μL of 1 M H_2SO_4. Absorbance at 490 nm was measured by spectrophotometry (Powerwave 200; BioTek, Winooski, VT) and normalized by the absorbance value of the sample treated without primary antibody. Samples with absorbance values above 0.1 for statherin and 0.2 for α-amylase were considered positive, respectively. These thresholds were determined to minimize the effect of non-specific absorbance. Statistical analysis was performed by one-way ANOVA with Scheffé's multiple-comparison test.

Phadebas® amylase test

The Phadebas® amylase tube test was performed following the manufacturer's instructions attached to the reagent, with a slight modification to reduce the scale. A Phadebas® amylase test tablet (0.2 g) was crushed into powder with a mortar. A volume of 5 μL of neat body fluid, 1:100 diluted saliva, or negative control (distilled water) was placed into a 1.5-mL tube. Then, 200 μL of sterile distilled water and 10 mg of powdered Phadebas® reagent was added to each tube and mixed, to give final dilutions of saliva and other body fluids of 1:4,000 and 1:40, respectively. The samples were incubated at 37°C for 30 min. The reactions were terminated by addition of 50 μL of 0.5 M NaOH. Samples were centrifuged at 10,000×*g* for 1 min. The absorbance of the supernatant at 620 nm was measured by spectrophotometry (UVPC-2100, Shimadzu, Kyoto, Japan) and normalized by that of a blank sample. Absorbance values were converted to α-amylase activity in international units (IU) per liter using the standard curve supplied with the reagent. The threshold was determined to be an absorbance of 0.1 (90 IU/L) on the basis of the lowest point of the supplied standard curve.

Results

Saliva and other body fluids

ELISA tests for detection of statherin and α-amylase were performed in 50 μL of diluted saliva and other body fluids. In the detection of statherin by ELISA, as shown in Fig. 1a, although absorbance values were moderately different among individuals, saliva showed high absorbance values compared with other body fluids even at a dilution of 1:6,400. The absorbance values of saliva at dilutions of 1:100 and 1:200 were significantly higher than those of blood, semen, vaginal fluid extract, sweat, and urine ($P<0.01$). The absorbance values of saliva at dilutions of 1:400 were significantly higher than those of semen, vaginal fluid extract, sweat, and urine ($P<0.05$). When samples with absorbance at 490 nm above 0.1 were considered positive, 23 of 24 diluted saliva samples tested positively by ELISA for detection of statherin (Table 1). On the other hand, as shown in Table 1, statherin was not detected in blood, semen, vaginal fluid extract, sweat, and urine. The limit of detection of statherin by ELISA equated to 0.03 μL (50 μL of 1:1,600 dilution) for pooled saliva samples.

In ELISA, for detection of α-amylase, all 24 saliva samples showed high absorbance values compared with other body fluids, even at a dilution of 1:1,600 (Fig. 1b). When samples with absorbance at 490 nm above 0.2 were considered positive, all 24 saliva samples tested positively by ELISA for detection of α-amylase (Table 1). However, some samples of blood, semen, vaginal fluid extracts, sweat, and urine also showed positive results (Table 1). The limit of detection of α-amylase by ELISA equated to 0.5 nL (50 μL of 1:102,400 dilution) for pooled saliva samples.

The semi-quantitative Phadebas® amylase tube test was also performed in saliva and other body fluids. As shown in Table 1, α-amylase activity was observed in all saliva and blood samples. Variable levels of α-amylase activity were observed in sweat and urine samples, and one of five semen samples showed high α-amylase activity (2100 IU/L). The α-amylase activity of all vaginal fluid extracts tested was below the detection limit (90 IU/L). Saliva samples showed at least 13.8-fold higher α-amylase activity than other body fluids. The limit of detection of the Phadebas® amylase tube test equated to 12.5 nL (5 μL of 1:400 dilution) for pooled saliva samples.

Saliva stains and mixed stains

ELISA tests were performed for the detection of statherin and α-amylase in extracts of saliva stains, aged saliva stains, mixed stains, and simulated casework samples. When tested by ELISA for detection of statherin, 21 of 24 saliva stains prepared by 5 μl of saliva (approximately 8 mm in diameter) and all aged saliva stains (1×1-cm square) showed positive results (data not shown). In addition, all samples of mixed body fluids and saliva showed positive results (Table 2). On the other hand, statherin was not detected in samples of mixed vaginal fluid and semen or sweat and semen (Table 2).

The results of ELISA detection of α-amylase for the extracts of saliva and body fluids stains were comparable to those for saliva and other body fluids (data not shown). All aged saliva samples also showed positive results (data not shown). As shown in Table 2, although all samples of mixed body fluids and saliva showed positive results on ELISA for detection of α-amylase, samples of mixed

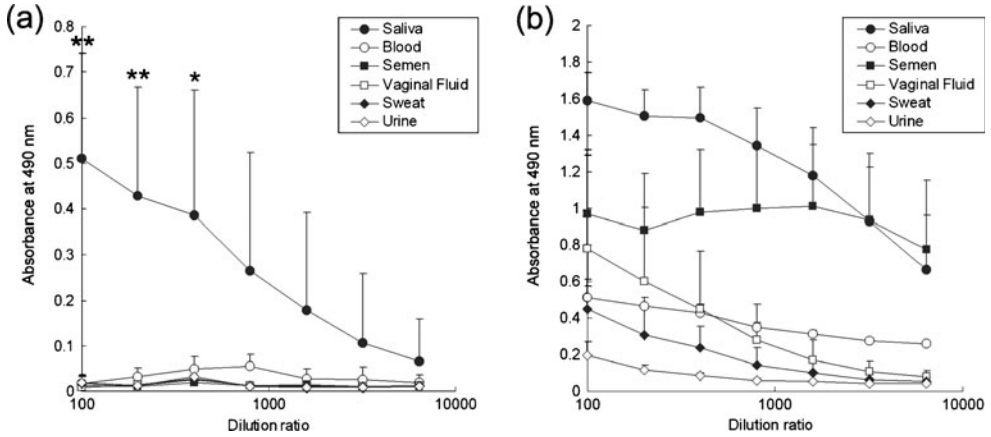

Fig. 1 Specificity of ELISA for detection of statherin (**a**) and α-amylase (**b**) in saliva and various body fluids. Body fluids were diluted from 1:100 to 1:6,400 with bicarbonate buffer, added to each well of microtiter plates, and incubated at 37°C for 1 h. After blocking, samples were incubated sequentially with goat anti-statherin and HRP-conjugated anti-goat IgG at 37°C for 1 h. *Ortho*-phenylenediamine solution was added to each well and incubated at room temperature for 5 min. Color development was terminated by addition of 1 M H_2SO_4, and absorbance was measured at 490 nm. Data are presented as mean±SD calculated from 24 (saliva) or five (other body fluid) samples. Absorbance at 490 nm from saliva was significantly higher than from blood, semen, vaginal fluid, sweat, and urine (**$p<0.01$), and significantly higher than from semen, vaginal fluid, sweat, and urine (*$p<0.05$) by one-way ANOVA with Scheffé's multiple-comparison test

vaginal fluid and semen, as well as sweat and semen, also showed positive results.

Simulated casework samples

To verify whether testing by ELISA for detection of statherin could be adopted for identification of saliva in forensic casework samples, ELISA tests for detection of statherin and α-amylase were compared in simulated casework samples. The ELISA test successfully detected statherin in the rolling paper and the filter tip of a cigarette butt, the inner surface of a face mask, and the lips of a bottle and a can (Table 3). The ELISA also successfully detected α-amylase in simulated casework samples (Table 3).

Discussion

In this study, to evaluate the applicability of statherin as a marker for the confirmation of forensic identification of saliva, an ELISA method for detection of statherin was developed and performed in body fluids, body fluid stains, aged saliva stains, mixed stains, and simulated casework samples. The results of this study showed that, although variable levels of statherin were detected among individual samples, almost all saliva and saliva stains showed positive results in ELISA tests for the detection of statherin. In addition, statherin was specifically detected in saliva but not in blood, semen, vaginal fluid extract, sweat, or urine. Furthermore, statherin was successfully detected in aged saliva stains, mixed stains of body fluids and saliva, and

Table 1 Comparison of specificity for saliva of ELISA tests for detection of statherin and α-amylase and Phadebas® amylase test

Body fluids	Number	Number of positive samples			α-Amylase activity (IU/L) [c]
		ELISA detection of statherin[a]	ELISA detection of α-amylase[a]	Phadebas® amylase test[b]	
Saliva	24	23	24	24	29,000–500,000
Blood	5	0	5	5	510–630
Semen	5	0	5	1	2,100
Vaginal fluid	5	0	4	0	–
Sweat	5	0	5	3	90–395
Urine	5	0	2	3	480–595

[a] Absorbance at 490 nm >0.1 for statherin or >0.2 for α-amylase in dilutions of 1:100 for each sample

[b] Absorbance of supernatant at 620 nm >0.1 in dilutions of 1:4,000 for saliva and 1:40 for other body fluids

[c] Absorbance values of positive samples for the Phadebas® amylase test were converted using the standard curve supplied with the reagent

Table 2 Results of ELISA detection of statherin and α-amylase in mixed stains

Samples	Results	
	Statherin	α-Amylase
Saliva+blood	+[a]	+
Saliva+semen	+	+
Saliva+vaginal fluid	+	+
Saliva+sweat	+	+
Saliva+urine	+	+
Semen+vaginal fluid	−	+
Semen+sweat	−	+

[a] Absorbance at 490 nm >0.1 for statherin or >0.2 for α-amylase for extracts of each sample at dilutions of 1:2

simulated casework samples. The results of testing mixed stains of body fluids and saliva indicated that there was no interference by other body fluids in the ELISA test for detection of statherin in saliva. Because the limit of ELISA detection of statherin equated to 0.03 μL, it is thought that ELISA detection of statherin can be performed using a small part of a stain without impairing followed DNA analysis. In previous studies, we and others have demonstrated that the expression of statherin mRNA was specific for saliva among body fluids [10–13]. Therefore, the results of the present and previous studies together indicate that both statherin polypeptide and mRNA are specific for saliva, and ELISA detection of statherin can be used as a sensitive confirmatory test for forensic identification of saliva and saliva stains prior to DNA analysis. The method for detection of statherin by ELISA developed in this study can be applied practically in forensic casework because all reagents are commercially available and cost-effective moderately, and the result can be obtained within 5 h.

It is generally believed that mRNA is unstable and rapidly degraded by endogeneous ribonuclease or various environmental conditions. We reported previously that the levels of histatin 3 mRNA in saliva stains were strongly affected by environmental conditions such as humidity and light [13]. On the other hand, Zubakov et al. identified stable mRNA markers for saliva identification and amplified these markers successfully in aged saliva stains stored at non-humid condition for 2–6 years [23, 24]. In the case of statherin, to determine which ELISA detection or mRNA-based analysis is suitable for each casework sample, further investigations using actual and simulated casework samples are required.

In this study, ELISA detection of α-amylase for body fluids was also examined. Although high absorbance values were observed in saliva samples, some blood, semen, vaginal fluid extracts, sweat, and urine samples also showed positive results for ELISA detection of α-amylase. Although the level of α-amylase in body fluids is normally much lower than in saliva [1, 4, 5], α-amylase in body fluids may be detectable by a highly sensitive method such as ELISA. On the other hand, in a previous study, Quarino et al. [18] reported that a sandwich ELISA using monoclonal anti-human salivary amylase antibody can be used as a confirmatory test for human saliva. Although some samples of semen, urine, feces, and sweat showed absorbance values in the sandwich ELISA, saliva samples showed 10-fold higher values than the other body fluids and could be distinguished from these other body fluids with a confirmatory threshold value [18]. The simple indirect ELISA with polyclonal antibody performed in the present study may be less specific than the sandwich ELISA with monoclonal antibody.

The results of the Phadebas® amylase test showed that, although all blood samples and some samples of semen, sweat, and urine exhibited α-amylase activity, saliva samples had at least 13.8-fold higher α-amylase activity than the other body fluids (Table 1). These results were consistent with previous studies [1, 4]. Consequently, the Phadebas® amylase test performed in sufficiently dilute samples or extracts of a small piece of stain may be relatively specific for saliva. The high level of α-amylase (2,100 IU/L) in one of five semen samples tested is in accordance with the results of previous studies [4, 5]. Hochmeister et al. [25] suggested that high levels of α-amylase in seminal fluids may represent a simple artifact such as saliva contamination in the collection process. However, because statherin was not detected in this semen sample, we conclude that the high level of α-amylase in this sample was not caused by saliva contamination.

In cases of sexual assault, it is important to discriminate between saliva and vaginal fluid mixed with semen to prove oral, as opposed to vaginal, intercourse. The result of

Table 3 Results of ELISA detection of statherin and α-amylase in simulated casework samples

Samples	Results	
	Statherin	α-Amylase
Rolling paper of cigarette butt[a]	+[c]	+
Filter tip of cigarette butt[a]	+	+
Inner surface of face mask[a]	+	+
Lip of bottle[b]	+	+
Lip of can[b]	+	+

[a] A 1×1-cm square piece of stain

[b] Wiped with 1×1-cm square of cotton cloth dampened with distilled water

[c] Absorbance at 490 nm >0.1 for statherin or >0.2 for α-amylase for extracts of each sample at dilutions of 1:2

this study showed that detection of statherin by ELISA was able to distinguish a mixed saliva–semen stain from a mixed stain of vaginal fluid and semen. On the other hand, detection of α-amylase by ELISA showed positive results for all mixed stains containing semen. In addition, one of five semen samples showed α-amylase activity in the Phadebas® amylase test. Therefore, the results of this study indicate that ELISA detection of statherin could be used for identification of saliva from semen mixed samples in cases of sexual assault.

In conclusion, the ELISA test for detection of statherin developed in this study could be an effective tool for the forensic identification of saliva because of its specificity for saliva among other body fluids. In contrast, the Phadebas® amylase test may not be specific enough to identify saliva; however, it is easy to use for screening forensic casework samples for α-amylase activity. Therefore, forensic casework samples should be tested for statherin by ELISA detection or mRNA-based analysis to supplement the presumptive tests for α-amylase such as the Phadebas® amylase test.

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