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Brief Communication

Detection of proline-rich proteins for the identification of saliva by enzyme-linked immunosorbent assay

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ABSTRACT

Saliva is one of the most common body fluids found at a crime scene. Therefore, identifying saliva is important in forensic science. However, the current protein marker assays used to identify saliva are not sufficiently specific. Although proline-rich proteins (PRPs) are highly specific for saliva, their forensic potential has not yet been investigated. In this study, we developed enzyme-linked immunosorbent assays (ELISAs) to detect acidic salivary PRP HaeIII subfamily 1/2 (PRH1/2) and basic salivary PRP 2 (PRB2). The specificity, sensitivity, and efficiency of the ELISAs for PRH1/2 and PRB2 were compared with those of the ELISA for statherin (STATH), a known protein marker for saliva. The levels of PRH1/2 were significantly higher in saliva and saliva stains than in other body fluids (nasal secretions, urine, semen, vaginal fluid, blood, and sweat). PRB2 and STATH were detected in both nasal secretions and saliva. The PRH1/2 ELISA showed sensitivity similar to that of STATH ELISA. The detection rate of PRH1/2 ELISA was almost similar to that of STATH ELISA, followed by the ELISA for PRB2. The PRH1/2 ELISA had higher specificity for saliva than STATH ELISA. Therefore, the PRH1/2 ELISA has potential as a method to identify saliva for forensic investigation.

1. Introduction

The identification of body fluids is important in forensic investigations, to help determine the events that took place and to interpret the DNA results. Saliva is often found at crime scenes, for example, on cigarette butts, on the rim of drink cans, or on the skin of victims in sexual assaults.

Methods to detect α-amylase are commonly used to identify saliva [1,2]. However, α-amylase is also present in other body fluids including semen, vaginal fluid, blood, sweat, and urine [3,4]. Recently, methods for detecting histatin 3 (HTN3) mRNA [5] and bacterial DNA [6] have been reported. Simple, quick, and cost-effective methods are indispensable for forensic investigation, because of the increasing numbers of samples requiring analysis.

Forensic institutes routinely use protein-based methods to identify body fluids. For example, hemoglobin and prostate specific antigens are used as protein markers for blood and semen, respectively [7,8]. Moreover, several immunochromatographic assay kits are commercially available. These kits enable simple and quick identification of some body fluids [9–13], but investigating novel protein markers is important to develop more convenient and cost-effective methods.

Statherin (STATH) is a known protein maker used to identify saliva [4]. However, the protein is also detected in nasal secretions [14]. Although STATH has higher specificity for saliva than α-amylase, more specific protein markers are needed. Proline-rich proteins (PRPs) are coded by a multigene family of seven genes. Differential RNA splicing and proteolytic cleavage after secretion result in more than 20 different PRPs [15,16]. PRPs are classified into acidic, basic, and glycosylated groups. Acidic PRPs are involved in typical oral processes such as mineral homeostasis and neutralization of toxic substances in the diet and are present only in saliva. Basic PRPs are present in saliva, nasal secretions, and bronchial mucus and may have a more general protective function. Although acidic PRPs are specifically expressed in salivary glands [17,18], to date, the proteins have not been investigated to identify saliva in forensic science.

Enzyme-linked immunosorbent assays (ELISAs) have been developed to identify body fluids such as semen, urine, and saliva [3,4,19–21]. These assays can be used to analyze crude samples that have not been subjected to processing and purification. In the present study, we used ELISAs to evaluate the expression of acidic salivary proline-rich protein HaeIII subfamily 1/2 (PRH1/2) and basic salivary proline-rich protein 2 (PRB2) in various body

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fluids (nasal secretions, saliva, urine, semen, vaginal fluids, blood, and sweat), and to determine whether these proteins could be used as markers for the forensic identification of saliva.

2. Materials and methods

2.1. Sample collection and treatments

2.1.1. Sample collection

All procedures involving human volunteers were approved by the Ethical Committee of Human Genome and Genomic Analysis from the Japanese Association of Forensic Science and Technology. Samples were collected from consenting adults. Nasal secretions ($n = 5$), saliva ($n = 20$), semen ($n = 5$), blood ($n = 5$), vaginal fluids ($n = 10$), urine ($n = 5$), and sweat ($n = 5$) were collected from volunteers aged 26 to 57 years. Blood samples were collected from the brachial vein into Venoject II tubes (TERUMO, Tokyo, Japan). Vaginal fluids were self-collected by the volunteers by swabbing the vaginal wall with sterile cotton swabs, regardless of the menstrual cycle. Sweat samples were collected from the facial region and arms after exercise, using filter paper strips. Saliva, nasal secretions, and other body fluids were collected in sterile plastic tubes. With parental consent, saliva samples were also collected from twelve children aged between 2 months and 12 years. Collected body fluids were stored at −80 °C until required for further analysis.

2.1.2. Stain preparation

Body fluid stains were prepared as follows. Five microliters of saliva were spotted onto filter papers (10 mm × 10 mm squares) and air-dried at room temperature for 1 week ($n = 20$) or 1 year ($n = 8$). Sterile cotton swabs with vaginal fluid samples ($n = 10$) were cut into 5 mm × 5 mm squares and air-dried at room temperature for 1 week. Filter papers with sweat samples ($n = 5$) were cut into 10 mm × 10 mm squares and air-dried at room temperature for 1 week. Other body fluids stains ($n = 5$ for each fluid) were prepared as for saliva stains and air-dried at room temperature for 1 week.

2.1.3. Mixed stains

Mixed stains were prepared by spotting 5 μl of saliva and semen onto filter papers (10 mm × 10 mm squares), and 20 μl of saliva were spotted onto sterile cotton swabs with vaginal fluid samples (3 mm × 3 mm squares). Both mixed stains were air-dried at room temperature for 1 week.

2.1.4. Simulated casework samples

Rolling papers from cigarette butts were cut into 10 mm × 10 mm squares. The rims of drink bottles and cans were wiped with sterile cotton swabs. The skin of volunteers' arms was licked and dried for 1 h, and the trace was wiped with sterile cotton swabs. The swabs were cut into 3 mm × 3 mm squares.

2.2. Enzyme-linked immunosorbent assay

2.2.1. Reagents

Goat polyclonal antibody against the near N-terminus of human PRH1/2 (anti-PRH1/2), purified by affinity chromatography, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody against the C-terminus of human PRB2 (anti-PRB2), purified by affinity chromatography, was purchased from Abgent (San Diego, CA, USA). Goat polyclonal antibody against the N-terminus of human STATH (anti-STATH; Santa Cruz, CA, USA), purified by affinity chromatography, was donated by Dr. Sakurada of the National Research Institute of Police Science. Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG was purchased from Sigma Aldrich (St. Louise, MO, USA), and HRP-conjugated goat anti-rabbit IgG was purchased from KPL (Gaithersburg, MD, USA).

Anti-PRH1/2 and anti-PRB2 were diluted (1:1000) with 0.05% Tween-20 in phosphate buffered saline (PBST), and anti-STATH was diluted (1:500) with PBST. The HRP-conjugated mouse anti-goat IgG and HRP-conjugated goat anti-rabbit IgG were diluted (1:1000) with PBST for PRH1/2 and PRB2 ELISA, respectively. The HRP-conjugated mouse anti-goat IgG was diluted (1:5000) with PBST for STATH ELISA.

2.2.2. Sample preparation

Vaginal fluid swabs were cut into 5 mm × 5 mm squares and the sample was extracted with 100 μl of 0.05 M bicarbonate buffer (BCB; pH 9.6). Body fluids and vaginal fluid extracts were diluted with BCB (1:100 to 1:6400). Filter papers with sweat samples were cut into 10 mm × 10 mm squares and extracted with 250 μl of BCB by pipetting. The extracts were diluted with BCB (1:2–1:64).

Vaginal fluid stain samples were prepared by extracting with 100 μl of BCB by pipetting. The extracted vaginal fluid samples (5 μl) were diluted (1:100) with BCB on ice. Other body fluid stains were extracted with 250 μl of BCB by pipetting on ice. The sample extracts (250 μl) were centrifuged at 7900*g* for 3 min at 4 °C. The supernatants were diluted with BCB (1:2–1:64).

2.2.3. ELISA

Diluted samples (50 μl per well) were added to 96-well multitier plates (SUMILON MS7296F; Sumitomo Bakelite, Tokyo, Japan) and incubated at 37 °C for 1 h. Each well was blocked with 200 μl of Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) at 37 °C for 1 h. The wells were then washed three times using 250 μl of PBST per well and 50 μl of diluted anti-PRH1/2 or anti-PRB2 were added to each well. The plates were incubated at 37 °C for 1 h. The plates were then washed three times with 250 μl of PBST per well and incubated with 50 μl of diluted HRP-conjugated rabbit anti-goat IgG or HRP-conjugated goat anti-mouse IgG per well at 37 °C for 1 h. The plates were then washed five times with 250 μl of PBST per well. In PRH1/2 and PRB2 ELISA, 50 μl of TMB + Substrate Chromogen (Dako Cytomation, CA, USA) were added to each well and incubated at room temperature for 3 min. Color development was stopped by the addition of 50 μl of 1 M H_2SO_4. Absorbance was measured at a wavelength of 450 nm using a Molecular Devices SPECTRA max PLUS 384 (Molecular Devices, CA, USA). In STATH ELISA, the procedure of color development and the measurement of absorbance value were followed according to the previous report [4]. Each absorbance value was normalized by subtracting the primary antibody blank absorbance value.

2.3. Data analysis

The ELISA data for each dilution ratio were statistically analyzed by one-way ANOVA with Scheffé's multiple-comparison test.

3. Results

3.1. Specificity and sensitivity of ELISA for detecting adult body fluids

The expression of PRH1/2 and PRB2 in body fluids and the expression of STATH in nasal secretions and saliva were evaluated by performing ELISA. The PRH1/2 absorbance values of saliva samples at dilutions ranging from 1:100 to 1:200 were significantly higher than those of other body fluids (nasals secretions, semen, vaginal fluid, urine, blood, and sweat; $p < 0.05$; Fig. 1A). Furthermore, the PRH1/2 absorbance values of diluted saliva (1:6400)