TECHNICAL NOTE

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Deoxyribonuclease I Phenotyping from Saliva Stains*

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ABSTRACT: Good typing results were obtained using a newly developed method for extraction and purification of deoxyribonuclease I (DNase I) from saliva stains. Previously, DNase I phenotyping from saliva stains has been unsuccessful because of low enzyme activity and heavy contamination. Salivary DNase I was extracted from stains using phosphate buffer containing Nonidet P-40. Extracts were purified using Phenyl Sepharose CL-4B gel. Electrophoresis was performed, and DNase I was successfully phenotyped. All of the DNase I phenotypes, which were obtained from saliva stains using this new method, were identical to the phenotypes determined from urine samples. Moreover, DNase I was correctly phenotyped from saliva stains that had been stored for over three months at room temperature or at 37°C. These results suggest that DNase I polymorphisms provide valuable information for forensic characterization of saliva stains.

KEYWORDS: forensic science, deoxyribonuclease I, dried agarose film overlay, isoelectric focusing, polymorphism, saliva stains

Deoxyribonuclease I (DNase I; E.C.3.1.21.1) activity is distributed widely in various tissues and body fluids (1,2). Protein polymorphism of human DNase I was first demonstrated in urine by isoelectric focusing in a thin layer of polyacrylamide gel (IEF-PAGE) (3). Subsequently, similar isozymes have been detected in serum (4), semen (5), saliva (6) and perspiration (7), and it has been demonstrated that there is perfect correlation among the phenotypes of four different samples taken from the same individual. DNase I is controlled by five codominant alleles on chromosome 16 (8,9); three common alleles, *DNASE1*1*, **2* and **3*, have been observed at the polymorphic level (gene frequencies in the Japanese population are 0.55, 0.44 and 0.01 (10), respectively, whereas two rare alleles, *DNASE1*4* and **5*, are found at the mutation level (gene frequencies of both are less than 0.01). Since DNase I has informative gene frequencies and high stability, it is one of the most useful biochemical markers for genetic, clinical, and forensic analyses. We have succeeded in phenotyping DNase I from small semen stains (11) and perspiration stains (12) found at crime scenes. However, we have not succeeded in typing DNase I from saliva stains, because of the low enzyme content and the presence of interfering substances in saliva. In this paper, we describe a novel DNase I phenotyping method for small saliva stains that comprises an effective extraction step and an efficient purification step.

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Materials and Methods

Chemicals

All chemicals were reagent grade. Phenyl-Sepharose CL-4B was purchased from Pharmacia Biotech (Uppsala, Sweden); 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), *Clostridium perfringens* sialidase (Type V) and salmon testis DNA (type III) from Sigma (St. Louis, MO); Triton X-100 and Tween 20 from Nacarai Tasque (Kyoto, Japan); Nonidet P-40 from Iwai Kagaku (Tokyo, Japan); and SYBR™ Green I from Molecular Probes Inc. (Eugene, OR).

Biological Samples

A set of whole saliva and urine samples was collected from 14 healthy Japanese donors. Whole saliva samples were collected without any stimulation and stored at −80°C until use. Urine samples were concentrated, dialyzed and lyophilized according to a method reported previously (13,14). A 0.1% (w/v) solution of the lyophilized urine, which corresponded to tenfold concentrated urine, was used for DNase I phenotyping. Artificial saliva stains of the three common DNase I phenotypes (1, 1–2 and 2) were prepared on filter paper (3MM Chr, Whatman Japan, Tokyo, Japan), air-dried for one day at room temperature, and stored at room temperature and at 37°C until required for the phenotyping.

Extraction and Purification of DNase I from Saliva Stains

Extraction and partial purification of DNase I from saliva stains were performed according to the procedure summarized in Fig. 1. A 5 mM potassium phosphate buffer (pH 6.7) containing 0.5 M ammonium sulfate (buffer 1) was used as the extraction buffer,

and several detergents (CHAPS, Triton X-100, Nonidet P-40 and Tween 20) were added to the buffer in various concentrations. The effects of the detergents on DNase I extraction from saliva stains were examined. We used 5 mM potassium phosphate buffer (pH 6.7) containing 0.5 M ammonium sulfate (buffer 1) and 5 mM potassium phosphate buffer (pH 6.7) (buffer 2) for adsorption and elution of DNase I, respectively. DNase I was eluted from the Phenyl Sepharose CL-4B gel with 250 μL of buffer 2 into a tube containing 10 μL of 1% (w/v) bovine serum albumin in order to protect the enzyme. The eluate was desalted by ultrafiltration using an Ultrafree C3 concentrator (Millipore Corporation, Bedford, MA), lyophilized, and used for subsequent IEF-PAGE analysis.

DNase I Phenotyping

IEF-PAGE was performed as described previously (6,7). Samples were treated with an equal volume of 2 units/mL of sialidase overnight at 4°C before electrophoresis. Visualization of DNase I was achieved using the dried agarose film overlay (DAFO) method according to our previous description (6,15) with modifications. The thin agarose film was prepared as described previously, except that 0.0005% (w/v) salmon testis DNA in 100 mM sodium cacodylate buffer containing 0.2 mM $CaCl_2$ and 20 mM $MgCl_2$, pH 6.5 was used as the reaction mixture. After the IEF-PAGE run, the agarose film was placed on top of the gel and incubated at 37°C for 2 to 3 h. After incubation, the agarose film was peeled off the gel and submerged in SYBR Green I solution (1:10 000 dilution in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 10 min and analyzed by a fluorescence imaging analyzer (FluorImager SI, Molecular Dynamics, Sunnyvale, CA).

Results and Discussion

A previously successful method for DNase I phenotyping from semen stains (11) was attempted on saliva stains. Each stain was extracted with a simple buffer, and electrophoresis was performed without any purification. However, it was difficult to perform accurate DNase I phenotyping on the extracts because of the presence of several extraneous bands having p*I* values similar to the DNase I type 2 bands, and also a high background (Fig. 2, lanes 4–6). These difficulties may have been due to the low DNase I content of the extracts (DNase I content of saliva is less than one-thousandth that of semen). However, a more likely explanation for the presence of extraneous bands was that saliva stain extracts are generally heavily contaminated. Therefore, we added two new steps to our procedure—an effective extraction step and a cleanup step—before IEF-PAGE analysis. In order to increase the efficiency of DNase I extraction, one of four detergents was added to the extraction buffer (buffer 1) at various concentrations, and the effectiveness was tested. A solution of 0.5% (v/v) Nonidet P-40 was the most effective detergent. A threefold-to-fivefold increase in the enzyme band intensity was observed when compared with

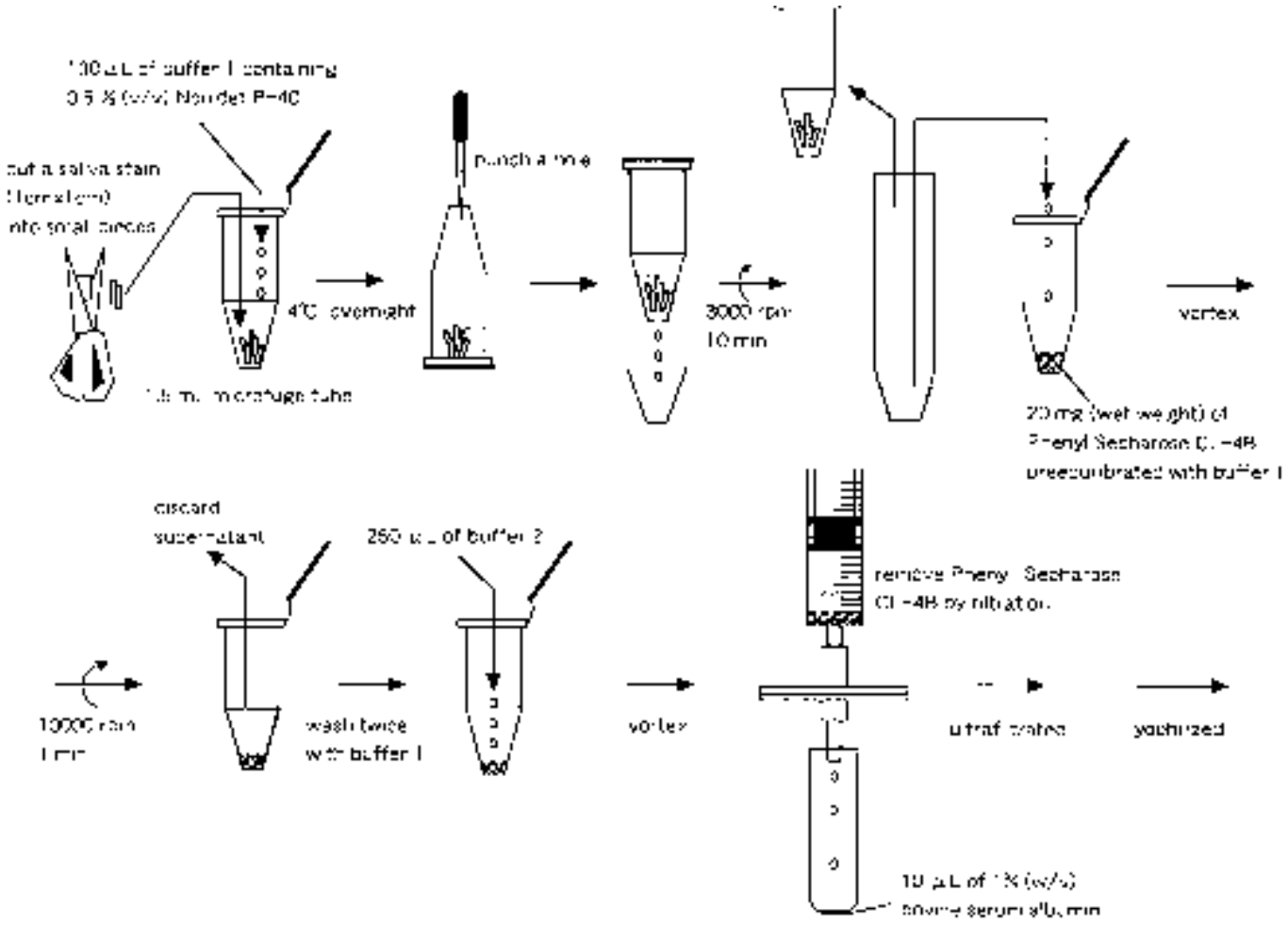

FIG. 1—*Flow diagram illustrating the method used for DNase I extraction and purification from saliva stains.*

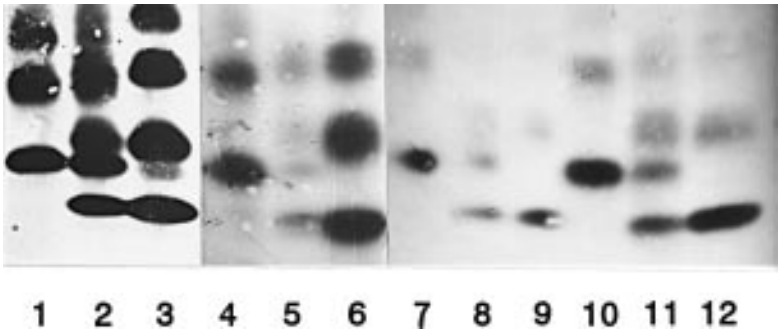

FIG. 2—*IEF-PAGE (pH 3.5 to 5) patterns of desialylated DNase I from urine (lanes 1–3) and saliva stains (4–12). Extracts from saliva stains were analyzed before (lanes 4–6) and after (lanes 7–12) purification using Phenyl-Sepharose CL-4B gel. Addition of 0.5% (v/v) Nonidet P-40 to the extraction buffer (lanes 10–12) increased the band intensity about threefold in comparison with that without detergent (lanes 7–9). Lanes 1, 4, 7 and 10, phenotype 1; lanes 2, 5, 8 and 11, phenotype 1–2; lanes 3, 6, 9 and 12, phenotype 2. The anode is at the top.*

TABLE 1—*DNase I stability study of saliva stains stored under different conditions.*

	Room temperature			37°c		
Phenotype	1	1–2	2	1	1–2	2
Number of samples	4	6	4	4	6	4
Storage period						
1 day	4*	6	4	4	6	4
1 week	4	6	4	4	6	4
1 month	4	6	4	4	6	4
3 months	4	6	4	4	6	4

* The value indicates the number of samples phenotyped correctly.

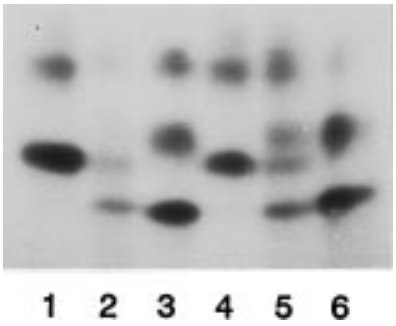

FIG. 3—*IEF-PAGE (pH 3.5 to 5) patterns of desialylated DNase I extracted from saliva stains stored for one month at room temperature (lanes 1–3) and at 37°C (lanes 4–6). Lanes 1 and 4, phenotype 1; lanes 2 and 5, phenotype 1–2; lanes 3 and 6, phenotype 2. The anode is at the top.*

the results obtained without detergent (Fig. 2, lanes 7–12). In order to remove contaminants from the extracts, we used Phenyl-Sepharose CL-4B gel pre-equilibrated with buffer 1, because it proved optimal in a rapid, effective and semi-specific purification of DNase I, and also because it was effective in removing the detergent from the extracts (16). By using this cleanup step, the number of extraneous bands and the high intensity of the background decreased markedly (Fig. 2, lanes 10–12). Hence, a combination method consisting of the effective extraction step with 0.5% (v/v) Nonidet P-40 and the purification step with Phenyl-Sepharose CL-4B gel was employed throughout the remainder of this study. The detailed procedure used for DNase I extraction and purification from saliva stains is shown diagrammatically in Fig. 1. Figure 2 shows that the IEF-PAGE patterns of DNase I obtained from the saliva stains (lanes 10–12) were similar to those from urine samples (lanes 1–3) using the above procedure. This agreement of the results indicates that saliva stains may be phenotyped with confidence.

We investigated the possibility of phenotyping DNase I from saliva stains stored for an extended period of time under different conditions. All stains stored at room temperature or at 37°C for up to one month were phenotyped correctly (Table 1 and Fig. 3, lanes 1–6). It was also possible to determine the DNase I phenotypes from all saliva stains stored over three months, although the development of phenotype 1–2 took about twice as long as that for phenotypes 1 and 2. The amount of saliva stain required for DNase I phenotyping was estimated to be a 0.25 to 0.5 cm^2 spot on filter paper (equivalent to 8 to 15 μL of liquid saliva) after one day of storage at room temperature, 0.3 to 0.6 cm^2 (equivalent to 10 to 20 μL) after one month of storage, and 0.5 to 1 cm^2 (equivalent to 15 to 30 μL) after three months of storage. These amounts of saliva are typical quantities obtained from items of evidence submitted in criminal investigations. However, it should be noted that the size of stain required for DNase I phenotyping may vary depending on the type of substrate on which the saliva has been deposited.

Most saliva samples found at crime scenes are dried stains; biochemical markers for individual identification applicable to dried saliva stains are limited. Only ABO phenotyping is used in practice, and is useful only if the stains have been deposited by secretor-type persons. Recently, PCR-based DNA typing methods from saliva stains have been reported (17,18). They are very useful because of their higher sensitivity and power of discrimination. However, there may be a place for DNase I typing along with ABO typing in some cases where saliva DNA is too badly degraded to be typed. Therefore, DNase I polymorphism, which can be detected by conventional but well-established methods, may serve as an effective, low-cost screening tool for saliva stains.

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