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TECHNICAL NOTE

ANTHROPOLOGY

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Age Estimation by Racemization Method in Teeth: Application of Aspartic Acid, Glutamate, and Alanine

ABSTRACT: Here, we report on an experimental approach of simultaneous determination of various amino acids racemization (AAR) rates in teeth. We evaluated the measurements of aspartic acid (Asp), glutamate (Glu), and alanine (Ala) isolated from dentin. Asx D/L rates from total amino acid fraction, generally used for age estimation, showed high correlation ($r = 0.98$) with age. As Glx and Ala showed very slow racemization kinetics in TA, we performed further analysis of the acid-soluble protein (SP) fraction. The results supported improved correlation between age and D/L rates for Glu ($r = 0.84$) and Ala ($r = 0.85$), as well as for Asp ($r = 0.98$). By providing further elucidation on dentin protein racemization, the technique offers a considerable opportunity to involve other amino acids in age estimation studies. As the process does not require additional separation steps, the method can be easily adapted to existing protocols.

KEYWORDS: forensic science, racemization, amino acid, dentin, age estimation, odontology

Age estimation by amino acids racemization (AAR) (among other methods available for aging by teeth, e.g., use of the translucent dentin) has been developed and employed in forensic practice during the last decades (1). Extensive research on AAR's applicability, accuracy, and reliability has defined relevant standards for forensic utilization of this method (2,3). As the first reports (4,5), those forensic investigations were mainly focused on bradytrophic tissues including teeth. In teeth, long-lived proteins have restricted, if any, turnover rates, thus nonenzymatic modifications as racemization effects are most pronounced.

Through racemization process, the native, protein-building L-enantiomer amino acids spontaneously convert into D-enantiomers in a time-dependent manner. The constant accumulation of the dextrorotatory forms has been related to aging and more recently to pathological conditions and diseases in various tissues. In the forensic field, measuring the alteration of the D/L ratio in biological samples allowed the development of AAR age estimation methods (6–9). Upon careful consideration of AAR limitations and internationally accepted quality controls (10), an impressively precise determination of the chronological age could be achieved with an approximate error of ±3 years (11). Among the mineralized tissues, dentin has been promoted (7,12) for most regular AAR measurements, because it represents the bulk material of teeth, and it is relatively protected from exogenous damage.

Historically, racemization analysis in forensic medicine, as well as in archeology and geochemistry, often involves experiment on aspartic acid as the fastest racemizing amino acid. D-Asp forms via transitional succinimide intermediates originated by Asx (Asp or asparagine) residues (13). Asx racemization as a first-order chemical reaction is influenced by numerous factors such as protein conformation, temperature, pH, and environmental water concentration. Hence, postmortem conditions (e.g., sample degradation, diagenesis, and climate), postmortem interval, as well as laboratory analytical protocols must be clarified before the assumption of actual age of death. In addition, certain variance in Asx D/L ratios has been reported by tooth type and by tooth region (14,15). Because of the inherent complexity of AAR, recent studies suggest that introduction of standard mixtures of D- and L-amino acids or new sampling strategies (3,16,17) would bring further improvement in forensic application.

Besides the evaluation of D-Asp accumulation, other D-amino acids were also indicated in connection with tissue aging, and their utilization was promoted to potential biomarkers. D-glutamate (forms through glutamic acid and glutamine residues called Glx) generation may be involved in cancer and neurodegenerative disorders (18,19), while D-alanine seems to accumulate in Alzheimer's disease and in renal dysfunction (20,21). Notably, in tooth dentin, D-serine and D-threonine could be detected along with D-Asx, D-Glx, and D-Ala (22). However, the traditional approach of AAR-based age estimation solely involves D-Asx detection in total amino acid (TA) fraction from dentin. The lack of published regression data on these amino acids led us to investigate rate constants for Glx and Ala. In this study, we attempted to stretch conventional utilization of AAR toward a more compound direction, where D-Glx and D-Ala were assayed simultaneously with D-Asx. Glx (9) and Ala residue racemization occurs considerably slower than that of Asx, and an earlier report (23) described difficulties in establishing a firm correlation between their D/L ratio and chronological age. To address this issue, we designed a novel experimental approach of Glx and Ala racemization analysis from dentin soluble fraction.

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Materials and Methods

A total of 24 permanent premolars (ranging from 13 years to 88 years) were collected with informed consent from patients under periodontal or orthodontics treatment. Information about the patient's age, gender, and extraction date was available for each tooth. Only teeth without macroscopically advanced caries were included in the analysis (C1 and C2 stages were acceptable).

After careful removal of the attached soft tissues, such as periodontal membranes, samples were cut with a low-speed saw (Isomet 11-1180, Buehler) under continuous water-cooling. Midline longitudinal sections in 1-mm thickness were prepared, and tissues other than dentin were eliminated. Next, fragments were treated serially by ultrasonication in 0.2 M hydrochloric acid, pure water (three times), ethanol, and ether, each for 5 min. After drying, the specimens were pulverized by a grinder to 74–297 μm particle size. Five milligrams of the dentin powder was used for the extraction of TA fraction. As the detailed procedure of amino acid analysis has been described already in previous reports (24,25), only a brief outline is provided as follows.

Dentin powder was subjected to hydrolysis in 6 M hydrochloric acid at 100°C for 6 h, collected by an ion-exchange resin (Dowex 50W-X8, USA) and then dried in a rotary evaporator. All chemicals were of analytical grade and purchased from Wako Pure Chemicals (Osaka, Japan). The derivatization technique included esterification and acylation steps with subsequent drying and chilling under nitrogenous ventilation. Modified amino acids from the TA fraction were finally dissolved in ethyl acetate and analyzed using gas chromatography (GC).

Acid-soluble peptide fraction was extracted by adding one milliliter of 1.0 M hydrochloric acid to 20 mg dentin powder under cooling centrifugation (4000 g) for 1 h at 4°C. The supernatant was then dried using an evaporator and used as the SP fraction for further steps. The D/L ratios of Asx, Glx, and Ala were measured in two fractions: TA and SP.

GC analysis was carried out on a Shimadzu GC 17A (Kyoto, Japan) instrument equipped with a hydrogen flame ionization detector and with a fused silica capillary column. The column (length 25 m × 0.3 mm inner diameter) was coated with Chirasil-L-Val for enantioseparation. Samples (1 μL) were injected onto the chiral phase, and the quantity of D- and L-amino acids was calculated from their peak areas (measurements were repeated from two to four times).

Results

The separation of chiral enantiomers of Asx, Glx, and Ala was confirmed on the chromatograms. A representative example of a TA fraction gas chromatogram is shown in Fig. 1*a*. The D- and L-forms of Ala, Asx, and Glx were eluted in that order. The most abundant L-amino acid content was detected for glycine (Gly), followed by proline (Pro), alanine (Ala), hydroxyproline (Hyp), and glutamic acid (Glu). SP fraction exhibited a similar elution pattern (Fig. 1*b*) with an even more apparent D-amino acid separation. D-enantiomers were found to be base line separated and well resolved from other amino acids. In the SP fractions, however, a larger amount of Asx and a lower amount of Hyp were detected compared to those TA fractions.

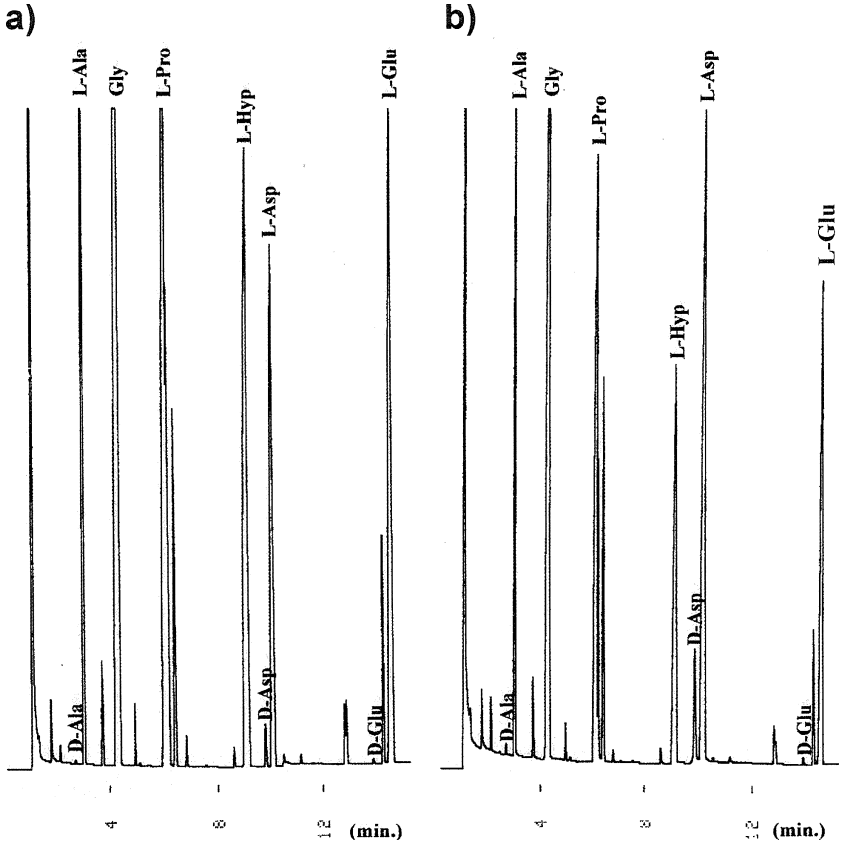

FIG. 1—*Representative gas chromatograms of dentin. (a) Total amino acid fraction, (b) acid soluble protein fraction. Well-resolved enantiomer peaks of Ala, Asp, and Glu eluted after 2 min, 8 min, and 14 min, respectively (Chromatographic conditions: carrier gas, helium; injector temperature, 220°C; detector temperature, 230°C; initial column temperature, 110°C and then raised to 200°C by time programming).*

TABLE 1—*Racemization rates of Asp, Glu, and Ala in human dentin.*

	Total Amino Acid Fraction		Soluble Peptide Fraction	
	Racemization Rate Equation	*r*	Racemization Rate Equation	*r*
Asp	$\ln[(1 + D/L)/(1 - D/L)]\ t = 0.001057\ t + 0.0481$	0.983	$\ln[(1 + D/L)/(1 - D/L)]\ t = 0.003855\ t + 0.0269$	0.986
Ala	$\ln[(1 + D/L)/(1 - D/L)]\ t = 0.000014\ t + 0.0063$	0.372	$\ln[(1 + D/L)/(1 - D/L)]\ t = 0.000296\ t + 0.0027$	0.850
Glu	$\ln[(1 + D/L)/(1 - D/L)]\ t = 0.000027\ t + 0.0097$	0.398	$\ln[(1 + D/L)/(1 - D/L)]\ t = 0.000432\ t + 0.0027$	0.838

t, age; *r*, correlation coefficient.

D/L ratios were calculated for each amino acid from TA and soluble peptide fraction. In Table 1, we summarized racemization rate constants and correlation coefficients for Asx, Glx, and Ala. Linear least square (LLS) analysis revealed a strong association between actual age and ln[(1+D/L)(1-D/L)] values for Asx in both TA and SP fractions. Asx correlation coefficients rated above 0.98, and this correlation was significant at the level of $p < 0.001$. However, Glx and Ala TA fractions represented a significantly slower racemization rate compared to Asx; therefore, a significant correlation between chronological age and racemization rate could not be established. On the other hand, using the acid soluble fraction for Glx and Ala racemization determination, an indicative correlation was detected ($r = 0.83$ and $r = 0.85$, respectively). LLS lines for Asx, Glx, and Ala are illustrated in Fig. 2. Accordingly, Ala and Glx displayed an improved and almost linear increase in the D/L ratio by aging in SP fractions. The estimated standard errors for Asx, Glx, and Ala were calculated as ± 0.57 years, ± 2.36 years, and ± 2.31 years, respectively.

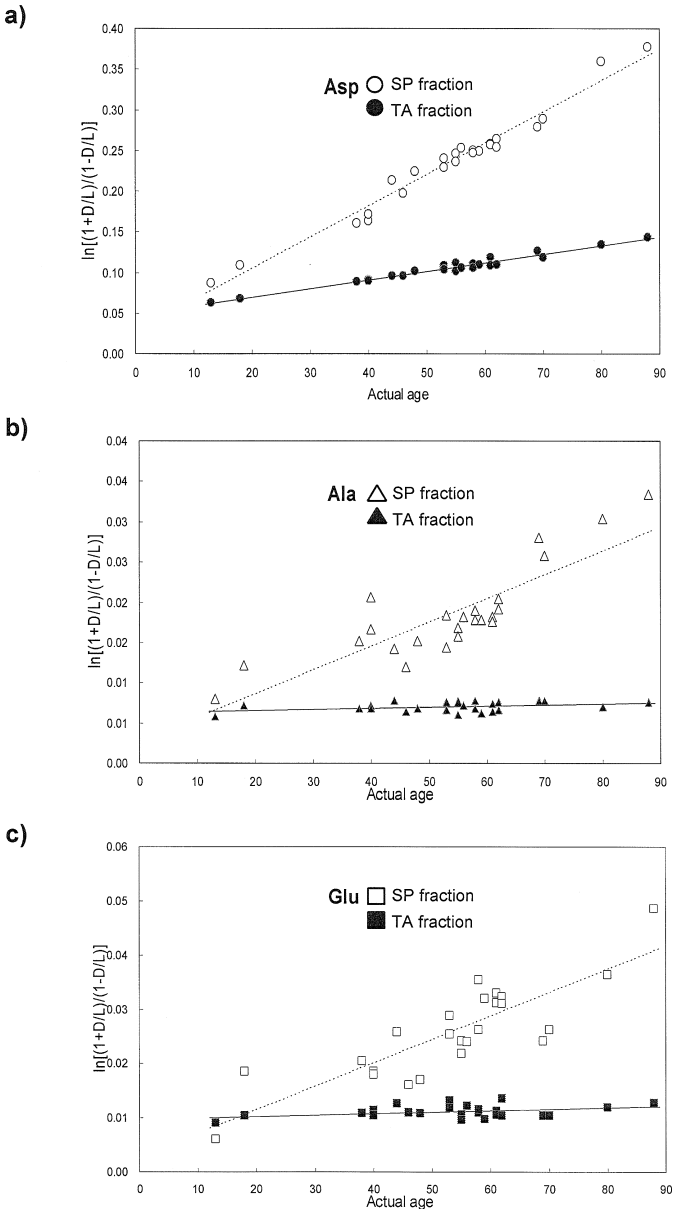

FIG. 2—*Scatterplots of the correlation between chronological age and D/L-amino acid ratios expressed from gas chromatography peak areas. TA, total amino acid fraction; soluble protein (SP), and acid SP fraction. Linear regression lines represent Asp (a), Ala (b), and Glu (c) analysis from permanent tooth dentin.*

Discussion

The protocol followed in this study allowed us to obtain information about racemization rates of three different amino acids in tooth dentin. The relation between Asx D/L ratio and age of death has been studied over the last decades, and it has proven its efficiency for forensic purposes. Our data corresponded well to earlier reports on Asx racemization in dentin, and we explored the potential of using other amino acids beyond Asx. The utilization of Glx and Ala racemization, based on previous pioneer efforts, has not gained affirmation for age estimation, which could be mostly attributed to the recorded poor correlations with aging (ranging from 0.1616 to 0.806 for Glx) (9,23) and to the difficulties in explicit quantification of their D-forms (22) from dental samples. To circumvent these problems, we adopted a synchronous analysis of Asx, Glx, and Ala from the acid soluble fraction of dentin by GC. Acid soluble fraction, isolated as noncollagenous supernatant from pulverized dentin, is composed mainly of noncollagenous proteins (NCPs). NCPs are extracellular matrix constituents (10%) of dentin, while the mineralized dentin primarily (90%) consists of collagen (predominantly type I collagen). Because of the different protein contents, AAR kinetics and degree of racemization differ in TA and in SP dentin fraction (correspondingly, our chromatographic analysis reflected this variance in amino acid content). Asx racemization progresses rapidly in SP fraction (26) throughout life, thus yielding an unequivocally strong correlation coefficient ($r > 0.99$) and an improved accuracy for age estimation (11). It is noteworthy to add that a recent study by Griffin et al. (27) also demonstrated a good correlation between aging and racemization from enamel SP fraction extracted by a novel, less-invasive technique. In our approach, with the employment of SP fraction from dentin, we achieved a significant improvement in quantification of Glx and Ala racemization. We must add that our investigation of a confined pool of samples (80% of the samples were collected from individuals with chronological age between 40 and 70 years) might contribute to a deduced, better correlation with age. Moreover, we must emphasize that, during the production of SP fraction by acid extraction, any variance in the method may result in an altered racemization rate; thus, sample preparation needs to be

carried out in vigorously uniform manner. We published our concerns over the importance of the sample washing procedure in bone samples in a previous study (28). Analogously, Waite et al. (29) suggested proceeding with particular caution with SP fraction preparation attributed to its unsteady protein conformation. Likewise, the utilization of SP fraction in cases of a prolonged postmortem interval seems to be problematic.

We also evaluated the performance of a commercially available chiral separation column, and our results supported its capability for assessing D/L rates for the selected amino acids. The authors believe that the technological intervention of the separation ability of chiral columns (30), as well as the latest developments in GC systems and measurement conditions, provided a technological base for improved separation compared to earlier research. The ability to perform additional calculations on other amino acids during the same analysis procedure contributes to a fast and simple strategy for combination measurements. In this combination method, we analyzed Glx as well as Ala racemization and demonstrated that our protocol delivers comparable dentin D/L ratios from various ages.

It must be noted that D-Glx levels might be affected by bacterial contamination of the dental tissue. Ideally, caries-free, intact teeth could serve as appropriate specimens for age estimation, but healthy teeth are not the regular findings in forensic practice. Different research groups (31,32) have evaluated the effect of caries on amino acid composition, and a statistically significant decrease in the concentration of Glx, Ser, Thr, Arg, and Ala has been recorded from carious teeth. However, Griffin et al. (27) did not demonstrate correlation between caries advancement and Asx racemization. Our suggestion is that teeth with C1 or C2 lesions could be used for age determination with the emphasis on measuring error and on reflection of variability.

In the case of Ala, according to previous reports, an apparent experimental complication (un-resolved peaks) in the detection of D-, L-Ala was observed (22) by HPLC. Moreover, an age-related increase in Ala was argued to be the possible result of transamination and accompanying racemization. Given our refined separation, those implications facilitate the design of future AAR studies for further analysis of amino acids beyond Asx.

Our described methodology is accessible and well suited to the simultaneous measurement of D/L ratios of Asx, Glx, and Ala from tooth dentin samples. We attempted to shed light on a poorly investigated subject, i.e., racemization of other amino acids with respect to multiple advances from numerous studies of AAR. Desirably, accurate age estimation by a reproducible, cost-effective method should be accessible in forensic laboratories. Current works recommend the investigation of some other amino acid such as Ser (22) in dentin phosphophorins. Here, we demonstrated corrected values for Glx and Ala racemization in human dentin SP fractions, which may, therefore, require the reconciliation of their utilization along with Asx in age estimation. Combination analysis of various amino acids from teeth (considering the inclusion of younger teeth under age of 40 for future studies is required) expected to offer insights into the complex mechanism of AAR. In conclusion, reproducibility and circumspect application in adequate cases create an optimal basis for actual age assessment by various amino acid racemization in teeth.

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