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ORIGINAL ARTICLE

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Age estimation based on aspartic acid racemization in elastin from the yellow ligaments

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Abstract The yellow ligaments of the spine are characterized by an exceptionally high content of elastin, a protein with a proved longevity in several human tissues. This unique biochemical composition suggested a suitability of yellow ligaments for age estimation based on aspartic acid racemization (AAR), which was tested by determination of AAR in total tissue specimens and in purified elastin from yellow ligaments of individuals of known age. AAR was found to increase with age in both sample sets. The purified elastin samples exhibited a much faster kinetics than the total tissue, with ca. 3.7–4.6-fold higher apparent rates. The relationship between AAR and age was much closer in the purified elastin samples (r =0.96–0.99) and it can therefore be used as a basis for biochemical age estimation. The analysis of total tissue samples cannot be recommended since the AAR values can be strongly influenced even by slight, histologically non-detectable variations in the collagen content. Age estimation based on AAR in purified elastin from yellow ligaments may be a valuable additional tool in the identification of unidentified cadavers, especially in cases where other methods cannot be applied (e.g. no available teeth, body parts).

Keywords Age estimation · Aspartic acid racemization · Yellow ligaments · Elastin

Introduction

Biochemical age estimation based on aspartic acid racemization (AAR) is gaining acceptance in forensic practice as a method which is both highly accurate and reproducible, as stated by numerous publications of different groups (e.g. Fu et al. 1995; Mörnstad et al. 1994; Ogino et al. 1985; Ohtani 1995a, 1995b; Ohtani and Yamamoto 1987; Ritz et al. 1990, 1993, 1995; Ritz-Timme 1999; Ritz-Timme et al. 2000a; Rösing and Kvaal 1998). AAR is an inevitable consequence of the physiological ageing of proteins (for details see Ritz-Timme and Collins 2002). It represents one of the major types of non-enzymatic covalent modification of proteins which are synthesized using only one optically active form of amino acids, laevo or L-amino acids. In vivo racemization can be observed in numerous proteins of different human tissues (Ritz-Timme and Collins 2002). It leads to an age-dependent accumulation of D-aspartic acid in long-living human proteins[1] which is the basis of the biochemical age estimation.

Routinely, age estimation based on AAR is performed by analysis of dentine. Dentine is an "ideal" tissue for biochemical age estimation because of its high content of long-living proteins and its relatively stable biochemical composition. However, in forensic practice teeth are not always available. The suitability of non-dental tissues for age estimation based on AAR has been tested. In the tissues investigated so far, biochemical age estimation in total tissue specimens and crude protein extracts is associated with a much reduced accuracy in comparison to dentine (Ohtani et al. 1998; Pfeiffer et al. 1995a, 1995b; Ritz and Schütz 1993; Ritz et al. 1994). Accurate results can only be achieved by analysis of purified long-living proteins, which usually requires highly sophisticated methodologies (Ritz et al. 1996). The necessity of protein purifi-

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[1] In proteins the so-called racemization of aspartic acid (AAR) involves both aspargine and aspartic acid that decompose via a succimide ring to the same four residues, namely L-aspartyl, D-aspartyl, L-isoaspartyl and D-isoaspartyl, all of which are in chemical equilibrium via the succimide ring (for overview see Ritz-Timme and Collins 2002). Asparingyl, aspartyl, isoaspartyl and succinimidyl residues, are all converted to free aspartic acid during acid hydrolysis, a preparative step in chromatographic amino acid analysis for biochemical age estimation.

cation in non-dental tissues for accurate results can be attributed to the more complex biochemical compositions of non-dental tissues and/or to their lower contents of metabolically stable proteins, as compared with dentine.

In contrast to the non-dental tissues investigated so far, the yellow ligaments of the spine exhibit a less complex structure and a high content of long-living protein. The unique composition of these ligaments is characterized by an extremely high content of elastic fibres, they consist of ca. 80% elastic fibres and ca. 20% collagen fibres (Viejo-Fuertes et al. 1998). The major component of the elastic fibres is elastin (comprising up to 90% of the mature fibre), which appears to be an extremely long-living, if not permanent protein of the human organism. Shapiro et al. (1991) and Powell et al. (1992) both observed an age-dependent accumulation of D-aspartic acid in elastin, purified from lung parenchyma and arteries, respectively. The close relationship between AAR and age indicates a lifetime residence for mature elastin in human lung parenchyma and arteries (for details see Ritz-Timme and Collins 2002). It could be assumed that elastin is permanent and exhibits an in vivo racemization also in the bradytrophic yellow ligaments. If this is true, the yellow ligaments with their extremely high content of elastin may represent a tissue well suited for age estimation based on AAR.

To test this assumption, AAR was determined in total tissue samples and purified elastin from yellow ligaments of individuals of known age.

Materials and methods

AAR was determined in total tissue samples and in purified elastin samples from yellow ligaments of different lumbal segments of the spine. Total tissue specimens (L1/2: n =10, L2/3: n =19, L3/4: n = 17) from 46 individuals aged between 20 and 80 years were analysed. Elastin was purified from 24 yellow ligaments from the segments L1/2 (n =6), L2/3 (n =9), and L3/4 (n =9) of individuals between 20 and 76 years old.

Preparation of yellow ligament specimens

Yellow ligament specimens were collected during autopsy. Samples (1×1×0.3 cm) were prepared avoiding contamination with adjacent tissues.

Aliquots of all samples were examined histologically (paraffin sections, Hematoxylin-Eosin stain and Elastica van Gieson stain). Cases with signs of advanced degeneration such as fibrocartilaginous proliferation and calcification in the histological sections were excluded from the study.

All samples were washed in 15% NaCl solution containing protease inhibitors (2.5 mM benzamidine HCl, 50 mM ε-amino-η-caproic acid, 0.5 mM N-ethylmaleimide, and 0.3 mM phenylmethylsulfonylfluoride) overnight at 4°C. After rinsing in cold water the samples were defatted in ethanol/ether (3:1) for 15 min at 4°C and rinsed again in cold water. Total tissue samples were freeze-dried and stored at –20° until further analysis.

Purification of elastin

Elastin purification was performed by enzymatic removal of the contaminating collagen. The washed and defatted samples were digested twice with bacterial collagenase (Sigma type 7, 250 U/100 mg tissue in 1.25 ml 50 mM tricine buffer with 10 mM calcium chloride and 400 mM sodium chloride, pH 7.5 at 25°C) for 24 h at 37°C. After each digestion the samples were repeatedly rinsed in cold water. The samples were freeze-dried and stored at –20° until further analysis.

Assessment of the quality of elastin purification

The quality of the elastin purification was assessed by histological examination and by amino acid analysis. For histological examination paraffin sections of the purified elastin samples were stained as described and checked for the presence of collagen. Amino acid analyses were performed on aliquots of the purified elastin and an elastin standard (elastin from bovine neck ligament, Sigma). The samples were hydrolysed in 6 M HCl for 24 h at 110°C; analyses were performed by ion-exchange chromatography on a Eppendorf Biotronic LC 3000.

Determination of AAR in purified elastin and in total tissue specimens; evaluation of the relationship between AAR and age

The extent of aspartic acid racemization was determined as described earlier (Ritz et al. 1993; Ritz-Timme 1999), taking into account defined quality standards (Ritz-Timme et al. 2000b). Briefly, the dried samples were hydrolysed in 6 N HCl at 100°C for 6 h. Hydrochloric acid and water were removed in a vacuum and the hydrolysate was esterified with isopropanol/sulfuric acid (10:1) for 1 h at 110°C. After alkaline extraction with dichloromethane, acetylation was performed with trifluoroacetic anhydride (TFA) at 60°C for 15 min. The amino acids were now present as TFA-isopropylesters. The ratio of D-aspartic acid to L-aspartic acid was determined after separation of the amino acids by gas chromatography on a chiral capillary column (Chirasil-L-Val, Chrompack) using a flame ionization detector, with hydrogen as carrier gas. The relationship between AAR {as $\ln[(1+D/)/(1-D/L)]$}[2] and age was evaluated by linear regression analysis.

Results

The purity of the analysed elastin samples

The histological examination of the purified elastin revealed no detectable collagen contaminations on the morphological level. Figure 1 presents the results of amino acid analysis of purified elastin from yellow ligaments in comparison with the data for the elastin standard and a predicted amino acid composition of human elastin, calculated from the cDNA of tropoelastin (Daamen et al. 2001). The amino acid composition of the purified elastin samples was reproducible, independent from the anatomical location of the analysed ligament, and in good agreement with the composition of the elastin standard as well as with the predicted amino acid composition of human elastin.

[2] The kinetics of AAR is described by the equation $\ln [(1+D/L)/(1-D/L)]=2$ kAsp t+constant (where *kAsp* is the first order reversible rate constant for aspartic acid); for derivation of this equation see Bada and Schroeder (1975). Theoretically, the equation is valid only for free amino acids in aqueous solution but is has been used by the majority of groups working on age estimation based on AAR; to enable direct comparison it is also used here.

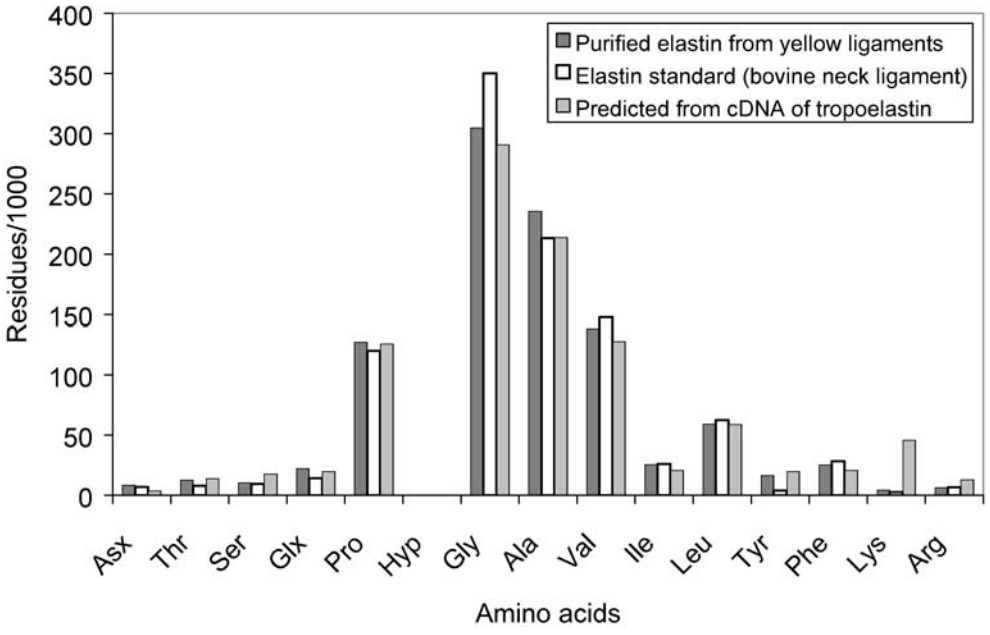

Fig. 1 Amino acid compositions (residues per 1000 residues) of the purified elastin from yellow ligaments and of the elastin standard from bovine neck ligament, compared with the amino acid composition of human elastin, predicted from the cDNA of tropoelastin (Daamen et al. 2001) (posttranslational modifications are not taken into account and therefore particularly the Lys value is lower than predicted from the sequence)

Aspartic acid racemization in purified elastin from yellow ligaments

AAR increased with age both in the total tissue specimens and in the purified elastin samples (Fig. 2). The purified elastin samples exhibited much faster kinetics than the total tissue, with ca. 3.7–4.6-fold higher rates. The relationship between AAR and age could be described by the equations presented in Table 1. It was closer in the purified elastin samples (r =0.96–0.99) than in the total tissue specimens (r =0.84–0.92). The regression lines for purified elastin from the different segments (L1/2, L2/3, L3/4) were nearly identical (see eqs. 5–7 in Table 1); the evaluation of all data for purified elastin (independent of the anatomical location of the analysed ligaments) as total group revealed a close relationship between AAR and age (r =0.97, eq. 8 in Table 1).

Discussion

AAR in total tissue samples of the yellow ligaments

Analysis of total tissue samples revealed a relatively slight increase of AAR with age, even though the yellow ligaments have a high elastin content, which has already been identified as permanent protein with in-vivo racemization in other tissues (Powell et al.1992; Shapiro et al. 1991). The low racemization rate can be explained by very low levels of Asx in elastin (see Fig. 1), as compared with collagen (Asx 48/1000), which is the second main structural protein of the yellow ligaments. Racemization in collagen is very slow because of the conformational constraint exerted by the triple helix which prevents succinimide formation (Collins et al. 1999; Van Duin and Collins 1998). Thus the total tissue values are dominated by the low racemization rate of collagen Asx.

Because of the very high Asx content of collagen as compared with elastin, it is likely that the AAR values in total tissue samples are strongly influenced even by slight, histologically non-detectable variations in the collagen content. The greater scattering of values in the total tissue samples as compared to purified elastin is obviously due to differences in the elastin/collagen proportions in the analysed samples. Thus it is doubtful that analysis of total tissue specimens provides a sufficient precision of age estimation in the forensic context. To eliminate the influence of contaminating proteins (notably collagen) elastin was purified from the yellow ligament samples.

The purity of the analysed elastin samples

Purification of the highly stable and insoluble crosslinked elastin is performed by solubilization and removal of all other proteins. Purification methods producing the purest elastin samples are usually also the harshest procedures, producing the most degraded products (Soskel and Sandberg 1983). Since the kinetics of AAR depend on the molecular structure of a protein (degradation enhances racemization) and on temperature, we had to choose a purification method which combines a low risk of elastin degradation, minimises heating and yet produces an acceptable purity of product, all without being methodologically too sophisticated for an application in forensic practice. For purification of elastin from more complex tissues with low elastin contents, elaborate multi-step procedures are usually employed (for overview see Daamen et al. 2001). In less complex tissues with high elastin contents like the yellow ligaments, elastin purification is less complicated (Rosenbloom 1984). Thus we employed a minimized purification procedure, consisting of the described washing steps and an enzymatical removal of the contaminating collagen.

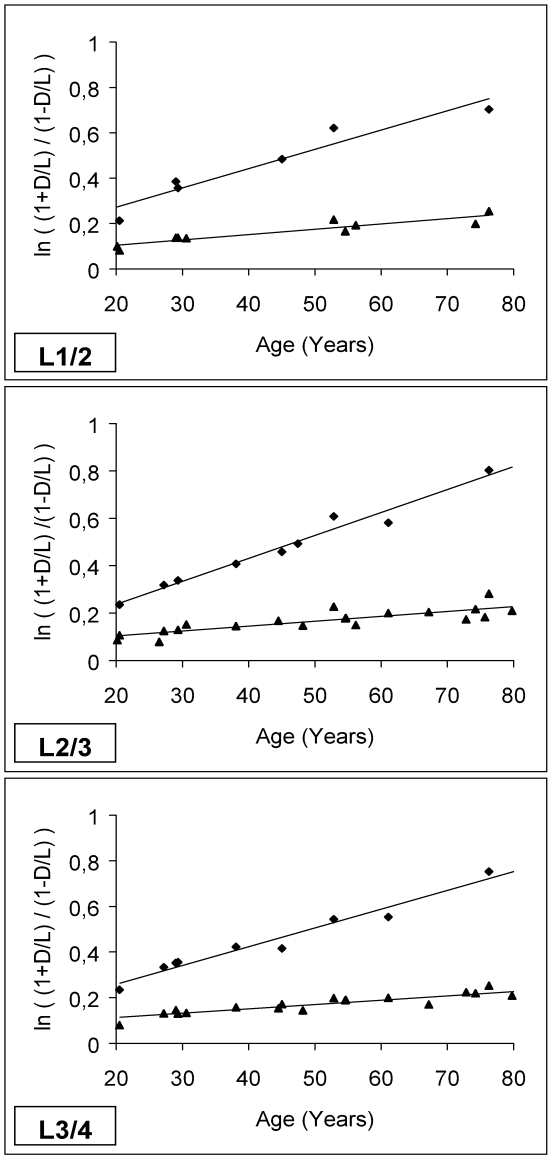

Fig. 2 AAR {as ln[(1+D/L)/(1–D/L)]; D/L=D-aspartic acid/L-aspartic acid} in total samples (*triangles*) and in purified elastin (*rhombuses*) from yellow ligaments in relationship to age. The close relationship between AAR in purified elastin and age can be used as basis for biochemical age estimation

Our purification procedure produced an elastin preparation with a reproducible amino acid composition in close agreement with the composition of the elastin standard and with the amino acid composition of human elastin predicted from sequence data (Fig. 1). The lack of hydroxyproline indicated a lack of collagen contamination which was confirmed histologically. Thus the elastin preparation appeared to be suitable for age estimation based on AAR, a fact confirmed by the AAR data.

AAR in purified elastin from the yellow ligaments

Analysis of the purified elastin revealed a relatively rapid accumulation of D-Asp and a close correlation with age (Fig. 2). These data prove a longevity of elastin also in the yellow ligaments (for details see Ritz-Timme and Collins 2002); the age-dependent accumulation of modified aspartic acid residues is obviously a common feature in the ageing elastin, independent of the tissue source. In fact, in adult healthy tissue, elastin production is suppressed by a posttranscriptional mechanism mediating a rapid decay of the tropoelastin mRNA (Parks 1997; Zhang et al. 1999). However, reinitiation of elastin production has been observed after tissue damage (Rucker and Dubick 1984). This is an apparent contradiction to the assumption that elastin is a permanent protein without relevant turnover, as indicated by the AAR data. Newly synthesized elastic fibres have been described as highly disorganized, non-cross-linked, immature, and of a low elastin content (Rucker and Dubick 1984); such deficient fibres should be removed by the purification steps employed (see also Powell et al. 1992).

Age estimation based on AAR in elastin from the yellow ligaments

The close relationship between AAR in elastin from yellow ligaments (r =0.96–0.99) and age can be used as basis for biochemical age estimations. The attainable accuracy is apparently comparable with the accuracy of age estimation based on AAR in dentine; however, it remains to be determined by a final calibration based on a sufficient number of cases.

Table 1 Equations describing the relationship between AAR and age in total tissue samples and in purified elastin samples from ligamenta flava (*D/L* D-aspartic acid/L-aspartic acid, *t* age at death, *r* correlation coefficient)

Samples	Equations	No.
Total tissue samples		
L1/2 (n=10)	ln[(1+D/L)/(1–D/L)=0.0023 t+0.0576 (r=0.92)	1
L2/3 (n=19)	ln[(1+D/L)/(1–D/L)=0.0021 t+0.0625 (r=0.84)	2
L3/4 (n=17)	ln[(1+D/L)/(1–D/L)=0.0019 t+0.0752 (r=0.90)	3
Total group: L1/2, L2/3 and L3/4 (n=46)	ln[(1+D/L)/(1–D/L)=0.0021 t+0.0640 (r=0.88)	4
Purified elastin samples		
L1/2 (n=6)	ln[(1+D/L)/(1-D/L)=0.0085 t+0.1023 (r=0.96)	5
L2/3 (n=9)	ln[(1+D/L)/(1-D/L)=0.0097 t+0.0431 (r=0.99)	6
L3/4 (n=9)	ln[(1+D/L)/(1-D/L)=0.0082 t+0.0933 (r=0.98)	7
Total group: L1/2, L2/3 and L3/4 (n=24)	ln[(1+D/L)/(1-D/L)=0.0088 t+0.0785 (r=0.97)	8

To minimize influences of contaminating proteins, samples with signs of advanced degeneration should not be analysed. In case of doubt the reproducibility and the quality of the elastin preparation should be controlled by determination of the amino acid composition.

Elastin is characterized by its insolubility and its considerable resistance to harsh chemical and physical influences; most proteolytic enzymes do not attack elastin (Robert 2002). Thus it can be assumed that the molecular integrity of elastin is preserved post-mortem for a relatively long time. However, in cases of advanced decomposition, degradation of elastin may influence the quality of the purified product. In such cases the product must be controlled by amino acid compositional analysis, where an abnormal amino acid composition indicates an insufficient purity of the elastin sample. If so, age estimation based on AAR in elastin cannot be recommended. In burnt bodies the deeply buried ligamenta flava are (in contrast to the teeth) relatively better protected against heat, a factor known to accelerate post-mortem AAR. However, at least prolonged heating will also impact the proteins of the ligamenta flava, and further work would be required to establish the utility of AAR in the elastin of the ligamenta flava for age estimation in burnt bodies.

Age estimation based on AAR in elastin from yellow ligaments may be a valuable additional tool in the identification of unidentified cadavers, especially in cases where other methods cannot be applied (e.g. no available teeth, body parts). The necessity of elastin purification for age estimation based on AAR in yellow ligaments makes the method more sophisticated than the analysis of dentine. Even more than for dentine, the use of quality standards (Ritz-Timme et al. 2000b) is essential to exclude variation due to the lack of standardized preparative and analytical procedures (Ohtani 2002). However, the elastin purification protocol is relatively simple and straightforward and should be readily applicable in forensic practice.

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