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DETECTION OF ASPARTIC ACID ENANTIOMERS BY CHIRAL CAPILLARY GAS CHROMATOGRAPHY

DETERMINATION OF *IN VIVO* RACEMIZATION AND REDUCTION OF METAL-INDUCED BACKGROUND

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SUMMARY

Racemization of aspartyl residues in proteins is a post-translational process, related to ageing. A method is presented for the detection of aspartic acid enantiomers in protein hydrolysates, based on chiral capillary gas chromatography. It is fast, easy and preferable to the usual diastereomeric dipeptide technique. We present evidence that traces of metals that are extracted from the glassware during acidic hydrolysis are the main cause for high background racemization, which often troubles accurate measurements. Effective ways to reduce this background and its standard deviation to acceptable levels are discussed, and a mathematical approach to correct for background racemization is given. Hydrolysates of aged human eye lens proteins were used to demonstrate the enantiomeric separation.

INTRODUCTION

The biochemistry of living organisms exhibits a strong enantioselectivity[1]. Protein synthesis is highly discriminative to amino acid enantiomers and uses only L-amino acids. Therefore, the finding of D-aspartyl residues in tooth enamel proteins from living humans was very surprising[2]. However, especially because of the work of Bada and co-workers[3–5], aspartic acid racemization is now accepted as a fundamental post-translational process. Because of its low rate constant, *in vivo* aspartic acid racemization can only be detected in long-living tissues, such as the eye lens[6–8], tooth enamel and dentine[2,9] and white brain matter[10,11]. These studies have shown that racemization is a spontaneous, slow but continuous process with a rate constant of *ca.* 0.14% $year^{-1}$ (ref. 12). In proteins from nuclear parts of old normal human lenses, the D-enantiomer of aspartic acid can comprise up to 15% of the total aspartate content[13]. There is a strong belief that racemization plays an important role in ageing processes[3,5,8,14].

Until now, aspartic acid racemization in proteins has mostly been detected by

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the method of Manning and Moore[15]. Diastereomeric dipeptides are prepared by coupling the enantiomeric amino acids with L-leucine-N-carboxyanhydride, and then separated by ion-exchange chromatography on a regular amino acid analyser. Since the peaks of L-Leu-D-Asp and L-Leu-L-Asp overlap with other dipeptides, a pre-purification of aspartic acid by ion-exchange chromatography is essential and makes this method very laborious and time-consuming.

Two decades ago, chiral gas chromatographic (GC) liquid phases, capable of separating amino acid enantiomers, were reported by Gil-Av *et al.*[16] and Chang *et al.*[17]. The low thermal stability of these chiral peptide phases, however, generally made them unsuitable for less volatile amio acids. (For a review on enantioselective GC phases, see ref. 18).

Chiral diamide phases, derived from L-valine bound to cross-linked polysiloxane, exhibit greater efficiency, higher enantiomeric separation factors, shorter retention times and better thermal stability. Three such phases are now available in fused-silica capillary columns. These are Chirasil-Val (Chrompack and Alltech) developed in the group of Bayer and Frank[19,20], RSL-007 (Alltech) from Saeed *et al.*[21,22] and XE-60–*S*-valine–*S*-α-phenylethylamide (Chrompack) from the group of Koenig[23,24]. The third of these exhibits the best enantiomeric separation factors for most amino acids, including aspartic acid. Because of their good thermal stability, all common amino acids can be resolved by analysis of a total protein hydrolysate in a temperature-programmed run. Aspartic acid enantiomers can be detected without pre-purification. These columns meet the demand that the elution time of D-amino acids is a little shorter than that of their optical antipodes. Only then is a good quantification of small amounts of a D-amino acid in the presence of an excess of the L-form possible.

Catalysis of racemization by metal ions is known as a notorious problem in peptide synthesis[25,26], but has been given little attention in the literature on aspartic acid racemization. Nevertheless, great effort should be given to the reduction of the concentrations of metals, since especially during hydrolysis they can cause a very high background and so obscure results.

In this paper we describe the detection of aspartic acid enantiomers in protein samples by a chiral capillary GC method. Furthermore, advice is given on how to reduce hydrolysis-induced background racemization and how to correct data mathematically for this background.

EXPERIMENTAL

Preparation of protein samples

The isolation of proteins from human lens fractions by extraction and gel chromatography has been described[8]. Bovine serum albumin (Sigma, A-8002) was used to determine the background racemization. In order to remove traces of metal ions, the protein solutions were dialysed for 24 h against 10 m*M* EDTA in water of Milli-Q quality and subsequently against Milli-Q water for 76 h. The Milli-Q system is from Millipore. The dialysis solutions were changed three times a day. The samples were lyophilized prior to acidic hydrolysis. L-Asp and D-Asp used in the reproducibility tests were purchased from Sigma.

Acidic hydrolysis

Single-use Duran 50 borosilicate hydrolysis tubes (Schott) were pre-extracted for 48 h with 6 *M* hydrochloric acid (Merck, p.a.) at 100°C to remove acid-extractable metal ions. Hydrolysis was performed for 6 h at 110°C under vacuum in 0.5 ml of 6 *M* hydrochloric acid (Merck, Suprapur) in tubes that were sealed in a flame. The hydrolysates were lyophilized prior to derivatization.

Preparation of amino acid derivatives

Dry protein hydrolysates were esterified using 0.6 ml of 4 *M* hydrochloric acid in 2-propanol in closed reaction vials at 110°C for 50 min. After evaporation under nitrogen at 40°C, acylation was performed with a mixture of 0.5 ml of trifluoroacetic acid anhydride and 0.5 ml of dichloromethane for 1 h at room temperature. The redundant reagents were evaporated at 30°C under nitrogen. The derivatized hydrolysates were dissolved in dichloromethane.

Gas chromatographic analysis of aspartic acid enantiomers

Samples were injected onto a fused-silica capillary column (20 m × 0.22 mm I.D.) coated with the chiral polysiloxane phase XE-60–*S*-valine–*S*-α-phenylethylamide (Chrompack, Middelburg, The Netherlands). The Hewlett-Packard 5710A gas chromatograph was used in the split mode, with helium as carrier gas and a flame ionization detector. The oven temperature was kept at 135°C until both aspartic acid enantiomers had eluted from the column (8 min), after which the less volatile compounds were removed by heating the column to 180°C for 4 min. The injector and detector temperatures were 250°C, the carrier gas pressure was 1.7 bar, the vent 80 ml min^{-1} and the gas flow-rate in the column was 1% of the vent. After every hundred analyses, a few centimetres were broken off from both ends of the column and the septum was changed. Peak heights were automatically recorded by a Hewlett-Packard 3390A integrator. The percentage racemization is expressed as the $D \times 100/(D+L)$ ratio. The reproducibility of the determination of this ratio was tested by ten-fold analysis of standard samples containing both aspartic acid antipodes in a 35%, 5% and 0.6% ratio.

Determination of metal ion concentrations

In order to quantify the extraction of metal ions from the hydrolysis tubes during the acidic hydrolysis step, 0.5 ml of 6 *M* hydrochloric acid (Merck, Suprapur) was heated at 110°C for 6 h in six pre-extracted and six non-treated tubes. These twelve samples and three fresh Suprapur hydrochloric acid samples were analysed with a Jarrell-Ash Atom Comp Model 975 inductively coupled argon plasma multi-channel atomic emission spectrometer system equipped with a scanning monochromator. Specifications of the main characteristics of the system have been given elsewhere[27].

Amino acid analysis

Samples were analysed on a LKB 4151 Alpha Plus amino analyzer, with post-column ninhydrin derivatization.

RESULTS

Gas chromatographic separation of D- *and* L-*aspartic acid*

The separation of the aspartic acid enantiomers of a human eye lens protein hydrolysate with a chiral capillary column is shown in Fig. 1. D-Asp elutes before L-Asp. The resolution was calculated to be 1.42 at retention times of *ca.* 7.5 min.

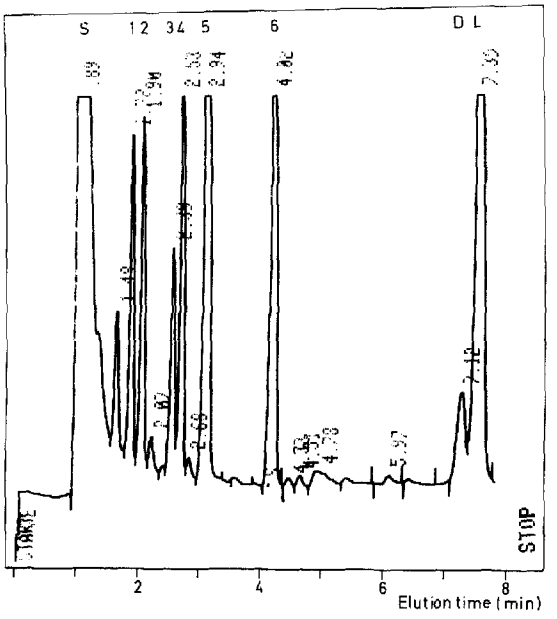

Fig. 1. Enantiomeric separation on a chiral capillary column. The applied sample is a hydrolysate of the cortical urea-soluble protein fraction of a 55-year-old human eye lens. The analysis revealed an aspartic acid racemization of 10.5%. Peaks: S = solvent; 1 = L-Ala; 2 = L-Val; 3 = L-Thr; 4 = L-Ile; 5 = L-Leu; 6 = L-Pro; D = D-Asp; L = L-Asp.

The reproducibility of the ratio determination is shown in Table I. The coefficient of variation (C.V.) is *ca.* 8%. Because of large relative errors in the case of small amounts of D-Asp, the coefficient is much higher for small percentages of D-Asp (0.6%). However, one should realize that owing to the acidic hydrolysis, samples

TABLE I

REPRODUCIBILITY OF THE D × 100/(D + L) ESTIMATION BY CHIRAL GC ANALYSIS OF ASPARTIC ACID ENANTIOMERS

Mixtures of D- and L-Asp were derivatized and chromatographed as mentioned in Experimental. Results of each mixture are based on ten analyses.

Mean D × *100/(*D + L*) ratio*	*S.D.*	*C.V. (%)*
34.82	2.91	8.36
4.93	0.41	8.32
0.62	0.13	20.97

contain at least 2% racemized aspartic acid. We wish to emphasize that we investigated the reproducibility of the determination of the D × 100/(D+L) ratio and not that of the GC analysis, which of course has a C.V. much less than 8%.

The detection limit, defined as a signal-to-noise ratio of 2, is *ca.* 250 pmol at a splitting ratio of 1%, in which case only 2.5 pmol are actually applied to the column.

The lifetime of the column is extremely good. Only a slight decrease in retention time due to bleeding of the liquid phase, but no detectable loss of resolution were noticeable after 6 months of continuous operation.

Racemization during acidic hydrolysis

Since the racemization rate is enhanced in strong acid[28–31], the hydrolysis time should be kept as short as possible. We therefore investigated the time needed for total liberation of aspartic acid. Acidic hydrolysis of bovine serum albumin was carried out as described for varied periods of time. The amounts of free Asp, as determined by amino acid analysis, are depicted in Fig. 2. Obviously, 6 h are sufficient to release all aspartate residues.

The metal ions that catalyse racemization in the hydrolysis medium can originate from the sample and from the glass of the hydrolysis tubes. The remedy for the first source is exhaustive dialysis against an EDTA solution, followed by ultra-pure water. Leaching of the glass during the hydrolysis step can greatly be reduced by a 48-h pre-extraction of the tubes with 6 *M* hydrochloric acid at 100°C. Concentrations of thirteen metal ions, present in the hydrochloric acid after a dummy hydrolysis in treated and non-treated tubes, are shown in Table II. Obviously, the pre-treatment reduces the total amount of acid-extractable metals dramatically. Furthermore, the standard deviation (S.D.) of their concentration is also greatly decreased.

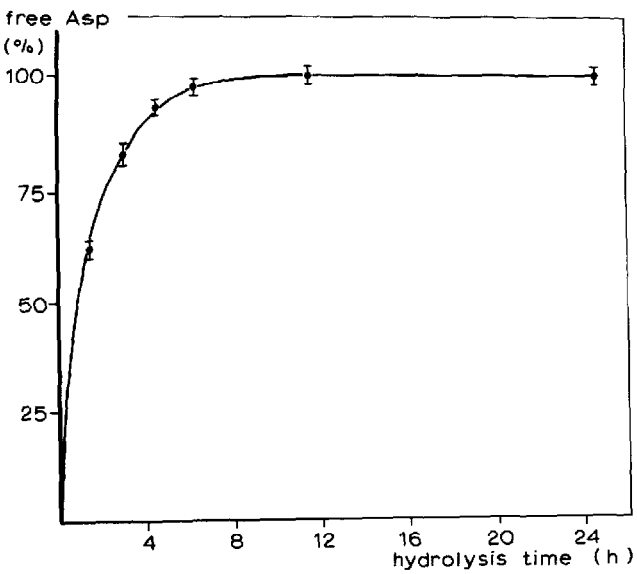

Fig. 2. Release of aspartic acid as a function of duration of hydrolysis. The amount of free aspartic acid was measured on an amino acid analyser after hydrolysis in 6 *M* hydrochloric acid at 110°C for the times indicated.

TABLE II

METAL CONCENTRATIONS IN HYDROCHLORIC ACID AFTER HYDROLYSIS IN PRE-EXTRACTED AND NON-EXTRACTED TUBES

Concentrations are presented as parts per billion (10^9). The upper row shows the normal background concentrations in the Suprapur hydrochloric acid used in this experiment. Standard deviations are given in parentheses. The composition of the Duran 50 glass is 80.5% SiO_2, 12.9% B_2O_3, 3.8% Na_2O, 2.2% Al_2O_3, and 0.4% K_2O.

	Zinc	*Lead*	*Cadmium*	*Aluminium*	*Iron*	*Manganese*
Hydrochloric acid, fresh ($n=3$)	<4	53 (13)	4 (1)	<25	47 (6)	1 (0.5)
Hydrochloric acid after hydrolysis in pre-extracted Duran 50 tubes ($n=6$)	17 (5)	74 (9)	4 (2)	190 (30)	320 (300)	5 (4)
Hydrochloric acid after hydrolysis in non-treated Duran 50 tubes ($n=6$)	22 (10)	83 (13)	5 (2)	1600 (200)	165 (75)	5 (1)

The effect of reduction of metal concentration on the background racemization is shown in Fig. 3. Clearly, the removal of acid-extractable metals from the tubes is very effective in reducing the background and its S.D. Sample dialysis contributes only slightly to the reduction of the racemization background in the case of commercial bovine serum albumin (Fig. 3). For protein extracts the effect may be expected to be higher. Derivatization-induced racemization, measured with optically pure, only derivatized L-Asp, was undetectable. Furthermore, it turned out that the amount of protein played no role in the racemization.

DISCUSSION

Chiral capillary GC is a suitable technique for the analysis of aspartic acid racemization *in vivo*. It offers various advantages over the classical diastereomeric dipeptide method, originally introduced by Manning and Moore[15] and used in most studies in this field[4,7]. Because of the high resolution of the individual amino acids and the moderate enantioselectivity offered by the GC technique, the aspartic acid enantiomers can be detected in a sample as complex as a protein hydrolysate (Fig. 1). This eliminates the need to isolate the aspartic acid from the other amino acids. Furthermore, the analysis time is well reduced. Elution times in the diastereomeric method exceed 1 h, whereas on a 20-m capillary GC column retention times are as short as 7.5 min. The 250-pmol detection limit is about the same for both methods. $D \times 100/(D+L)$ ratios can be determined with a C.V. of 8% (Table I). The reproducibility of the GC analysis as such is of course much better. Therefore, this method is more accurate than the dipeptide analysis, for which Bada mentioned a "reproducibility of ± 5–10%"[4].

Background racemization caused by the hydrolysis step has been given little attention in the literature. The problem is seldom acknowledged, and quantitations are scarce and vary considerably. We have convincingly shown that metal ions, originating from the sample and especially from the glassware, play a major role in the racemization during the hydrolysis step (Fig. 3). Although well-documented reports on that role are not available, there is some information on the catalytic properties

Chromium	*Magnesium*	*Copper*	*Calcium*	*Boron*	*Sodium*	*Potassium*	*Total*
8 (1)	<4	6 (1)	28 (31)	21 (7)	<4	<100	310 (40)
16 (1)	58 (22)	20 (8)	300 (200)	1400 (200)	1400 (300)	<100	3900 (600)
20 (2)	126 (38)	45 (22)	500 (150)	12600 (1300)	7300 (3500)	1320 (350)	24000 (4000)

of metals and metal complexes on the racemization rate of amino acids. Metals that have been positively identified as catalysts include: cobalt[32–36], copper[32,35–42], iron and aluminium[39], magnesium[35,38], zinc[35,36,41] and nickel[35,36,43]. The concentrations of all those metals, except iron, were all increased during hydrolysis in non-treated tubes (Table II). If precautionary steps such as dialysis of the samples and pre-extraction of the tubes are undertaken, the background racemization and its S.D. are reduced to acceptable levels (Fig. 3).

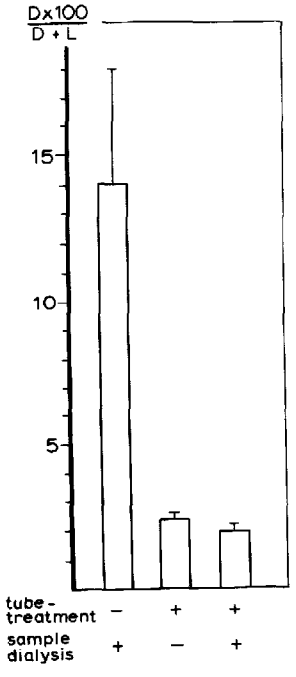

Fig. 3. Effect of tube-treatment and sample-dialysis on the background racemization of aspartic acid. Bovine serum albumin, dialysed and non-dialysed, was hydrolysed in extracted and non-extracted tubes. Racemization and standard deviation were calculated from five individual hydrolyses.

For samples treated in this way, the S.D. of the background is of the same order of magnitude as that of the GC analysis. Therefore, if extreme accuracy is required, this cannot be achieved by multiple GC analysis only. The sample should also be hydrolysed in duplicate or triplicate. We have achieved relative standard deviations (R.S.D.) of 1% by hydrolysing eye lens protein fractions in triplicate and triplicate analysis of each hydrolysate[13]. We have some preliminary results, indicating that this accuracy can also be achieved by the HPLC separation of fluorescent diastereomeric amino acid derivatives, as described by Aswad[44].

At this point we should comment on the question of how measured $D \times 100/(D+L)$ ratios may be corrected mathematically for background racemization. Simply subtracting this background, as is often done, is not correct since it neglects the facts that not only L-Asp but also D-Asp racemizes during hydrolysis and that the equimolar enantiomeric ratio of a racemic mixture does not change on hydrolysis. Therefore, after subtracting the background from the measured ratio, the resulting value has to be multiplied by a factor of 50/(50 − background). Let us consider a hypothetical experiment, in which the background amounts to 2% racemization. In that case, a realistic measured amount of racemization of, *e.g.*, 15.0%, would be corrected to 13.5%, in contrast to 13.0% if the background were simply subtracted.

We have described here a method for the analysis of aspartic acid enantiomers, based on chiral capillary GC. The simplicity of the method and the short analysis time might give a new impulse to the investigation of *in vivo* racemization of aspartic acid, its implications for protein structures and its role in ageing processes.

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