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Short communication

Preparative isolation of nine indispensable amino acids from a single sample

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Abstract

In order to measure low isotopic labelling of amino acids with an acceptable precision, the isolation of large amounts of amino acid is required. When this is the case, the size of the original sample can be a limitation and usually labelling in only one amino acid can be measured; the rest going to waste. By modifying existing methods, we have developed a method in which nine indispensable amino acids can be isolated by ion-exchange chromatography from a single sample. Protein hydrolysates containing up to 10 μmol lysine were fractionated on a system that isolated lysine and histidine. The amino acids not resolved in this system were also collected and methods for the isolation of valine, methionine, isoleucine, leucine, threonine, tyrosine and phenylalanine from the same sample are described. © 1997 Elsevier Science B.V.

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1. Introduction

The labelling of amino acids with stable isotopes can be measured with relatively quick methods like GC–MS [1–3] or by GC–combustion isotope ratio mass spectrometry (C-IRMS) [4]. Although these methods are very useful when the isotopic labelling in the amino acids is relatively high, their precision is not accurate enough for low isotopic labelling (i.e., 0.002–0.005 atoms ^{15}N % excess or 0.2–0.7 dpm/μmol). When high precision for this sort of labelling is required, the only way in which this can be achieved is by using the tedious traditional methods in which large amounts (i.e., 10 μmol) of the amino acid of interest are isolated by preparative ion-exchange chromatography. The labelling can then be measured by IRMS or by liquid scintillation counting. In the case of ^{15}N, the equivalent to 500 μg of N are required. In the case of radioactive labelling with ^{14}C, the amount of amino acid required will depend on the degree of labelling (a minimum of 20 dpm over background are desirable).

During studies on the incorporation of inorganic ^{15}N (as $^{15}NH_3$) and of ^{14}C from labelled carbohydrates into indispensable amino acids [5], a method was required for the accurate determination of isotopic labelling of amino acids. We required the preparative isolation of relatively large amounts (i.e., 10 μmol per run) of nine indispensable amino acids from biological samples, in which the size of the sample was a limitation (i.e., tissues, plasma free pool, microbial fraction of digestive contents, etc.). However, most methods for the preparative isolation of amino acids are focused on the isolation of a single amino acid, with the remaining amino acids going to waste [6,7]. In order to overcome the limitation of the size of the samples, by modifying

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existing methods [8], we have developed a method in which nine indispensable amino acids can be isolated from a single sample.

2. Experimental

2.1. Reagents

Hydrochloric acid, sulphuric acid, sodium hydroxide, disodium tetraborate (borax), trisodium citrate, lithium citrate, sodium acetate, ethanol, methanol and mercaptoethanol (all AnalaR Grade) were obtained from BDH, Poole, UK. *ortho*-Phthalaldehyde (OPA; analytical-reagent grade) was obtained from ICN Biochemicals, Thame, UK.

2.2. Sample hydrolysis

The protein samples were hydrolysed under reflux with distilled constant boiling 6 *M* HCl at 137°C for 18 h. The acid was removed by rotary-evaporation and the hydrolysates were taken to pH 11 with 10 *M* NaOH and left in a desiccator under reduced pressure in the presence of concentrated H_2SO_4 for at least 1 h. This was to reduce the quantity of highly enriched $^{15}NH_3$ present which could interfere with the [^{15}N]Lys enrichment measurements. The samples were acidified to pH<2 with concentrated HCl before loading onto an ion-exchange column, the effluent of which was collected in a fraction collector.

2.3. Identification of amino acid peaks

The amino acid content of each fraction was measured using post-column derivatisation with OPA by the modification of an existing method [9]. By using OPA, the identification of the amino acid peaks was carried out using only a very small amount of the amino acids isolated. The OPA solution (16 mg OPA in 400 μl methanol, 20 ml 0.3 *M* borax (saturated), 40 μl mercaptoethanol) was prepared freshly every day. A 50 μl sample from each fraction was transferred into an Immulon 4 MicroELISA plate (Dynatech Labs., West Sussex, UK) and 80 μl of OPA solution were added. The plate was left at room temperature for 2 min and the absorbance measured at 340 nm using a Titertek Multiskan MCC/340 (ICN Biochemicals). The OPA reaction described above, needs to be conducted at pH 8–10. Therefore, when amino acid peaks eluted with a hydrochloric acid solution were identified, a calculated equimolar amount of NaOH was added to the OPA solution in order to neutralise the HCl.

2.4. Separation of lysine, histidine and arginine

Samples of protein hydrolysate containing about 10 μmol lysine were loaded onto an automated ion-exchange chromatography system, (Locarte, London, UK) fitted with a 9 mm internal diameter column filled with 400 mm resin bed (Locarte long column resin). The amino acids were eluted with a 0.13 *M* trisodium citrate buffer (pH 4.18) at 29°C, at a flow-rate of 30 ml/h. The column was operated on a 12 h cycle basis (amino acid elution for 11 h, regeneration with 0.3 *M* NaOH for 20 min and equilibration with the eluting buffer for 40 min). This system was preferred to others commonly used because it resolved lysine from ornithine [8], which could be highly labelled. Fractions (3 ml/6 min) were collected using a fraction collector. Arginine may be obtained by adding a second buffer, e.g., 0.13 *M* trisodium citrate, pH 5.5, (not shown in Fig. 1a) before the NaOH regeneration.

Fractions containing lysine and histidine were pooled (Fig. 1a). The peaks containing tyrosine+phenylalanine and acidic and neutral amino acids (threonine, leucine, isoleucine and valine amongst others) were also collected (Fig. 1a) and kept for further processing. When pooling the fractions, a few extra fractions before and after the peak were included in order to allow for the possible displacement of labelled relative to unlabelled amino acids [10–12].

2.5. Desalting of lysine

To desalt such large samples, an adaptation of a chromatographic method (using HCl) [13] was used. The pooled fraction containing lysine was loaded onto a 150×18 mm column of Dowex-50 (H^+ form, 100–200 mesh, 8% cross-linked; Sigma, St. Louis, MO, USA). After loading the sample (approximately 10 μmol lysine, pH<2), the citrate was removed

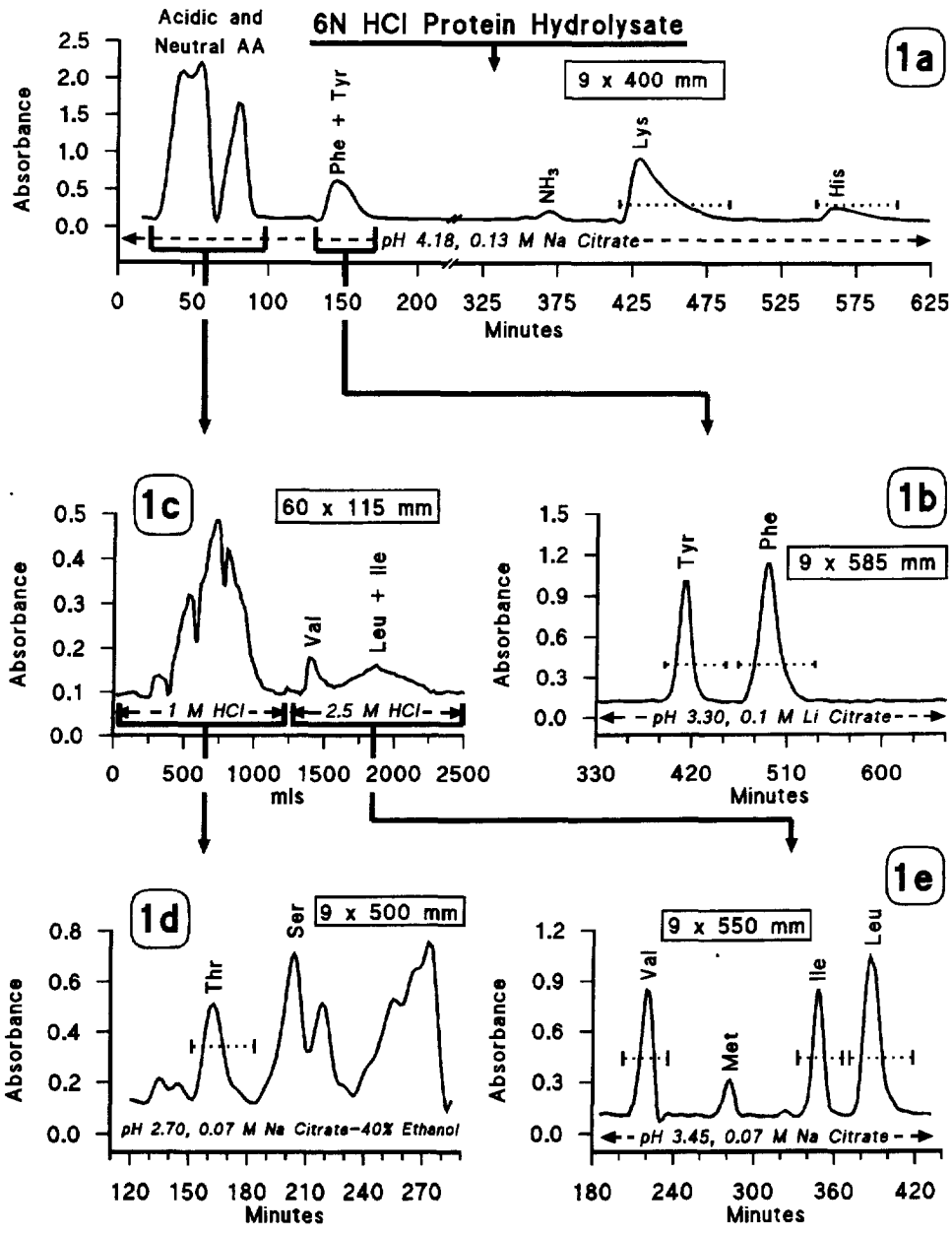

Fig. 1. Isolation of nine indispensable amino acids by ion-exchange chromatography.

with deionised water, and the column eluted with 100 ml 2.5 *M* HCl, followed by 150 ml 6 *M* HCl. Na^+ and any possible contaminating NH_4^+ were eluted with the 2.5 *M* HCl solution, whereas lysine appeared in the 6 *M* HCl eluent. The considerable retention time difference between lysine and NH_4^+ in the HCl system was very convenient, since ammonia could have interfered with the [^{15}N]lysine measurements and other systems do not separate them to the same extent. After each sample the desalting column was regenerated as follows: excess HCl was displaced with deionised water, and the column was

washed with 100 ml 2 *M* NaOH. The NaOH in excess was removed with deionised water and the column was converted back to the H^+ form with 100 ml 6 *M* HCl. Two additional washes with deionised water were applied (until the column effluent was of neutral pH) before loading the next sample.

2.6. Desalting of histidine

An Amberlite CG-120 (H^+ form, 200–400 mesh, 8% cross-linked; BDH) column (45×18 mm) was used. Fractions containing approximately 18 μmol histidine were loaded, the salt was removed with 100 ml 0.5 *M* HCl and the bound histidine eluted with 300 ml 6 *M* HCl. The column was regenerated between samples as described for lysine.

2.7. Desalting of tyrosine and phenylalanine

Fractions containing tyrosine+phenylalanine (40 μmol; Fig. 1a) were desalted before further separation, using an Amberlite CG-120 (H^+ form), 50× 18 mm resin bed. Salt was removed with 100 ml 0.5 *M* HCl, and tyrosine+phenylalanine were eluted with 300 ml 6 *M* HCl. The column was regenerated with 2 *M* NaOH between samples.

2.8. Separation of tyrosine and phenylalanine

Desalted tyrosine and phenylalanine (approximately 10 μmol of each per run) were separated with an ion-exchange column (Locarte), containing a 585×9 mm resin bed, using 0.1 *M* Li^+ citrate buffer pH 3.30 at 55°C, at a flow-rate of 30 ml/h (Fig. 1b). The system was operated on a 12 h cycle. Fractions (6 min) were collected, and amino acid contents were determined with OPA. Pooled tyrosine and pooled phenylalanine fractions were desalted as described above.

2.9. Separation of branched-chain amino acids from the acidic and neutral fractions

The acidic and neutral amino acid fraction obtained previously (Fig. 1a) was loaded onto a 120× 60 mm resin bed with Amberlite CG-120 resin (H^+ form; Fig. 1c). After thorough washing with deionised water, the first fraction containing aspartate, serine, threonine, glutamate, glycine and alanine was obtained by washing the column with 1250 ml 1 *M* HCl. A second fraction containing valine, proline, methionine, isoleucine and leucine was eluted with 1000 ml 6 *M* HCl. The column was regenerated after each sample with 500 ml 2 *M* NaOH, and 500 ml 6 *M* HCl.

2.10. Separation of threonine and serine

Threonine was isolated from the first fraction shown in Fig. 1b by a two step procedure: firstly aspartic and glutamic acids were removed by chromatography on a 200×20 mm resin bed of weak anion-exchanger AG3-X4A, 200–400 mesh (BioRad Labs., Richmond, CA, USA) in 0.2 *M* sodium acetate pH 5.0, at room temperature (profile not shown). And secondly, there was a complete separation of threonine from serine on a 500×9 mm bed of Locarte long column resin at 60°C in 0.067 *M* sodium citrate, pH 2.70 containing 40% (v/v) ethanol at a flow-rate of 25 ml/h (Fig. 1d).

2.11. Separation of valine, leucine, isoleucine and methionine

After removing the HCl from the branched-chain amino acid fraction obtained in Fig. 1b, valine, methionine, isoleucine and leucine were resolved on a 550×9 mm resin bed, using a 0.067 *M* trisodium citrate buffer pH 3.45 at 55°C, at a flow-rate of 30 ml/h, on an 8 h cycle (Fig. 1e). Fractions (6 min) were collected and amino acids identified with OPA.

2.12. Desalting of valine, leucine, isoleucine and methionine

A two-step procedure was required to desalt fractions containing valine, isoleucine, leucine and methionine because these amino acids have a relatively low affinity for a cation-exchange resin, which is similar to that of Na^+ (i.e., it is difficult to resolve them), and some amino acid would be partially lost if Na^+ was removed with 0.5 *M* HCl as described for tyrosine and phenylalanine. The first step was to remove the citrate anion; a sample containing 40 μmols amino acid was loaded onto a 50×18 mm Amberlite CG-120 (H^+ form) column. Citrate was

removed with 100 ml deionised water, and the bound amino acid and Na^+ was recovered with 250 ml 6 *M* HCl. After each sample the column was regenerated with 100 ml 2 *M* NaOH and 100 ml 6 *M* HCl. The second step was to remove the Na^+ cation; after removing the 6 *M* HCl by rotary-evaporation, the sample (pH>10) was loaded onto a 30×40 mm Dowex-1 column (OH^- form, 200–400 mesh, 8% cross-linked; Sigma, St. Louis, MO, USA). The Na^+ was washed through with 100 ml deionised water and the amino acid obtained with 200 ml 6 *M* HCl. Between samples the column was washed with deionised water and then reconverted to the OH^- form by adding 100 ml 2 *M* NaOH: the excess NaOH was removed with deionised water.

3. Results and discussion

The system described (Fig. 1) gave a good preparative isolation of the indispensable amino acids. Conventional ion-exchange chromatographic analysis of the pooled fractions confirmed the sole presence of the isolated amino acid. It is well documented that when labelled amino acids are separated by ion-exchange chromatography a degree of displacement between the labelled and unlabelled amino acids occurs [10–12]. The degree of displacement in our lysine system was checked by running a standard solution containing 10 μmol lysine and 45 000 cpm L-[U-^{14}C]lysine. The lysine peak was identified with OPA as described above and the radioactivity in a 0.5 ml sample of each fraction was measured. A slight displacement of about one fraction was observed (Fig. 2), which indicates the importance of the complete collection of all the fractions forming the amino acid peak. It also illustrates the convenience of including a few extra fractions at either side of the peak in the pooled samples.

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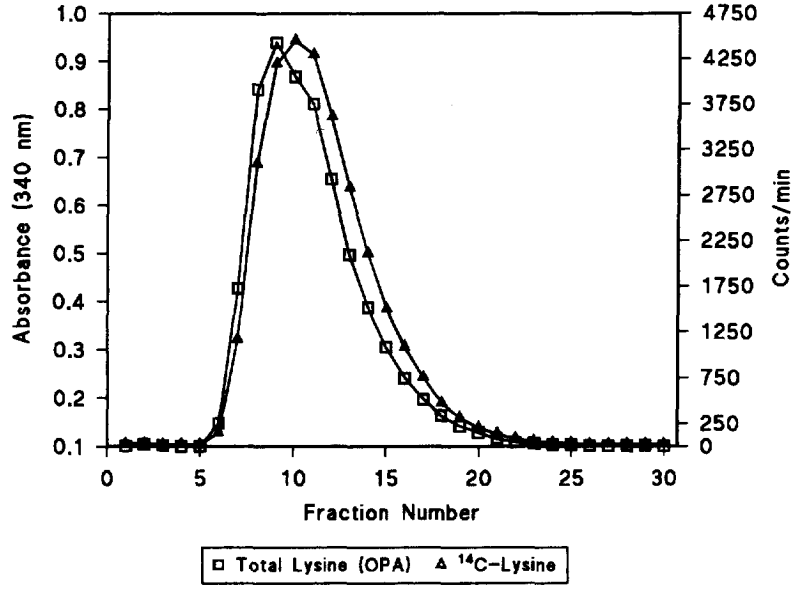

Fig. 2. Displacement of L-[U-^{14}C]lysine relative to total lysine in the lysine ion-exchange chromatography system. Total lysine was detected with OPA; [^{14}C]lysine was detected by liquid scintillation counting.

References

[1] A.G. Calder, S.E. Anderson, I. Grant, M.A. McNurlan, P.J. Garlick, Rapid Commun. Mass Spectrom. 6 (1992) 421.
[2] G.E. Lobley, A. Connell, M.A. Lomax, D.S. Brown, E. Milne, A.G. Calder, D.A.H. Farningham, Br. J. Nutr. 73 (1995) 667.
[3] B.W. Patterson, F. Carraro, R.R. Wolfe, Biol. Mass Spectrom. 22 (1993) 518.
[4] C.C. Metges, K.J. Petzke, J. Mass Spectrom. 31 (1996) 367.
[5] D. Torrallardona, C.I. Harris, E. Milne, M.F. Fuller, in: W.B. Souffrant, H. Hagemeister (Eds.), Proceedings of the VIth International Symposium on Digestive Physiology in Pigs, Bad Doberan, October 1994, FBN, Dummerstorf, 1994, p. 245.
[6] D. Torrallardona, C.I. Harris, M.E. Coates, M.F. Fuller, Br. J. Nutr. 76 (1996) 689.
[7] D. Torrallardona, C.I. Harris, M.F. Fuller, Br. J. Nutr. 76 (1996) 701.
[8] D.H. Spackman, W.H. Stein, S. Moore, Anal. Chem. 30 (1958) 1190.
[9] F.C. Church, D.H. Porter, G.L. Catignani, H.E. Swaisgood, Anal. Biochem. 146 (1985) 343.
[10] F.H. Spedding, J.E. Powell, H.J. Svec, J. Am. Chem. Soc. 77 (1955) 6125.
[11] M.K. Gaitonde, R.W.K. Nixey, Anal. Biochem. 50 (1972) 416.
[12] G.A. Nicholas, G.E. Lobley, C.I. Harris, Br. J. Nutr. 38 (1977) 1.
[13] W.H. Stein, S. Moore, Cold Spring Harbor Symposia on Quantitative Biology 14 (1950) 179.