Iranian J,Publ. Health
1983, Vol.12, No 1-4 (32-42)

ESTIMATION OF METHIONINE AND LYSINE IN PROTEIN BY THIN-LAYER CHROMATOGRAPHY

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Key words: Methinonine, Lysine, thin-layer chromatograhy

ABSTRACT

A thin-layer chromatography method has been developed and evaluated for the separation and determination of lysine and methonine in a mixture of amino acids obtained from acid or enzyme hydrolysis of protein. The solvent systems were(a) ethanol-ether,(b) phenol-water, (c) ethyl methyl ketone-pyridine-distilled water - glacial acetic acid.

Procedurs in all the three stages were in the same dimension. Results of casein analysis by this method showed ± 15% fluctuations when compared with standard amino acid analyser.

INTRODUCTION

The determination of amino acid composition of food proteins has been a research topic for more than a century. Increased attention paid to this question in recent years, is mainly due to the recognition of the specific nutritive role played by certain amino acids in growth,reproduction, lactation and maintenance.

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Food processing has a direct effect on the nutritive value of the protein and the biological availability of the amino acids, especially lysine and methionine. Interaction between protein and reducing sugars, for example, results in the formation of a lysine-sugar complex which hydrolyses to furosine. Alkali damage results in the formation of lysino-alanine bound (2). Heat damage at a pH near neuhral results in the formation of internal links between the epsilon-amino groups of lysine and react ion with glucose(25). Over heating of casein lowers the biological availability of this amino acid (9). A reaction between glucose and the free amino groups in casein has been reported (11,12). Gossipol reacts with the free amino groups of lysine in albumine or cotton-seed protein(13).

Oxidation leads to conversion of methionine to sulphoxde which is destroyed during acid hydrolysis but may be found after alkaline hydrolysis of the protein (2). Methionine also changes during processing to methionine Sulphone and methionine sulphoxide or other compounds which are poorly utilized (5,21,22).

The numerous and immense difficulties in developing reliable analytical procedurs are actually best attested to the vast literature on the subject. Considerable progress was made after the introduction of microbiological and chromatographic methods during the last two decades (15).

Thin-layer chromatography of amino acids start with hydrolysis by enzyme, followed by separating of hydrophilic substances such as amino acids. This technique is being utilized because it is both simple and inexpensive. Quantitative analysis of mixtures of amino acids by this technique is, however, best with several difficulties. Lysine, arginine, and histidine of basic amino acids, and methionine, valine and isoleucine of neutural amino acids show very close values for most of the solvent systems and techniques reported, and this is the major hurdle in the quantitative separation of these amino acids(17,1,10)

in the present work the amino acid content of protein were estimated after acid or enzyme hydrolysis.

MATERIALS AND METHODS

Reagent used included,(a) ethanol-water, freshly distilled ethanol was mixed with distilled water in the proportion of 70:30 (V/V);(b) phenol-water,freshly distilled phenol or pure phenol was mixed with distilled water in the proportion of 78:22(V/V);(c) methyl ethyl ketone/pyridine/distilled water/glacial acetic acid(90:30:30:3)(3); (d) ninhydrin-spray reagent, 1 gm of ninhydrin was dissolved in 250 ml of 1-butanol and mixed with 8 ml of glacial acetic acid.

PROCEDURE

(A) Acid hydrolysis of samples: Samples containing about 15 mg of protein were hydrolysed in a sealed tube with 6 N HCl (8) at 110±2 C. for 24 hr. The hydrolysates were cooled and filtered. The residue was washed for several times in small quantities of distilled water. Filterates plus washings were evaprorated to dryness under vacuum at 45 C. (14). The residue was solved in 5 ml of distilled water and re-evaporated. This process is repeated once more in order to remove most of HCl,(28).Samples were then extracted three times with 5 ml portions of 10% propanol and the supernatants pooled. The pH of the pooled samples was adjusted with N/100 sodium hydroxide to7-7.2. The solution were declorized with activated charcoal and centrifuged. The samples were concentrated on a water bath (45 C.) in vacuo and made up to 5 ml.

(B) Enzymatic hydrolysis: A portion of samples containing about 15 mg protein were dissolved in 5 ml of 0.05 M -phosphate buffer (Na_2HPO_4-HCl ,pH 8.2) and 0.1 ml toluene and 0.1 ml of solution containing 10 mg pancreatopeptidase (Elastase E.C.No.3,4,21,11) E/ml were then added. The mixture was incubated at 40 C. for 24 hr with continiuous shaking. Then cooled and centrifuged at 3000 g. for 10 min. The supernatant fraction was removed and the volume was adjusted to 5 ml with distilled water (18).

(C) Thin-lyer chromatography:Both ready-made aluminum TLC plates (E.Merck) and preparation of plate: Silica gel (30 g) (E.Merck Type 60) was vigorously shaken with 60 ml of water for 1 min. in a closed Erlen meyer flask and

applied to 20X20 cm. glass plates by means of Stahl,s applicator (Shandon Scientific Co., London , England) . A layer thickness of 0.25 mm was used.The plates were dried in air over-night in a harizontal position. Heating the plates at 100 C before applying the sample dose not make any improvement in results. Suitable aliquots of amino acids (acid and enzyme hydrolysates) 2-6 µl of final Solutions were applied on TLC plates with the help of 10 µl Hamilton syringe, using a cut point niddle No.50. Casein was used as a standard protein.

Standards: 20 mg of pure methonine and lysine were dissolved in 100 ml of 0.01 N HCl (or hydrochloride amino acids in distilled water). 2,4 and 6 µl of each solution were applied to the plates. A series of standards of each amino acids was run on each plate since the standards vary from plate to plate.

Rectangular chambers (Shandon Scientific Co.) were used for development of the plates. Approximately 150 ml of freshly prepared solvent was put into the chamber by pouring down the sides to saturate the filter paper lining. The plates were first developed in ethanol-water solvent system, A good separation was achived in 3 hr.At the end of this period the plates were removed from the chamber and dried, (at room temprature for 20 min.) , then redeveloped in a phenol-water solvent system using the same ascending technique for 4 hr to complete the separation of lysine, histidine and arginine.At the end of this period the plates were removed from the chamber and dried with the help of hair-dryer (or could be left in a fume cupboard over-night). The plates were redeveloped for the third time in methyl ethyl Ketone / pyridine / distilled water/acetic acid solvent system using the same ascendig technique for 2 hr. After drying,the plates were sprayed with ninhydrin reagent and colour developed at80° C for 15 min.

(D) Estimation of amino acids: The intensity of colour of the amino acid ninhyrin spot was read by reflected light using a Chromoscan 200/20 system (Joyce -Lobel Limited, Team Valley Gateshead NELL OUJ England).

RESULTS AND DISCUSSION

(a) Separation of a mixture of amino acids obtained from the hydrolysis of casein, soybean and skimmed milk powder on silica gel G is shown in Figure 1. Lysine remained at the bottom of the plate very near to the point of application and arginine and histidine separated as distinct spots above it. The three spots near the top of the plate show the isoleucine, methionine and valine respectively. In each plate between 12-16 samples could be applied.

(b) Solvents: For a good separation of these amino acids the solvents used were the best , as many samples were applied with different solvent systems or one solvent with different prportion and those mentioned solvents after all were used. Solvent butanol/acetic acid/water(60 :15:15) and phenol/water (75:25) has been previously used (16) for the separation of free amino acids in plasma , however, it's application for complete separation of lysine from arginine and histidine has not been successful (20). Isopropanol/water (70:30) has also been used on silica gel G layers (27) to separate amino acids in the extracts of rat brain. This solvent caused overlap between methionine and isoleucine.

(c) pH: At pH range of 7-7.2 for a solution of amino acids and a propotion of phenol/water solvent78:22mixture the lysine was separated from arginine and histidine , as it is shown in Figure 1. At more acidic or alkaline pH these three amino acids overlapped.

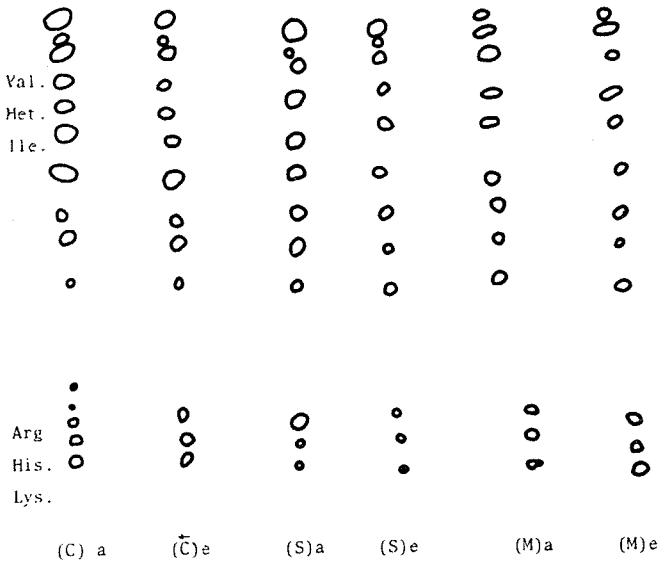
(d) Time: A total of nine hours(three hours for first developing, four hr for the second one,and two hr for the third developing) is required for this separation method. 2 hr mentioned by Bahl, et al(1)is simply not sufficient.

(e) Developing: A minimum temprature of 80° C and 15 min. time was required for developing the colour of the amino acid spots. The colour was found to be stable for about 2 hr, after which a gradual progressive reduction in intensity began.

(f) Reading and Calculation: The density of spot colour should be read when the plate is completely cold . Tailing spot, can be calculated by reading each amino acid spot in two dimensions or the whole spots together and estimate the test by comparison with standards.

Figure1

Chromatogram of acid(a) and enzyme(e) hydrolysis of casein(c) soybeab(s) and skimmed milk powder (M) on silica gel G.



Figures 2 and 3 show the standard curves of lysine and methionine respectively. Area of each peak calculated by digital calculator (Instrument) . For obtaining the curve, squar root of area of each standard amino acid was ploted against their weight logarithm (23).

Salts have little effect on separation of amino acids on TLC. However for better detection and determination of the quantity of amino acids in leguminous seeds (grain) it would be better to remove salts by a reliable technique after hydrolysing with acid or enzyme(26).Electrodialysis apparatus, such as that described by Consden, et al, (4), or suitable ion exchange system,(19) is also useful. Electrolytic demineralisation partially converts arginine into ornithine and 10-30% of the histidine,lysine, methionine, proline and tyrosine will be lost (24). An ion exchange procedure, based on a method of Dreze et al, (6, 7) using Dowex-2 in column chromatography may also be used. Although the final solution of enzyme hydrolysis contains some amount of salts (buffer), it has no serious effect on this technique.

Washing the residue three times with distilled water and re-evaporating in acid hydrolysis removes partially HCl, and the pH of solution also is not far from neutral, so adding a few drops of dilluted NaOH for adujting the pH to 7-7.2 dose not make much salt in final solution.

In a sample of casein, the mean level of lysine was determined to be 7.53 (n = 7, S.D. = ± 1.07).

Recovery test:

To four samples of 15 mg casein 1,2,3 and 4 mg pure lysine were added. The reproducibility was ± 15% . The results of methionine show 37% difference in acid from enzyme hydrolysis. The reason for this big difference will be discussed in future paper.

All the samples were sent for analysing by amino acid analyser (Tropical Products Institute, 56-62 Gray's Inn Rd.London), the results on the casein did not show more than ± 15% differences.

Figure 2 Standard Curve of Lysine

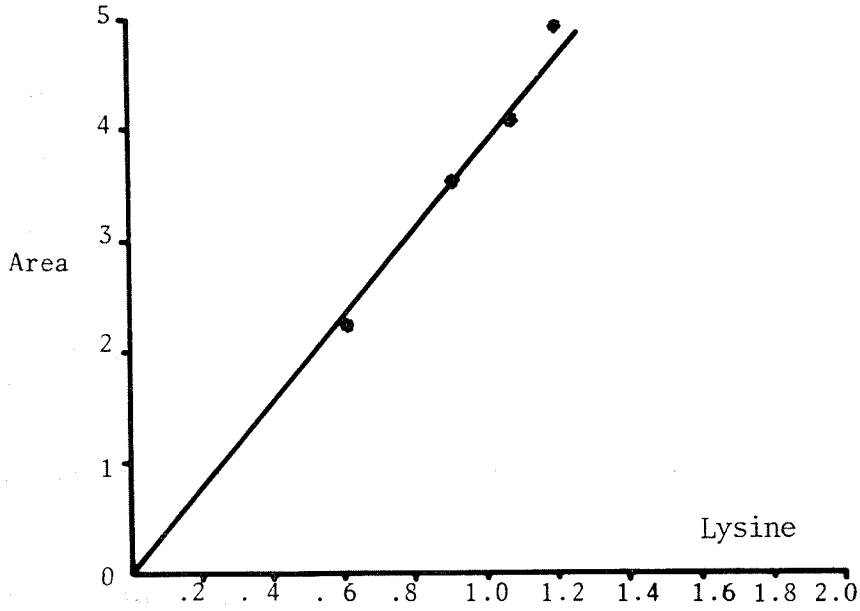
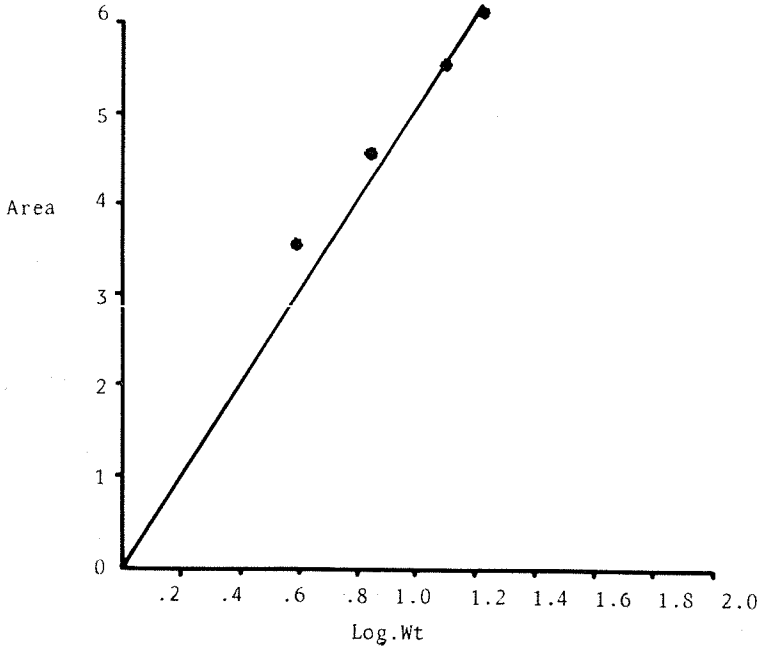


Figure 3 Standard Curve of Methionine



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Received : Mey 1980