CHROM. 4182

AN ELECTROPHORETIC METHOD FOR THE DETERMINATION OF 'BOUND' ε-AMINOLYSINE IN PROTEINS

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(Received May 8th, 1969)

SUMMARY

A simple method is described, using high-voltage electrophoresis, for the estimation of lysine in proteins in which the ε-amino groups are protected by either hydrolytically unstable bonds or by location in the ordered regions of insoluble proteins. The method is shown to give results which are in good agreement with those previously obtained by other techniques. Examples are given of the uses of this method in the quantitative determination of the formation of hydrolytically unstable bonds with the ε-amino group of lysine, during protein modification.

INTRODUCTION

The amount of 'available' lysine in proteins (*i.e.*, lysine in which the ε-amino group is free) is of importance in nutritional studies. Various methods have been developed to determine this quantity[1,2], mainly based on reactions of the intact protein with reagents which form derivatives of the ε-amino group of lysine which are stable to hydrolytic conditions. Many of these methods are based on the reaction of 1-fluoro-2,4-dinitrobenzene (FDNB) with the protein[1,3,4]. These usually rely on the column separation and subsequent estimation of the ε-dinitrophenyllysine from the protein hydrolysates. Some direct spectrophotometric methods have been devised, a very recent one used 2,4,6-trinitrobenzenesulphonic acid as the reagent[5].

At the same time, with structural proteins 'bound' lysine (*i.e.*, lysine in which the ε-amino group is not available for reaction with FDNB) is of some interest. Usually this quantity is determined by difference using a FDNB method.

Such techniques as chromatography by nylon powder columns[6,7] require specialist knowledge and equipment. Also, in order to determine the 'bound' lysine, two determinations must be made, *i.e.*, (a) total lysine in the protein, and (b) 'available' lysine, whereas a direct method for the determination of this quantity would give the result by one estimation.

The present paper describes a method for the direct estimation of 'bound' lysine in native and modified proteins. The procedure is based on the estimation, by high voltage electrophoresis, of lysine present in DNP-protein hydrolysates. Electro-

phoretic techniques, though possibly not as accurate as ion-exchange methods for the determination of lysine, have the advantages of rapidity and ease of determination.

EXPERIMENTAL

Materials

Australian Merino 64's and Lincoln wools were cleaned by solvent extraction and after being rinsed in distilled water were dried to constant weight and conditioned *in vacuo* at room temperature.

The silk fibroin used was prepared by the method of DRUCKER *et al.*[8].

Wheat gluten, insulin (bovine) and lysozyme (egg white) were obtained from British Drug Houses Ltd.

N^ϵ-DNP-lysine was prepared by the method of PORTER AND SANGER[9].

α-, β- and γ-keratoses were prepared from purified Australian Merino wool according to the method of ASQUITH AND PARKINSON[10], using performic acid as the oxidising agent.

Methods

Wool keratin and silk fibroin were dinitrophenylated according to the procedure of MIDDLEBROOK[11]. The DNP-proteins were washed with distilled water and extracted with acetone and then conditioned in a desiccator. DNP-derivatives of wheat gluten and β-keratose were prepared in the same way. DNP-insulin, lysozyme and α- and γ-keratoses were prepared by the method of SANGER[12].

Hydrolysis procedure. All the proteins were hydrolysed with 6 N hydrochloric acid at 110° in sealed tubes for 24 h. The hydrolysates were then cooled and freeze-dried, and the hydrochloric acid was removed by repeated freeze-drying with distilled water.

Electrophoretic estimation of 'bound' lysine in DNP-protein hydrolysates. A band of hydrolysate (100 μl) was applied to a strip (100 cm × 15 cm) of Whatman No. 3MM chromatography paper. Another band (50 μl) of a standard solution of L-lysine was applied alongside that of the DNP-protein hydrolysate. Electrophoresis was carried out according to the method of ATFIELD AND MORRIS[13] in a buffer of pH 5.2 (pyridine–acetic acid–water, 41:18:1941) at 75 V/cm for 1.5 h at 10°. The paper was dried, dipped in a cadmium–ninhydrin solution[14] and allowed to develop for 24 h in an ammonia-free atmosphere in the dark. After development the areas corresponding to the lysine in the hydrolysate and the standard were cut out, and the colour eluted with methanol (10 ml). Then the optical density was measured at 500 nm and the concentration of the lysine determined.

Treatments of modified proteins

Dry heating. Merino wool (100 mg) was heated in a sealed tube; internal volume 12 ml, for 48 h at 140°. The samples were dinitrophenylated and hydrolysed as described.

Setting of wool fibres. Bundles of Lincoln wool fibres (100 mg) were stretched and set on setting frames as originally described by SPEAKMAN[15] using a borax buffer

solution of pH 9.2 as used by ZIEGLER[16]. The set fibres were then treated with FDNB and hydrolysed by the method previously described.

Reactive dye on wool fibre. The dyeings were carried out using 3% on weight of fibre Levafix Brilliant Red E-2B at pH 4.0 under reflux at the boil for 90 min. The dyed samples were then rinsed with water and 'washed off' at the boil for 20 min in 1 g/l Lissapol N. Finally the dried and conditioned samples were treated with FDNB and hydrolysed as before.

RESULTS AND DISCUSSION

The estimation of lysine by high-voltage electrophoresis on a series of proteins shows that the method is reproducible and gives results which are in good agreement with those of previous authors, who used other techniques (Table I).

It can be seen from Table II that if N^ε-DNP-L-lysine is subjected to acid hydrolysis it does not decompose to give free lysine. Based on these findings a relatively easy method for the determination of 'bound' lysine in proteins can be evolved.

Within a protein the ε-amino group of lysine may be in any of the following states with respect to its possible reaction with FDNB: (a) Free or 'available', the product of reaction giving on hydrolysis N^ε-DNP-L-lysine. (b) Chemically bound in

TABLE I

ESTIMATION OF TOTAL, 'BOUND' AND % REACTED ε-AMINOLYSINE GROUPS IN VARIOUS PROTEINS BY HIGH-VOLTAGE ELECTROPHORESIS AND COMPARISON WITH COLUMN METHODS FROM THE LITERATURE

Values are given in μmoles/g.

Sample	Total lysine			'Bound' lysine	% reacted lysine		
	HVE	Column	Ref.	HVE	HVE	Column	Ref.
Wool Merino	230.0	233.0	6	48.0	79.2	80.0	3
Wool Lincoln	222.0	220.0	6	46.0	79.3	80.0	3
Silk fibroin	41.0	38.0	22	8.0	80.6	79.0	19
α-Keratose	264.0	250.0	23	29.0	89.0	—	
β-Keratose	370.0	358.0	23	164.0	55.6	—	
γ-Keratose	56.7	53.6	23	8.3	89.0	—	
Insulin	181.0	174.0	24	4.0	97.8	—	
Lysozyme	446.0	419.0	25	16.8	96.2	—	
Wheat gluten	85.0	90.0	26	20.3	76.0	—	

some way, the bond being hydrolytically stable, *e.g.*, lysinoalanine and returned on hydrolysis of the protein as the amino acid derivative. (c) Chemically bound as in amide cross-links and hence unavailable for reaction with FDNB. The amide bonds are, however, hydrolytically unstable and so on hydrolysis of the protein give free lysine. (d) In insoluble proteins the amino acid may be sterically blocked, in the ordered regions of the molecule, and so the FDNB cannot penetrate and hence react, again on hydrolysis lysine is returned. (e) Following the reaction of FDNB with the protein, as well as reacting with the ε-amino groups of lysine reaction may occur with

TABLE II

AMOUNT OF LYSINE RECOVERED ON HYDROLYSIS OF N^{ε}-DNP-L-LYSINE

Hydrolysis with 6 N HCl in sealed tubes at 110°. Values are given in μmoles/g.

	Time of hydrolysis of N_{ε}-DNP-L-lysine (h)			
	12	24	36	48
Lysine recovered	0.00	0.00	0.00	trace

the carboxylic groups of aspartic and glutamic acids. As a result of this DNP-esters may be formed which can act as acylating agents for any lysine groups in the same region. Such a formation of ω-ε-peptide bonds would decrease the possible number of amino groups of lysine available for dinitrophenylation[17,18]. Again on hydrolysis any unreacted ε-amino groups would be returned as free lysine. This state (e), is probably very important in the simpler proteins as the amount of 'bound' lysine in these is very small.

Total lysine in a protein and stable, chemically bonded lysine can therefore be estimated directly as the original amino acid, or in the latter case, its chemically bonded product lysinoalanine. However, lysine groups which are hydrolytically unstably bonded, e.g. with reactive dyes or with amide cross-links, cannot be so detected. Previous methods have relied upon estimating in these cases the 'available' lysine as the DNP-derivative and subtracting this figure from the total lysine content of the protein. The present method is a system for determining the 'bound' lysine content directly.

Table I shows the amount of lysine in a series of proteins, which is recovered as lysine on hydrolysis of the DNP-proteins. It can be seen that with the soluble proteins only a small amount of lysine remains unreacted and this apparent incomplete reaction may be due to the formation of DNP-esters as previously explained.

With the structural proteins, however, a considerable proportion of the total lysine remains unreacted with the FDNB and hence on subsequent hydrolysis this amount of lysine can be estimated by means of high-voltage electrophoresis. In cases where this 'bound' lysine or non-reactive lysine has been found by difference by other workers, the amounts are similar to the results obtained by this technique.

Thus for wool keratin[3] and silk fibroin[19] ZAHN has shown that 20% and 21% respectively of the ε-amino groups of lysine were unavailable for reaction with FDNB. The states of the ε-amino groups of proteins with respect to their reaction to FDNB has already been discussed. The method described cannot differentiate between states (c) and (d) in native proteins. When, however, the protein is modified or denatured in some way which yields new cross-links of type (c), an increase in the amount of 'bound' lysine would be reflected in an increase in the free amino acid after dinitrophenylation and hydrolysis. Hence deduction as to the degree of the reaction which has occurred may be made.

Three examples of the use of this method are given in Table III. Firstly, it can be seen that with set wool there is no increase in the unbound lysine. This is in agreement with previous findings[20]. It was shown[21] that on setting wool in a borax solution 26% of the available lysine was rendered inert to reaction with FDNB.

TABLE III

ESTIMATION OF 'BOUND' LYSINE IN WOOL KERATIN AFTER VARIOUS TREATMENTS
Values are given in μmoles/g.

Sample	Total lysine in protein	Lysine on hydrolysis after treatment	'Bound' lysine after hydrolysis of treated DNP sample
Wool Merino, control	230.0	—	48.0
Set wool 40% stretch, 0.1 M borax	222.0	222.0	53.0
Dyed wool, Levafix E-2B	230.0	—	72.0
Heated wool, 140° for 48 h	230.0	204.0	87.5

Whilst, later ZIEGLER[16] showed that 23.8% of lysine was converted to lysinoalanine under the same conditions. Consequently little or no possible alternative reaction could occur. Secondly, when treating wool with reactive dyes it is shown that after hydrolysis of the DNP-protein more of the lysine is returned as the free amino acid, indicating that whilst the reactive dye has attached itself to the ε-amino group of lysine the resultant bond is one which is hydrolytically unstable. This is shown in the increase of free amino acid after hydrolysis. Finally it can be seen that when wool is heated it seems to undergo some reaction in which the ε-amino groups of lysine become more inert to the reaction with FDNB. Possibly the ε-amino groups of lysine condense with nearby glutamic or aspartic residues to form amide cross-links. Thus after dinitrophenylation and hydrolysis the amount of lysine returned is far greater than in unheated wool.

This method therefore is valuable for determining the amount of 'bound' lysine in native proteins and the amount and possible nature of any ε-amino groups of lysine in which this group has become bound by a hydrolytically unstable link during treatment. It has the advantages of direct estimation, rapidity, and requires little starting material for analysis.

ACKNOWLEDGEMENT

REFERENCES

1 K. J. CARPENTER, Biochem. J., 77 (1960) 604.
2 S. R. RAO, F. L. CARTER AND V.L. FRAMPTON, Anal. Chem., 35 (1963) 1927.
3 E. SIEPMANN AND H. ZAHN, 3e Congr. Intern. Rech. Textile Lainiere, Paris, Vol. 1, 1965, p. 303.
4 B. REXEN AND B. CHRISTENSEN, European Technicon Symp. "Automation in Analytical Chemistry," Paris, 1966.
5 M. L. KAKADE AND I. E. LIENER, Anal. Biochem., 27 (1969) 273.
6 H. BEYER AND U. SCHENK, J. Chromatog., 39 (1969) 482.
7 H. BEYER AND U. SCHENK, J. Chromatog., 39 (1969) 491.

8 B. DRUCKER, R. HAINSWORTH AND S. G. SMITH, *Shirley Inst. Mem.*, 26 (1953) 196.
9 R. R. PORTER AND F. SANGER, *Biochem. J.*, 42 (1948) 287.
10 R. S. ASQUITH AND D. C. PARKINSON, *Textile Res., J.*, 36 (1966) 1064.
11 W. R. MIDDLEBROOK, *Biochim. Biophys. Acta*, 7 (1951) 547.
12 F. SANGER, *Biochem. J.*, 39 (1945) 507.
13 G. N. ATFIELD AND C. J. O. R. MORRIS, *Biochem. J.*, 81 (1961) 606.
14 J. HEILMAN, J. BAROLLIER AND E. WATZKE, *Z. Physiol. Chem.*, 309 (1957) 219.
15 J. B. SPEAKMAN AND C. S. WHEWELL, *J. Soc. Dyers Colourists*, 52 (1936) 335.
16 KL. ZIEGLER, *J. Biol. Chem.*, 239 (1964) PC2713.
17 D. HEIKENS, P. H. HERMANS AND P. F. VAN VELDEN, *Nature*, 174 (1954) 1187.
18 H. BEYER AND H. ZAHN, *Textile Res. J.*, 38 (1968) 1069.
19 H. ZAHN AND H. ZUBER, *Textil-Rundschau*, 9 (1954) 119.
20 R. S. ASQUITH AND J. B. SPEAKMAN, *Proc. Intern. Wool Textile Conf. Australia, 1955*, C 302.
21 R. S. ASQUITH AND J. B. SPEAKMAN, *Nature*, 170 (1952) 798.
22 W. A. SCHROEDER AND L. M. KAY, *J. Am. Chem. Soc.*, 77 (1955) 3908.
23 M. C. CORFIELD, A. ROBSON AND B. SKINNER, *Biochem. J.*, 68 (1958) 348.
24 A. P. RYLE, F. SANGER, L. F. SMITH AND R. KITAI, *Biochem. J.*, 60 (1955) 541.
25 R. E. CANFIELD AND C. B. ANFINSON, in H. NEURATH (Editor), *The Proteins*, Vol. 1, 2nd Ed., Academic Press, New York, 1963, Chap. 4.
26 J. H. WOYCHIK, J. A. BUNDY AND R. J. DIMLER, *J. Agr. Food Chem.*, 9 (1961) 307.