CHROM. 5527

DETERMINATION OF DINITROPHENYLENE AMINO ACIDS IN STRUCTURAL PROTEINS BY CHROMATOGRAPHY ON NYLON POWDER COLUMNS*

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(First received April 13th, 1971; revised manuscript received June 28th, 1971)

SUMMARY

A procedure is described for the quantitative determination of the water-soluble dinitrophenylene (DPE) amino acids in structural proteins by column chromatography using nylon powder as adsorbent. Native and reduced wool were treated with 1,5-difluoro-2,4-dinitrobenzene (FFDNB), hydrolysed and chromatographed. Six out of ten peaks (S,N_ε-DPE-cysteine-lysine, N_ε-FDNP-lysine, O,O'-DPE-bis-tyrosine, N_ε,O-DPE-lysine-tyrosine, N_ε,N_ε'-DPE-bis-lysine, N_ε-OHDNP-lysine) of the native wool and six of thirteen peaks (S,S'-DPE-bis-cysteine, S,N_ε-DPE-cysteine-lysine, N_ε-FDNP-lysine, O,O'-DPE-bis-tyrosine, S,O-DPE-cysteine-tyrosine, N_ε-OHDNP-lysine) of the reduced wool were estimated quantitatively. The estimation was carried out at 30° and a flow rate of 12 ml/h using 0.01 *N* hydrochloric acid as eluent.

INTRODUCTION

The classical studies of SANGER[1] using 1-fluoro-2,4-dinitrobenzene (FDNB) for the estimation of N-terminal amino acids in proteins showed that lysyl and tyrosyl residues are involved in the dinitrophenylation procedure. It was concluded from these results that the bifunctional 1,5-difluoro-2,4-dinitrobenzene (FFDNB), first synthesized by SWARTS[2], would react in a similar manner, and accordingly it was introduced into protein chemistry as a cross-linking reagent[3,4].

In order to apply a cross-linking reagent in protein chemistry and to study the amount of cross-linked amino acids produced, the following conditions must be met: (1) the two reactive groups of the reagent and the functional side-chain groups of the protein must form a covalent bond, which should remain stable during acid hydrolysis; (2) the cross-linked amino acids must be detectable in the hydrolysate by analytical methods; (3) the cross-linked amino acids must be separable from the free amino acids.

* 12th Communication on the reactivity of the side chain of amino acids (11th communication, K. ZIEGLER, *J. Appl. Polym. Sci.*, in press).

The 2,4-dinitrophenylene (DPE) amino acids were almost completely stable during hydrolysis. Their detection and separation from the free amino acids were achieved by chromatography on nylon powder columns.

In the past, many bifunctional reagents have been synthesized and used in various studies with soluble and insoluble proteins[5]. FFDNB is one of the most commonly used bifunctional reagents, and its application to studies with insulin[6], ribonuclease[7,8] and synthetic polypeptides[9] has shown that it is useful for the clarification of the structural aspects of proteins in solution. Therefore, the development of methods for the quantitative estimation of DPE-amino acids is required.

An automatic method of analysis for the estimation of DPE and dinitrophenyl (DNP) derivatives of lysine and tyrosine has been described[10]. However, since a column chromatogram of DPE-wool is more complicated, a selective adsorbent is essential for good resolution.

For the estimation of DNP-amino acids in α-keratins, exact and reproducible values have been obtained by the use of nylon powder as adsorbent prepared as described in ref. 11. The same adsorbent was therefore used for the determination of DPE-amino acids in native and reduced wool. The present paper describes the use of nylon powder columns for the determination of the water-soluble DPE-amino acids in merino wool, lincoln wool and South African mohair.

EXPERIMENTAL

Chromatographic methods

The preparation of the nylon powder, the columns and the chromatographic procedure of this analytical method have been described in detail in a previous communication[11]. The column (90 × 1.5 cm) was maintained at 30° and filled with the nylon powder to a height of 80 cm. The eluent used was 0.01 *N* hydrochloric acid at a flow rate of 12 ml/h.

Structural proteins studied

For these investigations, three α-keratins of different mean diameter were used: merino wool (20.9 μm), lincoln wool (38.4 μm) and South African mohair (33.5 μm). All samples were washed isoionic prior to extraction in acetone.

Complete amino acids analysis was carried out by ion-exchange chromatography[12].

TABLE I

LYSINE, TYROSINE, CYSTEINE AND CYSTINE CONTENTS (μmoles/g) OF THE STRUCTURAL PROTEINS USED

Sample	*Amino acid*			
	Lys	*Tyr*	*Cys*	$(Cys)_2$
Wool, merino	233	287	27.5	458
Wool, lincoln	229	201	26.0	408
Mohair, South African	249	254	24.0	465

The cystine and cysteine contents were estimated by the polarographic method[13]. These values are summarized in Table I.

Control DPE-amino acids for column equilibration

For the identification and the quantitative estimation of peaks in the nylon powder chromatogram various DPE-derivatives were prepared, as listed in Table II

TABLE II

DPE-AMINO ACIDS AND DERIVATIVES SYNTHESIZED

Abbreviations: DPE = 2,4-dinitrophenylene; FDNP = 1-fluoro-2,4-dinitrophenyl; OHDNP = 1-hydroxy-2,4-dinitrophenyl.

Compound	*Abbreviation*	*Reference (which describes the synthesis)*	*Correction factor*
S,S'-2,4-Dinitrophenylene-bis-cysteine	S,S'-DPE-bis-cysteine	14	1.0
S,N_ε-2,4-Dinitrophenylene-cysteine-lysine	S,N_ε-DPE-cysteine-lysine	14	1.0
1-Fluoro-5-N_ε-lysyl-2,4-dinitrobenzene	N_ε-FDNP-lysine	6,9	1.035
O,O'-2,4-Dinitrophenylene-bis-tyrosine	O,O'-DPE-bis-tyrosine	6,9	1.06
N_ε,O-2,4-Dinitrophenylene-lysine-tyrosine	N_ε,O-DPE-lysine-tyrosine	6,9	1.0
S,O-2,4-Dinitrophenylene-cysteine-tyrosine	S,O-DPE-cysteine-tyrosine	14	1.0
N_ε,N_ε'-2,4-Dinitrophenylene-bis-lysine	N_ε,N_ε'-DPE-bis-lysine	6,9	1.23
1-Hydroxy-5-N_ε-lysyl-2,4-dinitrobenzene	N_ε-OHDNP-lysine	7	1.21

together with their reference numbers and correction factor sfor losses during hydrolysis.

Dinitrophenylenation of the samples

1 g of each sample was treated with a solution of 4 g of sodium bicarbonate in 25 ml of water and 2 g of FFDNB, dissolved in 50 ml of acetone at 40° over 48 h with constant stirring. The samples were then washed in water and extracted with acetone. 150 mg of the DPE-wool were hydrolysed in 30 ml of 6 *N* hydrochloric acid in a sealed tube for 16 h at 95°. The hydrolysate was then diluted, extracted with ether and brought to a volume of 10 ml. 1 ml of this hydrolysate was applied to the pre-equilibrated column. The chromatographic analysis was performed at 313 nm, 30° and at a flow rate of 12 ml/h.

Reduction of wool

Reduced wool was prepared by shaking 1 g of wool in 100 ml of 20% (v/v) *n*-propanol in the presence of 1.24 g of toluene-ω-thiol at pH 6.4 for 48 h (ref. 15).

The reduced wool was washed twice with 50 ml of 50% (v/v) *n*-propanol and

with 50 ml of acetone. The samples obtained were dinitrophenylated to estimate the degree of reduction via the estimation of S-DNP-cysteine, or treated with FFDNB as described above.

RESULTS

The water-soluble DPE-amino acids in native wool

The reaction between FFDNB and a protein appears to be more complicated than that between FDNB and a protein. If a second reactive side-chain group in the protein is inaccessible, FFDNB may also react monofunctionally and the remaining fluorine atom then reacts with the solvent in a second step. The ideal reaction gives rise to symmetrical bridges provided two of the same reactive groups are linked together, and to unsymmetrical bridges when two different groups are cross-linked. The synthesis of all possible reaction products between FFDNB and the side-chain groups of wool has not yet been completed.

Fig. 1 shows the elution profile of a hydrolysate of DPE-wool[16], revealing ten peaks. Six of these peaks could be identified and estimated quantitatively. The identification of the isolated derivatives was achieved by comparison of the R_F values

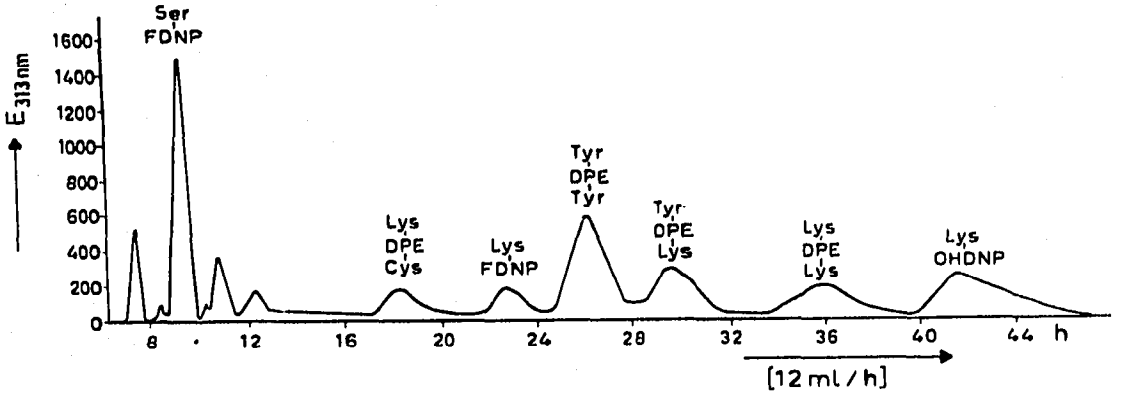

Fig. 1. Nylon powder chromatogram from the hydrolysate of native wool treated with FFDNB. ———, d = 1.0 cm.

and the UV and IR spectra with those of model compounds. From these data, the presence of S,N_ε-DPE-cysteine-lysine, N_ε-FDNP-lysine, O,O'-DPE-bis-tyrosine, N_ε,O-DPE-lysine-tyrosine, N_ε,N_ε'-DPE-bis-lysine and N_ε-OHDNP-lysine was established. The second peak was identified as O-FDNP-serine, but since no synthetic O-FDNP-serine was available, its quantitative estimation was not feasible. The three remaining peaks could not be identified. The values for DPE-merino wool, DPE-lincoln wool and DPE-mohair are given in Table III, corrected for losses during hydrolysis.

The water-soluble DPE-amino acids of reduced wool

When reduced wool is treated with FFDNB, new DPE-derivatives are observed. Since in reduced wool the sulphydryl group of the cysteinyl residues is the most reactive, the formation of S,S'-DPE-bis-cysteine predominates. The amount of the S,N_ε-DPE-cysteine-lysine is increased and in addition, S,O-DPE-cysteine-tyrosine is formed. On the other hand, formation of N_ε, N_ε'-DPE-bis-lysine and N_ε,O-DPE-

TABLE III

ESTIMATION OF THE WATER-SOLUBLE DPE-AMINO ACIDS OF DIFFERENT α-KERATINS (μmoles/g UNTREATED SAMPLE)

Sample	*Cys* | *DPE* | *Lys*	*Lys* | *FDNP*	*Tyr* | *DPE* | *Tyr*	*Tyr* | *DPE* | *Lys*	*Lys* | *DPE* | *Lys*	*Lys* | *OHDNP*
Wool, merino	3.5	47.7	40.3	24.5	8.7	40.5
Wool, lincoln	4.3	27.6	26.1	29.6	7.4	45.8
Mohair, South African	2.9	27.2	25.3	22.8	7.1	35.3

lysine-tyrosine was not observed. In addition to these six peaks, seven others were noted which could not be identified. In Fig. 2, the nylon powder chromatogram of reduced wool treated with FFDNB is shown[17]. Table IV gives the values for the derivatives, which are corrected for losses during hydrolysis.

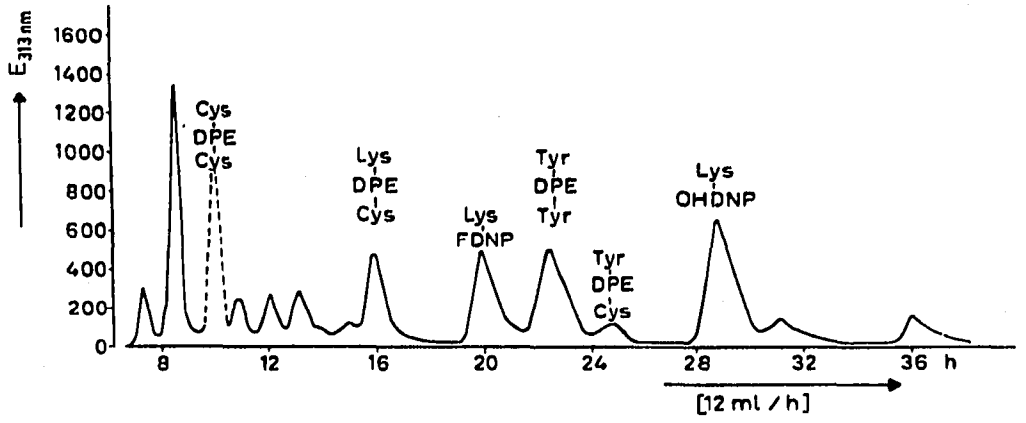

Fig. 2. Nylon powder chromatogram from the hydrolysate of reduced wool treated with FFDNB. — — —, d = 0.25 cm; ------, d = 1.0 cm.

TABLE IV

ESTIMATION OF THE WATER-SOLUBLE DPE-AMINO ACIDS FROM REDUCED MERINO WOOL (μmoles/g UNTREATED SAMPLE)

Sample	*Cys* | *DPE* | *Cys*	*Cys* | *DPE* | *Lys*	*Lys* | *FDNP*	*Tyr* | *DPE* | *Tyr*	*Cys* | *DPE* | *Tyr*	*Lys* | *OHDNP*
Wool, merino	215.0	18.9	96.6	10.7	8.0	33.8

DISCUSSION

FFDNB is one of the classical bifunctional reagents which reacts under mild conditions with N-terminal and side-chain amino acids in proteins to form covalent

bonds. On the other hand, the reactivity of FFDNB is so high that it reacts not only with cysteinyl, lysyl and tyrosyl residues in keratin fibres but also with histidyl and seryl residues. The identification of the seryl and histidyl derivatives was not achieved, since the synthesis of the respective model compounds has not yet been successful.

If the reaction between wool and FFDNB is considered from a quantitative point of view, it is necessary to differentiate between the preferential formation of bridged amino acids, given at a low ratio of wool to FFDNB, and maximum reaction of the side-chain amino acids, given at a high ratio of wool to FFDNB. In this investigation, the results estimated for four lysine derivatives in native wool showed that only 57% of the lysyl residues, and in reduced wool only 50% of the cysteinyl residues, had reacted whereas the estimation of S-DNP-cysteine showed a rate of reduction of 97%. An overall quantitative analysis is thus made difficult not only by the large number of possible DPE-derivatives but also by possible side-reactions[18]. Therefore, further studies of the reaction between α-keratins and FFDNB are required to identify the unknown peaks of the chromatogram.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. H. ZAHN for many helpful discussions. Thanks are also due to Dipl.-Chem. H. KNITTEL for the cystine and cysteine estimations. The work was supported by grants from the Arbeitsgemeinschaft Industrieller Forschungsvereinigungen (AIF) and the Forschungskuratorium Gesamttextil and the Landesamt für Forschung of Land Nordrhein-Westfalen.

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