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DETERMINATION OF DINITROPHENYLAMINO ACIDS IN STRUCTURAL PROTEINS BY CHROMATOGRAPHY ON NYLON POWDER COLUMNS

II. THE ETHER-SOLUBLE DINITROPHENYLAMINO ACIDS

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SUMMARY

A procedure is described for the quantitative determination of the ether-soluble dinitrophenyl (DNP)-amino acids in structural protein by column chromatography using nylon powder as adsorbent. The DNP-derivatives of the amino acids: aspartic acid, glutamic acid, serine, threonine, glycine, alanine and valine were estimated quantitatively on such columns by eluting with phosphate buffer, pH 8.0, at 60° and at a flow rate of 30 ml per hour. In addition, DNP-cysteic acid, the oxidation product of N,N'-bis-DNP-cystine, was estimated with citrate buffer, pH 3.0, at 30° at a flow rate of 30 ml per hour.

INTRODUCTION

The classical reagent for estimating N-terminal and side-chain amino acids in proteins is SANGER's 1-fluoro-2,4-dinitrobenzene, FDNB[1]. Studies using other reagents have been reported[2–4]. However, most of the work concerning the estimation of N-terminal amino acids in structural proteins has been done with FDNB.

Seven N-terminal DNP-amino acids, DNP-aspartic acid, DNP-glutamic acid, DNP-serine, DNP-threonine, DNP-glycine, DNP-alanine and DNP-valine have been detected in a hydrolysate of dinitrophenylated wool[5–10]. The corresponding amino acids are present in the keratin with a free α-amino group and were therefore designated as the N-terminal amino acids in wool. In a more recent investigation by THOMPSON[11] it has been shown that in addition to these seven amino acids, cystine was also present as an N-terminal amino acid in α-keratins. DNP-cysteic acid, the oxidation product of N,N'-bis-DNP-cystine, belongs to the water-soluble DNP-amino acids, but as it was found as an additional N-terminal amino acid it should be mentioned here.

A method is now described for the quantitative determination of ether-soluble DNP-amino acids in structural proteins using nylon powder as adsorbent, and the corrected values for the amounts of the eight N-terminal amino acids present in the series of wools and silk fibroin tested are reported. The accuracy of the method was also investigated.

EXPERIMENTAL

Structural proteins similar to those described in part I of this series were used for these investigations. The conditions and apparatus used are also as described in Part I. Only changes in the details given in Part I will be mentioned here.

Preparation of the nylon powder columns

The filling of the column was done at 60° and the column was then eluted twice with phosphate buffer, pH 8.0. The buffer was prepared from 2.7 g KH_2PO_4 and 55 g $Na_2HPO_4 \cdot 2\ H_2O$. This was made up to a volume of 5 l with distilled water.

Control DNP-amino acids for equilibrating the columns

The synthesis of the seven DNP-amino acids was carried out according to RAO AND SOBER[12]. DNP-cysteic acid was synthesised as reported by BETTELHEIM[13]. Standard solutions of these DNP-amino acids were prepared by dissolving in phosphate buffer, pH 8.0. DNP-cysteic acid was dissolved in citrate buffer, pH 3.0.

Dinitrophenylation of the sample

Dinitrophenylation was performed as described previously. 500 mg of the dinitrophenylated sample, dried for 2 h at 105°, were hydrolysed with 100 ml 6 *N* hydrochloric acid in a sealed tube for 16 h at 95°. The hydrolysate was diluted with 100 ml water and extracted four times with 100 ml ether. The combined ether extracts were washed with 100 ml 0.01 *N* hydrochloric acid and evaporated to dryness. After this, dinitrophenol was removed by vacuum sublimation[14] because dinitrophenol complicates the estimation of DNP-valine. The residue was dissolved in 1 ml acetone and diluted with 50 ml ether. This solution was extracted six times with 5 ml 0.1 *M* sodium bicarbonate solution. This was then neutralised with 0.75 ml of a 3 *M* phosphoric acid solution and evaporated to dryness. The resulting residue was dissolved in buffer, pH 8.0, and made up to a volume of 10 ml with this buffer. In the case of silk fibroin 1 g DNP-silk fibroin was hydrolysed, and after working up the hydrolysate as described above, the residue was made up to a volume of 5 ml with buffer, pH 8.0.

For quantitative estimations it was necessary to determine the losses of each DNP-amino acid during hydrolysis. This was done as described previously and the factors for correction were found to be:

Amino acid	DNP-Asp	DNP-Glu	DNP-Ser	DNP-Thr	DNP-Gly	DNP-Ala	DNP-Val	DNP-$CySO_3H$
Correction factor	1.09	1.10	1.12	1.02	1.26	1.05	1.30	1.48

Chromatography of the ether-soluble DNP-amino acids

The chromatographic analysis of 2 ml of the hydrolysates of the DNP-keratins was performed at 366 nm, pH 8.0 and a flow rate of 30 ml per h. For the estimation of the ether-soluble DNP-amino acids in silk fibroin 2 ml portions which corresponded to 400 mg DNP-silk fibroin were applied on the column. It was found necessary to regenerate the nylon powder after each run.

Chromatography of DNP-cysteic acid

The DNP-wool had to be oxidised with performic acid[11] for the estimation of

DNP-cysteic acid. The performic acid solution was prepared by mixing of 27 ml 98–100 % formic acid and 3 ml 35 % hydrogen peroxide. This solution was allowed to stand for 1 h at room temperature. After cooling to 0° 1 g DNP-wool was added. The oxidation process was carried out at 0° for 1 h giving a 90 % conversion of cystine to cysteic acid. The reaction mixture was dialysed, concentrated by means of a rotary evaporator (20°) and lyophilsied. Then 1 g oxidised DNP-wool was hydrolysed with 200 ml 6 *N* hydrochloric acid, extracted with ether and the water-soluble part evaporated to dryness. The residue was dissolved in citrate buffer, pH 3.0, and made up to a volume of 10 ml. 1 ml of this solution was chromatographed on a column (20 × 1 cm). The total length of the filled column was 13 cm. The chromatographic analysis was

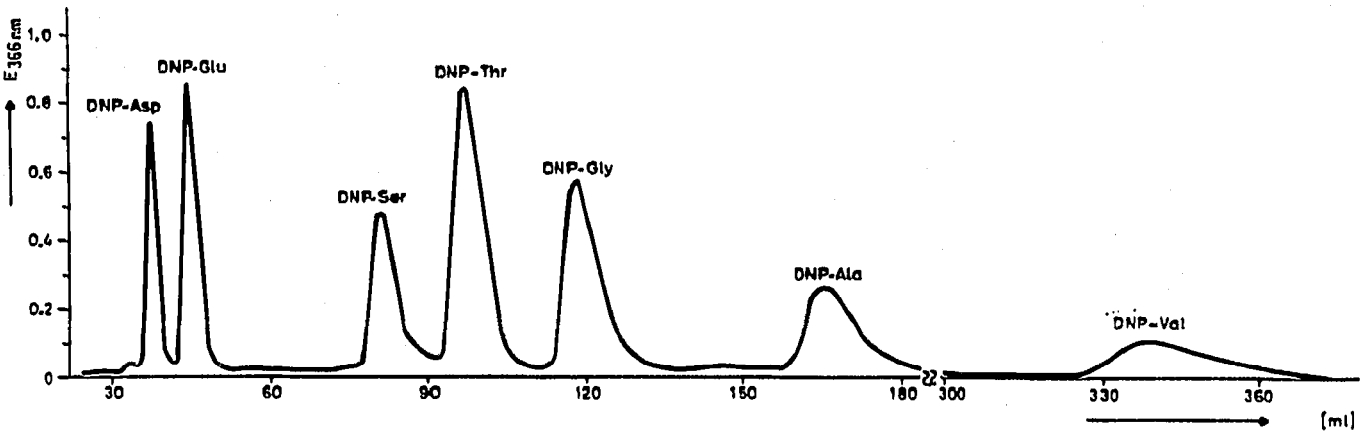

Fig. 1. Nylon powder chromatogram of the ether-soluble DNP-amino acids in the hydrolysate of DNP-wool.

performed at 366 nm, 30° and at a flow rate of 30 ml per h. After 300 min DNP-cysteic acid was eluted from the column.

RESULTS

The ether-soluble DNP-amino acids of α-keratins

Fig. 1 shows the chromatogram of the ether-soluble part of a hydrolysate of DNP-wool containing seven peaks. The positions of the peaks were identified by STEUERLE AND HILLE[10]. The position of the water-soluble DNP-cysteic acid, determined at 366 nm with citrate buffer, pH 3.0, on a short column corresponded to its position on a long column mentioned in part I. The values given in Table I are the average of four estimations together with their standard deviations. They have been corrected for losses during hydrolysis.

The ether-soluble DNP-amino acids of silk fibroin

Silk fibroin was prepared according to DRUCKER *et al.*[15]. The same N-terminal amino acids as in wool were found in this sample and the corresponding results are summarised in Table II. The values are the average of four estimations together with their standard deviations, and have been corrected for losses during hydrolysis.

TABLE I

ESTIMATION OF THE N-TERMINAL DNP-AMINO ACIDS OF DIFFERENT α-KERATINS

Values are given in μmole/g untreated sample.

Sample	*DNP-Asp*	*DNP-Glu*	*DNP-Ser*	*DNP-Thr*	*DNP-Gly*	*DNP-Ala*	*DNP-Val*	*DNP-$CySO_3H$*
Wool, merino	0.34 ± 0.02	0.68 ± 0.015	1.22 ± 0.035	3.20 ± 0.065	1.13 ± 0.035	0.67 ± 0.035	1.23 ± 0.10	3.07 ± 0.06
Wool, lincoln	0.64 ± 0.035	1.62 ± 0.09	1.72 ± 0.05	2.98 ± 0.035	3.22 ± 0.035	1.05 ± 0.02	2.60 ± 0.17	4.76 ± 0.09
Wool, New Zealand	0.39 ± 0.06	1.07 ± 0.07	1.24 ± 0.05	2.37 ± 0.06	1.51 ± 0.015	0.80 ± 0.04	1.79 ± 0.09	3.19 ± 0.07
Wool, German land merino	0.43 ± 0.003	0.87 ± 0.025	1.37 ± 0.15	2.90 ± 0.05	1.72 ± 0.19	0.72 ± 0.05	1.41 ± 0.04	3.32 ± 0.08
Wool, merino SW 293	0.32 ± 0.004	0.63 ± 0.01	1.60 ± 0.025	3.10 ± 0.02	1.62 ± 0.045	0.67 ± 0.035	0.93 ± 0.06	3.17 ± 0.06
Wool, lincoln SW 308	0.36 ± 0.015	0.64 ± 0.03	0.98 ± 0.035	2.04 ± 0.045	1.12 ± 0.04	0.71 ± 0.015	0.91 ± 0.03	3.13 ± 0.055
Wool, corriedale SW 295	0.19 ± 0.03	0.28 ± 0.025	1.41 ± 0.035	2.34 ± 0.10	1.15 ± 0.10	0.46 ± 0.015	0.44 ± 0.04	2.64 ± 0.075
Mohair, South Africa	0.35 ± 0.015	0.63 ± 0.02	0.80 ± 0.055	2.95 ± 0.13	0.70 ± 0.03	0.64 ± 0.055	1.84 ± 0.08	2.43 ± 0.095
Human hair	0.27 ± 0.02	0.31 ± 0.003	0.55 ± 0.02	1.67 ± 0.04	0.63 ± 0.025	0.30 ± 0.03	3.84 ± 0.13	1.19 ± 0.06

TABLE II

ESTIMATION OF THE N-TERMINAL DNP-AMINO ACIDS IN SILK FIBROIN

Values are given in μmole/g untreated silk fibroin.

Sample	*DNP-Asp*	*DNP-Glu*	*DNP-Ser*	*DNP-Thr*	*DNP-Gly*	*DNP-Ala*	*DNP-Val*
Silk fibroin	1.02 ± 0.03	0.095 ± 0.004	1.23 ± 0.02	0.11 ± 0.03	0.77 ± 0.02	1.02 ± 0.01	0.37 ± 0.015

DISCUSSION

These investigations were performed in order to estimate the known end groups in structural proteins; N-terminal cystine was also included in these investigations. The instability of N,N'-bis-DNP-cystine to acid hydrolysis has been shown[17], therefore, DNP-wool was oxidised and the resulting DNP-cysteic acid estimated[11]. The qualitative results obtained here on α-keratins agreed with those of previous workers, but it is not possible to compare the quantitative results obtained in this paper with those published earlier. This is because some results published earlier were not corrected for losses during hydrolysis; others have been corrected, but discrepancies occurred because these factors for losses during hydrolysis varied. Another factor, as shown in this paper, was that the amount of the N-terminal residues differed from one type of wool to another. In view of the known variations in crystallinity, the difference in origin and growth, and other factors, these differences are not surprising. A more detailed description of the reaction of FDNB with wool will be published later. The existence of N-terminal amino acids in silk fibroin was not surprising[18].

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