QUANTITATIVE DETERMINATION OF N-TERMINAL AMINO ACIDS IN SOME SERUM PROTEINS

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SUMMARY

A method for quantitative determination of N-terminal amino acids of proteins is described. Results obtained from different serum protein fractions are given and compared with published data.

INTRODUCTION

During a chemical study of different serum proteins it was found desirable to have a reliable method for the quantitative determination of their N-terminal amino acids. Hitherto, the DNP-method of SANGER[1] has been generally used[2-7]. Although excellent results have been obtained by SANGER's technique, it has the disadvantage of giving low recoveries because of losses of the DNP-amino acids during acid hydrolysis. EDMAN's thiohydantoin method[8], however, has proved satisfactory for the sequence determination of peptides, but this method has been infrequently used with higher molecular weight proteins, because of the reported low recoveries of N-terminal amino acids. The losses depend partly on destruction of the PTH's during the cyclization step, and also on difficulties with the determination of the amino acids released.

This paper reports an adaptation of the EDMAN technique for various serum proteins and has so far given quantitative recoveries of the N-terminal amino acids. The final quantitative determination of the PTH's has been performed using the paper chromatographic technique recently described by SJÖQUIST[9].

MATERIAL AND METHODS

Human serum albumin was prepared according to a modified COHN method 10. It moved as a homogeneous peak in free electrophoresis. Nitrogen content: 15.0 %.

Human γ-globulin (COHN fraction II) was prepared according to the modified procedure of DEUTSCH *et al.*[10]. It contained about 2 % each of albumin and β-globulin. Nitrogen content: 15.5 %. The γ-globulin was used without further purification.

Porcine γ-globulin (COHN fraction II) was a commercially available preparation

Abbreviations: DNP, dinitrophenyl-; PTH, phenyl thiohydantoin-; PTC, phenyl thiocarbamyl-.

from Pentex Inc., Kankatee, Ill. (U.S.A.), which was homogeneous by paper electrophoresis. Nitrogen content: 15.5 %.

Bovine prothrombin was prepared essentially according to the method of SEEGERS[11] and further purified by chromatography on Amberlite XE 64 according to the method of MILLER[12]. Nitrogen content: 13.3 %.

Human β_1-iron binding globulin (transferrin) was prepared by a modification of COHN fraction IV:7 and further purified by chromatography on DEAE-cellulose[13]. The preparation was homogeneous by starch gel electrophoresis[13]. Nitrogen content: 14.4 %.

Methylethyl ketone (Merck *p.a.*) was refluxed with $KMnO_4$ and distilled.

Pyridine (Merck *p.a.*) was refluxed over phthalic acid anhydride and distilled.

Other reagents used in the present investigation were purified as described in a previous paper[14].

Nitrogen determinations were performed using a micro Kjeldahl method.

Extinction readings were performed in a Beckman DU spectrophotometer.

Assay of N-terminal amino acids

Thick walled Pyrex tubes (length 13 cm, outer diameter 2.5 cm, provided with a male joint B29) designed to fit a lyophilizer were used at all times. Washings and other mixing operations were achieved with the aid of a Teflon coated magnet which was left in the tube through the whole experiment.

The protein sample (5–20 mg depending on molecular weight and computed amount of N-terminal amino acids) was dissolved in 1 ml saline and 2 ml of a solution of pyridine–triethylamine–phenyl isothiocyanate (100:3:1) was added. After mixing, the tube was placed in a water bath at 40° for 1.5 h. The solution, now containing the protein as PTC derivative, was thoroughly washed five times with 5-ml aliquots of benzene–ethylene chloride (3:1) previously saturated with 0.1 *N* NaOH. After centrifugation the benzene–ethylene chloride phase was discarded. The tube was subsequently placed at 40° in a gentle stream of N_2 to remove the last traces of organic solvent. In order to split off the N-terminals from the PTC-protein, 1 ml water and 2 ml hydrogen chloride saturated acetic acid were added with thorough mixing and the tube was kept at 40° for 2 h. Subsequently, the sample was lyophilized. Lyophilization was interrupted before the sample was completely dry, thereby preventing the protein's adherence to the glass walls. The residue was suspended in 2 ml water previously saturated with ethyl acetate–methylethyl ketone (2:1). The PTH's were extracted from the aqueous phase by four 2-ml extractions with ethyl acetate–methylethyl ketone (2:1) saturated with water. The extracts were combined and taken to dryness at 40° in a stream of N_2. The residue was dissolved in 0.1 ml 90 % acetic acid and suitable aliquots (10–40 μl) were used for paper chromatography according to the method recently described[9]. The PTH's were then eluted from the chromatograms at 40° with 2 ml 95 % ethanol for 1 h. The amounts of N-terminal amino acids present were calculated from the u.v. absorption at 269 (or 320) mμ. In these calculations the coefficients for recovery of each amino acid were used which were specified in a preceding paper[9].

No estimations of arginine or histidine were performed, as the PTH derivatives of these amino acids are water soluble and thus remain in the aqueous phase during the extraction.

RESULTS

Several attempts were made to find out the optimal conditions for the PTC-coupling and cyclization as well as washing and extractions. As test protein in these experiments human γ-globulin generally was used as it is known to have predominantly N-terminal aspartic acid and glutamic acid. Fig. 1 is a representative experiment, and shows the final recoveries of N-terminal aspartic acid and glutamic acid after different cyclization times. It is evident that a cyclization time of 2 h is sufficient. The values also indicate that prolonged cyclization does not destroy the PTH's to any appreciable extent. However, cyclization, lyophilization and extraction of the PTH derivatives should be performed during the same day. In some instances, considerable losses of the PTH derivatives occurred when the sample after lyophilization was kept at —20° overnight. On the other hand, after PTC coupling and washing, the sample can be kept at —20° overnight without losses.

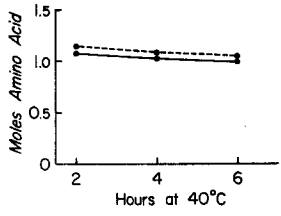

Fig. 1. Recoveries of N-aspartic acid, ———, and N-glutamic acid, – – – –, from human γ-globulin after different cyclization times. The values are expressed as moles amino acid/160,000 g γ-globulin.

Fig. 2 shows a paper chromatogram of the PTH amino acids obtained from human γ-globulin, as well as PTH derivatives of known amino acids for comparison. Fig. 3 compares the u.v. absorption spectra of the eluted spots with the spectrum of authentic PTH aspartic acid. (The spectra of PTH aspartic acid and PTH glutamic acid are virtually identical.) Undoubtedly, the eluted spots are PTH derivatives of glutamic acid and aspartic acid originating from the N-terminals of the γ-globulin fraction. The identity was also checked by hydrolyzing the PTH's and subsequently identifying the free amino acids by paper chromatography.

The small peak at 320 mμ of the eluted spot (Fig. 3) is due to a small but significant amount of PTH serine migrating with PTH aspartic acid on the chromatogram. Although these two PTH derivatives have the same R_F on the chromatogram, each can be individually determined because of the extra absorption of PTH serine at 320 mμ. Details for calculation of the serine content are given in a preceding paper[9].

The reproducibility of this method is shown in Table I, where eight different analyses on the same γ-globulin were performed.

Table II shows the N-terminal amino acids of the different serum proteins studied. No other N-terminal amino acids were detected than those indicated. The molar content of each amino acid was calculated from the published mean molecular weight of each protein fraction. As expected, human albumin had 1 mole aspartic acid/mole protein. Human γ-globulin contained 1 mole each of aspartic acid and glutamic acid and consistently a small amount of serine. Porcine γ-globulin, however, had 1 mole alanine and 2 moles of glutamic acid/mole of protein. Human transferrin showed only

valine as an N-terminal amino acid in a ratio of 1 mole/mole protein. Bovine prothrombin contained 1 mole of alanine/mole protein. These figures are rounded off to the nearest stoichiometric value although the protein fractions used were not completely homogeneous.

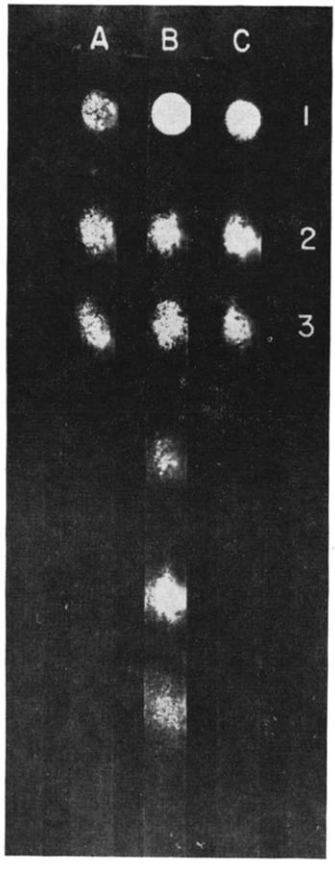

Fig. 2. Paper chromatogram of N-terminal amino acids from human γ-globulin (A and C). B denotes test strip. The figures indicate the following: 1, application point; 2, PTH-aspartic acid and PTH-serine; 3, PTH-glutamic acid.

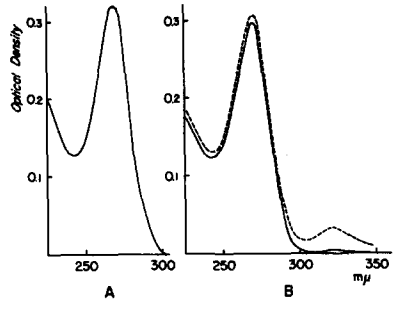

Fig. 3. Absorption spectra of: A, authentic PTH-aspartic acid. B, ------, PTH-aspartic acid and PTH-serine together; ———, PTH-glutamic acid eluted from the paper chromatogram.

TABLE I

MOLES N-ASPARTIC ACID AND N-GLUTAMIC ACID OBTAINED FROM HUMAN γ-GLOBULIN

The values have been calculated from an assumed molecular weight of 160,000.

Expt. No.	*γ-globulin (mg)*	*Moles N-terminal amino acids per 160,000 g protein*	
		Aspartic acid	*Glutamic acid*
1	16.95	1.15	1.22
2	16.00	1.11	1.12
3	28.25	1.05	1.20
4	21.01	1.13	1.20
5	21.01	1.07	1.15
6	21.01	1.15	1.20
7	21.01	1.18	1.18
8	21.01	1.10	1.14
Mean		1.12	1.18

TABLE II

N-TERMINAL AMINO ACIDS OF DIFFERENT SERUM PROTEINS

The values have been expressed as moles amino acid/mole protein.

Protein	*Assumed molecular wt.*	*Number of determinations*	*Alanine, moles*	*Aspartic acid, moles*	*Glutamic acid, moles*	*Serine, moles*	*Valine, moles*
Albumin (human)	69,000	4	—	1.03	—	—	—
γ-globulin (human)	160,000	8	—	1.12	1.18	0.10	—
γ-globulin (porcine)	160,000	4	1.04	—	1.99	—	—
Prothrombin (bovine)	62,700	2	1.10	—	—	—	—
Transferrin (human)	88,000	2	—	—	—	—	0.98

DISCUSSION

The N-terminal amino acid composition of human albumin has been studied by DESNUELLE *et al.*[2] who identified aspartic acid as the N-terminal amino acid by SANGER's technique. Their results were later confirmed by THOMPSON[3] in a comparative study of EDMAN's and SANGER's technique. THOMPSON found aspartic acid by both methods. However, with the EDMAN technique only 5 % of N-terminal aspartic acid could be recovered whereas SANGER's technique gave 64 % on the assumption that human albumin contains 1 mole of aspartic acid/mole albumin. By the present method we have found 1 mole of human albumin to contain 1.03 moles aspartic acid. This value is the mean of 4 different analyses. These results suggest the method to give a quantitative recovery.

The N-terminal composition of γ-globulin from various species has been thoroughly investigated. VAN VUNAKIS[4] has reported human γ-globulin to contain at least seven different N-terminals as determined by the DNP-method. However, this finding could not be confirmed by SMITH AND MCFADDEN[5]. These authors found only glutamic acid, aspartic acid, and serine when using the same technique. Certain differences were found to exist between subfractions of human γ-globulin. Thus fraction II:1,2 gave 1.06 moles aspartic acid, 1.82 moles glutamic acid and 0.10 moles serine whereas fraction II:3 contained 1.01 moles, 1.06 moles and 0.17 moles respectively. The values were based on the recoveries of the DNP-aspartic acid (60 %), DNP-glutamic acid (56 %) and DNP-serine (81 %) and on an assumed molecular weight of γ-globulin of 160,000. Recently LAY AND POLGLASE[7] have determined both the N-terminal and C-terminal amino acids of a human γ-globulin fraction. After correction for losses of the DNP-amino acids during hydrolysis they report one mole each of N-aspartic acid and N-glutamic acid. No N-serine was found.

In our investigation the human γ-globulin revealed 1.12 moles N-aspartic acid, 1.18 moles N-glutamic acid and approx. 0.1 moles N-serine. In this respect our results

agree well with the findings of SMITH AND MCFADDEN for fraction II:3 and with those of LAY AND POLGLASE (except for the serine content). However, our values are not corrected for losses as we assume the procedure to give quantitative recoveries of the N-terminal amino acids also from human γ-globulin.

The porcine γ-globulin studied has been found to contain 1.04 moles alanine and 1.99 moles glutamic acid. COLACICCO[15] has studied the N-terminal composition of this protein fraction (Pentex Inc.) by the DNP-method. He has found the ratio between N-glutamic acid and N-alanine to be 2:1. After correction for losses of the DNP amino acids by 30%, the protein contains 2 moles N-glutamic acid and 1 mole alanine. However, he found small amounts of serine, threonine, valine and isoleucine which we cannot verify in our protein preparation.

We cannot compare our finding of 1 mole N-valine/mole transferrin to other published data as the only data available is that of PUTMAN[16] who has reported human transferrin to contain N-valine, but did not give quantitative data.

Assuming a molecular weight of 62,700 for bovine prothrombin we found 1.10 moles alanine/mole protein. This value is higher than the results of MAGNUSSON[17] who found 0.95 moles N-alanine in the same preparation/mole protein. He used a modification of EDMAN's method[18] and corrected for losses during hydrolysis from added known amounts of alanine.

The method presented appears to give quantitative recovery of the N-terminal amino acid from proteins. This presumably depends on the mild conditions for splitting off the N-terminals and on the reliability in the estimation of the PTH amino acids by the paper chromatographic technique. The method is not restricted to the determination of the N-terminal amino acids of a single protein. It could be used as a suitable method for checking the purity during fractionation of protein as well as a simple technique for molecular weight estimate. We have applied this method for determination of all of the N-terminal amino acids of dialyzed whole serum from normal and pathological sera. This has given interesting results which will be published.

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