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GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF TRYPTOPHAN IN PROTEINS*

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SUMMARY

A gas-liquid chromatographic (GLC) method for the analysis of tryptophan in proteins using an acidic hydrolysis method of 6 *N* HCl with 5 % thioglycollic acid (TGA) at 110° for 20 h was developed. It is necessary to remove the TGA from the hydrolysate prior to GLC analysis to avoid chromatographic interferences. A cation-exchange resin was used to remove interferences. The hydrolysis method itself was evaluated on tripeptides containing tryptophan residues as model systems. The molar ratios of amino acids found in these tripeptides agreed with theory. There was no significant effect of TGA on the molar response values of the protein amino acids with the exception of arginine and cystine. GLC analyses of hydrolyzed lysozyme and trypsinogen were demonstrated on the simultaneous dual-column and single-column chromatographic systems of GEHRKE *et al.* This method can be used for the analysis of tryptophan by GLC as well as other protein amino acids.

INTRODUCTION

Ideally, the amino acid analysis of proteins would include the complete conversion, without destruction, of the protein to its component amino acids and the use of an accurate analytical method for the quantitative determination of the resultant amino acids. Currently, gas–liquid (GLC) and ion-exchange chromatography are the preferred techniques for amino acid analysis. Nevertheless, only ion-exchange techniques have been employed for the determination of tryptophan in proteins following either basic or acidic hydrolysis.

Acidic hydrolysis methods are widely used for conversion of proteins to the free amino acids, but acidic conditions lead to the decomposition of certain amino acids, with tryptophan suffering by far the greatest breakdown.

For the analysis of tryptophan in proteins, several methods have been studied by a number of investigators, and include acidic hydrolysis with thioglycollic acid

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(TGA) as a protective reagent[1], *p*-toluenesulfonic acid[2], alkaline hydrolysis[3–5], enzymatic hydrolysis[6], and intact protein methods[7–9].

It has been shown that alkaline hydrolysis of proteins results in much less destruction of tryptophan than hydrolysis under acidic conditions. In 1938, LUGG[10] recommended a solution of 5 % stannous chloride in 5.5 *N* sodium hydroxide as an alkaline medium for the hydrolysis of tryptophan in proteins. Eleven years later, SPIES AND CHAMBERS[3] found that analyses for tryptophan in the intact proteins resulted in significantly higher tryptophan values than after alkaline hydrolysis followed by ion-exchange chromatography. Further, they reported the presence of cystine, lanthionine, serine, and threonine in the sample increased the breakdown of tryptophan during hydrolysis. They concluded, therefore, that alkaline hydrolysis of proteins should not be conducted prior to tryptophan analysis, as the amount of tryptophan destroyed is dependent on the amino acid composition of the protein.

In 1962, NOLTMANN *et al.*[11] reported on the use of 4 *N* barium hydroxide for the hydrolysis of tryptophan, however, this method resulted in the complete destruction of arginine, serine, threonine, cystine, and cysteine, thus precluding the use of this approach for amino acid analysis.

Recently, thiodiglycol[4] and starch[5] have been used as protective reagents in basic hydrolysis of proteins followed by amino acid analysis with a starch column; this column separated tryptophan from the other protein amino acids.

SPIES AND CHAMBERS[3] reported that the other amino acids present in proteins do not interfere in the methods for determining tryptophan in the intact protein. Although the intact protein methods gave accurate tryptophan values in many cases, it was later shown that high values[12] and abnormal coloration[13] were limitations to this approach. HOLIDAY[14], GOODWIN AND MORTON[7], and BENCZE AND SCHMID[15] have also reported on the spectrophotometric determination of tryptophan in intact proteins.

Tryptophan can be determined quantitatively from enzymatic hydrolysates, but enzymatic hydrolysis may be far from complete with regard to the other amino acids.

Two of the most promising methods for the analysis of tryptophan in acidic hydrolysates are those reported by MATSUBARA AND SASAKI[1] and LIU AND CHANG[2]. TGA has been reported to prevent the destruction of tryptophan during hydrolysis with 6 *N* HCl[1], and the use of *p*-toluenesulfonic acid containing 3-(2-aminoethyl)-indole has resulted in the successful analysis of tryptophan in proteins.

A major purpose in this research was the removal from protein hydrolysates of TGA and associated products by ion-exchange cleanup, followed by quantitative GLC analysis of the amino acids by the dual-column and single-column separation methods of GEHRKE *et al.*[16]. The recovery of tryptophan from cation-exchange cleanup and the effect of TGA on the response for the other nineteen amino acids was investigated. Lysozyme, trypsinogen, and tripeptides were used as the model systems.

EXPERIMENTAL

Apparatus

A Bendix 2500 Series gas chromatograph with a four-column oven bath, four hydrogen flame detectors, two differential electrometers, a linear temperature pro-

grammer, and a Varian Model A-20 dual-pen recorder were used for this study. An Infotronics Corporation CRS-104 digital integrator was used for determining peak areas. Filters for the hydrogen, air, and nitrogen carrier gas to the chromatograph were obtained from the Packard Instrument Company and contained Linde 5-A molecular sieve and drierite. For removal of particulate matter from the gas streams, "F" series inline 7-μ sintered stainless-steel filters were obtained from the Nupro Company, Cleveland, Ohio, and fitted in the lines. A Bio-Cal Company BC-200 Series amino acid analyzer equipped with an automatic sample injector and an Infotronics CRS 110-A digital integrator were used for ion-exchange chromatographic amino acid analyses. The apparatus for hydrolysis of proteins consisted of a 25 × 150 mm thick-walled Pyrex glass tube fitted with an O-ring seal and stopcock which allowed the removal of air from the sample, and use of nitrogen sweep, prior to hydrolysis. Solvents were removed from the samples with a CaLab rotary evaporator, "cold finger" condenser, and a Welch Duo-Seal vacuum pump. Pyrex 16 × 75 mm glass screw top culture tubes (Corning No. 9826) with PTFE-lined caps were used as the reaction vessels for the acylation reactions. For the removal of TGA and other interfering materials from the hydrolysate prior to GLC analysis, AG 50W-X8 cation-exchange resin was obtained from Bio-Rad Laboratories, Richmond, Calif., and packed in 300 × 11 mm I.D. glass chromatographic columns with a bed size of 5 ml of resin.

Reagents and materials

All of the amino acids used in this study were obtained from Mann Research Laboratories, Inc., New York, N.Y., or from Nutritional Biochemicals Corporation, Cleveland, Ohio, and were chromatographically pure. Lysozyme from egg white, three times crystallized, dialyzed and lyophilized, and trypsinogen from bovine pancreas, once crystallized, were obtained from Sigma Chemical Company, St. Louis, Mo. Four tripeptides, L-lysyl-L-tryptophyl-α-L-lysine, α-L-glutamyl-L-tryptophyl-L-glutamic acid, glycyl-L-tryptophyl-glycine, and L-leucyl-L-tryptophyl-L-leucine, were purchased from Mann Research Laboratories, New York, N.Y.

TGA was purchased from BDH Chemical Ltd., Poole, Great Britain. The trifluoroacetic anhydride was obtained from Distillation Products Industries, and was an "Eastman Grade" chemical. *n*-Butanol and methylene chloride were redistilled from an all-glass system and were refluxed over $CaCl_2$ before distillation. Anhydrous HCl gas, for preparation of 3 *N* HCl in *n*-butanol, was generated by the slow addition of 250 ml of reagent grade hydrochloric acid to 500 ml of concentrated sulfuric acid. The HCl gas was passed through two drying towers containing concentrated sulfuric acid and then bubbled into the *n*-butanol until 3 *N* in HCl.

Method of hydrolysis

(a) Protein samples (*ca.* 6 mg) were weighed into 16 × 120 mm pyrex glass screw top culture tubes.

(b) 5 ml of constant boiling 6 *N* HCl containing 5 % thioglycollic acid were added to the sample.

(c) After dissolution of the sample, the uncapped tube containing the sample

was then placed in the thick-walled Pyrex glass hydrolysis tube containing *ca.* 5 ml of constant boiling HCl (Fig. 1).

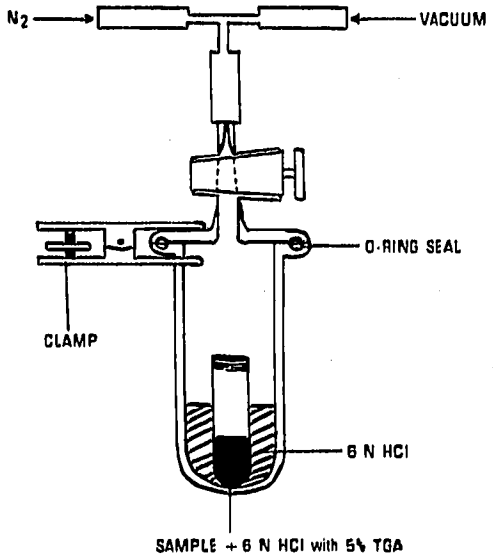

Fig. 1. Hydrolysis apparatus for proteins.

(d) A "T" arrangement of vacuum tubing which allowed a vacuum to be applied, and purified nitrogen to be flushed into the tube was used to remove the air both from the sample and the air dissolved in the 6 *N* HCl. The pressure inside the tube was reduced to *ca.* 0.2 mm Hg via a vacuum pump, the line between the pump and hydrolysis tube closed, and purified nitrogen gas was flushed into the tube. Then, the nitrogen gas line was closed and the vacuum reapplied. This process was repeated five times.

(e) After the final flushing with nitrogen gas, the sample tube was placed in a 110° oven for 20 h, then removed from the oven, and allowed to cool at room temperature.

(f) The hydrolysate was transferred into a 125-ml $\bar{\S}$ flat-bottom flask, dried by rotary evaporation, and the residue redissolved in 5 ml of 0.1 *N* HCl.

Removal of interferences

As it was necessary to remove TGA and associated products from the hydrolysates prior to GLC, thus cleanup was investigated. Five milliliters of cation-exchange resin (AG 50W-X8, 50–100 mesh, H form) were placed in the glass columns mentioned earlier, giving a resin capacity of 8.5 mequiv.

The samples dissolved in 5 ml of 0.1 *N* HCl were quantitatively transferred to the resin column with 10 ml of additional 0.1 *N* HCl, and passed through the column at a flow-rate of 0.5 ml per min. Three portions (10 ml each) of distilled water were used to wash the resin, and the effluent and washings were discarded. The amino acids were eluted from the resin with 20 ml of 3 *N* NH_4OH at a flow-rate of 1 ml

per min. The alkaline effluent was collected and evaporated to dryness on a rotary evaporator at 60°, and analyzed by the method of GEHRKE *et al.*[16,17].

Recovery of tryptophan from cation-exchange columns

To determine the quantitation of the cation-exchange cleanup procedure for removal of interferences, 0.255 mg standard samples of tryptophan in 0.1 *N* HCl were taken through the method described above. These experiments were conducted at both room temperature and in a cold room at 3°.

Recovery experiments of tryptophan taken through both the hydrolysis with 6 *N* HCl containing TGA and cation-exchange cleanup were made. For analysis by GLC, 4-(aminoethyl)cyclohexanecarboxylic acid and stearic acid were used as internal standards. The dual-column separation system described by GEHRKE *et al.*[17] was used for GLC analysis, and consisted of 1.5 m × 4 mm I.D. glass columns of 0.65 % stabilized EGA on 80–100 mesh Chromosorb W, and 1.5 m × 2 mm I.D. glass columns of 2 % OV-17/1 % OV-210 mixed phase on 100–120 mesh Gas-Chrom Q.

The recovery for tryptophan taken through the cleanup cation-exchange columns was then determined by comparison with standard samples directly injected into the gas chromatograph.

Effect of thioglycollic acid on other amino acids

Standard amino acid solutions containing 0.20 mg of each of nineteen protein amino acids were used to determine the effect of TGA during hydrolysis, and comparisons were made with samples subjected to hydrolysis with 6 *N* HCl, and with the initial standard solutions. After cation-exchange cleanup and derivatization, GLC analyses were made for the twenty amino acids with the dual-column system[17]. The chromatographic response values were calculated relative to hydroxyproline which was arbitrarily assigned the value of 1.000.

Applications of the hydrolysis methods

In this study, four tripeptides, lysozyme, and trypsinogen were investigated. The peptides were: L-lysyl-L-tryptophyl-α-L-lysine, α-L-glutamyl-L-tryptophyl-L-glutamic acid, glycyl-L-tryptophyl-glycine, and L-leucyl-L-tryptophyl-L-leucine. 1.0 mg of each was hydrolyzed individually with 3 ml of constant-boiling 6 *N* HCl containing 5 % TGA at 110° for 24 h by the techniques described earlier. 1.5-ml aliquots of each hydrolysate were dried under a nitrogen stream at 60°, then dissolved in 3 ml of pH 2.20 buffer solution containing 0.50 mg norleucine as internal standard for amino acid analysis by cation-exchange chromatography.

To study the 6 *N* HCl-TGA hydrolysis procedure further, duplicate hydrolysates of both lysozyme and trypsinogen were prepared; also, an equimolar standard solution of amino acids was taken through the hydrolysis method. The hydrolysis conditions and cleanup procedures used were as described above. After passing the samples through the cation-exchange cleanup column, the eluates containing the amino acids were collected and divided into two equal portions, for both GLC and ion-exchange amino acid analysis. The *RMR* values obtained for the amino acids in the standard solution were used in the calculations for the number of residues of amino acids in lysozyme and trypsinogen.

Calculations

The following equation was used to calculate the relative number of residues of each amino acid in lysozyme and trypsinogen from the GLC data.

Relative number of amino acid residues

$$= \frac{A_{\text{a.a.}}}{RMR_{\text{a.a./pro.}}} \Big/ \frac{\frac{A_{\text{pro.}}}{RMR_{\text{pro./pro.}}}}{R_{\text{pro.}}} = \frac{A_{\text{a.a.}}}{\text{Factor}} \Big/ \frac{A_{\text{pro.}}}{R_{\text{pro.}}}$$

where $A_{\text{a.a.}}$ = area in counts of the amino acid peak, $A_{\text{pro.}}$ = area in counts of the proline peak, $RMR_{\text{a.a./pro.}} = A_{\text{a.a.}}/A_{\text{pro.}}$ = Factor, calculated from the analysis of an equal molar standard solution, and $R_{\text{pro.}}$ = the number of proline residues from literature value.

The above equation states, in the numerator, that the counts for an amino acid are given in equivalent counts of proline; the denominator gives the counts of proline for one residue of proline. Thus, the number of residues for an amino acid can be calculated relative to proline.

RESULTS AND DISCUSSION

In 1969, MATSUBARA AND SASAKI[1] reported a procedure for the analysis of tryptophan in proteins by ion-exchange chromatography after hydrolysis with 6 *N* HCl containing 4 % of TGA.

This hydrolysis method was studied with the aim of developing a GLC technique for the analysis of tryptophan in proteins. Initial GLC studies showed that use of 6 *N*

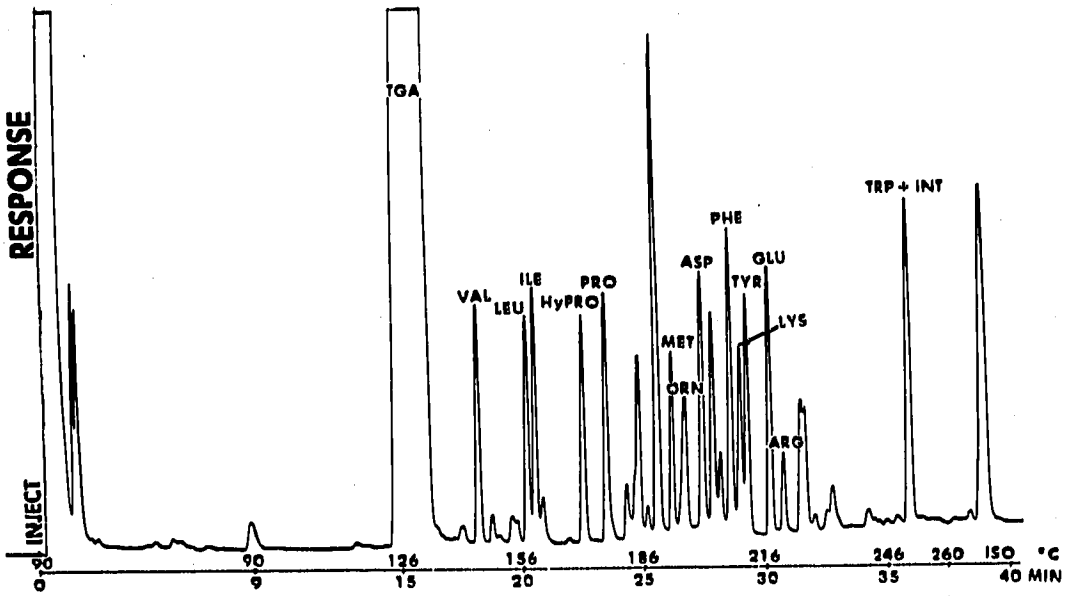

Fig. 2. Single-column GLC separation of the N-TFA *n*-butyl esters of amino acids — without clean-up. Column: 10% (w/w) Apiezon M on 80–100 mesh HP Chromosorb W, 2.5 m × 2 mm I.D. glass. Sample: 0.2 mg each. Hydrolysis: 5 ml 6 *N* HCl with 5 % TGA, 21 h, 110°. Instrumental conditions: initial temperature 90°, delay 9 min, 6°/min, and final temperature 260°.

HCl–5 % TGA would require removal of the TGA from the hydrolysate prior to analysis in order to avoid the large number of chromatographic interferences and the incomplete esterification and acylation of the amino acids resulting from the presence of TGA in the hydrolysate. Figs. 2 and 3 show chromatograms for the protein amino acids without removal of TGA and their associated interfering peaks.

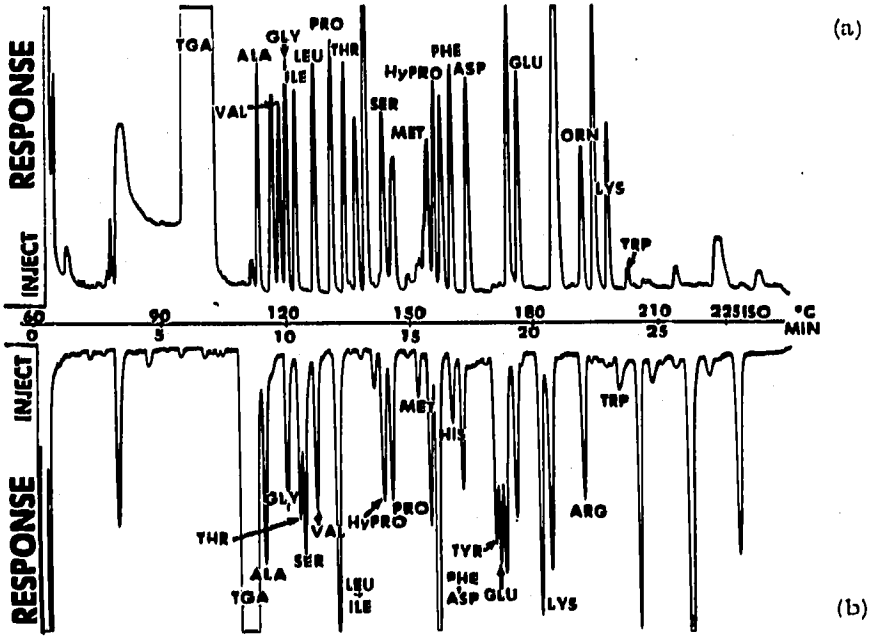

Fig. 3. Simultaneous GLC separation of the N-TFA *n*-butyl esters of amino acids — without cleanup. Columns: (a) 0.65% (w/w) EGA on 80–100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass; (b) 2.0% (w/w) OV-17, 1.0% (w/w) OV-210 on 100–120 mesh Gas-Chrom Q, 1.5 m × 4 mm I.D. glass. Sample: 0.2 mg each. Hydrolysis: 5 ml 6 *N* HCl with 5% TGA, 21 h, 110°. Instrumental conditions: initial temperature 60°, 6°/min, and final temperature 225°.

Cation-exchange was evaluated first as a cleanup procedure after hydrolysis of the proteins. Anion-exchange was also considered for use as a cleanup technique, but TGA could not be removed from the hydrolysate without losses of aspartic acid on elution of the amino acids from the anion column due to the close K_a values of TGA and aspartic acid, which are 2.0×10^{-4} and 1.1×10^{-4}, respectively.

As the use of a cation-exchange resin column for removal of interferences resulted in losses of tryptophan of 30 to 35 % on the resin column (Table I), thus the effect of temperature during the cleanup procedure was investigated as ORESKES AND KUPFER[18] had reported that glutamine could not be quantitatively recovered from a cation-exchange column at elevated temperatures.

As seen in Table I, no significant differences in recovery were observed at 3 ° or 25 °, indicating that the loss of tryptophan was not influenced by the temperature of the resin column. Our studies also showed that the recovery of tryptophan from the resin was not significantly changed by doubling the amount of resin used. The ratio of the molar equivalents of exchange capacity of the resin used to tryptophan was about 10^4 for 5 ml of resin and 2×10^4 for 10 ml of resin. A large excess of exchange

TABLE I

RECOVERY OF TRYPTOPHAN FROM CATION-EXCHANGE CLEANUP

Sample solutions containing 0.255 mg of tryptophan. Cation-exchange column volume: 5 ml.

	Trp/IS-1[a]			*Recovery* (%)	*Trp/IS-2*[b]			*Recovery* (%)
	1	*2*	*Av.*		*1*	*2*	*Av.*	
Uncleaned	1.70	1.70	1.70		1.47	1.52	1.50	
Cleanup[c] A	1.13	1.12[d]	1.13	66.5	1.03	1.02	1.03	68.7
Cleanup[c] B	1.15	1.17	1.16	68.2	1.00	1.02	1.01	67.3
Cleanup[c] C	1.10	1.06	1.08	63.5	0.99	0.95	0.97	64.7

[a] 4-(Aminoethyl)cyclohexanecarboxylic acid as internal standard.
[b] Stearic acid as internal standard.
[c] Cleanup A carried out at 25°; cleanup B carried out at 3°; cleanup C carried out at 25° after hydrolysis procedure with 5% TGA in 6 *N* HCl at 110° for 20 h.
[d] Cation-exchange column volume: 10 ml.

resin to tryptophan was present with the resulting constant loss of a fraction of the tryptophan.

A study was next made to determine the effect of TGA on the response of other protein amino acids, and as seen in Table II, no significant changes were observed with the exception for arginine and cystine. From the observation of a cysteine peak

TABLE II

EFFECT OF THIOGLYCOLLIC ACID ON THE RELATIVE WEIGHT RESPONSE OF THE PROTEIN AMINO ACIDS

Amino acid	*Relative weight response*[a]			
	Std.[b]	*6 N HCl*[c]	*6 N HCl–TGA*[c]	
		RT 21 h	*RT 21 h*	*110°, 21 h*
Alanine	1.079	1.105	1.114	1.084
Valine	1.190	1.165	1.166	1.149
Glycine	1.063	1.050	1.058	1.051
Isoleucine	1.138	1.115	1.134	1.128
Leucine	1.126	1.153	1.132	1.114
Proline	1.116	1.132	1.114	1.133
Threonine	1.003	0.983	0.996	0.962
Serine	0.937	0.967	0.964	0.926
Methionine	0.826	0.761	0.779	0.798
Hydroxyproline[a]	1.000	1.000	1.000	1.000
Phenylalanine	1.189	1.166	1.152	1.156
Aspartic acid	1.186	1.195	1.198	1.217
Glutamic acid	1.191	1.201	1.187	1.222
Tyrosine	0.822	0.828	0.832	0.849
Lysine	0.947	0.935	0.923	0.914
Histidine	0.463	0.485	0.480	0.515
Arginine	0.640	0.641	0.643	0.338
Tryptophan	0.799	0.563	0.555	0.551
Cystine	0.513	—	—	0.348

[a] Hydroxyproline used as internal standard.
[b] Standard solution without cleanup.
[c] Cleaned by cation-exchange.

on the chromatogram, a part of the cystine is reduced to cysteine during acid hydrolysis with TGA. This reduction was not found to be reproducible. In a comparison of hydrolysates using different reagents, 3 *N* *p*-toluenesulfonic acid and 3 *N* methanesulfonic acid significantly affected the Met, Tyr, His, Trp, and Cys response values even though tryptamine was added to the solutions as a protective reagent.

To evaluate the hydrolysis procedure itself, four tripeptides containing tryptophan and basic, neutral, and acidic amino acids were hydrolyzed in constant-boiling 6 *N* HCl with 5 % TGA at 110° for 24 h. The tripeptides were: L-lysyl-L-tryptophyl-α-lysine, α-L-glutamyl-L-tryptophyl-L-glutamic acid, glycyl-L-tryptophyl-glycine, and L-leucyl-L-tryptophyl-L-leucine. The molar ratios of amino acids from these tripeptides are presented in Table III and the results are seen to be in good agreement

TABLE III

ANALYSIS BY ION-EXCHANGE CHROMATOGRAPHY OF TRIPEPTIDES AFTER HYDROLYSIS WITH 6 *N* HCL–5% TGA

Hydrolysis: 1 mg of each tripeptide in 3.0 ml constant-boiling 6 *N* HCl–5% TGA, at 110° for 24 h.

Tripeptide[a]	*μmoles of amino acid*[b]	*μmoles of tryptophan*	*Amino acid/ tryptophan*
Lys-Trp-Lys	0.193	0.095	2.04
Leu-Trp-Leu	0.402	0.210	1.91
Glu-Trp-Glu	0.412	0.203	2.07
Gly-Trp-Gly	0.597	0.305	1.96

[a] No cleanup procedure was carried out prior to analysis by ion-exchange chromatography.
[b] Norleucine was used as internal standard; μmoles of amino acids except tryptophan.

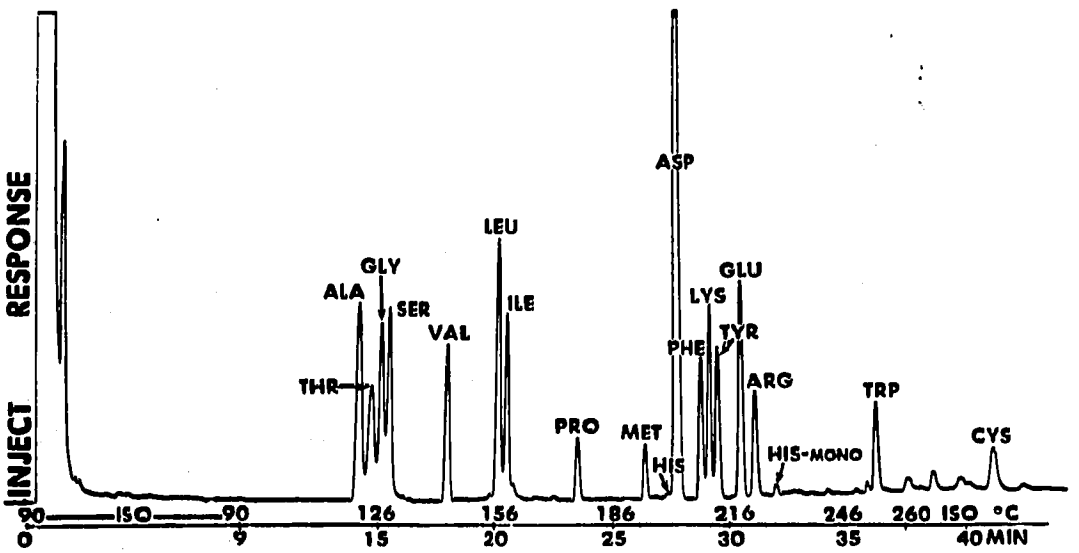

Fig. 4. Single-column GLC analysis of the N-TFA *n*-butyl esters of lysozyme — with cleanup. Column: as in Fig. 2. Sample: 6.0 mg lysozyme. Hydrolysis: 5 ml 6 *N* HCl with 5% TGA, 21 h, 110°. Instrumental conditions: initial temperature 60°, delay 9 min, 6°/min, and final temperature 260°.

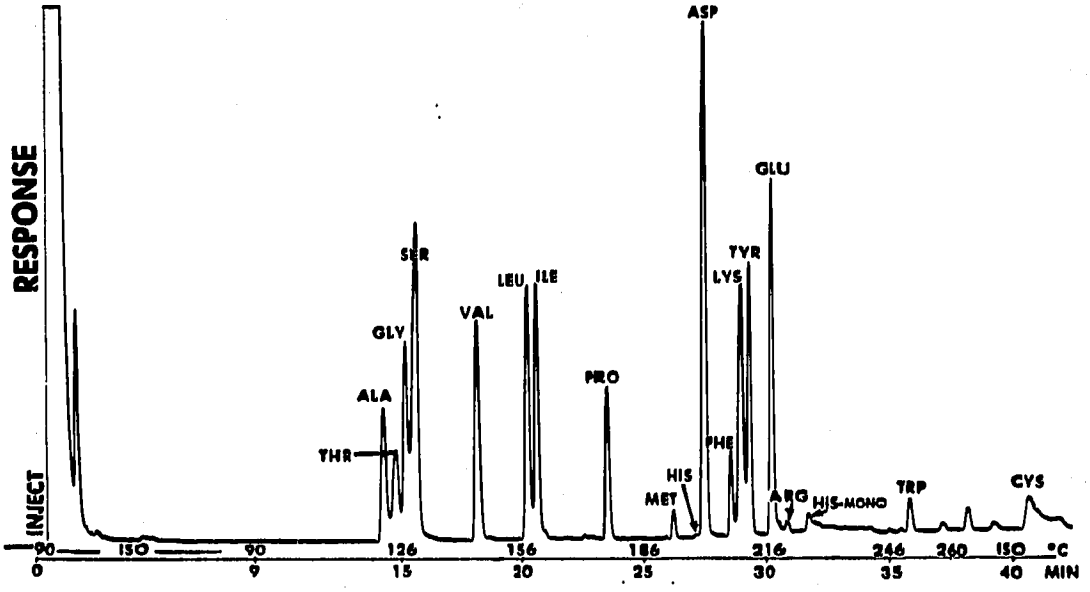

Fig. 5. Single-column GLC analysis of the N-TFA *n*-butyl esters of trypsinogen — with cleanup. Column: as in Fig. 2. Sample: 6.0 mg trypsinogen. Hydrolysis: 5 ml 6 *N* HCl with 5% TGA, 21 h, 110°. Instrumental conditions: as in Fig. 4.

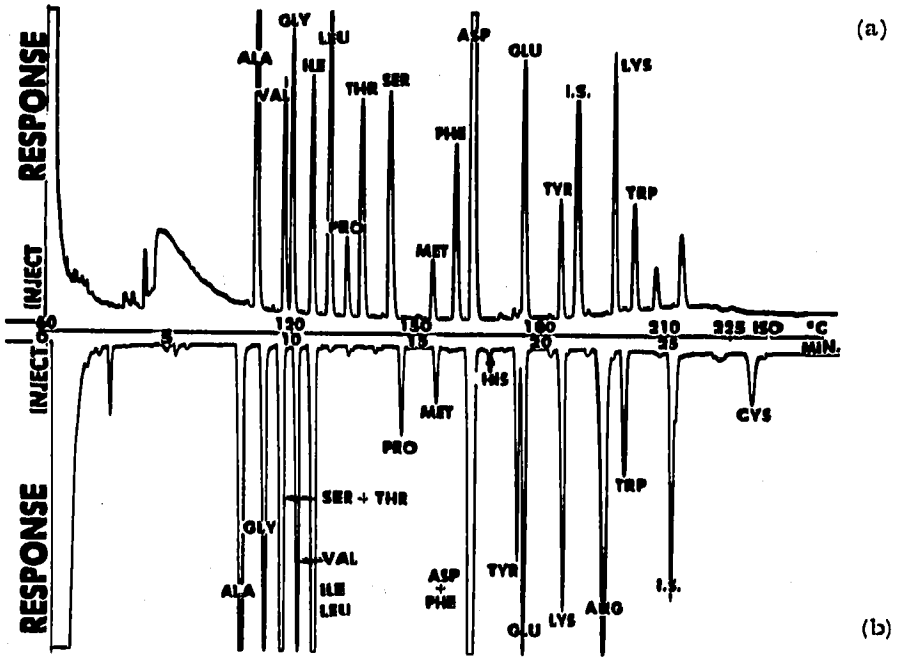

Fig. 6. Simultaneous GLC analysis of the N-TFA *n*-butyl esters of lysozyme — with cleanup. Columns: as in Fig. 3. Sample: 6.0 mg lysozyme. Hydrolysis: 5 ml 6 *N* HCL with 5% TGA, 21 h, 110°. Instrumental conditions: as in Fig. 3. Internal standard: *n*-butyl stearate.

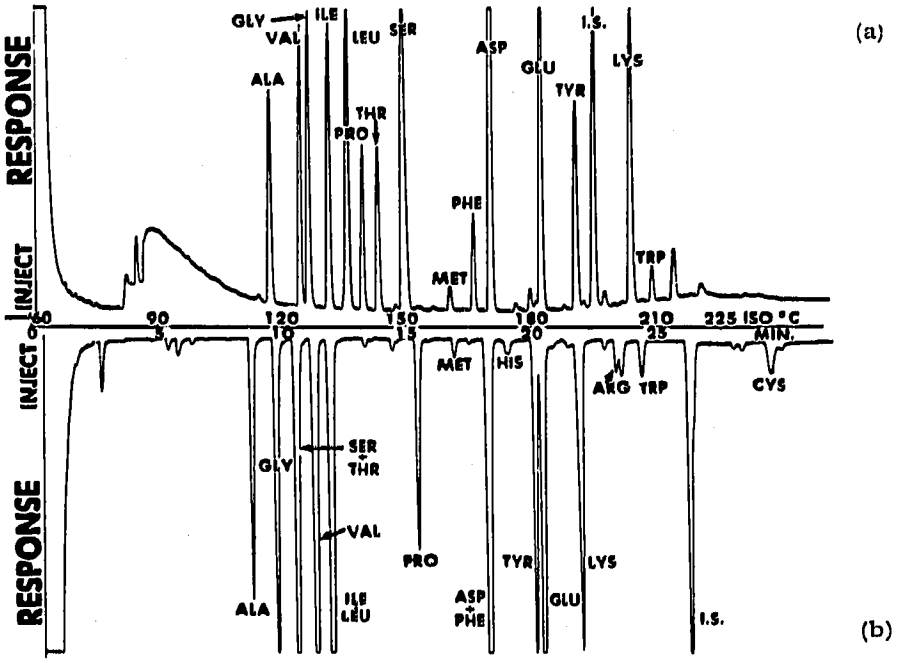

Fig. 7. Simultaneous GLC analysis of the N-TFA *n*-butyl esters of trypsinogen — with cleanup. Columns: as in Fig. 3. Sample: 6.0 mg trypsinogen. Hydrolysis: 5 ml 6 *N* HCl with 5% TGA, 21 h, 110°. Instrumental conditions: as in Fig. 3.

TABLE IV

RELATIVE NUMBER OF AMINO ACID RESIDUES IN LYSOZYME

6.0 mg of lysozyme hydrolyzed with 5 ml of 6 *N* HCl–5% TGA at 110° for 20 h.

Amino acid	*GLC*[a]		*Av.*	*Ion-exchange chromatography*[b]	*Literature (ref. 19, p. 221)*
Alanine	13.0	12.5	12.7	12.0	12
Threonine	7.5	7.2	7.3	6.5	7
Glycine	12.9	12.6	12.7	12.0	12
Serine	10.2	9.6	9.9	9.7	10
Valine	5.6	5.2	5.4	4.8	6
Leucine	8.3	8.3	8.3	7.1	8
Isoleucine	5.3	5.0	5.2	4.7	6
Proline	2.0	2.0	2.0	2.1	2
Methionine	2.0	2.0	2.0	1.4	2
Histidine	1.5	1.0	1.2	1.0	1
Aspartic acid	22.1	21.0	21.5	20.0	21
Phenylalanine	3.3	3.1	3.2	2.7	3
Lysine	6.2	5.9	6.0	5.3	6
Tyrosine	3.5	3.2	3.4	2.8	3
Glutamic acid	5.4	5.0	5.2	5.1	5
Arginine	7.6	9.0	8.3	8.4	11
Tryptophan	5.6	5.2	5.4	5.7	6
Cystine	5.0	4.3	4.7	5.8	8

[a] Proline value was assumed as two residues for GLC calculations.
[b] Glycine value was assumed as twelve residues for ion-exchange calculations.

with theory, indicating that both the hydrolysis was efficient, and that no serious destruction of tryptophan had occurred.

Figs. 4 and 5 present chromatograms of hydrolyzed lysozyme and trypsinogen on the single column of 10 % (w/w) Apiezon M. Also, the same samples were injected on the dual-column system developed by GEHRKE *et al.*[17] (Figs. 6 and 7).

In Tables IV and V, the relative number of residues of each amino acid in lysozyme and trypsinogen calculated from both GLC and ion-exchange chromatography are presented. As will be noted, good agreement of the GLC and ion-exchange data was obtained, as well as agreement with the reported literature values. The analysis of tryptophan in these two enzymes was also near the expected values.

TABLE V

RELATIVE NUMBER OF AMINO ACID RESIDUES IN TRYPSINOGEN

6.0 mg of trypsinogen hydrolyzed with 5 ml of 6 *N* HCl–5% TGA at 110° for 20 h.

Amino acid	*GLC*[a]		*Av.*	*Ion-exchange chromatography*[b]	*Literature (ref. 19, p. 205)*
Alanine	13.9	14.1	14.0	14.4	14
Threonine	9.6	9.7	9.7	9.1	10
Glycine	24.2	24.2	24.2	25.0	25
Serine	29.9	30.2	30.1	31.5	34
Valine	13.2	13.4	13.3	12.6	18
Leucine	13.7	13.8	13.8	12.7	14
Isoleucine	12.5	12.6	12.6	11.7	15
Proline	8.0	8.0	8.0	8.5	8
Methionine	1.8	1.7	1.8	1.4	2
Histidine	2.8	3.0	2.9	2.5	3
Aspartic acid	23.9	25.8	24.8	22.6	26
Phenylalanine	3.3	3.1	3.2	2.9	3
Lysine	14.0	13.4	13.7	13.5	15
Tyrosine	10.5	10.1	10.3	8.9	—
Glutamic acid	15.0	14.9	15.0	14.5	14
Arginine	1.7	1.7	1.7	1.1	2
Tryptophan	3.4	3.8	3.6	—	4
Cystine	7.8	5.5	6.7	6.6	12

[a] Proline value was assumed as eight residues for GLC calculations.
[b] Glycine value was assumed as twenty-five residues for ion-exchange calculations.

CONCLUSIONS

The objective of this research was the development of a GLC method for the analysis of tryptophan in proteins. As tryptophan is destroyed during protein hydrolysis with 6 *N* HCl alone, we evaluated the hydrolysis method reported by MATSUBARA AND SASAKI[1], whereby the addition of thioglycollic acid prevents tryptophan destruction.

Four tripeptides and two enzymes were studied, with both GLC and ion-exchange amino acid analyses conducted after hydrolysis with 6 *N* HCl–5 % TGA. A cation-exchange procedure was used for the removal of TGA and related interferences from the hydrolysate prior to GLC analysis.

The analyses of the four tryptophan-containing tripeptides showed the destruction of tryptophan was less than 5 %. Extension of the method to two enzymes, lysozyme and trypsinogen, resulted in the successful analysis of tryptophan by GLC, as well as the other protein amino acids. TGA had no effect on the *RWR* values of the protein amino acids except arginine and cystine, with cystine being partially reduced to cysteine during hydrolysis in the presence of TGA.

The cation-exchange procedure for removal of thioglycollic acid resulted in a recovery of 66.5 % for tryptophan. The recovery data were reproducible and allowed accurate calculation of the tryptophan values in the tripeptides and proteins.

A GLC method is presented whereby tripeptides and proteins are hydrolyzed by 6 *N* HCl–5 % TGA to the amino acids, derivatized to the N-TFA *n*-butyl esters, and chromatographically separated simultaneously on dual-column and single-column systems. The method is useful for the analysis of tryptophan as well as the other protein amino acids.

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