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Gas chromatography of tryptophan together with other amino acids in hydrochloric acid hydrolysates

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ABSTRACT

The classical hydrolysis of proteins with hydrochloric acid using tryptamine [3-(2-aminoethyl)indole] as additive revealed that tryptophan can be measured without destruction together with other amino acids by gas chromatography. An extensive study was made to establish the optimum conditions for protein hydrolysis (time and temperature of hydrolysis, amount of tryptamine) and for the derivatization of amino acids. The amino acid contents (including tryptophan) of standard proteins such as lysozyme, bovine and human albumin, human γ-globulin, casein and α-chymotrypsin and protein matrices (meat and fish meals, sunflower) were determined, after hydrochloric acid hydrolysis (4 h, 145°C) in the presence of tryptamine, as N,O,(S)-trifluoroacetyl isobutyl esters with SE-30 as the stationary phase. The reproducibility of the measurements was 4.6% (relative standard deviation) or less.

INTRODUCTION

Modifications of existing analytical methods and the development of new methods are important areas of tryptophan research [1–10]. According to common methods [11–34], tryptophan is measured by chromatography in hydrolysates obtained by reaction with organic acids [11–15], bases [16–25] or hydrochloric acid plus additives [16–30].

The aim of this work was to improve and extend the use of 3-(2-aminoethyl)indole (tryptamine) as the additive in the classical hydrochloric acid hydrolysis [30]. Tryptamine has been found to be an excellent oxygen scavenger during the hydrolysis of proteins with methanesulphonic acid [11] or *p*-toluenesulphonic acid [12]. The common hydrolysis with bases [16–25] is appropriate for the exclusive determination of the tryptophan contents of proteins, requiring classical hydrochloric acid hydrolysis in parallel for the assay of other amino acids. Also, it is well known that using organic acids as catalysts one must take into consideration that when applying the common hydrolysis time for protein (20–24 h) leucine, isoleucine and valine segment are not released quantitatively, but if the hydrolysis time is increased then decreased amounts of threonine, serine, methionine and cystine/cysteine can be expected [33].

Hence, in the light of the above, it seemed useful to undertake an exhaustive study to optimize the classical hydrochloric acid hydrolysis in the presence of

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tryptamine in order to provide the possibility of the joint determination of tryptophan with all other amino acids as N,O(S)-trifluoroacetyl isobutyl esters [N,O,(S)-TFAIBE] [31,32] by gas chromatography (GC).

EXPERIMENTAL

Materials and reagents

All chemicals used were of analytical-reagent grade obtained from Reanal (Budapest, Hungary), Serva (Heidelberg, F.R.G.), Merck (Darmstadt, F.R.G.), Sigma (St. Louis, MO, U.S.A.) and Applied Science Labs. (State College, PA, U.S.A.). Protein matrices were purchased from commercial sources.

Apparatus

A Chromatron G.C.H.F. 18.3 gas chromatograph (VEB Chromatron, Berlin, G.D.R.), equipped with a flame ionization detector and a 2 m × 4 mm I.D. stainless-steel column, was used. Nitrogen was the carrier gas at a flow-rate of 60 cm^3/min. The column packing consisted of 3% SE-30 on Chromosorb W (100–120 mesh) (Supelco, Bellefonte, PA, U.S.A.). The column temperature was increased from 105 to 250°C at 6°C/min. The temperatures of the injector and detector were 270 and 270°C, respectively.

Hydrolysis

To a 1–2-cm^3 volume of a stock solution of amino acids (each 1.5–5 mg/cm^3) in 1 *M* hydrochloric acid, after evaporation to dryness, or to various amounts of standard proteins or protein matrices[a], 10 cm^3 of distilled hydrochloric acid and 0.02 g of tryptamine were added. The solution of amino acids (or heterogeneous samples of proteins) were made oxygen free by bubbling nitrogen (99.99%) through for 10 min. The screw-capped bottles were then closed immediately and placed in an oven at 145 ± 0.5°C for 4 h. Thereafter the hydrolysates were filtered into 25-cm^3 vessels, which could be fitted with either a vacuum distillation device or a reflux condenser, via a ground-glass joint. For filtration glass-fibre paper (grade GF/A; Whatman, Maidstone, U.K.) was used and the insoluble residues were washed with 3 × 1 cm^3 of 0.1 *M* hydrochloric acid. The supernatants were evaporated to dryness for derivatization.

Derivatization

To the evaporated residues 1 cm^3 of isobutanol (containing 1 *M* thionyl chloride) was added. A reflux condenser was then fitted to the flask and the apparatus placed in an oil-bath. Esterification took place at 110°C for 60 min. After cooling to room temperature, the solution was evaporated to a syrupy consistency under vacuum in a water-bath at 60°C. The residue was transferred quantitatively with 5 × 0.1 cm^3 dichloromethane into a 5-cm^3 Pierce Reacti-Vial. A 1.0-cm^3 volume of trifluoroacetic

[a] Weighed with analytical accuracy as follows: 0.02–0.08 g of lysozyme or α-chymotrypsin, 0.04–0.16 g of human γ-globulin (HGG), 0.10–0.20 g of bovine serum albumin (BSA) or casein and 0.20–0.40 g of human serum albumin (HSA), fish meal (FM), meat meal (MM) and sunflower meal (SM).

acid anhydride (TFAA) was added and acylation was carried out for 10 min at 150°C. Thereafter, the solution of the acylated derivatives was transferred quantitatively into a glassstoppered, calibrated test-tube (also connected to the vacuum evaporator). The solution of the N,O,(S)-TFA esters was evaporated to dryness in an ice-bath.

To the residues of the samples dichloromethane and TFAA were added, and a stock solution of 0.3 cm^3 was prepared from each. Aliquots of 5–10 μl were injected into the gas chromatograph.

RESULTS AND DISCUSSION

The results of our extensive studies on the effect of tryptamine on tryptophan measurement after classical hydrochloric acid hydrolysis are presented in Tables I–III and can be summarized as follows.

Tryptamine (in the concentration range 5–20 mg of tryptamine per 60 mg of amino acids) effectively protects tryptophan from destruction (Table I). An additional advantage of the use of tryptamine is that it protects methionine from destruction, probably under the conditions of the derivatization procedure. Model studies revealed that the methionine detector response can be increased by 40% when tryptamine is used in the hydrochloric acid hydrolysis. These results complement interlaboratory studies [34] which showed that the amounts of methionine measured by GC were about the half of those obtained by ion-exchange chromatography from the same hydrochloric acid hydrolysate.

The only disadvantage in the application of this additive is that the trifluoroacetyl isobutyl derivative of tryptamine elutes together with the N,O,(S)-TFAIBE of lysine. Therefore, in order to determine the lysine content of the hydrolysate, the tryptamine residue must be separated by an ion-exchange method. The optimum conditions for this ion-exchange procedure are under investigation.

The results of a reproducibility study with various amounts of eighteen amino acids in model solutions derivatized immediately and after hydrolysis in the presence

TABLE I

RECOVERY OF TRYPTOPHAN TOGETHER WITH OTHER AMINO ACIDS IN MODEL SOLUTIONS[a] WITHOUT AND AFTER HYDROLYSIS IN THE PRESENCE OF TRYPTAMINE, MEASURED AS N,O,(S)-TFAIBE

Procedure[b]	Amount of tryptamine added (mg)	Tryptophan yield (%)[c]
A	–	100
B	–	60
B	2.5	85
B	5.0	101
B	10.0	99
B	20.0	100

[a] Containing 3 mg of each amino acid, including tryptophan.
[b] Model solution derivatized (A) immediately or (B) after hydrolysis.
[c] Expressed as the tryptophan percentage obtained without hydrolysis (A).

TABLE II

REPRODUCIBILITY OF THE DETERMINATION OF VARIOUS AMOUNTS OF AMINO ACIDS, MEASURED AS N,O,(S)-TFAIBE, IN MODEL SOLUTIONS DERIVATIZED IMMEDIATELY (A VALUES) AND AFTER HYDROLYSIS IN THE PRESENCE OF 20 mg OF TRYPTAMINE (B VALUES)

Amino acid	Amount injected (μg)	Integrator units per μg amino acid			Standard deviation	Relative standard deviation (%)
		Single values		Mean[a]		
		A	B			
Alanine	7.21	83	91	88	3.13	3.6
	14.42	87	87			
	28.86	90	91			
Glycine	7.16	114	121	116	2.75	2.4
	14.31	114	114			
	28.62	115	116			
Threonine	7.14	163	171	164	4.09	2.5
	14.28	164	164			
	28.56	159	161			
Serine	7.18	98	104	98	3.03	3.1
	14.36	98	98			
	28.72	95	97			
Valine	7.50	89	85	85	2.68	3.2
	15.00	86	86			
	30.00	82	82			
Leucine + isoleucine	12.72	115	121	116	3.49	3.0
	25.44	116	116			
	50.88	112	113			
Cysteine + cystine	6.75	333	355	348	9.67	2.8
	13.50	344	344			
	27.00	357	357			
Proline	8.64	119	123	119	2.60	2.2
	17.28	119	119			
	34.56	116	116			
Hydroxyproline	6.82	265	259	260	3.37	1.3
	13.63	261	261			
	27.26	258	255			
Methionine	4.66	421	419	414	4.69	1.1
	9.32	413	413			
	18.64	411	409			
Aspartic acid	11.75	113	111	111	1.41	1.3
	23.50	112	112			
	47.00	109	111			
Phenylalanine	10.70	99	101	101	1.41	1.4
	21.40	102	102			
	42.80	103	101			
Ornithine	7.49	390	394	383	11.08	2.9
	14.98	388	388			
	29.96	370	368			
Glutamic acid	10.80	117	122	122	2.68	2.2
	21.60	123	123			
	43.20	122	125			
Tyrosine	6.21	178	192	195	8.89	4.6
	12.42	201	201			
	24.84	200	196			

(Continued on p. 197)

TABLE II *(continued)*

Amino acid	Amount injected (μg)	Integrator units per μg amino acid			Standard deviation	Relative standard deviation (%)
		Single values		Mean[a]		
		A	B			
Arginine	8.43	(169)	(178)	234	4.24	1.8
	16.86	237	237			
	33.72	234	228			
Tryptophan	3.54	380	385	382	3.18	0.83
	7.08	378	388			
	14.16	385	378			
	28.32	381	386			
Histidine	9.79	89	94	93	2.88	3.1
	19.58	95	95			
	39.16	(64)	(64)			

[a] Excluding data in parentheses.

TABLE III

TRYPTOPHAN CONTENT OF VARIOUS AMOUNTS OF PROTEINS, MEASURED AS N,O,(S)-TFAIBE IN HYDROCHLORIC ACID HYDROLYZATES, IN THE PRESENCE OF 20 mg OF TRYPTAMINE

Protein	Weighed (mg)	Tryptophan (%, w/w)		Standard deviation	Relative standard deviation (%)	Measured by the acid ninhydrin method [19–21] (%, w/w)
		Single values	Mean			
Lysozyme	22.5	6.94	6.94	0.102	1.5	6.97
	19.3	6.80				
	43.8	6.98				
	43.6	6.98				
	82.5	7.08				
	81.9	6.84				
α-Chymotrypsin	21.9	6.06	5.86	0.255	3.8	6.87
	22.2	6.01				
	42.0	5.91				
	42.3	6.01				
	81.2	5.53				
	80.0	5.62				
HGG	41.8	2.57	2.48	0.095	3.8	2.69
	80.8	2.49				
	163.5	2.38				
HSA	201.0	0.331	0.324	0.010	3.1	0.384
	199.8	0.317				
BSA	101,2	0.720	0.707	0.019	2.8	0.726
	100.0	0.719				
	202.4	0.709				
	201.0	0.680				

TABLE III *(continued)*

Protein	Weighed (mg)	Tryptophan (%, w/w)		Standard deviation	Relative standard deviation (%)	Measured by the acid ninhydrin method [19–21] (%, w/w)
		Single values	Mean			
Casein	97.7	1.24	1.30	0.042	3.2	–[a]
	98.0	1.34				
	199.0	1.30				
	196.9	1.30				
MM	200.8	0.363	0.367	0.003	0.94	0.333
	199.6	0.369				
	401.0	0.367				
	398.3	0.371				
FM	201.4	0.619	0.604	0.014	2.3	0.653
	200.0	0.585				
	401.4	0.606				
	400.8	0.606				
SM	200.4	0.238	0.230	0.009	4.1	–[a]
	200.8	0.236				
	399.6	0.217				
	400.0	0.229				

[a] No data available.

of tryptamine are given in Table II. It can be seen that the hydrolysis conditions do not affect the detector responses of amino acids, including tryptophan; the relative standard deviations of the measurements were 4.6% or less.

Regarding the tryptophan contents of various proteins and protein matrices, both the reproducibility of the results and their agreement with tryptophan contents obtained by the acid ninhydrin method [9,10] are good (Table III).

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