Journal of Chromatography, 632 (1993) 57-61
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2618

High-performance liquid chromatography of tryptophan and other amino acids in hydrochloric acid hydrolysates

I. Molnár-Perl, M. Pintér-Szakács and M. Khalifa

Institute of Inorganic and Analytical Chemistry, L. Eötvös University, P.O. Box 32, H-1518 Budapest 112 ***(Hungary)***

ABSTRACT

Tryptamine [3-(2-aminoethyl)indole] as an additive to 6 A4 HCl largely prevented the decomposition of tryptophan in proteins, and also in gas-phase hydrolysis, using a Pica-Tag Work Station, at 145°C for 4 h. This procedure proved to be excellent for amino acid analysis using conventional high-performance liquid chromatography with derivatization with phenyl isothiocyanate. The recovery of tryptophan from model solutions and from proteins was 80–98%. The results proved to be as good as any other obtained by the analysis of special alkaline or organic acid hydrolysates, most of which are suitable exclusively for the determination of tryptophan. The most important advantage of tryptamine was that it did not affect the quantitative recovery of other amino acids of proteins, as shown by the composition of lysozyme hydrolysates obtained in the absence and presence of various amounts of tryptamine. The reproducibility of the measurements was 3.7% (relative standard deviation) or less.

INTRODUCTION

It is well known that, under the common conditions of protein hydrolysis (6 *M* HCl, 100°C for 24 h or 145°C for 4 h), a considerable amount tryptophan is destroyed. A number of procedures have been published for the determination of tryptophan, including (i) the time-consuming and tedious alkaline hydrolyses suitable exclusively for tryptophan determination [l-lo], (ii) organic acid [11–15], with the limitation that they are successful in total amino acid analysis by ion-exchange chromatography only [the non-volatile organic acids inhibit the derivatization of amino acids for both gas chromatography (GC) and high-performance liquid chromatography (HPLC)] and (iii) the common HCI hydrolyses with additives [16–23], such as thioglycolic acid [16,191, mercaptoethanesulphonic acid [17], ethanedithiol [18], mercaptoethanol [21], phenol [22] or tryptamine [20,23], which are expected to be the most promising for the subsequent simultaneous assay of amino acids, including tryptophan, in protein hydrolysates.

Recently, it has been found [22] that the addition of 1–3% of phenol to 6 *M* HCl acid largely (*ca.* 80% recovery) prevents the destruction of tryptophan in peptides and proteins during liquid-phase hydrolyses at 110, 145 and 160°C for 22 h, 4 h and 25 min, respectively. However, from gas-phase hydrolyses only 10–20% of tryptophan could be recovered even with 3% phenol containing 6 *M* HCl.

The aim of this work was (i) to study the inhibitory activity of [3-(2-aminoethyl)indole] (tryptamine) also in gas-phase hydrochloric acid hydrolysis and (ii) to demonstrate its utility in the determination of amino acids including tryptophan in hydrolysates as their phenylthiocarbamyl (PTC) derivatives by HPLC [24].

EXPERIMENTAL

Materials

Triethylamine (TEA), phenyl isothiocyanate (PITC), tryptamine, amino acids and proteins were obtained from Sigma (St. Loius, MO, USA) and

Correspondence to: I. Molnár-Perl, Institute of Inorganic and Analytical Chemistry, L. Eötvös University, P.O. Box 32, H-1518 Budapest 112, Hungary.

0021-9673/93/$06.00

Serva (Heidelberg, Germany). HPLC-grade acetonitrile and methanol were purchased from Reanal (Budapest, Hungary). All other reagents were of the highest analytical purity. Hydrolysis and derivatization tubes were supplied with the Pica-Tag Work Station (Millipore-Waters, Chromatography Division, Milford, MA, USA).

Hydrolysis of proteins

Standard proteins (0.014–0.016 g, weighed with analytical accuracy) were dissolved in 10 cm^3 of 0.1 *M* HCl. Standards of free amino acids (AA) in a mixture containing 5 $\mu mol/cm^3$ of each were prepared in 0.1 *M* HCl. Tryptamine was dissolved in distilled water (2 $\mu g/\mu l$). Into a 50 × 6 mm tube, 5 μl of AA solution or 20 μl of standard protein solution were injected and various amounts of tryptamine were added (3.75–15 μl) and up to twelve tubes were placed in a vacuum vial. The vial was then attached to the Work Station manifold and the solvent removed under vacuum. After drying, the vacuum was released and 75 μl per tube of constant-boiling HCl were pipetted into the bottom of the vacuum vial. The vacuum vial was then re-attached to the manifold. First the vacuum valve was opened until it showed about l-2 Torr (1 Torr = 133.322 Pa) and the HCl began to bubble (20-30 s). Thereafter, after closing the vacuum valve, the nitrogen valve was opened, purged for 30 s, and closed again. The vacuum-nitrogen cycles were repeated three times. After the last cycle, the vacuum valve was opened and the cap of the vial was closed under vacuum and put into the Pica-Tage Work Station oven (145°C for 4 h). After hydrolysis, the sample tubes were carefully removed from the vial and the HCl was wiped from the outside of each tube. The tubes were then transferred into a fresh reaction vial, attached to the manifold and evaporated to the constant minimum reading (cu. 65 mTorr). The hydrolysed samples were then ready for derivatization.

*Derivutization of umino acids with **PITC***

A 5-μl volume of AA solution, used for comparison of the hydrolysed samples, was injected into 50 × 6 mm tubes and dried under vacuum. Hydrolysed AA (free amino acids) and hydrolysed proteins were redried by adding 10 μl of ethanol-water-TEA (2:2:1) to each tube. Thereafter to each redried sample 20 μl of derivatization reagent [ethanol–TEA–water–PITC (7: l: 1: I)] were added and mixed by vortex mixing. Subsequently they were treated according to the Waters Pica-Tag Work Station manual.

The derivatized standards were dissolved in 600 μl and the hydrolysed and derivatized proteins in 300 μl of 0.05 *M* sodium acetate solution (pH 7.2). Thus, the 20-μl aliquots of AA solution contain 800-1000 pmol of each AA and the 20-μl protein samples represent, in total, 1.2 2.0 μg of AA.

Chromatography

The Liquochrom Model 2010 liquid chromatograph (Labor MIM, Budapest, Hungary) used consisted of two Liquopomp 312/1 solvent-delivery pumps and a Type OE-308 UV detector with a wavelength range of 195-440 nm. Samples were injected in 20-μl volumes using an injector supplied by Labor MIM. The columns (BST, Budapest, Hungary) were 150 mm × 4.00 mm with Hypersil ODS bonded phase (5 μm) (Shandon). Eluents were kept under a blanket of nitrogen. The solvent system consisted of two eluents: A = 0.05 *M* sodium acetate (pH = 7.2) and B = 0.1 A4 sodium acetate-acetonitrileemethanol (46:44: 10) (mixed according to their volume ratios and titrated with glacial acetic acid or 50% sodium hydroxide to pH 7.2). A gradient which was optimized for the separation was from 0% to 100% B in 22 min. For 5 min a washing step with 100% B was applied, then after 100% A was applied for 2 min. After an additional elution for 5 min with solvent A, the system was ready for the next injection.

RESULTS AND DISCUSSION

Based on our earlier experience, *i.e.*. the facts that (i) tryptamine proved to be a powerful additive for the protection of tryptophan in classical HCl hydrolysis in the liquid phase [23] and (ii) gas-phase HCl hydrolysis in the Pica-Tag Work Station with subsequent determination of PTC-amino acids by HPLC can be regarded as one of the most promising methods in protein analysis [24], the extension of the inhibitory activity of tryptamine to gas-phase HCl hydrolyses seemed to be worthy of investigation.

The results of our studies on the effect of tryptamine on tryptophan measurement after HCl hy-

TABLE I

EFFECT OF AMOUNT OF TRYPTAMINE ON THE RECOVERY OF AMINO ACIDS IN MODEL SOLUTIONS (AA) BY HYDROLYSIS WITH 6 *M* HCl AT 145°C FOR 4 h IN THE GAS PHASE, MEASURED AS PTC-AMINO ACIDS BY HPLC

No.	Amino acid	Detector responses: peak area (arbitrary units/pmol AA)					Reproducibility data		
		Tryptamine added (μg per 70 μg AA)					Average	S.D.	R.S.D. (%)
		0[b]	0	7.5	15	30			
1	Aspartic acid	182	180	188	192	180	184	5.4	2.9
2	Glutamic acid	208	204	214	217	215	212	5.4	2.5
3	Hydroxyproline	207	203	209	214	207	208	4.0	1.9
4	Serine	160	150	151	161	162	157	5.8	3.7
5	Glycine	194	208	200	212	198	202	7.4	3.7
6	Histidine	170	178	177	183	175	177	4.7	2.7
7	Threonine	176	173	184	181	178	178	4.3	2.4
8	Alanine	200	199	211	211	212	207	6.5	3.1
9	Proline	245	240	236	246	240	241	4.1	1.7
10	Arginine	210	200	205	212	213	208	5.4	2.6
11	Tyrosine	193	191	202	203	195	197	4.8	2.5
12	Valine	207	205	200	204	212	208	6.0	2.9
13	Methionine	183	180	182	185	188	184	3.1	1.7
14	Cyst(e)ine	140	144	141	150	143	144	3.9	2.7
15	Isoleucine	192	184	182	190	188	187	4.2	2.2
16	Leucine	204	198	198	206	197	201	4.1	2.1
17	Phenylalanine	184	190	183	189	188	187	3.1	1.7
18	Tryptophan	198	53	151	169	188			
		(100)	(27)	(76)	(85)	(95)			
19	Lysine	280	290	290	297	281	288	7.1	2.5

[a] Single values represent the averages of six separate tests.
[b] Tests without hydrolysis; in parentheses, percentage of the theorerical values.

drolyses in the gas phase are presented in Tables I and II and Fig. 1.

Tryptamine (in the concentration range 15–30 μg per 70 μg of AA or 30 μg of protein) largely protects tryptophan from decomposition. In model solutions the inhibitory effect proved to be 80–95% (Table I) whereas with lysozyme 80% of the tryptophan content could be measured (Table II).

Applying 30 μg of tryptamine per 30–40 μg of α-chymotrypsin, bovine albumin and human albumin for their gas-phase hydrolysis by HCl, 76, 84 and 98% tryptophan **contents were** measured, respectively.

Regarding the effect of tryptamine on the other amino acids, it can be stated that their presence did not affect either the response values of the other components in model solutions (Table I) or the number of residues found in hydrolysates of lysozyme (Table II, Fig. 1.) The repeatability for all other amino acids, besides tryptophan, obtained with model solutions or after hydrolyses performed with various amounts of tryptamine or without it proved to be good, *i.e.*, ($\leqslant$ 3.7% (relative standard deviation) reproducibility data are given in Tables I and II.

These results are important with respect to the advantage of the use of tryptamine as an additive over phenol, even in liquid-phase hydrolysis. The presence of phenol seriously decreased the response of cystine [22]; on using 1–3% phenol-containing HCl for hydrolysis in model tests, the cystine response decreased to 40–50% of its original value.

Concerning the mechanism of the inhibitory effect of tryptamine (tryptamine being a decarboxylated tryptophan and present in a considerable excess over tryptophan), it is very likely that instead

TABLE II

EFFECT OF AMOUNT OF TRYPTAMINE ON THE RECOVERY OF AMINO ACIDS IN HYDROLYSATES OF LYSOZYME BY HYDROLYSIS WITH 6 A4 HCl AT 145°C FOR 4 h IN THE GAS PHASE. MEASURED AS PTC-AMINO ACIDS BY HPLC

No.	Amino acid	Moles amino acid per mole protein[a]					Reproducibility data		
		Literature data	Tryptamine added (μg per 30 μg lysozyme)				Average	S.D.	R.S.D. (%)
			0	7.5	15	30			
1	Aspartic acid	20	19.7	19.8	19.5	19.6	19.6	0.14	0.7
2	Glutamic acid	5	5.1	5.1	5.1	5.0	5.1	0.06	1.2
4	Serine	8	8.2	8.0	7.8	8.0	8.0	0.16	2.0
5	Glycine	12	11.9	11.7	12.0	11.9	11.9	0.13	1.1
6	Histidine	1	1.09	1.09	1.01	1.08	1.05	0.04	3.5
7	Threonine	7	6.5	6.4	6.6	6.5	6.5	0.08	1.3
8	Alanine	12	11.9	12.0	12.0	12.1	12.0	0.08	0.7
9	Proline	2	2.04	2.04	2.11	2.08	2.08	0.04	1.8
10	Arginine	11	11.2	11.1	10.8	11.1	11.1	0.18	1.6
11	Tyrosine	3	2.95	3.15	3.20	3.11	3.13	0.11	3.6
12	Valine	6	6.1	6.0	6.0	6.0	6.0	0.05	1.0
13	Methionine	2	2.25	2.20	2.10	2.16	2.18	0.06	2.9
14	Cyst(e)ine	8	x.5	8.2	8.4	x.0	x.3	0.22	2.7
15	Isoleucine	6	6.2	6.2	6.0	6.1	6.1	0.10	1.6
16	Leucine	8	7.x	7.9	7.1	7.7	7.8	0.1	1.3
17	Phenylalanine	3	2.99	3.03	3.00	2.94	2.98	0.04	1.3
18	Tryptophan	6	0.6 (10)	3.4 (57)	4.x (80)	4.8 (80)			
19	Lysme	6	6.0	6.3	6.2	6.1	6.2	0.14	2.3

[a] As in Table I, literature data: [25].

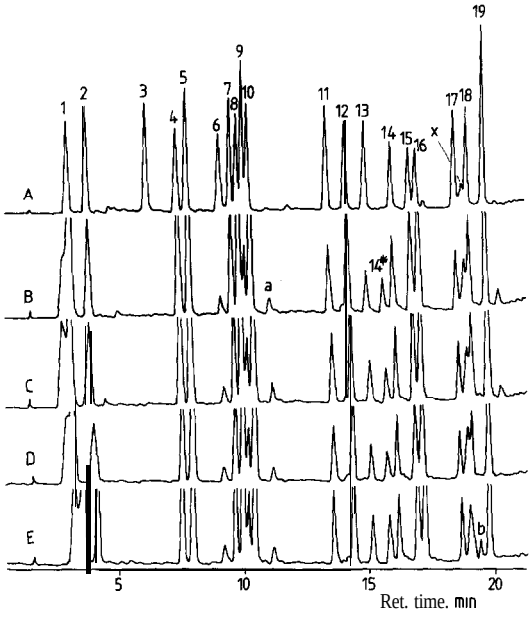

Fig. 1. Chromatograms of AA solution (A, detailed data in Table I: first column of data) and those of lysozyme hydrolysates (B-E, detailed data in Table II) obtained by HCI hydrolysis in the presence of(B) 30, (C) 15 and (D) 7.5 μg of tryptamine and (E) without additive. Peak identification as in Tables I and II; also, a = unknown component of lysozyme; b = decomposition product of tryptophan; x = reagent impurity: in AA solution (0.6 cm^3 volume) proved to be the half compared to those in lysozyme hydrolyzates (0.3 cm^3 volume). 14* = Cystine separates into two peaks, as a result of racemization during hydrolysis. Note: aspartic acid in large amounts elutes as two peaks.

of tryptophan, tryptamine takes part in those undesirable interactions which lead to the decomposition of tryptophan.

In conclusion, tryptamine as an additive in gas-phase HCl hydrolysis, providing 76–98% recovery of tryptophan from a single hydrolysate with one injection together with other amino acids with 100% recovery, can be utilized as a powerful protocol in the rapid analysis of protein hydrolysates.

REFERENCES

1 J. C. Huet and J. C. Pernollet, ***J. Chromatogr., 355 (1986) 451.***
2 H. K. Nielsen and R. F. Hurrell, ***J. Sci. Food Agric.,*** 36 (1985) 893.
3 H. Sato, T. Seino, T. Kobayashi, A. Murai and Y. Yugar, ***Agric. Biol. Chem., 48 (1984) 2961.***
4 R. L. Levine, ***J. Chromatogr., 236 (1982) 499.***
5 P. J. Buttery and J. B. Soar, ***J. Sci.*** Food Agric., 26 (1975) 1273.
6 A. P. Williams, D. Hewitt and P. J. Buttery, ***J. Sci. Food Agric.,*** 33 (1982) 860.
7 T. E. Hugli and S. Moore, ***J. Biol. Chem., 247 (1972) 2828,***
8 R. Know, G. 0. Kohler, R. Palter and H. G. Walker, ***Anal. Biochem.. 36 (1970) 136.***
9 E. L. Miller, ***J. Sci. Food*** Agric., 18 (1967) 381.
10 J. R. Spies, ***Anal.*** Chem., 39 (1967) 1412.
II R. J. Simpson, M. R. Neuberger and T. Y. Liu, *J. Biol.* Chem., 251 (1976) 1936.
12 T. Y. Liu and Y. H. Chang, ***J. Biol.*** Chem., 246 (1971) 2842.
13 J. Gundel and E. Votisky, *Tagungsber.* ***Akad.*** *Landwirtschaftswiss.* ***DDR, 124 (1974) 59.***
14 B. Penke, R. Ferenczi and K. Kovács, ***Anal. Biochem., 60 (1974) 59.***
15 R. J. Richard, M. R. Neuberger and T. Y. Liu, ***J. Biol. Chem., 251 (1976) 1936.***
16 R. B. Ashworth, ***J. Assoc. Off. Anal.*** Chem., 70 (1987) 80.
17 M. L. G. Gardner, ***Anal. Biochem., 141 (1984) 429.***
18 P. Felker, ***Anal.*** Biochem., 76 (1976) 192.
19 H. Matsubara and R. M. Sasaki, ***Biochem. Biophys. Res.*** *Commun.*, ***35 (1969) 175.***
20 L. C. Gruen and P. W. Nicholls, ***Anal. Biochem., 47 (1972) 348.***
21 L. T. Ng, A. Pascaud and M. Pascaud, ***Anal. Biochem.,*** 167 ***(1987) 47.***
22 K. Muramoto and H. Kamiya, ***Anal.*** Biochem., 189 (1990) 223.
23 V. Fabian, M. P. Szakács and I. M. Perl, ***J. Chromatogr., 520 (1990) 193.***
24 M. Morvai, V. Fabian and I. M. Perl, ***J. Chromatogr.,*** 600 ***(1992) 87.***
25 R. L. Heinrikson and S. C. Meredith, ***Anal. Biochem., 136 (1984) 65.***