HIGH RECOVERY OF TRYPTOPHAN FROM ACID HYDROLYSATES OF PROTEINS

Hiroshi Matsubara and Richard M. Sasaki

Space Sciences Laboratory, University of California, Berkeley,Calif. 94720

Received February 23, 1969

Moderate concentrations of thioglycolic acid largely prevented the destruction of tryptophan during acid hydrolysis of proteins. A new method for the microanalysis of tryptophan by acid hydrolysis is proposed.

It is known that tryptophan is destroyed to a considerable extent when proteins are hydrolysed with acid. Several methods are used for tryptophan determination which avoid acid hydrolysis (Light and Smith, 1963; Moore and Stein, 1963; Barman and Koshland, 1967). Recently thioglycolic acid has been used to prevent the oxidation of methionine, tyrosine, or carboxymethylcysteine during chromatography or acid hydrolysis of peptides and proteins (Tuppy and Kreil, 1962; Sletten et al., 1968; Keresztes-Nagy, Perini and Margoliash, 1968; Matsubara, Sasaki and Tsuchiya, 1968).

This paper concerns the use of the reagent for the study of protein composition in small-scale experiments under various conditions.

EXPERIMENTAL

Materials: Purified thioglycolic acid was purchased from Mann Research Lab., Inc. and kept in a refrigerator. Chicken lysozyme, bovine α-chymotrypsin, and bovine ribonuclease A were from Worthington Biochem. Corp.; mercury-papain was from Calbiochem.; bovine cytochrome c and spinach ferredoxin were prepared as described (Hagihara et al., 1958; Tagawa and Arnon, 1962); the protein from tobacco mosaic virus(TMV) vulgare, and E. coli tryptophanase (apo) were the generous gifts of Dr. C.A.Knight, and Dr. H. Kagamiyama, respectively. L-tryptophan, obtained from Calbiochem., was dissolved in 0.02 N HCl.

Hydrolysis of Proteins and Analysis of Tryptophan: Proteins(0.1-0.2 mg) or tryptophan(0.02 μmole)were hydrolysed in an evacuated, sealed tube with 6 N HCl

(0.2 ml) containing 0.5-6%(v/v) thioglycolic acid. This solution was prepared just before hydrolysis of samples, which was for 24 to 64 hours at 108-110°. Hydrolysis tubes were evacuated to 25 to 50 microns. Hydrolysates were dried in a rotary evaporator at 60° within 15-20 minutes. Other details of the process were as previously described (Moore and Stein,1963). A Spinco amino acid analyzer, 120B, was used for the determination of tryptophan on the short column (7 cm height), with the buffer at pH 5.28, according to Hubbard (1965). Tryptophan emerges just before lysine (Moore and Stein,1963). The recovery of tryptophan was calculated on the basis of molar ratio to other amino acids and expressed as percentage of the theoretical value obtained from the literature.

Identification of the Peak on the Column as Tryptophan: It is stated that tryptophan and its destruction product emerge in two peaks on the longer resin column, just before the lysine peak (Moore and Stein,1963). We examined the material emerging at the position corresponding to the tryptophan peak, to determine whether it contained only tryptophan, or a mixture of tryptophan and its degradation products. About 20 mg of lysozyme were hydrolysed with 5 ml of 6 N HCl containing 2% thioglycolic acid as described above. The analysis of tryptophan showed approximately 84% recovery (see Table I). We have not studied the fate of the 16% of the tryptophan lost. In a separate experiment on a preparative scale, the material corresponding to the tryptophan peak was collected from the short column, with pH 5.28 buffer. A sample of the recovered material was rechromatographed, and emerged at the same position; this was assumed to represent the total amount of tryptophan. The following criteria were then used to compare the unknown sample with a tryptophan standard. (a) Ultraviolet absorption spectra at pH 5.28 and pH 13, recorded by Beckman spectrophotometer, Model DB. (b) Fluorescence spectra at pH 5.28 recorded by Aminco-Bowman fluorospectrophotometer. (c) Paper chromatography with butanol-acetic acid-water(100:15:37.5,v/v) and butanol-pyridine-acetic acid-water (15:10:3:12, v/v). (d) Chromatography on a long column of Spinco cnalyzer at pH 4.28. (e) Assay by E. coli tryptophanase (kindly carried out by Dr. H. Kagamiyama) (Newton and Snell, 1962).

Table I

Amino Acid Compositions of Proteins after Acid Hydrolysis in the Presence and Absence of Thioglycolic Acid

	Lysozyme			Cytochrome c		TMV protein	
Thioglycolic acid	+		-	+	-	+	-
Lysine	6.08	5.97	6.07(6)	18.0	18.0 (18)	2.11	2.06(2)
Histidine	1.00	1.01	0.87(1)	3.07	3.14(3)	0.00	0.00(0)
Arginine	10.9	10.9	12.0 (11)	1.94	1.80(2)	11.0	10.6(11)
Tryptophan	5.04	5.45	3.59(6)	0.86	0.19(1)	2.45	1.44(3)
Aspartic acid	21.1	21.1	20.5 (21)	8.24	8.35(8)	18.2	17.7 (17)
Threonine	7.35	6.90	6.92(7)	7.76	7.43(8)	14.6	15.1 (16)
Serine	9.77	8.60	9.64(10)	1.12	1.32(1)	15.4	15.6(16)
Glutamic acid	4.98	5.21	4.63(5)	12.0	12.1 (12)	15.8	15.6 (16)
Proline	6.33	6.70	3.23(2)	5.26	4.90(4)	8.72	8.31(8)
Glycine	12.2	12.0	11.6 (12)	14.0	14.3 (14)	6.40	5.90(6)
Alanine	12.2	12.5	11.4 (12)	6.15	6.06 (6)	14.2	14.1 (14)
Valine	3.87	6.04	3.82(6)	2.42	2.18(3)	9.44	9.59(14)
Methionine	1.93	2.04	1.70(2)	1.54	1.87(2)	0.00	0.00(0)
Isoleucine	4.52	5.65	4.41(6)	4.63	4.41(6)	5.68	5.63(9)
Leucine	7.65	7.90	7.74(8)	5.97	5.92(6)	11.4	11.2 (12)
Tyrosine	3.24	3.03	3.18(3)	3.84	3.77(4)	3.84	3.63(4)
Phenylalanine	2.90	2.91	3.48(3)	3.34	3.41(4)	7.63	7.48(8)
Recovery of Tryptophan (%)	84	91**	60	86	19	82	48
Reference for Theoretical Values*	Canfield and Liu, 1965			Nakashima et al, 1966		Funatsu & Fraenkel-Conrat, 1964	

* The theoretical values are shown in parentheses.

** Sixty-four hour hydrolysate with 4% thioglycolic acid.

We also examined the material emerging at the tryptophan position on the column after treatment of free tryptophan as described in the previous section. Only the criteria, (a) and (c), were applied in this case for identification of tryptophan

RESUTLS AND DISCUSSION

Analyses on the column showed 95-100% recovery as tryptophan after heating free tryptophan with acid in the presence and absence of thioglycolic acid when good evacuation of the hydrolysis tube was obtained. The ultraviolet absorption curve and Rf value on paper chromatography (butanol-acetic acid-water) of the sample corresponding to the tryptophan peak were the same as those of free tryptophan. When tryptophan was treated with acid without thioglycolic acid and evacuation, the product showed no peak at the tryptophan position on the short column, but a peak was detected at or just after the tyrosine position on the long column. Paper chromatography of the hydrolysate showed a major spot with an Rf value equal to that of histidine. The absorption spectrum of this material did not show the 280 mμ maximum. A detailed study of the breakdown products was not made.

All of the procedures, (a) to (e), for the hydrolysate of lysozyme showed that the peak corresponding to the tryptophan position on the column was composed solely of tryptophan. (a) Standard tryptophan and the unknown sample both showed two distinctive peaks at 288 and 280 mμ with a shoulder at 272 mμ ,and: (b) Both showed the same emmision spectra with 350 mμ maximum by 280 mμ exitation. (c) No difference was observed in chromatographic behavior between tryptophan and the experimental sample. Co-chromatography of these materials showed only one spot. (d) Tryptophan and the unknown sample were both eluted from the long column as a broad peak at about 5 hours. Co-chromatography showed only one peak. (e) The assay by tryptophanase fully confirmed the quantitative values obtained from column analyses.

On the basis of these observations and the experiments with free tryptophan, we carried out analyses of tryptophan in proteins. Table I gives the results obtained from three different proteins after acid hydrolysis in the presence and

absence of 2% thioglycolic acid in evacuated tubes at 50 microns. Without the reagent, some tryptophan was recovered, but the recovery varied with the type of protein. However, in the presence of thioglycolic acid, the recoveries of tryptophan from protein hydrolysates were nearly constant, about 85%. This result encouraged the use of thioglycolic acid for the microanalysis of tryptophan. It was also noted that the use of the reagent did not affect the recovery of other amino acids, except proline, which in this case was probably due to contamination with cysteine. However, this might be overcome by exposing the hydrolysates to air before analysis (Moore and Stein, 1963). The low recoveries of hydrophobic amino acids in analyses with and without the reagent after 24 hours hydrolysis may be due to the incomplete hydrolysis of peptide bonds involving these amino acids (Light and Smith,1963). When a higher concentration (4%) of thioglycolic acid and a higher vacuum (25 microns) were used in the hydrolysis of several proteins, the recoveries of tryptophan were increased to about 90% or more. Various concentrations of thioglycolic acid were examined in the hydrolysis of lysozyme and it was found that at 25 microns, 2% thioglycolic acid gave an improved recovery of tryptophan, but increasing the concentration of the reagent above 4% did not raise the recovery of tryptophan to more than 91%. Surprisingly, even after 64 hours hydrolysis there was no decrease of tryptophan recovery when 4% thioglycolic acid was used (Table I). Table II summarizes these results. Even with 4% thioglycolic acid there was no interference with the analyses of other amino acids in cytochrome c, ferredoxin, chymotrypsin, tryptophanase, and lysozyme (see Table I for lysozyme). Complete analyses were not carried out for TMV protein, Hg-papain and ribonuclease.

Lower concentrations of thioglycolic acid (0.05 -0.1%,v/v) did not fully protect tryptophan during the hydrolysis; at least 2% thioglycolic acid was required. Although the analysis of total composition could be carried out accurately in a single determination, it is probably best to analyze tryptophan in an additional experiment. The presence of high concentrations of thioglycolic acid produces a high peak between cysteic acid and aspartic acid and a small peak at

Table II

Analyses of Tryptophan in Proteins by Acid Hydrolysis
With Various Concentrations of Thioglycolic Acid

		Tryptophan	Lysine	Histidine	Arginine	Recovery(%)
Bovine cytochrome c	Thioglycolic acid (4%)	0.96(1)	17.9(18)	3.22(3)	1.85(2)	96
Spinach ferredoxin		0.89(1)	3.74(4)	1.02(1)	1.05(1)	89
Bovine chymotrypsin		7.10(8)	13.8(14)	2.24(2)	2.76(3)	89
TMV protein		2.79(3)	1.94(2)	0.00(0)	11.3(11)	93
Hg-papain		4.91(5)	10.1(9)	1.75(2)	7.60(11)*	98**
Tryptophanase		0.88(1)	16.5(17)	4.95(5)	14.6(14)	88
Bovine ribonuclease A		0.00(0)	10.3(10)	3.60(4)	4.30(4)	-
Chicken lysozyme	Thioglycolic acid (%) 0.5	4.78(6)	5.57(6)	1.13(1)	10.4(11)	80
	1.0	5.23	5.83	1.01	11.3	87
	2.0	5.56	5.87	1.01	11.2	93
	4.0	5.47	5.84	1.02	11.1	91
	6.0	5.46	5.77	1.03	11.1	91

Theoretical values are shown in parentheses. The references for cytochrome c, TMV protein, and lysozyme are as in Table I. Others are: spinach ferredoxin (Matsubara and Sasaki, 1968); bovine chymotrypsin (Hartley and Kaufmann, 1966); Hg-papain (Light et al, 1964); bovine ribonuclease (Smyth, et al, 1963); tryptophanase (Morino and Snell, 1967).

*No reason was found for this discrepancy. ** The average value of lysine and histidine was used for tryptophan calculation.

---

the carboxymethylcysteine position. We recommend making the column a little longer than that we used in these experiments to avoid interference of the front

base line of the tryptophan peak by a yellow color caused by the ninhydrin reaction with the reagent and to drain the first effluent into the waste line for several minutes before sending it into the reaction coil of the analyzer.

We found that when 50 molar excess of glucose was present in the protein sample, the protective effect of thioglycolic acid was lost and no tryptophan was recovered from any of the protein samples.

Recently, Blake and Li (1968) have used β-mercaptoethanol to protect tryptophan from destruction during the synthesis of a tryptophan peptide. This fact complements our finding.

ACKNOWLEDGMENT

This work was supported by grant HE 11553 from the National Institutes of Health and grant NGR 05-003-020 from the National Aeronautics and Space Administration to the University of California, Berkeley. The authors express their thanks to Dr. T. H. Jukes for his interest and support of this study and to Mrs. D. K. Tsuchiya for her technical assistance.

REFERENCES

Barman, T. E. and Koshland, D. E. Jr., J. Biol. Chem., 242, 5771 (1967)
Blake, J. and Li, C. H., J. Amer. Chem. Soc., 90, 5882 (1968)
Canfield, R. and Liu, A. K., J. Biol. Chem., 240, 1997 (1965)
Funatsu, G. and Fraenkel-Conrat, H., Biochemistry, 3, 1356 (1964)
Hagihara, B., Tagawa, K.,Morikawa, I., Shin, M. and Okunuki, K., J. Biochem., Tokyo, 45, 725 (1958)
Hartley, B. S. and Kaufmann, D. L., Biochem. J., 101, 229 (1966)
Hubbard, R. W., Biochem. Biophys. Res. Commun., 19, 679 (1965)
Keresztes-Nagy, S., Perini, F. and Margoliash, E., personal communication (1968)
Light, A., Frater, R., Kimmel, J. and Smith, E. L., Proc. Natl. Acad. Sci.,52, 1276 (1964)
Light, A. and Smith, E. L., in The Proteins, H. Neurath (editor), Acad. Press, New York, 1963, p. 1.
Matsubara, H. Sasaki, R. M., J. Biol. Chem., 243, 1732 (1968)
Matsubara, H., Sasaki, R. M. and Tsuchiya, D. K., unpublished result(1968)
Moore, S. and Stein, W. H., in Methods in Enzymology, S. P. Colowick and N.O. Kaplan (Editors), Acad. Press, New York, Vol. VI, 1963, p. 819.
Morino, Y. and Snell, E. E., J. Biol. Chem., 242, 5602 (1967)
Nakashima, T., Higa, H., Matsubara, H., Benson, A. and Yasunobu, K. T., J. Biol. Chem., 241, 1166 (1966)
Newton, W. A. and Snell, E. E., Proc. Natl. Acad. Sci. U.S., 48, 1431 (1962)
Sletten, K., Dus, K., deKlerk, H. and Kamen, M. D., J. Biol. Chem., 243, 5492 (1968)
Smyth, D. G., Stein, W. H. and Moore, S., J. Biol. Chem., 238, 227 (1963)
Tagawa, K. and Arnon, D. I., Nature, 195, 537 (1962)
Tuppy, H. and Kreil, G., Monatsheft fur Chemie, 93, 780 (1962)