INFLUENCE OF FORMIC ACID ON THE HYDROLYSIS OF TISSUE PROTEINS

A NEW AND RAPID METHOD OF HYDROLYSIS OF PROTEINS

by

S. U. GURNANI, U. S. KUMTA AND M. B. SAHASRABUDHE

Biology Division, Department of Atomic Energy, Government of India, Indian Cancer Research Centre, Bombay (India)

The existing methods of hydrolysis of proteins into amino-acids can be classified into two main categories: (i) hydrolysis by acids or alkalies and (ii) hydrolysis by proteolytic enzymes. However these methods are time consuming, often requiring anything from 6 to 24 hours[1-3]. In addition, such methods frequently suffer from a further handicap, in that some of the amino-acids are destroyed during the progress of hydrolysis[4-6]. In an earlier communication[7] it was suggested that CO–NH bond is not so resistant as to take such a protracted period of time for its cleavage in an acidic or alkaline medium. Steric configurations[8-12] presumably play a decisive part to account for time factor. From studies on molecular configuration of proteins in tissues by RUDALL[11], it would appear that tissue proteins are predominantly of the α or the folded type. If the α type of proteins could be unfolded into extended β-form, the accessibility of CO–NH bonds to hydrolytic agents might thereby be increased. Based on the work of AMBROSE AND ELLIOT[12] regarding $\alpha \rightleftharpoons \beta$ transformations through the mediation of formic acid, experiments on the hydrolysis of proteins by HCl were carried out by GURNANI AND SAHASRABUDHE[7] after pretreatment with formic acid and it was observed that the time of hydrolysis was markedly curtailed.

In the present communication an attempt is made to assess the relative efficiency of this new technique in terms of standard procedures.

MATERIALS AND METHODS

The material upon which the present work is based consists for the most part of tissues from various organs of experimental animals. The schedule for processing them is as follows: The tissue is minced in a suitable homogenizer (Potter and Elvehjem) and followed by boiling with 85% formic acid (2 ml per g of tissue). With this treatment the tissue rapidly goes into solution (2–3 minutes). This is immediately followed by the addition of 2 *N* HCl, making up a final volume of 20 ml per g of tissue. The mixture is then refluxed for two different time intervals: 2 and 6 hours respectively.

For comparison, standard methods of hydrolysis are employed without formic acid. This consists of using similar aliquots of the homogenates which are refluxed either by treatment with 2.5 *N* HCl or 5 *N* NaOH for 20 hours.

For recovery experiments at these intervals, parallel experiments are also carried out with 5 mg of the amino acids (L-enantiomorph) added to each g of tissue prior to hydrolysis. Amino-acids

References p. 557.

are estimated from these hydrolysates by the usual microbiological procedures, by employing the organisms *Lactobacillus arabinosus* 17/5[13–15], *Leuconostoc mesenteroides* P-60[15–17] and *Streptococcus fecalis* R[18]. The technique of culturing and harvesting the organisms is essentially the same as described by BARTON-WRIGHT[15].

Standard amino-acids are also refluxed with 2 ml of 85 % formic acid and 2 *N* HCl for 2 hours. This is carried out to see whether presence of formic acid would have any specific effect on the natural growth of the organisms.

This procedure is also extended to serum proteins to study the efficacy of this method in releasing the amino-acids from serum proteins.

RESULTS

From Table I it will be seen that the release of amino-acid is complete in a short span of 2 hours. Further hydrolysis of similar aliquots for 6 hours bring about no augmentation in yield whatever of the amino acids. In contrast the corresponding yield of amino-acids is obtained only after 20 hours treatment with the standard procedure.

The data on the recovery experiments are presented in Table II. It is apparent from this, that the recovery of amino-acids is complete in all the three series of experiment.

TABLE I

COMPARATIVE STUDY OF RELEASE OF AMINO-ACIDS BY FORMIC ACID-HCl METHOD AND STANDARD PROCEDURE

No.	*Amino-acid*	*Hydrolysis of formic acid treated tissue with 2 N HCl*		*Hydrolysis with 2.5 N HCl alone 20 hours*
		2 hours	*6 hours*	
1	Phenylalanine	5.85*	5.83*	5.85*
2	Isoleucine	6.50	6.81	6.36
3	Methionine	2.40	2.61	2.70
4	Lysine	13.35	13.36	13.35
5	Leucine	17.5	15.83	15.95
6	Histidine	4.73	4.90	5.04
7	Arginine	12.20	12.41	10.23
8	Threonine	8.30	8.04	8.52
9	Valine	8.18	8.66	8.25

* Values expressed in mg per g of tissue.

TABLE II

RECOVERIES OF AMINO ACIDS ADDED TO TISSUE HOMOGENATES BEFORE HYDROLYSIS

No.	*Amino-acid*	*Recovery in formic acid treated hydrolysis (%)*		*Recovery in hydrolysis with 2.5 N HCl alone (%) 20 hours*
		2 hours	*6 hours*	
1	Phenylalanine	106.8	106.0	100.57
2	Isoleucine	98.46	104.70	100.50
3	Methionine	98.34	96.58	93.50
4	Lysine	99.0	100.0	98.0
5	Leucine	98.0	104.0	104.10
6	Histidine	97.33	104.10	97.60
7	Threonine	100.50	107.3	95.0
8	Arginine	100.40	99.66	98.0
9	Valine	106.06	100.29	100.0

References p. 557.

Once again, the marked diminution in time for the completion of the process in formic acid treated samples becomes manifest, and there is no indication of any impairment of hydrolysis by the formation of stable formyl derivatives as would occur by treatment with formic acid alone[19].

Table III illustrates the influence of pretreatment of serum proteins with formic acid in accelerating the complete release of amino-acids in two hours. The percentage recoveries of amino-acids added are also within the limits of accuracy of microbiological technique.

TABLE III

COMPARATIVE STUDY OF RELEASE OF AMINO-ACIDS FROM *serum proteins* BY THE FORMIC AND ACID HCl METHOD AND STANDARD PROCEDURE

No.	*Amino-acid*	*Hydrolysis of formic acid treated serum with 2 N HCl*		*Hydrolysis with 2.5 N HCl alone 20 hours*
		2 hours	*6 hours*	
1	Lysine	4.46	4.8	4.8
2	Methionine	0.85	0.85	0.84
3	Isoleucine	1.66	1.66	1.84
4	Phenylalanine	3.23	2.75	3.33
5	Threonine	3.35	3.12	3.30
6	Arginine	4.24	4.25	3.0

Though the amino-acids studied above are well protected in acid hydrolysis, the case of tryptophane needs special consideration. It is interesting to point out that the lability of this amino-acid differs from those reported for other amino acids. In acid hydrolysate e.g. this amino-acid is completely or partially destroyed, while in alkaline hydrolysis racemization occurs[15]. The destruction of amino-acids can also occur from reactions with carbohydrates[19–22], aldehydes[19], and pyruvic[5] acid present in tissue material. As is clear from Table IV, though pure tryptophane is not destroyed by formic acid HCl treatment yet the recovery of this amino-acid added to tissue homogenate is only 45%. In the case of HCl hydrolysis however 100% destruction of tryptophane takes place. Alkaline hydrolysis is therefore the method chosen for estimating this amino-acid.

TABLE IV

INFLUENCE OF FORMIC ACID HCl HYDROLYSIS ON TRYPTOPHANE

	Formic acid-HCl for		*2.5 N HCl alone for 20 hours*	*5 N NaOH alone for 20 hours*
	2 hours	*6 hours*		
% recovery of pure tryptophane	98	—	55.4	100*
% recovery of tryptophane added to tissue extract	45.5	10.58	0.0	101.0*
Release of tryptophane from tissue (mg/g)	0.568	0.241	0.0	1.687*

* Values doubled to allow for the racemisation with NaOH hydrolysis.

It is also observed that pure amino-acids treated with formic-acid HCl give growth responses identical with the untreated standard amino-acids, thus showing that formic

References p. 557.

acid does not interfere with the normal growth response of the organisms to the amino-acids.

DISCUSSION

Except for the destruction of tryptophane, the technique outlined here obeys the general criteria of a good method for the hydrolysis of tissue proteins. The conditions of hydrolysis do not cause destruction of amino-acids in the presence of mixtures of other amino-acids simulating the protein hydrolysate. Further they can be quantitatively recovered *in toto* after hydrolysis. This hydrolytic procedure therefore has the unique advantage of curtailing the time of hydrolysis without sacrificing in any way the release of amino-acids from tissue proteins.

Though the mechanism of action of formic acid is still a matter of speculation two alternative possibilities suggest themselves: (i) formic acid probably acts as a physical agent in readily dissolving the tissue material thus facilitating the subsequent hydrolysis with HCl or (ii) the aldehydic group of formic acid probably combines with the NH group of the peptide chain producing an unfolding effect. This latter possibility appears to be nearer the truth in view of knowledge gained on the structure of proteins and polypeptides.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. V. R. KHANOLKAR and Dr. A. R. GOPAL AYENGAR for their keen interest in the work and gratefully acknowledge their encouragement throughout the investigation.

SUMMARY

1. A new method of hydrolysis of tissue proteins with formic acid HCl is described which possesses the specific advantage of curtailing the time of hydrolysis without impairing the release of amino-acids.

2. The efficacy of this new procedure over standard methods of hydrolyzing proteins has been established with the help of microbiological methods.

3. The applicability of this technique to serum proteins has also been tested with equal validity.

4. It is not advisable to use this method for tryptophane.

5. The question of the effect of steric factors on the rate of hydrolysis of proteins is also considered.

RÉSUMÉ

1. Une nouvelle méthode d'hydrolyse des protéines des tissus par un mélange acide formique-HCl, qui présente l'avantage d'écourter le temps d'hydrolyse sans diminution de la libération des amino-acides, est décrite.

2. La supériorité de cette nouvelle technique sur les méthodes standard d'hydrolyse des protéines a été établie à l'aide de méthodes microbiologiques.

3. Cette méthode peut également être appliquée aux protéines du sérum.

4. Cette méthode ne peut être utilisée pour le tryptophane.

5. La question de l'influence des facteurs stériques sur la vitesse d'hydrolyse des protéines est également discutée.

References p. 557.

ZUSAMMENFASSUNG

1. Es wird eine neue Methode zur Hydrolyse von Gewebsproteinen mittels Ameisensäure/HCl beschrieben, welche den spezifischen Vorteil besitzt, die Hydrolysenzeit zu verkürzen, ohne die Freisetzung von Aminosäuren zu hindern.

2. Die Vorteile dieser neuen Methode gegen über klassischen Methoden der Proteinhydrolyse, wurden mit Hilfe von mikrobiologischen Versuchen geprüft und bestätigt.

3. Die Anwendbarkeit dieser neuen Methode auf Serumproteine wurde ebenfalls erfolgreich untersucht.

4. Gegenüber Tryptophan ist mit dieser Methode Vorsicht geboten.

5. Die Frage, ob für die Hydrolysengeschwindigkeit von Proteinen sterische Faktoren eine Rolle spielen, wird erörtert.

REFERENCES

1 A. H. SCHEIN AND C. P. BERG, *Arch. Biochem.*, 11 (1946) 209.
2 H. E. SAUBERLICH AND C. A. BAUMANN, *Cancer Research*, 11 (1951) 67.
3 K. A. KUIKEN, C. N. LYMAN AND F. HALE, *J. Biol. Chem.*, 171 (1947) 551.
4 J. W. H. LUGG, *Biochem. J.*, 32 (1938) 775.
5 H. S. OLCOTT AND H. FRAENKEL-CONRAT, *J. Biol. Chem.*, 171 (1947) 583.
6 I. T. GREENHUT, B. S. SCHWEIGERT AND C. A. ELVEHJEM, *J. Biol. Chem.*, 162 (1946) 325.
7 S. U. GURNANI AND M. B. SAHASRABUDHE, *Current Sci. (India)*, 22 (1953) 269.
8 P. A. LEVENE, R. E. STAIGER AND A. ROTHERS, *J. Biol. Chem.*, 97 (1932) 717.
9 R. L. M. SYNGE, *Biochem. J.*, 39 (1945) 351.
10 W. L. BRAGG, J. C. KENDREW AND M. F. PERUTZ, *Proc. Roy. Soc. London*, 203A (1950) 321.
11 K. M. RUDALL, *Biochim. Biophys. Acta*, 1 (1947) 549.
12 E. J. AMBROSE AND A. ELLIOT, *Proc. Roy. Soc. London*, 205A (1951) 47.
13 E. C. BARTON WRIGHT, *Analyst*, 71 (1946) 267.
14 E. C. BARTON WRIGHT AND N. S. CURTIS, *Analyst*, 73 (1948) 330.
15 E. C. BARTON WRIGHT, *Microbiological Assay of the Vitamin B Complex and Amino-acids*. Ashe Laboratories, London.
16 B. F. STEEL, H. E. SAUBERLICH, M. S. REYNOLDS AND C. A. BAUMANN, *J. Biol. Chem.*, 177 (1949) 533.
17 L. M. HENDERSON AND E. E. SNELL, *J. Biol. Chem.*, 172 (1948) 15.
18 I. T. GREENHUT, B. S. SCHWEIGERT AND C. A. ELVEHJEM, *J. Biol. Chem.*, 162 (1946) 69.
19 R. A. GORTNER, *Outlines of Biochemistry*, John Wiley & Sons, Inc., 1950.
20 E. G. HILL AND A. R. PATTEN, *Science*, 105 (1947) 481.
21 A. MOHOMMAD, H. FRANKEL-CONRAT AND H. S. OLCOTT, *Arch. Biochem.*, 24 (1949) 157.
22 A. R. PATTEN AND E. G. HILL, *Science*, 107 (1948) 68.

Received November 15th, 1954