ENZYMATIC HYDROLYSIS OF HISTONES IN RAT KIDNEY MICROSOMES

W. K. Paik and H. W. Lee

Fels Research Institute and Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pa. 19140

Received November 17, 1969

Summary

The microsomal fraction of rat kidney contains an enzyme which hydrolyzes basic proteins such as histones and protamine. However, the enzyme is present in all the organs of the rat to some degree. The enzyme is most active toward protamine and histones, but slightly active to ribonuclease. Albumin and globulin are completely resistant to the action of the enzyme. The optimum pH was found to be around 8 - 9, depending on the proteins used as substrate. Evidence indicates that the enzyme is not one of the cathepsins.

We report in this paper evidence that there is an enzyme in rat kidney microsomes which hydrolyzes various histones and protamine most actively. The enzyme is present ubiquitously in various rat organs, but the highest amount is found in kidney. The enzyme was partly purified from rat kidney and was found to have an optimum pH around 8 - 9. Some of the implications for genetic regulation are discussed.

Materials and Methods

All the proteins used in Table III were purchased from Sigma Chemical Co., and the rest of the reagents from local sources.

Enzymatic assay One-tenth or 0.2 ml of tissue homogenate or enzyme preparation, 0.2 ml of substrate protein suspension (0.6 mg), 0.1 ml of 0.5 M Tris-HCl buffer at pH 9.0 in a total volume of 0.5 ml were incubated at 37° for 20 minutes. The reaction was terminated by addition of 0.5 ml of 10% trichloroacetic acid (TCA). For a control, the enzyme suspension was added after the reaction was stopped by TCA. The mixture was centrifuged at 39,000 x g for 10 minutes. A portion of the clear supernatant (usually 0.1 or 0.2 ml) was transferred to a Coleman spectrophotometer cuvett (19 x 105 mm) and a pre-determined amount of NaOH solution was added to bring the pH of the solution to about 5. The volume

was adjusted to 1.0 ml with water and the ninhydrin color was developed according to the method of Moore and Stein (1). The values reported in the following experiments are the average of duplicate determinations and have been corrected to allow for the control value. The enzyme activity was expressed as $A_{580\ m\mu}$/ 20 minutes/mg enzyme protein. One unit of $A_{580\ m\mu}$ corresponds to 0.24 μmoles of leucine. The enzyme activity, therefore, is the increase in ninhydrin color found in the 5% TCA-soluble fraction after reacting the substrate protein with the enzyme. Although not presented, the enzyme reaction was dependent on time of incubation and on the concentration of the enzyme.

Partial purification of the enzyme 2.4 g of rat kidney was homogenized in 9.6 ml of 0.25 M sucrose solution with the aid of glass homogenizer, and the homogenate was centrifuged at 10,000 x g for 10 minutes to remove nuclei and mitochondria. The supernatant was further centrifuged at 105,000 x g for 1 hour, the pellets were suspended in 5 ml of water, and an equal volume of 2% sodium deoxycholate was added to the suspension. After homogenizing, the mixture was centrifuged at 105,000 x g for 1 hour and the clear supernatant was used as the

Table I

Distribution of the enzyme among the various organs of rat

Organ	Enzyme activity ($A_{580\ m\mu}$/20 minutes/mg enzyme protein)
Brain	0.120
Spleen	0.132
Liver	0.168
Heart	0.216
Lung	0.450
Kidney	0.978

Organs were homogenized in 0.25 M sucrose by glass homogenizer as 20%, and the homogenates were filtered through a double layer of cheese-cloth by gravity. Two-tenth ml of purified F_2 fraction (slightly lysine-rich histone; 0.6 mg) was used as substrate. The rest of the procedures are described under Methods.

enzyme in the following experiments. Attempts to purify the enzyme beyond this stage were unsuccessful due to extreme instability of the enzyme.

Various histones were purified from histone type II-A of Sigma Chemical Co. according to the method described elsewhere (2) (histone type II-A is a mixture of various histones). Protein was determined by the method of Lowry et al (3), and subcellular fractionation of rat kidney was carried out according to the method of Schneider (4).

Results and Discussion

Table I lists the results on the enzyme in various rat organs. The enzyme is present in all the organs tested, and kidney has the highest activity.

Practically all the enzyme activity is found in the microsomal fraction (Table II). The results in Table II, therefore, strongly suggest that the

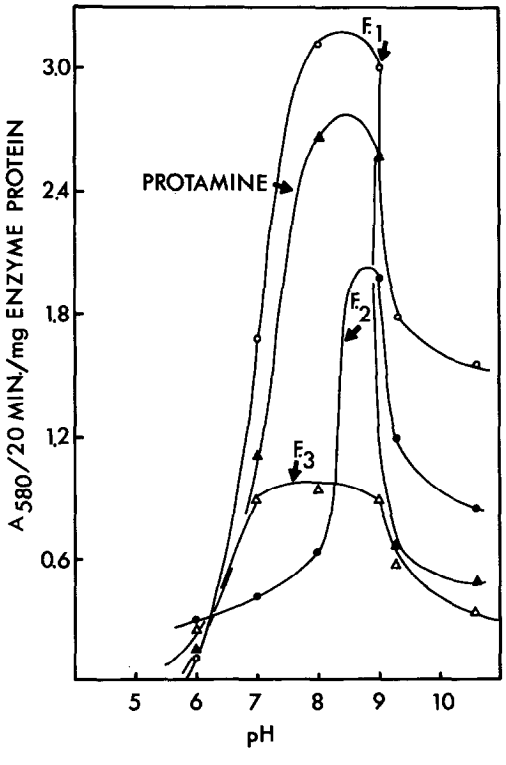

Fig. 1. pH-Curve Six-tenth mg of substrate protein and 1.34 mg of partly purified enzyme were used under the conditions described under Method. Phosphate buffer was used for pH 6 and 7; Tris-HCl for 8 and 9; borate for pH 9.3 and 10.6.

Table II

Distribution of the enzyme among various subcellular fractions of rat kidney

Fraction	Volume	Protein	Total protein	Enzyme activity	Total enzyme activity	Recovery	
						protein	enzyme
	(ml)	(mg/ml)	(mg)	($A_{580\ m\mu}$/20 min. /mg enzyme)	($A_{580\ m\mu}$/20 min)	(%)	(%)
Whole homog.	8.0	45.3	364	0.905	329	100.0	100.0
Nuclear	5.0	16.7	83.5	0.150	12.5	22.9	3.8
Mitochondrial	5.0	6.9	34.4	0	0	9.5	0
Microsomal	5.0	15.6	77.8	3.08	239	21.4	72.7
Soluble*	7.4	13.6	101	0	0	27.8	0

* Soluble fraction was obtained by centrifuging 8.0 ml of whole homogenate at 105,000 x g for 1 hour. Purified histone F_2 fraction was used. The rest of the conditions are the same as described under Table I.

enzyme concerned is not a cathepsin, since the latter is located in the particulate lyzosome fraction which sediments together with the mitochondrial fraction under these conditions (5).

pH Optima are slightly different depending on the species of basic proteins used as substrate (Fig. 1). However, optimum pH's, in general, lie around 8 - 9. It is most noteworthy that at pH 5.0 where cathepsins are most active (6) the hydrolysis of various histones or protamine is practically nil. This again suggets that the hydrolytic enzyme involved is not a cathepsin.

In order to examine the biochemical significance of the enzyme, the hydrolytic activity of the enzyme toward various purified proteins were measured

Table III

Specificity of the enzyme

Substrate	Enzyme activity *	
	7.0	9.0
Protamine	1.51	1.65
Very lysine-rich histone (F_1)	1.12	2.01
Histone type II-A	0.48	1.28
Arginine-rich histone (F_3)	0.47	0.56
Slightly lysine-rich histone (F_2)	0.31	1.64
Ribonuclease, 5 x recrystallized	0.45	0.56
Polylysine	0.19	0.55
Polyarginine	0.17	0.08
Lysozyme	0	0.41
Albumin	0	0
Globulin	0	0
Polyleucine	0	0
Polyglutamic acid	0	0

* A_{580}/20 min/mg enzyme protein.

The rest of the experimental conditions are described under Methods. Partially purified enzyme (1.34 mg protein) was used.

at two different pH's; at a physiological pH of 7.0 and optimal pH of 9.0 (Table III). The enzyme readily hydrolyzed the various histones and protamine at both pH's. Among the histones, the very lysine-rich histone is the most susceptible to the action of the enzyme. The fact that polylysine and polyarginine are hydrolyzed to a certain degree suggests that the enzyme preferably requires basic amino acids for the reaction. Albumin and globulin are completely resistant to the action of the enzyme.

Preliminary studies on the mechanism of enzyme action have been attempted (Fig. 2). When the reaction products of the enzyme on histone substrate are chromatographed on Bio-Gel P-2 (exclusion limit of 2,000) with 0.01 M phosphate

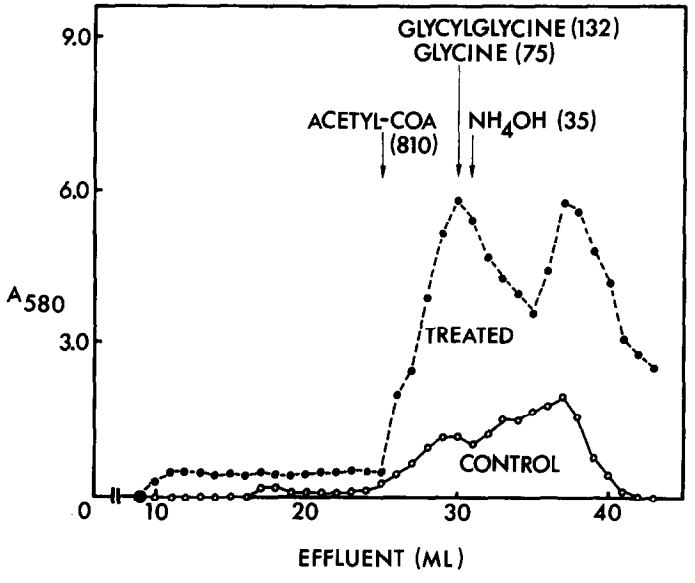

Fig. 2. Chromatographic analysis of the hydrolytic products on Bio-Gel P-2. 0.8 ml of histone type II-A (2.4 mg), 0.4 ml of 0.5 M Tris-HCl buffer at pH 9.0, 0.4 ml of partially purified enzyme (5.0 mg protein) and 0.4 ml of water were incubated at 37° for 3 hours, and the reaction was stopped by addition of 2 ml of 10% TCA. The control was prepared by adding the enzyme preparation after addition of TCA and was treated as the assay, otherwise. The incubation mixture was centrifuged at 39,000 x g for 20 minutes, and the supernatants were concentrated to 1.5 ml under reduced pressure. The whole sample was charged on Bio-Gel P-2 (0.8 x 78 cm) which had been previously equilibrated with 0.01 M phsophate buffer at pH 7.2. One ml fractions were collected at room temperature, and portions were determined for ninhydrin color. The arrows in the figure indicate the position of the compounds eluted, and the number in parenthesis indicates the molecular weight of the compound used for marker.

buffer at pH 7.2, peptides of various sizes appear, evidenced by elution of ninhydrin-positivity between exclusion and inclusion limit. Near the inclusion region two large peaks appear. Even though the control showed these two peaks with much less intensity, they may be due to contamination of the enzyme with free amino acids. When fractions of these peaks were pooled and were analyzed by the Beckman automatic amino acid analyzer, all the commonly occurring natural amino acids were found with no outstanding predominance of any particular amino acid. Since ninhydrin-positive compounds are continuously eluted in the earlier fractions on the column, indicating a continuous variation of the size of peptides which became soluble in TCA by the enzyme action, the enzyme concerned is most likely an exopeptidase. However, there still exists a possibility that the enzyme is an endopeptidase, and that the products are further hydrolyzed by contaminating exopeptidase. Why the second peak in Fig. 2 is formed is not clear at present; since no ninhydrin-positive compound of this size smaller than ammonia is possible and since amino acids were found in this peak, there might be an ion-exchange phenomena on Bio-Gel.

It is evident from the foregoing results that the enzyme is not one of the cathepsins, because of the difference in optimum pH and of location. The enzyme is not stimulated by any reducing agents, and it is inhibited by divalent metal ions (6)+. The enzyme is relatively specific for basic proteins, and albumin and globulin are completely resistant to the action of the enzyme.

Since the ratio of histone to DNA in the cell is remarkably kept at unity (7) and the amount of DNA per cell does not change, maintenance of constancy of the amount of histone should be under command of rigid homeostatic mechanisms. This could be achieved by delicate balance between anabolism and catabolism of the protein. Since recent evidence indicates that histone is synthesized in cytoplasm (8,9), the present enzyme might serve as one of the control mechanism on the amount of histone by hydrolyzing any excess amount of the protein.

+ Unpublished result by Paik and Lee.

Finally, the ease of destruction of histone during isolation previously reported (10,11,12) might have been due to the action of the enzyme presently discussed.

Acknowledgement

This work was supported by research grants AM 09602 from the National Institute of Arthritis and Metabolic Diseases, CA 07174 from the National Cancer Institute, and B7-0280R from the National Science Foundation.

References

1. Moore, S., and Stein, W. H., J. Biol. Chem., 176, 367 (1948).
2. Paik, W. K., and Kim, S., Arch. Biochem. Biophys., in press (1969).
3. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
4. Schneider, W. C., in Manometric Techniques, (edited by W. W. Umbreit, R. H. Burris and J. F. Stauffer), Burgess, Minneapolis (1959), p.188.
5. de Duve, C., in Subcellular Particles, (edited by T. Hayashi), The Ronald Press Co., New York (1959), p.128.
6. Fruton, J. S., and Simmonds, S., General Biochemistry, published by John Wiley & Sons, Inc., (1958), p.701.
7. Busch, H., Histones and Other Nuclear Proteins, Academic Press, New York, (1965), p.98.
8. Robbins, E., and Borun, T. W., Proc. Natl. Acad. Sci. U. S., 57, 409 (1967).
9. Gallwitz, D., and Mueller, G. C., Science, 163, 1351 (1969).
10. Murray, K., Ann. Rev. Biochem., 34, 209 (1965).
11. Crampton, C. F., Stein, W. H., and Moore, S., J. Biol. Chem., 225,363 (1957).
12. Stellwagen, R. H., Reid, B. R., and Cole, R. D., Biochim. Biophys. Acta, 155, 581 (1968).