THE ULTRAVIOLET INACTIVATION OF PEPSIN

by

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INTRODUCTION

The activity of the surface-denatured pepsin has been measured by many investigators[1,2,3]. Most of them concluded that surface-denaturation could not completely inactivate pepsin, though NEURATH[4] said that surface-denaturation is the most perfect denaturation.

Recently, the effect on the activity of pepsin of acetylation and deamination of its amino groups was studied[5]. It was found that this acetylation or deamination had no measurable effect on the peptic activity, and that further acetylation resulted in the decrease of activity. There was a correlation in the change in number or reactivity of tyrosine phenol groups and the alkali-labile acetyl radicals introduced and of these to the loss in activity. It was suggested that phenol groups of tyrosine in the enzyme are associated with its catalytic activity.

It was known for a long time that pepsin is inactivated by ultraviolet light. Then there comes a question, "are phenol groups also associated with this loss of activity?".

In order to clarify this question, the author has made the following experiments, and concluded that the inactivation by U.V. is due to the oxidation of phenol groups of tyrosine in the enzyme.

EXPERIMENTAL

Ultraviolet irradiation. For the U.V. inactivation a direct current super-high pressure quartz mercury lamp was used. The pepsin solution sealed in a quartz cell was irradiated with the mercury lamp, and it was found that pepsin lost all its activity after 30 minutes irradiation (Table II).

Molecular weight. The molecular weight of pepsin was determined surface-chemically by the method of BULL AND GUASTALLA[6]. The surface pressure was measured by the Wilhelmy type surface balance. The tray was of pyrex glass, and a saturated solution of sodium sulfate was used as the substrate solution.

Viscosity. The shape of the pepsin molecule was determined from the viscosity of its solution. The viscosities of pepsins before and after irradiation were measured by the capillary flow method using the Ostwald viscometer at 25°. The sample was dissolved in 0.1 *M* KCl solution to reduce the electroviscous effect. Five concentrations of pepsins (0.1, 0.2, 0.3, 0.4 and 0.5%) were used. The nitrogen contents of these solutions were determined by micro-Kjeldahl analysis. The results are shown in Table I.

Activity. The activity of pepsin was determined by NORTHROP's method[7]. Crystalline hemoglobin was used as the substrate and the activity was calculated from the value of colour developed by Folin's phenol reagent after the digestion of hemoglobin.

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For the determination of the activity of the surface-denatured pepsin, a somewhat modified method was adopted. Four ml of 2.5% pure hemoglobin solution were pipetted into a test tube, then 1 ml of 0.3 *N* hydrochloric acid was added from a pipette (final pH, 1.6) and the substrate solution was thus obtained. Pepsin was spread on the surface of solutions of varying pH and ionic strength. The spread film was then transferred onto the chromium plate. This plate was put in 5 ml of the substrate solution and the solution was stirred by whirling the plate. After 10 minutes 10 ml of 0.3 *N* trichloric acid was added, the tube was shaken vigorously, and the suspension was filtered. To 5 ml of the digestion filtrate were added 10 ml of 0.5 *N* NaOH and 3 ml of Folin's phenol reagent. The colour was read against the standard after 2–10 minutes. The number of activity units corresponding to the colour value of the digestion products in 5 ml filtrate was read as indicated above.

Spectrum. The Beckman spectrophotometer (Model D.U.) was used in studying the ultraviolet absorption of pepsin, tyrosine and dioxyphenylalanine (Dopa).

Paper chromatography. To demonstrate the existence of Dopa, the paper chromatography technique was used. About 10 mg of pepsin, before and after irradiation with U.V., were dissolved in 6 *N* HCl and digested at 100° for 24 hours. The hydrolysate was reduced in volume at 90° several times and the final syrup was cooled and chromatographed on paper using a butanol-formic acid-water system (4:1:5).

Sample. The pepsin used was the crystalline salt free product of Armour in U.S.A. The activity was about 0.27 hemoglobin unit.

RESULTS AND DISCUSSIONS

FA–F curves of the pepsin films before and after irradiation with U.V. are shown in Fig. 1. Both curves intersect at the same point, indicating that pepsins before and after irradiation have the same molecular weight.

The molecular weight can be calculated as about 35,000. On the other hand the two curves have different slopes. According to BULL[8] the slope is the direct measure of the co-area for the protein molecule. Since U.V. irradiated pepsin shows the larger co-area, it can be concluded that the U.V. irradiated pepsin molecule is more asymmetric than the native pepsin. This result is quite in agreement with that of viscosity measurement.

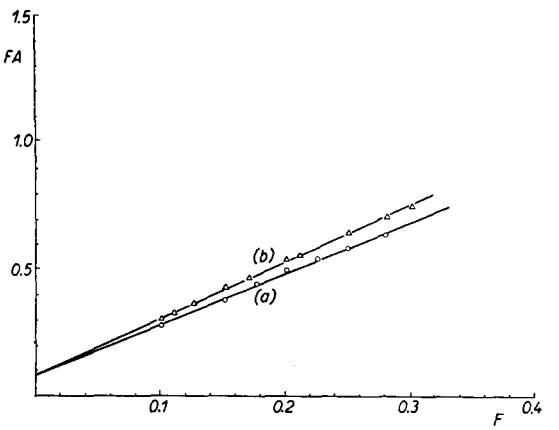

Fig. 1. FA *vs.* F plots of native and U.V. irradiated pepsins. Area expressed in square meters per milligram and pressure in dynes per centimeter. (a) native; (b) U.V. irradiated.

The viscosities of the pepsin solutions before and after irradiation are shown in Table I. The table shows that volume intrinsic viscosity $[\eta_\varphi]$ of the native pepsin is equal to 5.16 and by using Simha's relation the axial ratio (b/a) can be calculated to be 4.2. When pepsin is denatured by U.V., $[\eta_\varphi]$ increases to 18.5 and b/a is calculated as 13.2. It is quite obvious that the pepsin molecule becomes far more asymmetric after it is denatured by U.V. Since it is found that pepsin irradiated with U.V. for 30 minutes loses its activity completely (Table II), it can be supposed that this loss of activity is due to the disturbance of the structural pattern of the pepsin molecule as would appear from the results in Tables I and II. But that cannot be strictly correct.

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TABLE I

VISCOSITIES OF PEPSIN SOLUTIONS

Native

Concentrations				
%	φ	η_s	η_q	
0.065	0.00049	0.00252	5.17	
0.13	0.000975	0.00505	5.17	
0.195	0.00146	0.00767	(5.25)	
0.26	0.00195	0.0101	5.18	
		$[\eta_q]$ =	5.16	$b/a = 4.2$

U.V. Denatured

Concentrations				
%	φ	η_s	η_q	
0.075	0.000563	0.0122	21.6	
0.15	0.00113	0.028	24.9	
0.225	0.00169	0.0471	27.8	
0.30	0.00225	0.0664	29.5	
		$[\eta_q]$ =	18.5	$b/a = 12.4$

TABLE II

THE ACTIVITY OF NATIVE, SURFACE-DENATURED AND U.V. IRRADIATED PEPSIN

Sample	*Activity per mg of protein nitrogen*
Native	0.27
Surface-denatured at pH 3	0.24
Surface-denatured at pH 2	0.15
U.V. irradiated	0.008

Table II shows the activities of the native, U.V. irradiated, and surface-denatured pepsins. It is quite obvious that pepsins which are denatured at pH 2 and 3 preserve some part of their activities, while surface-denatured pepsin has lost its original structural pattern completely. Thus, the disturbance of the structural pattern cannot be the only cause of the inactivation of pepsin by U.V.

The action of U.V. on crystallised pepsin is very marked when studied spectroscopically (Fig. 2). In particular, the rise in the minimum at 250 mμ and the shift of maximum at 277 mμ to 278 mμ are characteristic of the action of U.V., as was reported about twenty years ago by GATES[9].

These characteristics are quite similar to those of the action of tyrosinase on pepsin, as has been observed by SIZER[10]. The increase and the shift of absorption maximum can be ascribed also to the oxidation of tyrosine residue into Dopa residue.

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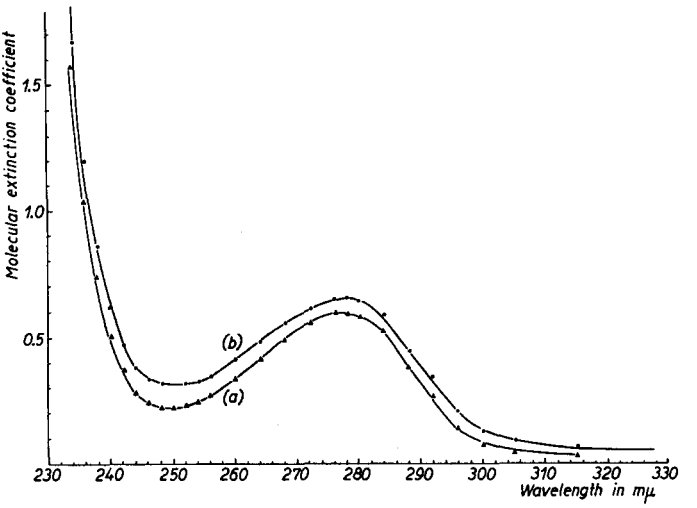

Fig. 2. Absorption spectra of native and U.V. irradiated pepsins. (a) native; (b) U.V. irradiated.

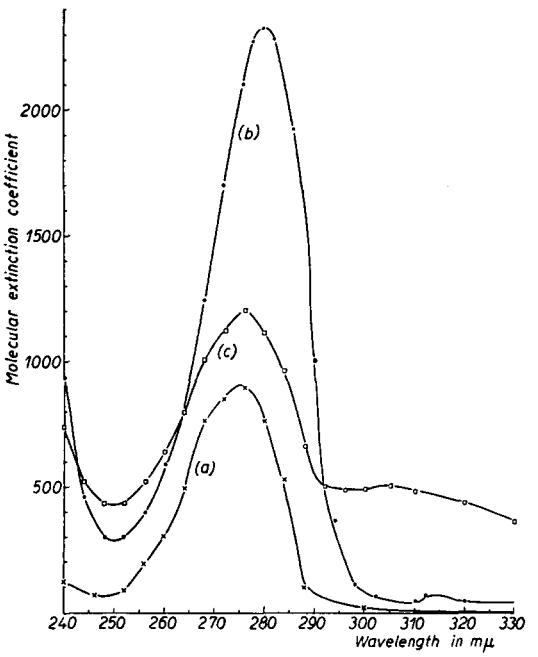

Fig. 3. Absorption spectra of tyrosine, Dopa, and U.V. irradiated tyrosine. (a) tyrosine; (b) Dopa; (c) U.V. irradiated tyrosine.

The absorption curve of Dopa and that of tyrosine are shown on Fig. 3. The peak of tyrosine appears at 276 mμ, while the Dopa peak appears at 280 mμ, and its molecular extinction coefficient is far larger than that of tyrosine. The curve (c) of Fig. 3 is the absorption curve of U.V. irradiated tyrosine. The increase and the shift of the peak caused by the U.V. irradiation are probably due to Dopa. Similarly, the increase and the shift of the peak of pepsin are probably due to Dopa, which is formed from tyrosine residues by the action of some oxidising agent.

This can be confirmed by paper chromatography. The hydrolysate of U.V. irradiated pepsin clearly contains as much Dopa as tyrosine, but that of native pepsin contains only a small quantity of Dopa, and this is probably formed by the action of HCl when pepsin is hydrolysed.

In any case, it is quite obvious that in U.V. irradiated pepsin some part of the tyrosine residues has been oxidised into Dopa residues. The inactivation of pepsin by U.V. is probably due to this oxidation.

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SUMMARY

1. It was found that the activity of pepsin was not completely lost by surface-denaturation.
2. The action of U.V. on pepsin was studied spectroscopically and it was found that there were many similarities between this action and that of tyrosinase.
3. Spectroscopic and paper-chromatographic evidence was obtained for the formation of Dopa groups in solutions of pepsin by U.V. irradiation.
4. The inactivation of pepsin by U.V. is probably due to the formation of Dopa from tyrosine.

RÉSUMÉ

1. On a trouvé que l'activité de la pepsine n'est pas complètement détruite par dénaturation superficielle.
2. L'action de la lumière ultra-violette sur la pepsine a été étudiée à l'aide de la méthode spectroscopique. On a trouvé qu'il y a une grande ressemblance entre l'action de la lumière ultra-violette et celle de la tyrosinase.
3. A l'aide des méthodes de spectroscopie et de chromatographie sur papier nous avons pu démontrer la formation de groupes Dopa dans une solution de pepsine irradié par la lumière ultra-violette.
4. L'inactivation de la pepsine par la lumière ultra-violette est peut-être due à la formation de groupes Dopa à partir de la tyrosine.

ZUSAMMENFASSUNG

1. Es wurde gefunden, dass die Pepsinaktivität durch Oberflächendenaturierung nicht ganz verloren geht.
2. Die Wirkung des ultravioletten Lichtes wurde mittels der spektroskopischen Methode studiert, und es wurde gefunden, dass die Wirkungen des Lichtes und der Tyrosinase viel Gemeinsames haben.
3. Spektroskopische und papierchromatographische Beweise für die Bildung von Dopa-Gruppen in Pepsinlösungen durch Beleuchtung mit U.V.-Licht wurden erlangt.
4. Die Inaktivierung des Pepsins durch U.V.-Licht beruht vielleicht auf der Bildung von Dopa aus Tyrosin.

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