The Structure of Surface-denatured Protein. VII. The Activity and the Shape of the Surface-denatured Pepsin Molecule

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The author was able to show that the serum albumin molecule which expanded on the extremely acid solution had an elongated shape, but the albumin molecule which expanded on the solution of its isoelectric point was rather round[1]. This observation indicated that many of the bonds of the albumin molecule which expanded on the acid solution were destroyed and the molecule showed the elongated shape but on the solution at the pH of its isoelectric point many of the intramolecular secondary bonds were still retained and the molecule could not take the elongated shape.

If this indication is correct, it would be possible to expect that the most part of the activity of the protein molecule will be lost when expanded on the extremely acid solution, but some part of the activity of the molecule on the solution at the pH of its isoelectric point will be reserved. The following experiments were planned with this expectation. The protein selected was pepsin.

Experimentals

(a) Sample.—The pepsin was purchased from The Armour Chem. Lab. of U. S. A. and it was the two times recrystallized enzyme of the salt free state. The serum albumin was purified by Dr. S. Nakamura and was the same sample as the author had used in the previous experiments.

(b) Solution and Apparatus.—Three acetate buffered solutions of pH=2, 3 and 5 were used as the underlying solutions. A Wilhelmy balance registered the film pressure.

(c) Size and Shape Determination.—The methods and techniques used for the determination of the size and shape of pepsin molecules on the solutions of various pH were quite the same as those used in the previous paper[1].

(d) Activity Determination.—The activity of the surface denatured pepsin molecule was determined by the following three methods.

(1) One of them used the time needed for the clotting of a standard skim milk solution into which the pepsin had been introduced. The milk solution containing 16.7 % of dry produced skim milk in 0.1 M acetate buffer adjusted to pH 5 was used. The pepsin unit can ordinarily be defined as the amount which causes the clotting of 6 cc. of this solution at 37°, or it can be expressed by the equation

$$U = V/6t,$$

where V is the volume of the used milk solution in cc., and t is the clotting time in minutes. V and t were measured by the following method. Pepsin was at first spread on the surface of solutions of various pH. The spread film was transfered onto a stainless-steel plate covered with a double layer of barium stearate, using the method of Blodgett. Then the thickness of the film was measured by an ellipsometer[2]. This plate was immersed for two minutes in 6 cc. of standard milk solution, during which time the milk was agitated continuously by gently shaking and rotating the tube. The plate was then removed and the time needed for the clotting was measured.

(2) *Hemoglobin Method.* This method is quite analogous to that of Northrop[3]. Four cc, of 2.5 per cent pure hemoglobin solution was added with pipette to a test tube and then 1 cc. of 0.3 N hydrochloric acid was added from a pipette. The final pH was 1.6. Thus the substrate solution was obtained. The built up film which was obtained by the method similar to that of (1), was put in 5 cc. of the substrate solution and the solution was mixed by whirling the plate. After ten minutes 10 cc. of 0.3 N trichloracetic acid was added, the tube was shaken vigorously, and the suspension was filtered.

To 5 cc. of the digestion filtrate were added 10 cc. of 0.5 N sodium hydroxide and 3 cc. of phenol reagent of Folin. The color was read against the standard after two to ten minutes. The number of activity unit corresponding to the colour value of the digestion products in 5 cc. of digestion filtrate was read from the curve shown in Northrop's book[3].

(3) *Mixed Film Method.* A given volume of 0.05 per cent pepsin solution was mixed well with the equal volume of 0.05 per cent serum albumin solution. A part of the mixed solution was put promptly on the surface of the solutions of various hydrogen ion concentrations. The mixed film of pepsin and serum albumin was obtained. If the film was kept under a constant surface area, the surface film pressure would increase gradually, as pepsin splits an albumin molecule into smaller ones. The more active the pepsin is, the more intensely would the surface pressure increase, and so if we plot the surface pressure against the time, the slope of the curve would indicate the activity of the pepsin. The relative activity of the pepsin could be obtained by this method.

1) K. Imahori, This Bulletin, **27**, 146 (1954).

2) Y. Yoneyama and K. Imahori, *Sci. Pap. College Gen. Educ. Univ. Tokyo*, 3, 145 (1953).

3) J.H. Northrop "Crystalline Enzyme", Columbia Univ. Press (1948), p. 303.

Results and Discussion

The molecular weight of pepsin which was expanded on the solution of pH=1, 3 and 5 was determined by the method of Bull and Guastalla[4]. The molecular weight thus obtained was 35,000 in all cases.

The limiting area per molecule and the shape of each molecule on pH=1, 2 and 3 are listed in the Table I.

TABLE I

pH	*Am*	f/f_0	*a/b*	*a*	*b*
1.0	5250 Å²	—	—	—	—
2.0	6100 Å²	1.5	15	342 Å	23 Å
3.0	5370 Å²	1.2	5.5	205 Å	37 Å
5.0	—	—	—	—	—

In the table, *Am* is the limiting area of one molecule in Å², f/f_0 the frictional ratio, *a/b* the axial ratio and *a* and *b* are the length of the longer and the shorter axes respectively. The data of pH=1 are lacking as the film on the substrate of pH=1 is hydrolyzed easily and the diffusion constant cannot be determined. The film on the substrate of pH=5 is not spread well and its data are also lacking. The result indicates that the pepsin molecule on pH=2 is more perfectly expanded and has a more elongated shape than that on pH=3.

The activities of pepsin expanded on pH=2 and 3 and measured by the earlier two methods are listed as Table II.

TABLE II

pH	Activity: Displacement method	Activity: Hemoglobin method
Native	9,000	0.27
2.0	3,900	0.15
3.0	7,900	0.24

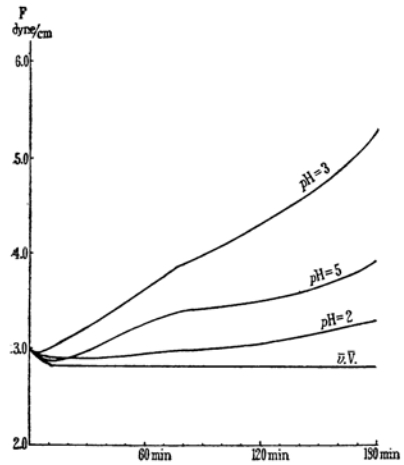

Fig. 1.

Table II shows that the pepsin film which has been built up from the solution of pH=3 is far more active than the film from pH=2. The result of the mixed film method, which is shown in Fig. 1 is quite in accordance with the above results. But as is obvious from the previous report of this series, the serum albumin on the substrate of pH 3 can be denatured more completely than that on the substrate of pH 5, so that the former will be digested more easily by pepsin than the latter. This would make also the mixed film on the substrate of pH 3 to have higher surface pressure than that on pH 5. Accordingly it cannot be concluded that the pepsin on the substrate of pH 3 is more active than that on pH 5. On the other hand, the albumin film on the substrate of pH 2 is denatured most perfectly and will be digested most easily and this fact verifies the fact that pepsin on the substrate of pH 3 is more active than that on pH 2. The curve U. V. in Fig. 1 which means that the mixed film is irradiated with the ultraviolet light from a mercury lamp shows that pepsin is completely inactivated by the ultraviolet light.

The above results show that the pepsin expanded on the solution of pH 2, which is more completely surface-denatured and has a more elongated shape has lost the larger part of its activity, while the pepsin expanded on the solution of its isoelectric point retains its activity to some extent. This result is in accord with the author's expectation. Yet, this activity disappears when the pepsin molecule is irradiated with the ultraviolet light. At any rate, it is quite interesting that pepsin monolayer retains its activity even on the solution of pH 2.

Summary

(1) The molecular weight, the size and the shape of the pepsin molecule on the substrates of pH 2 and 3 were determined. The molecule on the substrate of pH 3 (its isoelectric point) was of rather a round shape, but the molecule on pH 2 had a far more elongated shape.

(2) The enzymatic activity of the surface-denatured pepsin molecule on the solutions of various pHs was determined. The round-shaped molecule on the substrate of pH 3 was the most active, but the elongated molecule on pH 2 retained less activity.

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4) H. B. Bull, "Adv. in Protein Chem." Vol. III, p. 97.