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Effect of Acetylation on Specificity of Pepsin.

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The action of crystalline pepsin on synthetic substrates such as carbobenzoxy l-glutamyl l-tyrosine (C.G.T.) exhibits at least two well defined differences from its action on a protein like hemoglobin.[1] First, the action of pepsin on hemoglobin has a maximum rate at pH 2, while the action on C.G.T. has a maximum rate at pH 4. Secondly, it requires high concentrations of pepsin to effect rapid hydrolysis of C.G.T.

Since the action of pepsin on a protein can be quantitatively altered by acetylation of the enzyme, it was thought desirable to test the effect of acetylation on the action of C.G.T. and to compare this with the action on the typical protein substrate, hemoglobin.

This was done with 3 types of pepsin preparations. First, the crystalline N-acetyl derivative was prepared by ketene acetylation of crystalline pepsin. The crystalline pepsin was prepared from commercial Cudahy 1:10,000 pepsin by the method of Northrop.[2] The preparation and crystallization of the N-acetyl derivative was done according to the description of Herriott and Northrop.[3] This preparation is as active on hemoglobin as is unacetylated pepsin. Second, a series of more highly acetylated pepsins ranging in activity from 60% to 100% of that of regular crystalline pepsin were prepared by further treatment with ketene. No attempt was made to crystallize these preparations. Third, preparations were made by acid hydrolysis of the 0-acetyl groups in a 50% active preparation which causes the enzyme to regain its activity[3,4] as a result of an increase in the number of free phenolic hydroxyl groups of tyrosine.

The action of the enzyme on hemoglobin was determined according to the method of Anson and the results recorded in terms of hemoglobin units of specific activity.[5] The action on C.G.T. is recorded in terms of the percent hydrolysis after 24 hr incubation at 40°C of a solution .035 M in C.G.T. and .05 M in acetate buffer adjusted to pH 4.0 with dilute sodium hydroxide using a glass electrode. The extent of hydrolysis was determined by the volumetric method of Van Slyke for amino nitrogen and corrected for amino nitrogen in the enzyme preparations themselves. The results on the first two types of preparations are recorded in Table I.

To test the effect of acid hydrolysis of 0-acetyl groups, pepsin which had been acetylated to about 50% of its former activity was treated with normal sulfuric acid at 2°C. The results are recorded in Table II.

These results show that mild acetylation of pepsin has no influence on the activity of the enzyme towards C.G.T.; that more drastic acetylation inhibits this activity towards C.G.T. in the same manner as it does the activity towards hemoglobin. These experiments support the conclusion[3] that free amino groups in the enzyme are unnecessary for the action of pepsin and that free phenolic hydroxyl groups are necessary. If the percent hydrolysis of C.G.T. is plotted against the concentration of pepsin (in the range used in these experiments) a linear relationship is obtained. If points for the acetyl pepsin experiments are located on this graph by using, instead of pepsin concentration, the function acetyl pepsin concentration $\times \frac{\text{Sp. activity after acetylation}}{\text{Sp. activity before acetylation}}$ the points fall on the line. This is consistent with the interpretation that the hydrolysis of C.G.T. and hemoglobin by pepsin are similar processes. This is also consistent with the find-

[1] Fruton, J. S., and Bergmann, Max, *J. Biol. Chem.*, 1939, **127**, 627.

[2] Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 739.

[3] Herriott, R. M., and Northrop, J. H., *J. Gen. Physiol.*, 1934, **18**, 13.

[4] Herriott, R. M., *J. Gen. Physiol.*, 1935, **19**, 283.

[5] Anson, M. L., *J. Gen. Physiol.*, 1938, **22**, 79.

TABLE I.
Comparative Activities of Pepsin and Acetylated Pepsins.

Preparation	% hydrolysis C.G.T.	Specific activity on hemoglobin
Crystalline N-acetyl pepsin	55 a	.22
" " "	55 a	.22
2X cryst. pepsin	54 a	.23
" " "	55 a	.23
" " " acetylated for 2 hrs	52.5b	.23
3 "	44.6b	.22
4 "	43.2b	.21
7 "	41.3b	.15

a—1.2 mg pepsin N/ml. b—1.0 mg pepsin N/ml.

TABLE II.
Comparative Activities of 50% Active Pepsin Before and After Hydrolysis.

Hrs acid hydrolysis	Conc. pepsin used on synthetic substrate in mg protein/ml	% hydrolysis of C.G.T.	Specific activity on hemoglobin
0	0.8	36	.12
0	1.2	40.5	.12
48	0.8	39	.16
48	1.2	45	.16
100	0.8	45	.21
100	1.2	51.5	.21

ings of Tracy and Ross[6] that malonylation of pepsin with carbon suboxide does not effect the specificity of the enzyme.

The difference between C.G.T. and hemoglobin with respect to pH optima of hydrolysis is perhaps ascribable to the mechanism of the enzyme reaction. Bergmann[1] demonstrated the importance of carboxyl groups in synthetic substrates for pepsin; and Tracy and Ross,[6] found that the introduction of carboxyl groups into serum albumin by malonylation increases the rate and extent of peptic activity. If one assumes that pepsin catalyses the hydrolysis of appropriate substrate molecules with intact carboxyl groups (as against carboxyl ions) the difference in pH optima can be explained by the existence in the protein substrate of stronger acidic groups (which require a lower pH for their conversion to carboxyl groups) than in the peptide. The assumption is consistent with, but not uniquely demonstrated by, the data of Northrop,[7] who showed that the rate of peptic digestion runs parallel to the titration curve of protein substrates. Further data is of course necessary to test this point.

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Summary. Acetylation of the free amino groups of pepsin does not affect the hydrolysis of the peptide carbobenzoxy l-glutamyl l-tyrosine. Further acetylation decreases the activity towards this substrate. Acid hydrolysis of O-acetyl, leaving N-acetyl groups intact, restores the activity of the enzyme to its former value.

[6] Tracy, A. H., and Ross, W. F., *J. Biol. Chem.*, 1942, **146**, 63.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1922, **5**, 263.