*TRYPTIC ACTIVATION OF ACETYLATED CHYMOTRYPSINOGEN**

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In a previous paper[1] we have reported on a rather heavily acetylated α-chymotrypsinogen that could be activated by trypsin only in the presence of an electrolyte or, alternatively, by the action of unacetylated chymotrypsin prior to, or together with, that of trypsin. The present communication reports further observations on the preparation and composition of such an acetylated zymogen. The conditions for suitable acetylation are different in the presence and in the absence of acetate. After acetylation the solution must be slightly acid, at least for a short time. It also appears that the activation of the acetylated protein in water is prevented because some form of acetylated chymotrypsinogen inhibits the action of trypsin on the acetylated zymogen in the absence of salts. This inhibitory material has been concentrated as a fraction of the mixture that constitutes the product of acetylation.

Materials and Methods.—These have been previously described[1, 2] except for the following items.

Trypsin was measured in the same manner as chymotrypsin. The substrate was toluene-sulfonyl-L-arginine methyl ester in 0.001 *M* tris-maleate buffer[3] at pH 7.5.

The activity of C^{14}-labeled acetyl was measured in a liquid scintillation spectrometer. Protein (*ca.* 0.5 mg) was dissolved in 0.01 *N* NaOH. The amount of water was limited to 0.2 ml or less in a system containing 15 ml of a fluorescent medium and about an equal volume of finely divided silica.[4] Comparison with counts on the CO_2 produced by combustion of the protein (which were estimated to be 95% efficient) indicated 48% efficiency in the foregoing system.

The Effect of Acid on Acetylated Chymotrypsinogen.—The method of acetylation used in the past involved the addition (in several doses) of an equal weight of acetic anhydride to the protein in a solution of sodium acetate. During subsequent dialysis the solution of the protein became slightly acid. Dialysis against 0.001 *M* HCl was also done occasionally, as possibly conducive to greater stability of the protein. One preparation, however, was dialyzed against tap water, which is here alkaline. It was thus found that acidification of the solution of acetylated protein is necessary to produce an artifact that cannot be activated by trypsin in the absence of salts.

An experiment made on a preparation of acetylated protein that was never allowed to become acid shows (Fig. 1) that a sharp change in activability takes place just below pH 5.0; and (Fig. 2) that this change requires some time (although brief) to occur at pH 3.0. The appearance of a slight turbidity in acid solutions indicates the presence of a less soluble fraction. This is discussed later.

It is of interest that the change caused by exposure to acid is not a polymerization, for no difference in the ultracentrifugal behavior of the protein could be found. The change to inactivable protein is not reversed at pH 9 or under. Some reversal may occur at about pH 11, where in any case the protein may also be decomposed. Another matter of interest is that the protein does not lose acetyl on acidification.

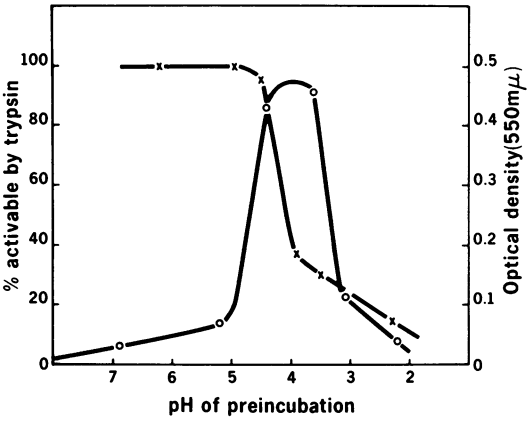

FIG. 1.—Exposure to pH levels below 5 renders the acetylated protein inactivable in the absence of salts. Solutions of 10 mg of unacidified acetylated protein in 2 ml water were kept for 30–35 min at the several recorded pH levels. Trypsin (1 part per 100 of acetylated protein) was added, the solution was suitably diluted with water, the pH brought to 7.0, and the activity (X) measured after 2 hr at 25°. The maximum activity found was taken at 100%. The acidification of such solutions also caused a faint turbidity which is plotted by (O) against optical density.

The specimen used in Figures 1 and 2 counted (*a*) 9.0, (*b*) 9.3 μ-equiv. of acetyl per 25 mg before being made pH 3.0, and counted (*a*) 8.9, (*b*) 9.1 thereafter. These values represent only about two thirds of the actual degree of acetylation because this preparation was made with sodium acetate. Nevertheless, the acetyl content was not changed by acidification.

Methods of Acetylation.—It was wished to employ C^{14}-labeled acetic anhydride as a means to determine the extent of acetylation of the product, but this precludes the use of acetate during the process because of the shift between acetate and acetic anhydride.[6] Sodium formate and sodium propionate as substitutes for acetate under the same general conditions gave unsatisfactory results. No other conditions have yet been found that lead to quite the same mixture of acetylated proteins as that obtained in the presence of acetate. However, a similar product with respect to activability was obtained by a scheme patterned on the Schotten-Baumann technique.

As an example, 1.0 gm of α-chymotrypsinogen (8×) was dissolved in 25 ml of 0.2 *M* phosphate and 25 ml of 0.05 *M* borate, and adjusted to pH 8.0. This system was vigorously stirred at 1–3°, and to it a total of 0.11 ml of acetic anhydride and 1.8 ml *N* NaOH, each in 6 equal doses, was added alternately every 5 min. (Total time thus was 55 min.) The pH during acetylation was checked with test papers, and appeared to vary only between 8.2 and 7.8. The final pH (glass electrode) was 7.9. After about 15 min of further stirring, the solution was allowed to come to room temperature and dialyzed to remove the salts. After most of the salts had been removed, the solution was brought to pH 2.8–3.0, and the dialysis then continued to com-

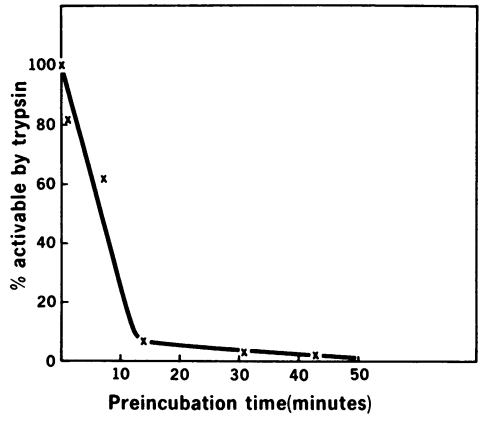

FIG. 2.—The change to inactivable protein requires a short time at pH 3.0. The experiment was made as in Fig. 1, except that the time of exposure to acid was varied instead of the pH level.

pletion. It will be noted that these conditions are quite different from those that are successful with sodium acetate in that the pH of the acylation is more alkaline and much less acetic anhydride is required.

The preparation just described will be referred to here as Prep. A. Three other preparations were studied in comparison. Prep. B was made in the same way as A, except that the pH range was 7.3–7.0, and was adjusted to pH 7.9 before dialysis. Prep. C was made in the same manner, but in half-saturated Na-nitrate containing 0.05 *M* maleate. The pH level was kept as nearly as possible at 5.5. Prep. D, however, was made in the same manner as those with Na-acetate (equal weights of anhydride and protein) except that Na-propionate was used instead of acetate. The pH was 7.0 at the start, 5.9 at the end. A comparison of the tryptic activation of these four preparations is shown in Table 1.

TABLE 1

TRYPTIC ACTIVATION* OF FOUR ACETYLATED CHYMOTRYPSINOGENS

Preparation	μ-Equiv. of acetyl per 25 mg protein	Specific activity† of chymotrypsin produced in: Hr	Water	Buffer‡
A	18.3	1	0	0.045
		20	0.005	0.058
B	13.7	1	0.024	0.025
		20	0.025	0.025
C	12.4	1	0	0.003
		20	0	0.003
D	8.4	1	0	0
		19	0	0

* The trypsin used was 1% of the zymogen; the latter was 1.0 mg/ml, pH 7.0.
† On L-tyrosine ethyl ester, 25°, calculated from the weight of zymogen used.
‡ 0.025 *M* tris-maleate.

As a result of these and other experiments it became evident that sodium acetate as used here in half-saturated solution[7] exerts a definite catalytic effect and probably also a definite directive effect on the acetylation of the protein. An extensive acetylation occurred in slightly acid medium without the formation of much insoluble protein. In the absence of acetate the extent of acetylation is obviously pH-dependent and also appears to be more indiscriminate. In order to introduce enough acetyl, more protein is made insoluble.

The Essential Role of Trypsin.—It has already been reported that natural chymotrypsin does not activate acetylated chymotrypsinogen, but produces a change in it which permits tryptic activation to take place thereafter in the absence of salts. More direct evidence that trypsin is the essential activating agent was seen in the response of the system to soybean trypsin inhibitor. Previously acid-treated acetylated chymotrypsinogen was exposed to 1/100 its weight of trypsin in water, as usual without activation. Part of the solution was then made 0.5 *M* to NaCl, whereupon the zymogen was activated, again as usual. To another part an excess

TABLE 2

PREVENTION OF ACTIVATION BY SOYBEAN INHIBITOR

Time after addition (min)	Specific Activity* NaCl only	NaCl + inhibitor
0	0	0
30	0.035	0
155	0.050	0

* Based on zymogen present.
Acetylated chymotrypsin pretreated with trypsin for 45 min in water; NaCl and inhibitor then added.

TABLE 3

INHIBITION OF TRYPTIC ACTIVATION BY LESS SOLUBLE FRACTIONS OF ACETYLATED CHYMOTRYPSINOGENS*

Insoluble fraction from	Prep. A					Prep. C					Prep. D				
Insoluble fraction added, μg	0	8	16	41	81	0	15	38	76		0	13	34	67	
Specific activity† of chymotrypsin produced in 1 hr	0.035	0.027	0.010	0.006	0	0.035	0.009	0	0.001		0.038	0.034	0.030	0	
Per cent. of maximum activity produced in 1 hr	92	71	26	16	0	92	24	0	2		(100)	90	79	0	
Specific activity produced in 1 hr after addition of tris-maleate buffer (to give a conc. of 0.025 *M*) to the above systems					0.045					0.047					0.034
Per cent. of maximum activity produced in 1 hr with buffer					96					(100)					72

* For details, see text.
† Based on weight of acetylated zymogen.

of crystalline soybean trypsin inhibitor was also added (10 times the weight of trypsin present). This portion developed no activity. The experiment is summarized in Table 2. It indicates that trypsin does not act on the acetylated protein in the absence of salt to produce a protein that is then activable by salt alone.

Concentration of a Tryptic Inhibitor from Acetylated Chymotrypsinogen.—It was observed that some of the protein acetylated as described for Prep. A was precipitated from aqueous solution at pH 3.0, although the precipitate was readily soluble at pH 7.0. When such a precipitate was removed, the remaining solution could then be activated, like natural chymotrypsinogen, in the absence of electrolyte. Return of the precipitate to the system restored its original behavior. The existence of an inhibitor of trypsin in the acid precipitate was thus indicated. A more concentrated form of this material was made by preparing that fraction of the protein that was insoluble in 0.1 *M* NaCl at pH 3.0, but soluble in water at pH 7.0. The extent of acetylation in such fractions was found to be slightly greater than that of the material from which they were derived.

It is obvious that such material cannot be a general inhibitor of trypsin, for it has little or no effect on the splitting of natural chymotrypsinogen or of acetylated chymotrypsinogen that has been exposed to natural chymotrypsin. Moreover, it appears to have no inhibitory effect whatever on trypsin in dilute salt solutions. It was therefore not surprising that, even with relatively large doses, the action of trypsin on casein or on toluene-sulfonyl-L-arginine methyl ester was not appreciably lessened. Such tests, by the way, are not easily carried out in a reliable manner. In such high dilutions trypsin is notoriously unstable at neutral pH, and still more so in the absence of calcium ion. One may assume, however, in view of the efficient activation of the chymotrypsinogens by traces of trypsin, that such substrates exert some protective action upon the enzyme. This was later found to be the case in the course of the following experiments. Trypsin was added to mixtures of the supposed inhibitor and another (less heavily) acetylated chymotrypsinogen (Prep.

B) that would have normally been activated without electrolyte. Thus, varying amounts of the material derived from three other acetylated chymotrypsinogens were added to a solution of 5.0 mg of Prep. B in 1.0 ml of water, adjusted to pH 7.0. Trypsin (50 μg in 0.050 ml water at pH 3.0) was then added and the whole diluted to 5.0 ml. After 1 hr at 25° the solution was assayed for chymotryptic activity with tyrosine ethyl ester as usual. This is admittedly an extremely delicate test for trypsin. The substrate used probably contains some inhibitor already, but not enough to prevent the activation. However, the addition thereto of a very small amount of the material isolated from other acetylated preparations was sufficient to stop completely the activity of the trypsin present. The conditions of the experiment did not destroy the activity of the trypsin, since subsequent addition of maleate buffer produced rapid activation to about the usual level achieved in this time interval (Table 3).

Summary.—An abrupt change in the activability of heavily acetylated chymotrypsinogen by trypsin occurs between pH 5 and 4. If maintained above this level, the protein may be activated in water like natural chymotrypsinogen. Once the protein has been acidified, tryptic activation requires the presence of salts (or a pretreatment with natural chymotrypsin). The change is not reversible at practical pH levels. A fraction of the protein, less soluble in acid solutions than the rest, is particularly inhibitory to the action of trypsin as activator of acetylated chymotrypsinogen.

Since no polymerization occurs as a result of acidification and no acetyl is lost, one is inclined to conclude that a structural change in the protein is responsible for its difference in behavior toward trypsin. This change could result in the production of inhibitory material or of indigestible substrate or quite likely of both, since both substances are patently of similar nature.

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[4] The medium "POPOP" consists of naphthalene, 2,5-diphenyloxazole, and 1,4-bis-2(5-phenyloxazolyl)benzene dissolved in dioxane.[5]

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