The Mechanism of Inhibition of Trypsin by Ovomucoids*

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It has been suggested that the mechanism of inhibition of bovine trypsin by chicken ovomucoid requires proteolytic activity of the enzyme and the formation of a specific covalent (enzyme-substrate) bond between the enzyme and the inhibitor, (Finkenstadt and Laskowski, 1965; Finkenstadt et al., 1965). We are now suggesting that proteolysis of ovomucoids is unnecessary and only coincidental and that several other explanations for proteolysis should be considered. Among these are the following: a) Proteolysis of free ovomucoid could be caused by free enzyme. b) Proteolysis of a limited number of bonds of complexed ovomucoid could be caused by free enzyme as indicated by Gorini and Audrian (1952). c) A restricted proteolysis of complexed ovomucoid could be caused by enzyme complexed with it and yet this proteolytic action would be completely unrelated to the formation of the complex. In this latter instance, proteolytic attack might be restricted to a few susceptible bonds proximal to the active site of the enzyme in the complex. The coincidental hydrolysis of such proximal bonds might thus be considered another example of "affinity labelling" (Wofsy et al., 1962).

Finkenstadt et al. (1965) treated chicken ovomucoid with trypsin in acidic solution and then with carboxypeptidase B at neutrality. They reported a restricted hydrolysis of one peptide bond with the release of arginine and the loss of inhibitory activity. We have confirmed that

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proteolysis of chicken ovomucoid with trypsin occurs, and that loss of activity occurs on subsequent treatment with carboxypeptidase B (Feinstein et al., 1966). However, similar treatments of two other ovomucoids, turkey and cassowary ovomucoids, gave less restricted proteolysis and no losses of activities. We have now examined the properties of these hydrolyzed ovomucoids in more detail.

Materials and Methods

Bovine trypsin (2X crystallized, salt-free, lot 6118), bovine α-chymotrypsin (3X crystallized, lot 6026), and porcine carboxypeptidase B were purchased from Worthington Biochemical Company. Other materials were similar to those used in this laboratory for related studies (Feeney et al., 1963; Stevens and Feeney, 1963; Simlot and Feeney, 1966).

In a typical enzymatic treatment with trypsin at low pH, 56 mg. of ovomucoid were dissolved in 5.5 ml of 0.1 M acetic acid (final pH 3.3). Trypsin, 0.1 ml of a 1% solution in 0.004 M acetic acid and 0.02 M $CaCl_2$, was added to the ovomucoid solution. The trypsin solution also contained 2×10^{-6} M of N-trans-cinnamoylimidazole (Schonbaum et al., 1961) to inhibit possible contamination by α-chymotrypsin in the trypsin samples. The reaction mixture was incubated at 38° for 15 hours. The pH remained constant throughout the incubation.

For the enzymatic treatment with carboxypeptidase B, the pH of the reaction mixture after treatment with trypsin was adjusted to pH 7.5 with 1.0 N NaOH. Sodium phosphate, 0.2 M at pH 7.5, was added to give a total volume of 9.9 ml. Carboxypeptidase B, 0.1 ml of 1% solution, was added and the solution incubated at 38° for various periods. The pH remained constant throughout the incubation. The reaction with carboxypeptidase B was stopped by addition of a few drops of concentrated HCl.

Samples of 0.5 ml were used for determination of free amino acids with a Technicon automatic amino acid analyzer. Starch gel electrophoresis

was done according to Lush (1964). Enzymatic assays were done spectrophotometrically (Feeney et al., 1963). The reduction and the alkylation of ovomucoids were done according to the method of Anfinsen and Haber (1961), using 2-mercaptoethanol and iodoacetamide.

Results and Discussions

There were marked differences among the inhibitory activities of the three ovomucoids after treatment with trypsin and carboxypeptidase B. Chicken ovomucoid lost about 30% of its inhibitory activity against trypsin after preincubation with trypsin at low pH. The loss was more than 90% after further incubation with carboxypeptidase B at pH 7.5. In contrast, turkey and cassowary ovomucoids retained essentially all of their inhibitory activity after both treatments.

Figure 1 shows the rate of appearance of the major amino acids released by carboxypeptidase B from trypsin-treated ovomucoids. Free amino acids were released in limited amounts from all three ovomucoids, but there were some marked differences. Primarily arginine was released from chicken ovomucoid, but tyrosine, leucine, and lysine were released in significant quantities as the incubation progressed. Arginine was primarily released from turkey ovomucoid during 5 minutes incubation with carboxypeptidase B. The release of lysine, however, followed that of arginine quite closely. The amounts of arginine and lysine released after 10 hours incubation were very close to one another, about 0.4 mole per mole of turkey ovomucoid. Extending the incubation period with trypsin from 15 hours to 36 hours, and increasing the ratio of trypsin to ovomucoid from 1:50 to 1:25, did not give higher amounts of these amino acids. Lysine was the main amino acid released from cassowary ovomucoid. The amount of lysine approached one mole per mole of ovomucoid. Small amounts of tyrosine and alanine were released and only very small amounts of arginine were released. In control

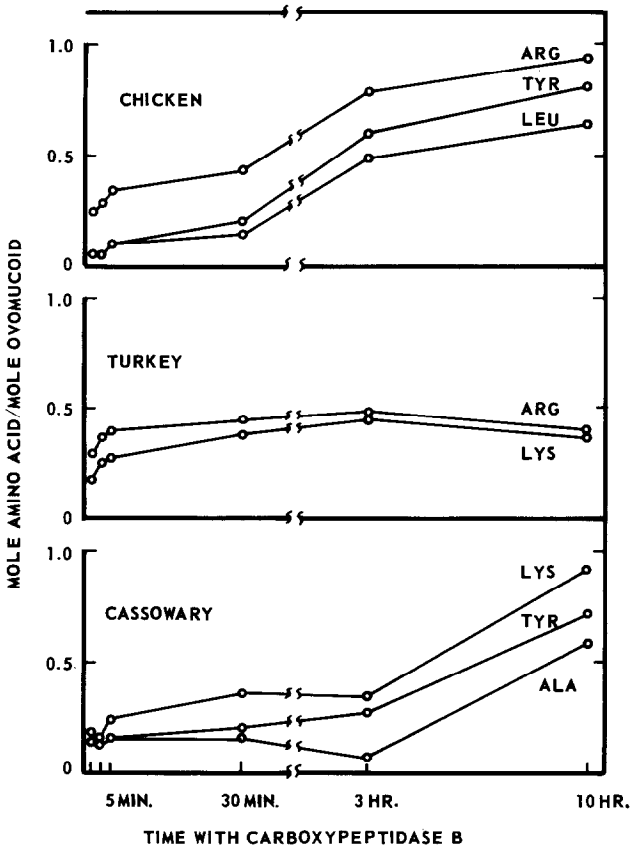

Figure 1. The rate of appearance of major free amino acids per mole chicken, turkey, and cassowary ovomucoids. Incubations were for 36 hr with trypsin (ratio of trypsin to ovomucoid, 1:25) at pH 3.3, and then for the various periods with carboxypeptidase B (ratio of enzyme to ovomucoid, 1:60) at pH 7.5. A Technicon automatic amino acid analyzer was used in determinations. Corrections for small amounts of nonspecific release of amino acids by carboxypeptidase B from chicken and turkey ovomucoids without previous trypsin treatment were made only for the 10-hr periods. The results with the cassowary ovomucoid were corrected for 3 and 10-hr periods.

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experiments, ovomucoids were incubated with either trypsin or carboxypeptidase B at pH 7.5 for 10 hours. Only small amounts of all amino acids were released.

The starch gel electrophoretic patterns of chicken and turkey ovomucoids before and after treatment with trypsin, and after treatment with both trypsin and carboxypeptidase B, showed that the trypsin

treatment gave rise to modified patterns. Starch gel patterns of chicken and turkey ovomucoids, which had been treated with trypsin and then reduced and alkylated, showed that trypsin treatment had caused extensive changes. Four or five additional bands appeared in both ovomucoids which had been treated with trypsin and reduced and alkylated, but there were only traces of the components corresponding to ovomucoids which had been reduced and alkylated without trypsin treatments.

The retention of complete inhibitory activity by turkey and cassowary ovomucoids after hydrolysis with trypsin and removal of the exposed carboxyl-terminal residues indicates that the formation of covalent bonds involving these specific amino acid residues is unnecessary for inhibition. Further studies on several aspects of these hydrolyses are underway. The absence of a requirement for the formation of an enzyme-substrate bond also appears to be proven by associated studies in which it has been demonstrated that enzyme-ovomucoid complexes are formed with enzymatically inactive trypsin and α-chymotrypsin derivatives (Feinstein *et al.*, 1966).

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