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# The Trypsin and Chymotrypsin Inhibitors from Avian Egg Whites*

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Studies of the comparative properties of proteins and enzymes from closely related species are receiving increasing attention as an approach to understanding the relationships between molecular structures and functions (1). Outstanding examples of these are the studies on the hemoglobins by Pauling *et al.* (2) and the studies on ribonucleases by Anfinsen *et al.* (3). The purpose of this approach is to utilize the similarities or differences provided by nature in the proteins or enzymes from closely related species as a tool. By so doing, the investigator may be able to differentiate between those parts of the molecule which are important for the biological, chemical, or physical characteristics of the molecule and those parts which are less important. Such an approach is similar in purpose to the use of chemical and physical means to modify proteins for study.

The eggs of different avian species offer an excellent area for comparative biochemical research. This is true not only because of the wide variety of species but also because a number of very similar eggs may be obtained from the same bird. Furthermore, considerable study has already been made of the biologically active proteins of chicken egg white (4, 5).

One of the more interesting constituents of egg white is ovomucoid. Chicken ovomucoid has been identified as an inhibitor of trypsin (6). Although it has not been crystallized, it is easily isolated in apparently essentially pure form and the assay of biological activity (trypsin inhibition) is readily conducted (7). Ovomucoid inhibits trypsin by the formation of an equimolar complex, and this complex is relatively stable for the periods which are necessary to conduct many biochemical experiments (6, 8). In addition to the ovomucoid in chicken egg white, Matsushima *et al.* (9) recently reported another inhibitor of a proteolytic enzyme which they named the ovoinhibitor. The ovoinhibitor was reported to inhibit microbial proteolytic enzymes prepared from *Bacillus subtilus* var. *biotecus* and an unidentified *Aspergillus.*

Indications as to the quantities of the ovomucoids, or materials resembling ovomucoids, in the whites of the eggs of different avian species have been given by the electrophoretic studies of Bain and Deutsch (10), and of Sibley and Johnsgard (11), and by the trypsin inhibitor assays of MacDonnell *et al.* (12). Apparently the only ovomucoid other than chicken which has received much study is that of the duck. These studies of chicken and duck ovomucoids concerned their relative stability to heat (13) and their comparative nutritional effect (14). The present work was stimulated by the recent report from this laboratory that duck eggs are more resistant than chicken eggs to deteriorative changes during incubation of infertile eggs (15).

In the present study, the ovomucoid fractions from the egg whites of eleven different avian species have been examined. The ovoinhibitors of chicken and turkey egg white have also been studied. Major differences were found in the biological properties of these proteins. In contrast to chicken ovomucoid, one ovomucoid was found to inhibit chymotrypsin primarily, others inhibited equal molar amounts of trypsin and chymotrypsin simultaneously, while still other ovomucoids were found to inhibit 2 moles of trypsin and 1 mole of chymotrypsin per mole simultaneously.[1]

## EXPERIMENTAL

*Sources of Egg Whites*—The eggs of the common domestic fowls were obtained locally and the eggs of the less common species were obtained at the San Diego Zoo, San Diego, California. All eggs were refrigerated within 24 hours of being laid. In most cases, the egg whites were separated within 2 days of refrigeration of the eggs, and the whites were shipped and stored in the frozen state. Preparation of the egg whites for isolation purposes consisted of carefully blending the whites followed by dialyzing against the starting buffer, 0.1 M acetic acid-ammonium hydroxide of the desired pH. The insoluble precipitate which formed was removed.

*Preparation and Use of Ion Exchange Agent*—CM-cellulose, containing 0.6 meq. of titratable groups per g, employed in this study was prepared by the method of Peterson and Sober (16). A wood cellulose, Solka-Floc BW200 and BW20 obtained from the Brown Company, was used in preparation of this exchange agent. DEAE-cellulose Type 20 containing 0.6 meq. of titratable groups per g was purchased from the Brown Company.

As previously described (5), purification of the proteins employing the cellulose ion exchange agents was accomplished by

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[1] This study constitutes one particular phase of a general program in the comparative biochemistry of the avian egg whites and egg-white proteins. More extensive and general studies will be published elsewhere.



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elution with buffers of stepwise changes in pH values predetermined by separate experiments using stepwise and gradient elution procedures.

*Analyses*—Total nitrogen was determined by micro-Kjeldahl analysis. Estimations of protein in fractions were routinely obtained by measuring the optical densities of the eluate at 280 mμ with a Beckman model DU spectrophotometer.

Tryptophan was determined chemically (17) and both tyrosine and tryptophan were determined spectrophotometrically (18). Sialic acid content of the proteins was determined by the method of Warren (19) and by use of Ehrlich reagent as given by Werner and Odin (20). The sialic acid used for a standard for these assays was a crystalline product prepared from *E. coli.*[2]

*Reagents*—The enzymes used in this study, trypsin and chymotrypsin, were purchased from the Worthington Biochemical Corporation. The trypsin was a twice crystallized product containing approximately 50% magnesium sulfate. It contained 47.6% trypsin as calculated on a nitrogen content of 16.1% for pure trypsin (21). Standard solutions of trypsin were prepared on the basis of this nitrogen content and were made up daily in 0.004 M acetic acid and 0.02 M $CaCl_2$. The chymotrypsin (α-chymotrypsin) was an activated, three times crystallized chymotrypsinogen preparation containing 15.0% N. The enzyme was used as received and made up daily in 0.004 M acetic acid and 0.02 M $CaCl_2$. The Nagarse crystalline proteinase was obtained from Nagase and Company, Ltd., Osaka, Japan, through the Biddle Sawyer Corporation, 20 Vesey Street, New York.

The trypsin substrate, TAMe,[3] and the chymotrypsin substrate, BTE, were purchased from Mann Research Laboratories, Inc. Part of the BTE used in this study was synthesized in this laboratory essentially according to the procedure of Fox (22) which he presented for the synthesis of benzoyl-L-diodotyrosine ethyl ester.

*Trypsin Inhibitor Assays*—Trypsin inhibitor assays were done with the use of a Beckman model DU spectrophotometer connected to a Bristol's Dynamaster recording potentiometer through a Beckman energy recording adapter, model 5800, as has been described (7) with slight modifications as given below. The reaction mixture in the cuvette of the spectrophotometer consisted of 0.3 ml of a solution containing 333 μg of trypsin per ml in 0.004 M acetic acid and 0.02 M $CaCl_2$, plus 0.0 to 0.7 ml of solution containing 150 μg per ml of inhibitor in 0.006 M Tris buffer, pH 8.2, and 0.7 to 0.0 ml of Tris buffer to bring the volume to 1.0 ml. Two milliliters of the substrate-buffer-indicator solution (0.02 M TAMe, 0.015 M Tris-HCl buffer, and 0.015% *m*-nitrophenol, respectively, with a final pH value of 8.2) were added to the cuvette just before starting the recorder. The speed of the chart was 4 inches per minute. Calculation of activities is as previously given (7).

*Chymotrypsin Inhibitor Assays*—Chymotrypsin assays were performed essentially by the same method as the trypsin inhibitor assays described above. The reaction mixture in the cuvette of the spectrophotometer consisted of 0.3 ml of a solution of chymotrypsin (333 μg per ml) in 0.004 M acetic acid and 0.02 M $CaCl_2$ plus 0.0 to 0.7 ml of a solution containing 150 μg of inhibitor per ml in 0.006 M Tris buffer at pH 8.2, and 0.7 to 0.0 ml of Tris buffer to bring the volume to 1.0 ml. To this were added 2 ml of substrate-buffer-indicator solution (0.008 M BTE, 30% methanol, 0.0075 M Tris-HCl buffer, and 0.01% *m*-nitrophenol with a final pH value of 8.2) and the recorder started. The speed of the chart was 2 inches per minute. Activities were calculated in the same manner as previously described for trypsin inhibitor activity (7).

*Protease Inhibitor Assay with Casein Substrate*—Inhibitor assays with casein as substrate were performed essentially as given by Sale *et al.* (23), and by Wu and Laskowski (24).

*Physical Analyses*—Diffusion experiments were carried out in a portable electrophoretic apparatus (American Instrument Company, Inc.) at 20–22°. Diffusion constants were routinely corrected to 20° and partial specific volumes were determined according to Bull (25). These determinations were made at 30°. Sedimentation analyses were performed in a Spinco model E ultracentrifuge. Paper electrophoretic analyses were performed with the use of horizontal strip apparatus employing a constant current for 16 to 20 hours.

## RESULTS

*Fractionation and Purification of Ovomucoid*—Initial fractionations of the egg whites of all species studies were performed on CM-cellulose. Columns of this exchange agent, consisting of 4 g of CM-cellulose per g of egg-white protein to be fractionated, were equilibrated with 0.1 M acetic acid titrated to pH 4.3 with $NH_4OH$. Quantities of 4 to 10 g of exchange agent were used as columns with a diameter of 2 cm and lengths appropriate to the amounts of CM-cellulose employed. Larger quantities of exchange agent (15 to 100 g) were used as short, compact columns on Buchner funnels of the appropriate size. In both cases, dialyzed egg white (0.1 M acetic acid-$NH_4OH$, pH 4.3) was run through the exchange agent. Elution was performed with the same buffer. With all the whites investigated, primarily only ovomucoid was unadsorbed and passed directly through the exchange column, whereas all of the other proteins were adsorbed. These ovomucoid fractions were then refractionated between pH 3.5 and 4.3 to separate different types of ovomucoids and to remove impurities. DEAE-cellulose was employed to remove small traces of apoprotein from the ovomucoid when present. The ovomucoid fractions equilibrated at pH 4.3 (0.1 M acetic acid-$NH_4OH$) were run through a short column of DEAE-cellulose (approximately 10 g of exchange agent per g of apoprotein). Under these conditions, the majority of the ovomucoid passed directly through the exchange agent unretained. The adsorbed apoprotein and ovomucoid containing a relatively high percentage of sialic acid were then eluted at pH 3.5 with 0.1 M acetic acid-$NH_4OH$ in 1 M NaCl. When this mixture was refractionated on CM-cellulose starting at pH 3.5, ovomucoid passed directly through the exchanger while the apoprotein was adsorbed.

The purified ovomucoids were subjected to paper electrophoretic analysis employing 0.1 M sodium acetate-acetic acid, pH 4.5, as the buffer and also at pH 6.9 employing 0.1 Γ/2 potassium phosphate buffer for 16 hours at 8 milliamperes. In all cases, the presence of contaminating proteins was not evident.

[2] Sialic acid (an acyl-substituted neuraminic acid) was kindly supplied by Dr. Saul Roseman and Dr. D. G. Comb, University of Michigan, and was prepared from a strain of *Escherichia coli*.

[3] The abbreviations used are: TAMe, *p*-toluenesulfonyl-arginine methyl ester; BTE, benzoyl-L-tyrosine ethyl ester.

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TABLE I

*Properties of chicken ovomucoids and proteinase inhibitor*

| Property | Ovomucoid prepared by | | | | Ovoinhibitor |
|---|---|---|---|---|---|
| | Trichloroacetic acid-acetone method | Elution from CM-cellulose | | | |
| | | pH 3.5[a] | pH 3.5-4.1 | pH 4.1-4.4 | |
| pH of peak elution | | 3.5 | 4.1 | 4.4 | 5.1 |
| N, % | 10.8 | 10.8 | 11.8 | 12.3 | 12.7 |
| Tyrosine, % (spectrophotometric) | 3.8 | 2.8 | 3.5 | 2.9 | 5.7 |
| Tryptophan, % (spectrophotometric) | 0.5 | 0.5 | 0.6 | 0.5 | 0.9 |
| Tryptophan, % (chemical) | <0.3 | <0.3 | <0.3 | <0.3 | |
| Sialic acid, %[b] | 0.5 | 4.1 | 0.6 | 0.4 | <0.1 |
| Relative trypsin inhibitor activity[c] | 1.0 | 0.9 | 1.1 | 1.1 | 0.8 |
| Relative chymotrypsin inhibitor activity[d] | <0.05 | <0.05 | <0.05 | <0.05 | 0.9 |

[a] Fraction passed through CM-cellulose unadsorbed at this pH value.

[b] The exact percentage of sialic acid in each fraction prepared by the use of CM-cellulose varied with the conditions of separation.

[c] Chicken ovomucoid prepared by the trichloroacetic acid method of Lineweaver and Murray (6) and corrected to 13.3% nitrogen was used as the relative standard. When the figures are multiplied by 0.9, the values obtained are approximately equivalent to the mg of trypsin inhibited per mg of inhibitor.

[d] Duck ovomucoid (Peking) was used as the relative standard. When the figures are multiplied by 0.9, the values obtained are approximately equivalent to the mg of chymotrypsin inhibited per mg of inhibitor.

TABLE II

*Physical properties of chicken and duck ovomucoid*

| Property | Ovomucoid[a] | |
|---|---|---|
| | Chicken | Duck |
| Sedimentation constant, $s_{20}$[b] | 2.49 S[c] | 2.50 S[c] |
| Diffusion constant (20°), $cm^2$ $sec^{-1}$ [b] | $7.7 \times 10^{-7}$ | $8.1 \times 10^{-7}$ |
| Apparent partial specific volume, ml $g^{-1}$ | 0.71 | 0.73 |
| Molecular weight, g $mole^{-1}$ | $27 \times 10^3$ | $28 \times 10^3$ |

[a] The chicken ovomucoid was a composite fraction eluted from pH 3.5 to 4.4. The duck (Peking) ovomucoid was unadsorbed on CM-cellulose at pH 4.1.

[b] Determinations were made on 1% solutions of the respective proteins; values were not corrected to infinite dilution.

[c] Averages of two determinations.

*Inhibitors from Chicken Whites*—When chicken egg white was fractionated on CM-cellulose starting at pH 3.5, four fractions were obtained which possessed trypsin inhibitor activity. These included three ovomucoid fractions and, the fourth, the ovoinhibitor recently reported by Matsushima (9). The principal difference detected among the ovomucoid fractions was their content of sialic acid as seen in Table I. This variation in sialic acid content was reflected in their respective pH values of elution from CM-cellulose. As initially eluted, the fraction unadsorbed at pH 3.5 comprised approximately 1% of the total ovomucoid and each of the other two fractions comprised approximately 50% of the remainder. The exact amount of sialic acid in each fraction varied slightly with each preparation and hence further work would be needed to prove homogeneity of each fraction. Somewhat larger quantities of the high sialic acid ovomucoid fraction (that unadsorbed on CM-cellulose at pH 3.5) were prepared essentially by the procedure recently reported for isolation of the apoprotein of the egg-white flavoprotein (26). This fraction of ovomucoid was reported there as an unidentified acidic protein. This procedure, which employed DEAE-cellulose, had several advantages. The main advantage was that much less exchanger was needed to isolate a gram of this fraction since primarily only the flavoprotein-apoprotein and the high sialic acid ovomucoid are retained on the DEAE-cellulose in the initial step while the other proteins of egg white are unadsorbed. When CM-cellulose was employed for direct isolation, only the high sialic acid ovomucoid was unadsorbed by this exchanger and all the other egg-white proteins had to be retained.

The fourth of the fractions possessing trypsin inhibitor activity was eluted between pH 4.9 to 5.5. In contrast to the ovomucoid fractions which were obtained in a nearly pure state in a single step, the ovoinhibitor contained three other proteins, none of which were any of the well characterized egg-white proteins. Refractionation on both CM-cellulose and DEAE-cellulose did not result in a completely homogeneous protein. As a result, trichloroacetic acid and acetone were employed as outlined by Matsushima (9) to obtain a protein which was homogeneous as indicated by paper electrophoresis in several buffers at different pH values.

Table I shows some chemical and biological properties of the chicken ovomucoid prepared by trichloroacetic acid-acetone precipitation, of the three ovomucoid fractions isolated, and of the ovoinhibitor. As can be seen, the ovomucoid fractions and the trichloroacetic acid-acetone prepared protein were all similar to each other except for differences in content of sialic acid. In contrast to the ovomucoids, the ovoinhibitor was eluted above pH 5 and had a higher tyrosine content, little or no sialic acid, and most significantly, was found to inhibit both trypsin and chymotrypsin.

*Inhibitor from Duck Egg White*—The ovomucoids isolated from both Peking duck egg white and from Khaki Campbell duck white were studied in detail. The ovoinhibitor present in chicken egg white appeared to be absent or present in very low concentration in duck white.

In Table II are given some physical properties of Peking duck ovomucoid compared to chicken ovomucoid. The mean sedimentation values, diffusion constants, and apparent specific volumes are similar. The calculated molecular weights of 27,000 and 28,000 for chicken and duck ovomucoid, respectively, are close to the values reported for chicken ovomucoid (7). Concentration of the proteins in the above sedimentation and diffusion analyses was 1%. The values given were not corrected to infinite dilution.

Chemical and biological properties of the duck ovomucoids are given in Table III. Comparison of the chemical properties with those of chicken ovomucoid as shown in Table I indicates a similarity in elution pH from CM-cellulose, in nitro-

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TABLE III

*Comparison of the ovomucoids of different avian species*

| Species | pH of elution from CM-cellulose | N | Spectrophotometric | | Chemical tryptophan | Sialic acid | Relative trypsin[a] inhibitor activity | Relative chymotrypsin[b] inhibitor activity | Approximate ratio of trypsin inhibitor activity to chymotrypsin inhibitor activity |
|---|---|---|---|---|---|---|---|---|---|
| | | | Tyrosine | Tryptophan | | | | | |
| | | % | % | % | % | % | | | |
| Peking duck | 4.1[c] | 12.7 | 5.7 | 0.8 | <0.3 | <0.1 | 2.0 | 1.0 | 2 |
| Khaki Campbell duck | 4.1[c] | 12.6 | 5.1 | 0.5 | <0.3 | <0.1 | 2.0 | 1.0 | 2 |
| Turkey | 3.5[c] | 12.4 | 2.9 | 0.6 | <0.3 | 4.4 | 0.8 | 0.9 | 1 |
| Turkey | 3.5–4.1 | 11.1 | 2.8 | 0.6 | <0.3 | 2.8 | 0.8 | 0.8 | 1 |
| Turkey | 4.1–4.3 | 11.7 | 3.4 | 0.4 | <0.3 | 1.9 | 0.9 | 0.9 | 1 |
| Guinea | 4.3[c] | 12.6 | 2.9 | 0.7 | | 0.3 | 0.9 | 1.0 | 1 |
| Goose | DEAE | | 4.7 | 0.7 | <0.3 | 1.7 | 1.5 | <0.05 | >30 |
| Goose | 4.1[c] | 11.6 | 4.8 | 0.6 | <0.3 | <0.1 | 1.7 | <0.05 | >30 |
| Emu | DEAE | 12.9 | 6.3 | 0.9 | 0.3 | 10.6 | 1.9 | 0.9 | 2 |
| Emu | 4.1[c] | 10.8 | 4.4 | 0.6 | 0.4 | 3.6 | 1.2 | 0.6 | 2 |
| Golden pheasant | 4.3[c] | 13.2 | 4.0 | 0.6 | | 0.6 | 0.3 | 1.0 | <0.3 |
| Cassowary | 4.1[c] | | 4.2 | 0.5 | <0.3 | 5.4 | 1.0 | <0.1 | >10 |
| Red jungle fowl | 4.3[c] | | 3.5 | 0.7 | <0.3 | 0.8 | 0.8 | <0.1 | >8 |
| California valley quail | 4.3[c] | | 4.9 | 0.7 | <0.3 | | 1.4 | 0.9 | 2 |
| Painted quail | 4.3[c] | | 4.0 | 0.6 | <0.3 | 0.4 | 0.7 | <0.1 | >7 |

[a] Chicken ovomucoid prepared by the method of Lineweaver and Murray (6), corrected to 13.3% N, was used as the standard. Figures for other ovomucoids expressed on a dry weight basis.

[b] Duck ovomucoid (Peking) was used as the standard. Figures expressed on a dry weight basis.

[c] Fraction passed through CM-cellulose unadsorbed at this pH value.

gen, and in low tryptophan content. However, the duck ovomucoids contained approximately twice the tyrosine content of chicken ovomucoids and very little or no sialic acid compared to variable amounts in the chicken ovomucoids.

Duck ovomucoid differed extensively from chicken ovomucoid in its inhibitory activity (Fig. 1). *Curves A* and *C* represent the curves obtained for chymotrypsin and trypsin inhibition by chicken ovomucoid. Extrapolation to the abscissa of the linear portions of these and other curves given below approximate the amount of inhibitor required to inhibit the amount of enzyme present. Since the molecular weights of chymotrypsin, trypsin, chicken ovomucoid, and duck ovomucoid (21) are approximately the same, the $\mu$g of enzyme inhibited per $\mu$g of inhibitor are approximately equivalent to the moles of enzyme inhibited per mole of inhibitor. As previously reported, chicken ovomucoid possessed very little or no chymotrypsin inhibitory activity (24) (*Curve A*). Also as previously reported (7), 100 $\mu$g of trypsin were inhibited by approximately an equal weight of chicken ovomucoid, indicating a 1:1 molar complex.

In this study, the results represented by *Curves B* and *D* were obtained for duck ovomucoid. *Curve B* indicated that 100 $\mu$g of chymotrypsin were inhibited by an equal weight of duck ovomucoid, again indicating a 1:1 molar complex. On the other hand, *Curve D* showed that 100 $\mu$g of trypsin were inhibited per 50 $\mu$g of duck ovomucoid. In this instance, a 2:1 molar complex of trypsin and duck ovomucoid was indicated. Thus, it appeared, in contrast to chicken ovomucoid which inhibited only trypsin as a 1:1 molar complex, duck ovomucoid would inhibit chymotrypsin as a 1:1 molar complex and would also inhibit trypsin by forming the 2:1 molar complex.

*Inhibitors from the Egg Whites of Different Avian Species*—The results given in the previous section concerning duck ovomucoid stimulated extension of this comparative biochemistry study to include the ovomucoids of 9 other avian species. Chemical characteristics and biological activities of these ovomucoids are presented in Table III. In general, the conditions for adsorption and elution of these ovomucoids from CM-cellulose or DEAE-cellulose were approximately the same as for chicken or duck ovomucoids. In addition, all of the ovomucoids had similar nitrogen contents and very low tryptophan contents. Some variations were noted in the tyrosine content and in the sialic acid content of the various ovomucoids. In addition, the fractions of ovomucoid from the egg white of certain species also varied in sialic acid content. For example, the three fractions of turkey ovomucoid varied in sialic acid content similarly to chicken ovomucoid, although the content was significantly higher.

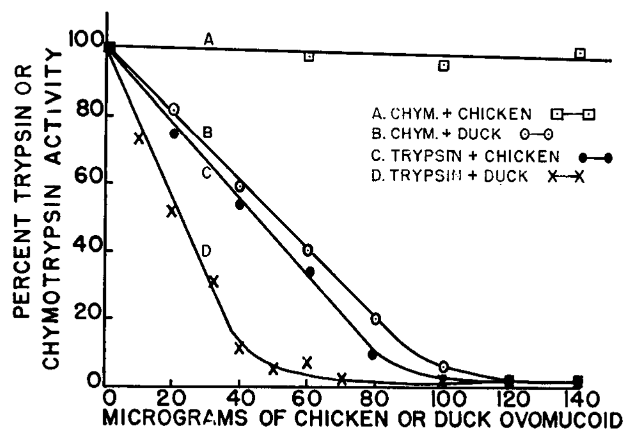


FIG. 1. Activity of 100 $\mu$g of trypsin or chymotrypsin in the presence of increasing amounts of chicken or duck ovomucoid. Conditions for assay are given under methods.

Extensive differences were observed in the biological prop-

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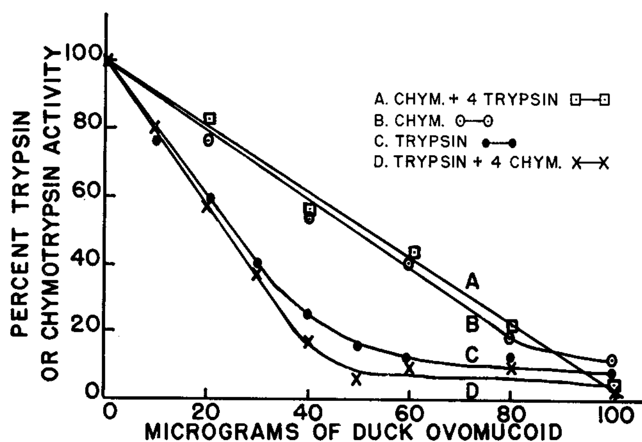


FIG. 2. Activity of 100 μg of trypsin or chymotrypsin in the presence of increasing amounts of duck ovomucoid. Four hundred micrograms of trypsin were added in the reaction mixture for *Curve A*, and 400 μg of chymotrypsin were added in the reaction mixture for *Curve D*. For details see text.

erties of the different ovomucoids. As apparent from Table III, the ovomucoids may be divided approximately into four classes on the basis of their inhibitory activities. First, the ovomucoids which inhibit primarily trypsin are those from goose, cassowary, red jungle fowl, painted quail, and chicken. Next, the only ovomucoid thus far found which inhibits primarily chymotrypsin was that from golden pheasant. Third, the ovomucoids which will inhibit approximately equal molar amounts of trypsin and chymotrypsin were those from turkey and guinea, and the fourth class, the ovomucoids which inhibit twice as much trypsin as chymotrypsin, were those from duck, emu, and California valley quail. Sedimentation constants obtained for the turkey, guinea, and golden pheasant ovomucoids indicate that the turkey and guinea ovomucoids form equal molar complexes with trypsin or chymotrypsin and that the golden pheasant ovomucoid forms an equal molar complex with chymotrypsin.

*"Multiheaded" Inhibitors*—As a result of finding that some of the ovomucoids would inhibit either trypsin or chymotrypsin, it was necessary to determine, if possible, whether the same inhibitor sites were involved or whether the site of trypsin inhibition was distinct from the site of chymotrypsin inhibition. One approach available was to determine the inhibition of one enzyme in the presence of high concentrations of the other enzyme. Such an approach was possible through the use of the specific synthetic substrates, TAMe and BTE for trypsin and chymotrypsin, respectively. Results of one such experiment are presented in Fig. 2. *Curves A* and *B* represent percentage activity of 100 μg of chymotrypsin in the presence of increasing amounts of duck ovomucoid. *Curve A* was obtained in the presence of 400 μg of trypsin. Similarly, *Curves C* and *D* represent percentage activity of 100 μg of trypsin. *Curve D* was obtained in the presence of 400 μg of chymotrypsin. These results indicate: (*a*) The sites of trypsin inhibition are distinct from the site of chymotrypsin inhibition, (*b*) both enzymes may be inhibited simultaneously, and (*c*) the molecular complex consists of two trypsins, one chymotrypsin and one duck ovomucoid. Extension of the investigation to the other ovomucoids indicated that those of turkey, guinea, emu, and California valley quail each have several distinct inhibitory sites. Inhibition of both trypsin and chymotrypsin occurs simultaneously, and excess of one enzyme does not effect inhibition of the other enzyme.

Further evidence for the simultaneous inhibition of trypsin and chymotrypsin by duck ovomucoid was obtained by the use of a mixed substrate of TAMe and BTE. The activity of the enzyme-inhibitor complex of two trypsin, one chymotrypsin, and one duck ovomucoid molecules was much lower than an equivalent amount of either enzyme on the mixed substrate.

*Physical Studies of Enzyme-Inhibitor Mixtures*—Further evidence for the enzyme-ovomucoid complexes was obtained through the use of paper electrophoresis. For these analyses, 0.1 Γ/2 potassium phosphate buffer at pH 6.9 was employed and the analyses were run at 8 milliamperes for 16 hours. In all cases, 0.02 ml of the enzymes, ovomucoids, or enzyme-ovomucoid mixtures prepared in the buffer was placed on the paper strips. The concentration of ovomucoid was 1% in all cases. The enzyme-ovomucoid mixtures given below contained additional amounts of enzymes on a weight basis.

A single spot for the chicken ovomucoid-trypsin (1:1) mixture was obtained. This was easily distinguishable from the spot for trypsin or chicken ovomucoid alone and moved to an intermediate position. Analyses of the chicken ovomucoid-chymotrypsin (1:1) mixture did not show a new spot but showed only the presence of two spots corresponding to those for chymotrypsin and chicken ovomucoid. Both the duck ovomucoid-trypsin (1:2) mixture and the duck ovomucoid-chymotrypsin (1:1) mixture gave single spots which were distinguishable from one another and the individual components of the mixtures. In the case of the duck ovomucoid-trypsin (1:2) mixture, however, a trace of a second component was observed which might correspond to the 1:1 duck ovomucoid-trypsin complex. The duck ovomucoid-trypsin-chymotrypsin (1:2:1) mixture gave a single spot which was indistinguishable from the duck ovomucoid-trypsin (1:2) mixture or to chymotrypsin alone. Finally, the duck ovomucoid-trypsin-chymotrypsin (1:1:1) mixture gave two spots. These corresponded to the duck ovomucoid-chymotrypsin (1:1) mixture and the duck ovomucoid-trypsin-chymotrypsin (1:2:1) mixture.

The golden pheasant ovomucoid-chymotrypsin (1:1) mixture gave one spot distinguishable from the spots of the chymotrypsin or golden pheasant ovomucoid alone as expected from the biological data. In contrast, the golden pheasant ovomucoid-trypsin (1:1) mixture gave two spots corresponding to trypsin and golden pheasant ovomucoid, respectively. These observations are the reverse of those obtained with chicken ovomucoid as would be expected from the enzymatic studies.

Further evidence for the enzyme-inhibitor complexes was furnished by ultracentrifugal analyses. Sedimentation patterns for duck, chicken, and golden pheasant ovomucoids and for several enzyme-ovomucoid mixtures are presented in Fig. 3. In all instances, the final protein concentration was 1%. The buffer employed for *Pattern A* and *B* was 0.1 M sodium acetate-acetic acid in 0.1 M KCl at pH 4.5. For the remaining analyses described, the buffer was 0.1 M Tris-HCl in 0.1 M NaCl at pH 8.5 except for *Pattern G* which was obtained in 0.01 M Tris-HCl in 0.01 M NaCl at pH 8.5. The speed of the rotor was 52,640 r.p.m. Only a single ultracentrifugal analysis was made in most cases. The sedimentation constants for the golden pheasant (*Pattern F*, 1.8 S) and for the turkey and guinea

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ovomucoids, 2.0 S and 2.1 S, respectively (not shown in Fig. 3), are somewhat lower than for duck or chicken ovomucoids (*Patterns A* and *B*, respectively). This does not necessarily indicate lower molecular weights in the former cases since calculations of molecular weights depend also on other physical measurements. Only single components were noted in each case, but more detailed studies would be necessary to prove homogeneity by this criterion.

Examination of the patterns obtained for the enzyme-ovomucoid mixtures indicates single peaks in all cases where complexes were expected from the biological and electrophoretic studies described above. This is apparent from *Pattern C* and *G*, duck ovomucoid, trypsin, and chymotrypsin in a 1:2:1 mixture; *Pattern D*, duck ovomucoid and trypsin in a 1:2 mixture; and *Pattern E*, chicken ovomucoid and trypsin in a 1:1 mixture. In addition, the calculated sedimentation constants increase in magnitude in the order which would be expected. The enzymes involved here have reported sedimentation constants which are similar to the ovomucoids (27). Another analysis not shown in Fig. 3 gave a calculated sedimentation constant for a duck ovomucoid-chymotrypsin mixture of 3.5 S and a single peak. This is in contrast to the results with the mixture of chicken ovomucoid and chymotrypsin where no complex was expected (*Pattern H*). No increase in sedimentation constant was obtained over chicken ovomucoid alone, and an unsymmetrical peak was obtained.

*Inhibitor Content of Various Avian Egg Whites*—In Table IV are given the percentage inhibitory activity against trypsin and

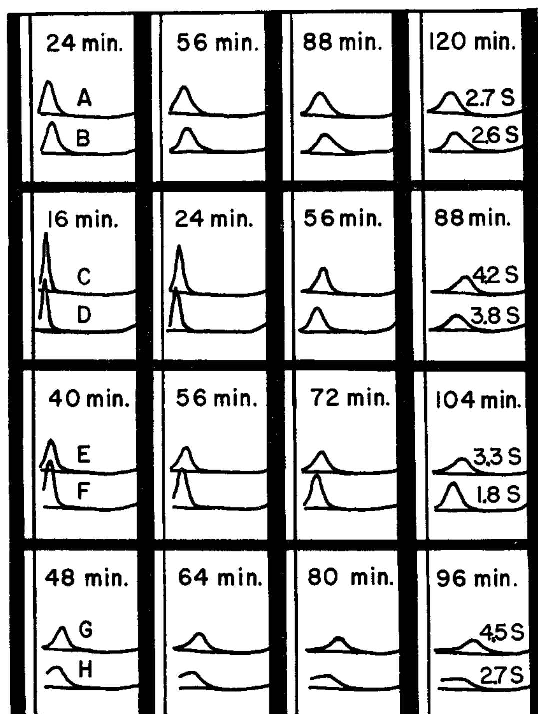


FIG. 3. Ultracentrifugal patterns for certain ovomucoids and enzyme-ovomucoid mixtures. See text for details. Patterns are as follows: *A*, duck ovomucoid; *B*, chicken ovomucoid; *C* and *G*, duck ovomucoid-trypsin-chymotrypsin (1:2:1); *D*, duck ovomucoid-trypsin (1:2); *E*, chicken ovomucoid-trypsin (1:1); *F*, golden pheasant ovomucoid; and *H*, chicken ovomucoid-chymotrypsin (1:1). Minutes indicated are the lengths of time run at 52,640 r.p.m. and figures on the patterns are the calculated sedimentation constants.

TABLE IV

*Trypsin and chymotrypsin inhibitor activity of egg whites of different avian species*

| Species | Relative trypsin inhibitor activity[a] | Relative chymotrypsin inhibitor activity[b] | Approximate ratio of trypsin inhibitor activity to chymotrypsin inhibitor activity | Approximate ovomucoid contents[c] |
|---|---|---|---|---|
| | % | % | | % |
| Chicken | 13 | 2.6 | 5 | 11 |
| Peking duck | 33 | 20 | 1.6 | 18 |
| Khaki Campbell duck | 39 | 18 | 2 | 19 |
| Turkey | 16 | 15 | 1 | 15 |
| Guinea | 23 | 20 | 1 | 21 |
| Goose | 24 | 1.9 | 12 | 15 |
| Emu | 47 | 22 | 2 | 22 |
| Golden pheasant | 8 | 19 | 0.4 | 19 |
| Cassowary | 30 | 3.2 | 9 | 30 |
| Rhea | 12 | 10 | 1 | |

[a] Chicken ovomucoid prepared by the method of Lineweaver and Murray (6), corrected to 13.3% N, was used as the standard. Figures calculated on a dry weight basis.

[b] Duck ovomucoid (Peking was used as the standard). Figures were calculated on a dry weight basis.

[c] Estimated from assays on whites and relative activities of preparations of ovomucoids.

percentage inhibitory activity against chymotrypsin of various avian egg whites employing chicken ovomucoid and duck ovomucoid as the respective standards. The percentage activities obtained in this study do not agree with those obtained in earlier studies on some of these whites (12, 14). These disagreements might be explained in that different substrates were used in these studies. Table IV also gives the ratios of inhibitory activities against trypsin and chymotrypsin for the different whites. In general, these ratios agree with the ratios obtained for the respective isolated ovomucoids (see Table III). The ovomucoid contents of the whites (calculated on the basis of the biological activity of the isolated ovomucoids) indicated chicken white to contain the least amount and cassowary white to contain the highest amount. The amount of ovoinhibitor of only the chicken and turkey were considered in these calculations since the amounts present in the other whites have not been established.

*Additional Observations*—A bacterial proteolytic enzyme marketed as Nagarse was found to be inhibited by duck ovomucoid as well as by the ovoinhibitor which had been previously reported (9). This enzyme has very slow activity on BTE compared to equal amounts of chymotrypsin and apparently no activity on TAMe. A study of the effect of Nagarse proteinase on the inhibition of trypsin or chymotrypsin by duck ovomucoid gave the following results: (*a*) Inhibition of trypsin in the presence of 4 times the amount of Nagarse (on a weight basis) was uneffected, (*b*) inhibition of chymotrypsin in the presence of 4 times as much Nagarse (on a weight basis) was reduced approximately 40%, and (*c*) equal amounts of Nagarse (on a weight basis) with chymotrypsin caused only an approximate 20% reduction in inhibition of chymotrypsin. Evidence supporting these data was obtained by paper electrophoresis with the methods described above for the other enzyme-inhibitor complexes.

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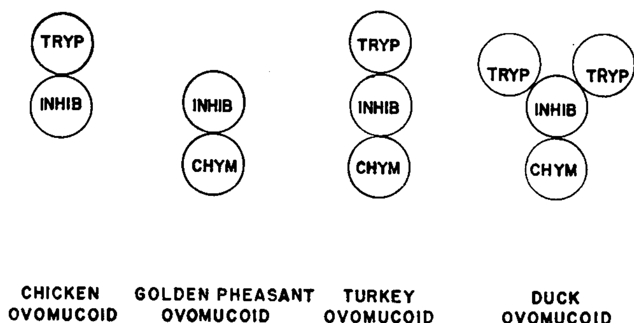


Sketch 1

Casein was also used as the substrate for trypsin or chymotrypsin instead of TAMe and BTE, respectively. In general, the same amounts of inhibitor were required to inhibit the enzymes as found with the synthetic substrates. Exceptions were found with guinea ovomucoid and chicken ovoinhibitor. Approximately 2 to 3 times as much of these inhibitors was required to inhibit chymotrypsin when casein was employed as a substrate as when BTE was employed. The significance of these observations has not been further investigated.[4]

## DISCUSSION

A variety of inhibitors of proteolytic enzymes has been studied including the chicken ovomucoid (5), chicken ovoinhibitor (9), pancreatic inhibitor (24), soybean trypsin inhibitor (24), the ascaris inhibitor (28), and the proteolytic enzyme inhibitors of animal sera (29). Most of these, however, have been found to be either inhibitors of trypsin or to have varying activities against trypsin and chymotrypsin and activity against bacterial proteolytic enzymes.

A "multiheaded"[5] inhibitor with simultaneous inhibitory activity against more than one proteolytic enzyme was implied by Wu and Laskowski (24) for soybean trypsin inhibitor. Even in this instance, inhibition of molar quantities of both trypsin and chymotrypsin B was not obtained. Thus, perhaps the duck ovomucoid is the first inhibitor of this type actually established. On the other hand, Green (28) has reported the isolation of an impure mixture of proteins as possibly containing the first example of a specific inhibitor of chymotrypsin. There is a question as to whether this preparation had two inhibitors present, as was suggested, or whether the inhibitor was of the multiheaded variety. Thus, the ovomucoid of golden pheasant egg white is at least one of the few protein inhibitors known to inhibit primarily chymotrypsin.

The existence of the multiheaded inhibitors appears well supported by biological, electrophoretic, and ultracentrifugal studies. However, future work perhaps should consider other criteria for the existence of these inhibitors. Diagrammatic presentation of the types of enzyme-inhibitor complexes demonstrated in this study are given in Sketch 1. Although this presentation is only diagrammatic, the equal size of the circles is indicative of the similarity in the molecular weights of the enzymes and inhibitors involved here. In addition, the lack of overlapping of the circles indicates that the site of inhibition of one enzyme is distinct from the site of inhibition of the other enzyme and that simultaneous inhibition of both enzymes may occur.

Other results indicated that both chymotrypsin and Nagarse proteinase are inhibited by duck ovomucoid but that these enzymes appeared to occupy the same site or closely adjacent sites on the inhibitor. This might be interpreted to mean that the combination site (active site?) of Nagarse proteinase is more similar to that of chymotrypsin than to that of trypsin. The comparative results with casein and BTE as substrates for chymotrypsin would indicate that the type of substrate, in certain cases, may effect the amount of inhibition caused by any particular inhibitor.

Variable amounts of sialic acid in the several fractions of chicken ovomucoid as well as in the ovomucoid fractions of other species have apparently not been reported previously. The results of a detailed investigation of the distribution of sialic acid in these fractions will appear elsewhere.

[4] Further studies have shown that duck ovomucoid may also inhibit less than two trypsins (between one and two) with casein as a substrate depending upon the conditions of the experiment.

[5] The term "multiheaded" inhibitor is used in a fashion similar to the term "multiheaded" enzyme as has been recently used by Racker and Krimsky (30).

## SUMMARY

The ovomucoids from eleven different avian species have been isolated and studied in detail. Particular attention has been focused on their respective biological properties. The ovomucoids were all somewhat similar as to the conditions for elution from carboxymethyl cellulose, low nitrogen content, and the low content or absence of tryptophan in these proteins. Variable amounts of sialic acid in the ovomucoid fractions prepared from the egg white of a given species have been found. The types of biological activity of the ovomucoids may be divided into four classes on the basis of their inhibitory activity: First, ovomucoids which inhibit primarily trypsin; second, ovomucoids which inhibit primarily chymotrypsin; third, ovomucoids which inhibit equal molar amounts of trypsin and chymotrypsin, separately or simultaneously; and fourth, ovomucoids which inhibit twice as much trypsin as chymotrypsin, separately or simultaneously. The latter two classes of inhibitors were considered to be "multiheaded" in their biological action.

The calculated ovomucoid contents of eight avian egg whites of different species are given.

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