Separation and Characterization of the Ovoinhibitors from Chicken Egg White*

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ABSTRACT: Chicken ovoinhibitor is heterogeneous with respect to carbohydrate content. Separation from egg white by ammonium sulfate fractionation and gel filtration followed by chromatography on DEAE-cellulose yields five fractions that contain from 2300 to 4800 g of carbohydrate per mole. The purified fractions do not differ in amino acid content or in inhibitory activity.

They contain glucosamine and hexose but no galactosamine, sialic acid, or tryptophan. Two moles of trypsin or chymotrypsin are bound per mole of ovoinhibitor. Nagarse and Pronase are inhibited by all fractions tested but papain is not. Sedimentation equilibrium in D_2O and H_2O gives a weight-average molecular weight of 49,000 and a $\bar{v}$ of 0.71. Since the molecular weight is unchanged in a denaturing reducing solvent, these ovoinhibitors contain only one polypeptide chain.

Chicken egg-white ovoinhibitor was distinguished from ovomucoid by Matsushima (1958a,b) on the basis of solubility and inhibitory activity toward bacterial and fungal proteinases. Rhodes *et al.* (1960) confirmed the presence of more than one trypsin inhibitor in chicken egg white and showed that ovoinhibitor inhibits bovine chymotrypsin. They also showed that the composition of ovoinhibitor was different from that of ovomucoid. Ryan and Clary (1964) and Ryan *et al.* (1965) found that ovoinhibitor inhibits avian trypsin and chymotrypsin. Feeney *et al.* (1963a) showed that the weak inhibition of chymotrypsin by commercial ovomucoid prepared by the Lineweaver and Murray (1947) method was due to contamination with ovoinhibitor. Amino acid composition, molecular weight, and other properties have been reported for ovoinhibitor separated from commercial ovomucoid (Tomimatsu *et al.*, 1966).

Matsushima (1958a,b, 1959), Rhodes *et al.* (1960), and Tomimatsu *et al.* (1966) have all observed activity of chicken ovoinhibitor against bacterial protease (subtilisin); Matsushima (1958b, 1959) and Feeney *et al.* (1963a) observed inhibition of fungal proteases (from *Aspergillus*) and Haynes and Feeney (1967) observed inhibition of components of Pronase (*Streptomyces*). Papain and pepsin were not inhibited (Matsushima, 1958b, 1959).

Tomimatsu *et al.* (1966) could distinguish three components in their ovoinhibitor preparation on starch gel electrophoresis. This finding is consistent with other reports of the occurrence of several molecular forms of various egg-white proteins (Feeney *et al.*, 1963b; Baker and Manwell, 1962). The heterogeneity of ovomucoid is particularly well documented (Melamed, 1966, 1967; Beeley and Jevons, 1965; Montreuil *et al.*, 1965; Feeney *et al.*, 1967).

This report describes the separation of chicken egg-white ovoinhibitor into five fractions and their characterization. The amino acid and carbohydrate analyses of these ovoinhibitor components were determined and they were compared with respect to their ability to inhibit proteolytic enzymes. Some physicochemical properties were also determined.

Experimental Procedures[1]

Separation of an Ovoinhibitor-Rich Fraction from Egg White. Egg white (2–4 l.) from 1- to 2-day-old eggs was blended and dialyzed against continuously changing deionized water for 2 days at 4°. The mucinous precipitate was centrifuged and discarded and the supernatant was brought to 0.40 saturation by addition of saturated ammonium sulfate. After centrifugation the supernatant was discarded, and the precipitate was dissolved in 200–300 ml of water and reprecipitated by addition of saturated ammonium sulfate to 0.40 saturation. The precipitate was dissolved in *ca.* 50 ml of water and the solution was passed through a Bio-Gel P-150 column (6 × 125 cm, 100 ml/hr, 0.1 M KCl). The effluent, monitored at 254 mμ, showed separation into three peaks. The first peak at 1.0 void volume was high molecular weight material; the second at about 1.5 void volume was ovoinhibitor and other globulins; and the third at 2.5–3 void volumes was lysozyme. The

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[1] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

second peak was concentrated to about 100 ml by ultrafiltration (Davis and Wiele, 1967) and brought to 0.30 saturation with ammonium sulfate. The precipitate of crude ovoinhibitor was collected and used as the starting material for further fractionation by ion-exchange chromatography.

Ion-Exchange Chromatography. The crude ovoinhibitor was subjected to ion-exchange chromatography on DEAE-cellulose with the aid of a Beckman Model 130 Spectrochrom. Effluents were monitored at 254, 280, and 450 mμ, and for pH and conductivity. Jacketed columns (1.9 × 160 cm) were maintained at 4° with refrigerated circulating water. Columns were packed from starting buffer with Whatman DE32 microgranular DEAE-cellulose to a height of 150–155 cm. Back pressures were generally in the range of 25–35 psi.

Ammonium phosphate–glycine buffers containing 0.01% sodium azide as a preservative were used throughout. J. T. Baker M798 glycine was sufficiently free of ultraviolet-absorbing impurities to use as supplied. Sodium azide (Eastman P2352) was recrystallized twice from water to eliminate material absorbing at 280 mμ; it has low absorption at 254 mμ. Reagent grade monobasic ammonium phosphate was used as supplied. Buffers were made up to required concentration of ammonium phosphate in 0.10 M glycine, 0.010% sodium azide, and adjusted to pH 6.50 with concentrated ammonium hydroxide. Concentrations therefore refer to phosphate ion only. All buffers were filtered through Millipore type HA membranes before use.

The DEAE-cellulose columns were equilibrated with starting buffer so that the effluent was identical with the input according to the monitoring system. The sodium azide preservative was the slowest of the buffer components to reach equilibrium. Two to three days at 40–50-ml/hr buffer flow were usually required to reequilibrate a column. Solutions of the crude ovoinhibitor for chromatography containing approximately 10% protein were dialyzed against buffer containing 70% as much phosphate as the starting buffer. Aliquots (2 ml) were layered under the buffer onto the top of the packing and chromatographed at 25 ml/hr with buffer 0.010 M in phosphate until the ovoinhibitor peaks were off the column. The phosphate concentration was then increased to 0.025 M *via* an exponential gradient (Beckman No. 324812) to clear contaminating proteins from the column. Effluent was collected in the refrigerated fraction collector of the Spectrochrom at 12 min/tube. The column was then reequilibrated with starting buffer for the next run. Two columns were used so that one could be equilibrated while the other was running. Under these conditions, it was not found necessary to repack the columns.

The individual ovoinhibitor peaks were rechromatographed with constant composition, constant pH buffers. For the first ovoinhibitor peak (A), pH 6.50, 0.0060 M phosphate–0.10 M glycine was used; for the remaining peaks, pH 6.50, 0.010 M phosphate–0.10 M glycine.

Materials. AcTyrEt[2] and *p*-toluenesulfonyl-L-arginine methyl ester were synthesized by Mr. E. F. Jansen or produced commercially (Calbiochem or Mann). BzArgEt was obtained from Mann and the corresponding methyl ester, BzArgMe, was synthesized by Mr. Jansen. Bovine hemoglobin was an Armour Laboratories product. *p*-Nitrophenyl *p*-guanidinobenzoate hydrochloride was synthesized according to Chase and Shaw (1967). *N-trans*-Cinnamoylimidazole was purchased from Mann and recrystallized from dry cyclohexane before use (Schonbaum *et al.*, 1961).

Bovine chymotrypsin was salt-free, two- or three-times-crystallized enzyme (Worthington CDI, Lot 6JF). Bovine trypsin was two-times-crystallized enzyme (Worthington TR 6138 or Sigma Lot 97B-8000). Papain (two-times crystallized, Lots PAP 61A and 71E) was obtained from Worthington. Pronase (purified *Streptomyces griseus* proteinase) was obtained from Calbiochem. Nagarse (Subtilopeptidase C; see Drenth and Hol, 1967) was obtained from Nagase and Co., Ltd., Osaka, Japan.

Chymotrypsin and trypsin were dissolved (1 mg of protein/ml) in 2 mM HCl and 2 mM HCl–20 mM $CaCl_2$, respectively, and kept at 4°. Pronase and Nagarse were dissolved in cold 50 mM Tris-acetate (pH 6.9). The Worthington (1967) absorption coefficients for trypsin and chymotrypsin were used for the spectrophotometric determination of their concentrations as protein.

Ovoinhibitors were stored at 4° in the elution buffers (0.1 M glycine–0.01 M NH_4 phosphate, pH 6.5) or in H_2O or 0.1 M KCl. Freezing or lyophilization did not significantly affect the inhibitory activity or behavior on polyacrylamide gel electrophoresis. Buffers contained NaN_3 (100 mg/l.) to prevent bacterial contamination.

D_2O was obtained from Bio-Rad Laboratories, Richmond, Calif. Guanidine hydrochloride was prepared from the carbonate, after exhaustive recrystallization by the method of Nozaki and Tanford (1967).

Enzyme Assays. Active enzyme in the preparations used was determined by active-site titrations, using *p*-nitrophenyl *p*-guanidinobenzoate for trypsin (Chase and Shaw, 1967) and *N-trans*-cinnamoylimidazole for chymotrypsin (Schonbaum *et al.*, 1961). Inhibition was determined by assay of residual esterase activity in mixtures of enzyme and ovoinhibitor. Enzyme and ovoinhibitor were incubated prior to assay for 10–15 min at room temperature in 9 mM Tris-HCl (pH 8.2)–0.30 M $CaCl_2$ in small test tubes. (Assays after incubation for 10–15 min at lower ionic strength in 0.05 M $CaCl_2$ gave essentially the same results.) For esterase assay, an aliquot of the incubated enzyme–inhibitor mixture, usually 0.33 ml, was added to 1.67 ml of substrate plus water and Tris-HCl, adjusted to pH 8.2, so that the final 2.0 ml assay mixture was 5 mM in Tris-HCl and 50 mM in $CaCl_2$. Substrates were 5 mM AcTyrEt for chymotrypsin and either 13 mM *p*-toluenesulfonyl-L-arginine methyl ester or 2 mM BzArgMe or BzArgEt for trypsin. Rates of hydrolysis were measured

[2] Abbreviations used are: AcTyrEt, *N*-acetyl-L-tyrosine ethyl ester; BzArgEt and BzArgMe, benzoyl-L-arginine ethyl and methyl esters, respectively.

titrimetrically at 25° in a thermostated reaction vessel. A Radiometer TTT1b titrator and SBU1a syringe buret was used as a pH-Stat and results were recorded on an SBR2c titrigraph. The pH was maintained by addition of 0.025 or 0.05 N NaOH. At high inhibitor to enzyme ratios, the enzyme and inhibitor were proportionally increased to give higher residual enzyme activity when feasible. Papain activity was assayed with 0.03 M BzArgEt essentially according to Sluyterman (1967), except that dithiothreitol replaced cysteine and that final activator concentrations in the assay were lower. All assays were corrected for blanks.

Protein digestion was measured with hemoglobin denatured by the method of Anson (Rick, 1963) as substrate. The final assay mixture included 0.05 M Tris-HCl (pH 8.2), 0.05 M $CaCl_2$, 5.4 mg/ml of hemoglobin (0.026 M borate–1.5 M urea, pH 7.8), enzyme, and inhibitor. Liberated peptides were measured by their absorbance at 280 mμ after precipitation of the protein by trichloroacetic acid.

Enzyme:inhibitor ratios were determined by measuring the residual enzyme activity at several different ratios of enzyme to inhibitor concentration. A least-squares fit to the linear portion of the curve was extrapolated to 100% inhibition to give the moles of enzyme bound per mole of inhibitor. It was assumed that only active trypsin or chymotrypsin was bound by ovoinhibitor. This is consistent with findings reported for other protease inhibitors (Green, 1953; Laskowski and Laskowski, 1954; Lebowitz and Laskowski, 1962).

Electrophoresis. Polyacrylamide gel disc electrophoresis was carried out as described by Ornstein (1964) and Davis (1964) for 60 min at 5 mA/tube at room temperature, in a 7.5% separating gel. Protein bands were detected by staining with Amido Black (Davis, 1964). Starch gel electrophoresis was carried out by the method of Poulik (1957), as described in Garibaldi *et al.* (1968).

Analytical. Amino acid analyses were performed by the method of Moore and Stein (1963) on samples hydrolyzed at 110 ± 1° in 6 N HCl in evacuated tubes. Prior to hydrolysis, samples were dialyzed against several changes of 0.01 M KCl to remove azide and ammonium ion. Retentates were centrifuged to remove insoluble material, and the concentrations of the sample solutions were determined spectrophotometrically at 280 mμ. Corrections for destruction of half-cystine, glucosamine, serine, threonine, and tyrosine and for incomplete hydrolysis of valine were obtained from 20-, 40-, and 70-hr hydrolyses of two of the ovoinhibitors. These corrections were applied to 20-hr hydrolyses for the other ovoinhibitors. Glucosamine, galactosamine, and the basic amino acids were determined on the 50-cm column for physiological fluids with pH 5.28, 0.35 N buffer (Maxfield and Stefanye, 1962). Hexose was determined by the orcinol method of Schönenberger *et al.* (1957), with galactose as the standard. Sialic acid was determined by the thiobarbituric acid method of Warren (1959), with N-acetylneuraminic acid as the standard. Samples were hydrolyzed in 0.1 N H_2SO_4 at 80° for 60 min to liberate sialic acid.

Physical Measurements. A Cary Model 15 or Beckman DK2a recording spectrophotometer was used to obtain ultraviolet spectra. When necessary, turbidity corrections were made as suggested by Leach and Scheraga (1960). A Beckman-Spinco Model E analytical ultracentrifuge, equipped with ultraviolet optics, photoelectric scanner, and multiplex was used for sedimentation velocity and equilibrium experiments.

Results

The separation of these ovoinhibitors depends upon the achievement of maximum resolution through the use of constant composition eluents (Sober *et al.*, 1965) and long, fully equilibrated DEAE-cellulose columns. Doubling the length of column by connecting two columns in series did not improve resolution sufficiently to justify more than doubling the time required for equilibration. The elution pattern (Figure 1) was highly reproducible so long as conditions were kept constant. A slight change in ionic strength of the eluent, as indicated by conductivity, apparently due to ions bound by the sample protein, caused distortion of the initial peak (A) in early chromatograms. This distortion was eliminated by dialyzing the sample solution against buffer containing 70% as much phosphate as the starting buffer instead of against starting buffer.

Figure 1 shows that the ratio of the chymotrypsin inhibiting activity to the optical density remained nearly constant as the components of ovoinhibitor were eluted. The increasing optical density at the end of the chromatogram is due to contaminating proteins in the crude preparation. These contaminants were identified by starch gel electrophoresis as other globulins. They showed no inhibitory activity.

The central portions of each of the five principal peaks (A–E) from several runs like the one shown in Figure 1 were rechromatographed on DEAE-cellulose as described above (Experimental Section) at least twice or until a single symmetrical peak was obtained for each component at an elution volume which was both reproducible and characteristic of that component.

In an attempt to confirm the apparent homogeneity suggested by the symmetry of the peaks obtained on elution, peaks A and D were divided in the middle and each half was again chromatographed. The peaks were symmetrical but eluted at different volumes. These peaks were again divided and the halves were chromatographed separately and again the front halves were eluted earlier than the back halves. Figure 2 showing this behavior for fraction D is typical also of fraction A. There was insufficient material from the other fractions to treat them in a similar manner but it seems likely that similar evidence of inhomogeneity would have been found. The elution conditions for these experiments were controlled with particular care. A single lot of buffer was made up in sufficient quantity for the entire subfractionation so that small differences in concentration or pH could not affect the elution volumes. The flow rate was controlled to within 1%.

Gel Electrophoresis. Starch gel electrophoresis of fractions from the chromatography of whole egg white

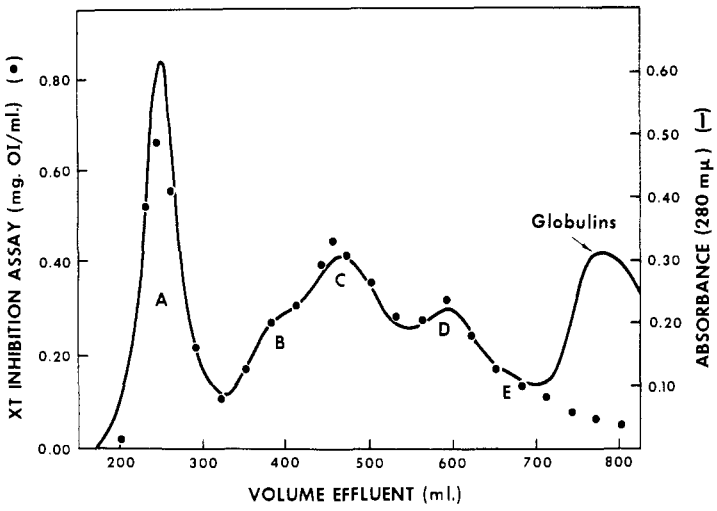

FIGURE 1: DEAE-cellulose chromatography of chicken ovoinhibitors. The line shows absorbance at 280 mμ (1-cm path length) and the points represent inhibitor activity against chymotrypsin as assayed with AcTyrEt.

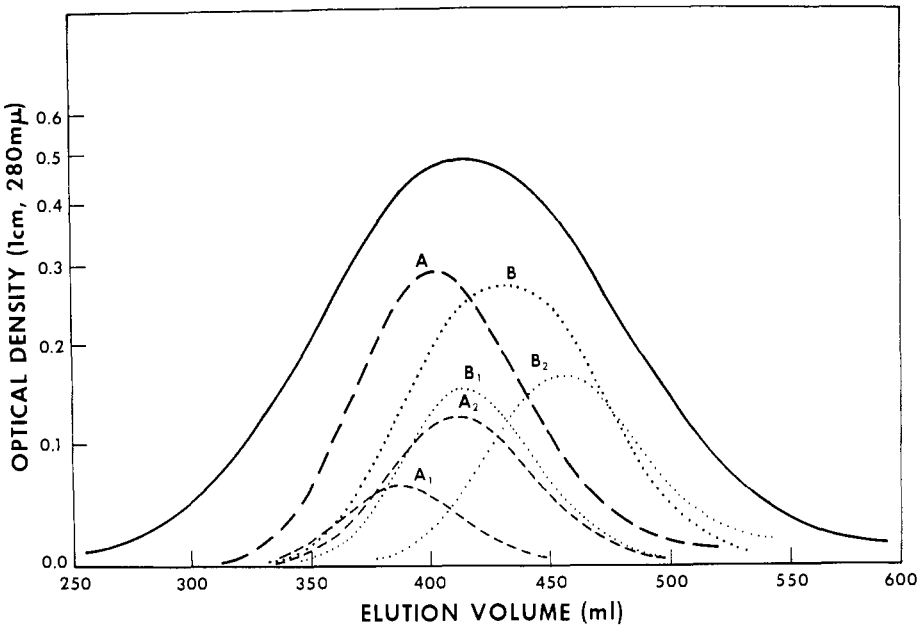

FIGURE 2: Results of rechromatography of ovoinhibitor D. The initial peak obtained by rechromatographing fraction D (Figure 1) gave A and B when the front and back halves were rerun. These in turn gave A_1 and A_2 and B_1 and B_2, respectively, on further chromatography. (A portion of A_1 was lost.)

gave the first indication that the components of the ovoinhibitor complex could be resolved on DEAE-cellulose columns. The resolution on starch gel, however, was poor compared with that obtainable with disc electrophoresis in polyacrylamide gel. Also, ovoinhibitor and conalbumin are not resolved under the conditions normally used for starch gel electrophoresis. Tomimatsu *et al.* (1966) found that they could be resolved by increasing the applied voltage approximately threefold and reducing the time to 3 hr, but the heat generated alters the gel so that it is difficult to handle during staining and destaining. Figure 3 shows the resolution of the ovoinhibitors by polyacrylamide disc electrophoresis and their separation from conalbumin. Some cross-contamination between successive ovoinhibitor peaks from the DEAE-cellulose column is apparent but no contamination by other unrelated egg-white proteins is detectable on either polyacrylamide disc or starch gel electrophoresis. It is evident from a comparison of Figures 1 and 3 that the ovoinhibitors retarded most strongly by DEAE-cellulose show the greatest electrophoretic mobility at pH 9.5.

Composition. Amino acid and carbohydrate analyses of the ovoinhibitor peaks discernable in Figure 1 are given in Table I. The fractions were purified as described above before analysis. The variations causing the comparatively high standard deviation for half-cystine appear to occur at random among replicates and do not reflect differences between ovoinhibitors. The data indicate that the amino acid compositions of the pro-

TABLE I: Composition of Ovoinhibitor Fractions in Residues per Mole.

Component	Fraction A[a]	B[b]	C[b]	D[a]	E[a]	Av ± Std Dev[c]	Nearest Integer
Lysine	22.8	23.8	23.0	23.0	22.6	23.1 ± 0.6	23
Histidine	14.1	14.6	14.2	14.5	14.1	14.3 ± 0.2	14
Arginine	20.2	20.9	20.6	20.5	20.2	20.5 ± 0.3	21
Aspartic acid	47.0	47.2	47.2	46.8	46.9	47.1 ± 0.3	47
Threonine	32.6	32.4	32.7	32.5	32.6	32.6 ± 0.3	33
Serine	27.6	26.8	26.7	27.0	27.3	27.1 ± 0.5	27
Glutamic acid	38.7	38.7	38.5	38.0	38.4	38.5 ± 0.4	39
Proline	17.8	17.5	18.1	18.4	18.3	18.0 ± 0.4	18
Glycine	32.5	32.1	32.3	32.5	32.2	32.3 ± 0.5	32
Alanine	19.6	19.7	19.7	19.4	20.2	19.6 ± 0.2	20
Half-cystine[d]	34.9	32.5	34.7	32.9	33.5	33.9 ± 1.6	34
Valine	26.1	26.9	26.6	26.7	26.9	26.5 ± 0.4	27
Methionine	3.9	4.0	3.9	3.9	4.0	3.9 ± 0.05	4
Isoleucine	16.9	17.4	17.3	17.2	17.3	17.2 ± 0.3	17
Leucine	21.9	21.2	22.0	22.2	22.4	21.9 ± 0.4	22
Tyrosine	16.6	17.1	17.0	17.6	16.5	17.0 ± 0.4	17
Phenylalanine	6.1	6.3	6.1	6.3	6.3	6.2 ± 0.1	6
Tryptophan[e]	<1	<1	<1	<1	<1	<1	<1
NH_3	36.5	38.4	36.8	37.5	35.5	37.1 ± 1.2	37
Glucosamine[f]	14.0 ± 2.0	15.2	11.0 ± 3.1	7.6 ± 0.2	9.0		
Hexose[f,g]	9.7 ± 1.4 (4)	10.2 (1)	7.7 (1)	5.6 ± 0.5 (4)			
Sialic acid[h]	0.2		0.4	0.5			
N recovery (%)	96.5	98.6	98.1	97.6	96.6	97.6 ± 1.5	

[a] Average of corrected 20-hr hydrolyses. Corrections for destruction of threonine, serine, half-cystine, tyrosine, and glucosamine, and for incomplete liberation of valine in 20 hr were obtained from hydrolyses of B and C for 20, 40, and 70 hr. [b] Extrapolated from 20-, 40-, and 70-hr hydrolyses. Two other samples of C were hydrolyzed for 20 hr. [c] Weighted average for 18 analyses (A = 5, B = 3, C = 5, D = 4, and E = 1). Extrapolated values were counted as three samples. Standard deviation given is that of a single measurement. The standard error of the mean can be obtained by dividing this by $\sqrt{18}$. [d] Range 30–35 residues. [e] Obtained from ultraviolet absorption spectra in neutral and alkaline solutions. No tryptophan is apparent. [f] No galactosamine was found. Standard deviations are shown where four or five analyses were made. [g] Numbers of samples analyzed are shown in parentheses. [h] Samples analyzed contained only 0.6–0.7 μg of sialic acid (as *N*-acetylneuraminic acid).

tein portions of the fractions are very similar if not identical. The only differences in composition found were for glucosamine and hexose, both of which tend to decrease in the order of elution. The small amount of sialic acid observed is in agreement with the results of Rhodes *et al.* (1960), and the apparent absence of tryptophan (Figure 4) confirms the finding of Tomimatsu *et al.* (1966).

Esterase Inhibition of Bovine Trypsin and Chymotrypsin. Trypsin activity as a function of enzyme:inhibitor ratio is shown in Figure 5. Enzyme:inhibitor ratios at 100% inhibition, obtained from plots of this type, are shown in Table II for peak fractions of ovoinhibitors A, C, and D eluted from this column. All ovoinhibitors tested inhibited both chymotrypsin and trypsin to about the same extent (Table II). To the nearest integer, the molar ratios of enzyme to inhibitor were 2:1. Complete inhibition curves were not obtained for ovoinhibitors B and E; however, their specific inhibitory activity for chymotrypsin (Figure 1) and trypsin measured on column effluents indicated no appreciable difference from A, C, and D in binding ratios. These ratios are based on active enzyme content of the preparations used. The trypsin used was 71% active enzyme and the chymotrypsin was 83% active, based on active site titrations.

TABLE II: Mole Ratios for Complete Inhibition of Esterase Activity of Trypsin and Chymotrypsin by Ovoinhibitors.[a]

Ovoinhibitor	Chymotrypsin: Ovoinhibitor	Trypsin: Ovoinhibitor
A	1.49	1.68
C	1.67	1.72
D	1.80	1.83

[a] Concentrations were calculated using $E^{1\%}_{1\,cm}$ at 278 mμ values of 6.7, 6.5, and 6.9 for ovoinhibitors A, C, and D, respectively.

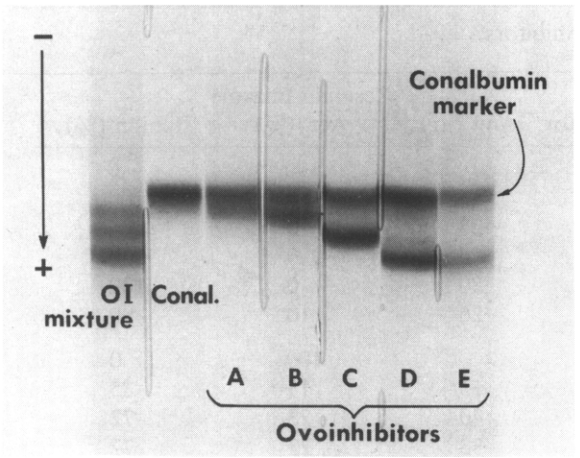

FIGURE 3: Polyacrylamide gel disc electrophoresis of chicken ovoinhibitors. Unfractionated ovoinhibitor is shown on the left. Note that this does not have a detectable amount of conalbumin. Conalbumin was added to the other samples as a marker. The samples were taken from peak tubes of the fractionation shown in Figure 1.

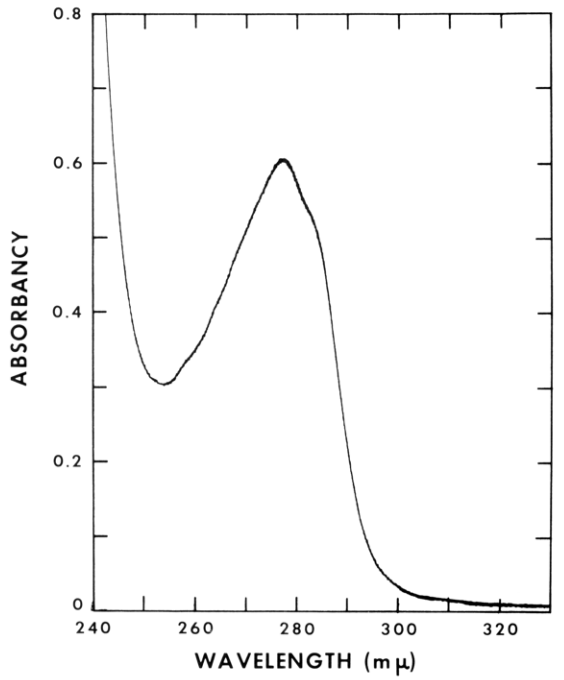

FIGURE 4: Absorption spectrum of unfractionated ovoinhibitors in 0.1 M KCl. The spectra of individual ovoinhibitors cannot be distinguished from the one shown.

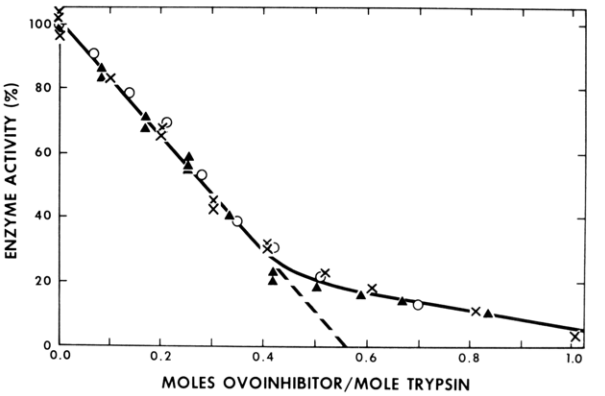

FIGURE 5: Effect of ovoinhibitor concentration on trypsin activity. BzArgMe was used as the substrate. Details of the assay are given in the text. Data for ovoinhibitors A (○), C (X), and D (▲) are shown. Molecular weights used were A = 48,800, C = 47,500, D = 46,500, and trypsin = 23,800. The abscissa is based on active trypsin. The line is an unweighted least-squares fit of all points below 0.42 mole of ovoinhibitor/mole of active trypsin.

TABLE III: Inhibition of Subtilisin (Nagarse) by Ovoinhibitors.[a]

Ovoinhibitor	Amt. (μg)	Residual[b] Enzyme Act. (%)	Inhibn. (%)
None		100	0
A	2.5	50	50
A	6.4	0	100
C	3.0	37	63
C	7.4	1	99
D	2.8	52	48

[a] Titrimetric assay, pH 8.2, 25°. Substrate: 5 mM AcTyrEt; enzyme: 12.5 μg. [b] 100% activity = 1.05 μmoles of H^+ liberated/min. Specific activity = 84 μmoles of H^+ liberated/min per mg of enzyme.

Proteinase Inhibition. Digestion of denatured hemoglobin by either trypsin or chymotrypsin was inhibited by ovoinhibitors A, C, and D. B and E were not tested.

Inhibition of Other Proteolytic Enzymes. Ovoinhibitors A, C, and D all inhibited the hydrolysis of AcTyrEt by Nagarse (Table III). Again, B and E were not tested. A, C, and D inhibited the esterase activities of Pronase, a mixture of proteinases and peptidases purified from *S. griseus*. Much less ovoinhibitor was required for complete inhibition of AcTyrEt hydrolysis by "chymotrypsin-like" enzymes present in Pronase than for complete inhibition of *p*-toluenesulfonyl-L-arginine methyl ester hydrolysis by "trypsin-like" enzymes present (Table IV). Papain was not inhibited by any of the highly purified ovoinhibitors; therefore, the preparations were free of the ficin and papain inhibitor found in egg white by Fossum and Whitaker (1968).

Inhibitor Constants. Inhibitor constants, K_i, were calculated assuming pure competitive or pure noncompetitive inhibition and no interaction between the binding of the first and second moles of enzyme. Approximate limits for K_i for three ovoinhibitors, calculated for a 1:1 complex (Table V), show no difference in affinity for either trypsin or chymotrypsin.

TABLE IV: Inhibition of Esterase Activities of Pronase by Ovoinhibitors.

Substrate	Enzyme (μg)	Ovoinhibitor	Amt (μg)	Residual Enzyme Act. (%)	Inhibn (%)
AcTyrEt (5 mM)	100	None		100	0
	100	A	13	0	100
	100	A	25	0	100
	100	C	12	0	100
	100	C	30	0	100
	100	D	26	10	90
p-Toluenesulfonyl-L-arginine methyl ester (6.5 mM)	50	None		100	0
	50	A	55	15	85
	50	C	40	28	72
	50	D	33	23	77

TABLE V: Inhibitor Constants for Ovoinhibitors.[a]

Enzyme	Substrate	Ovoinhibitor	K_i (M × 10^8) Competitive Inhibn	K_i (M × 10^8) Noncompetitive Inhibn
Chymotrypsin	AcTyrEt	A	1.2	4.8
		C	1.0	4.3
		D	0.96	4.2
Trypsin	BzArgMe	A	1.6	5.0
		C	1.5	4.8
		D	1.4	4.4

[a] Calculated for 1:1 binding and pure competitive or noncompetitive inhibition to simplify calculations. Lineweaver–Burk plots appear intermediate between competitive and noncompetitive inhibition.

Lineweaver–Burk (1934) plots obtained with one ovoinhibitor showed a mixed or intermediate type of inhibition; however, see Green (1953) and Laskowski and Laskowski (1954). The values shown in Table V are therefore approximate, and the assumption that the apparent K_i is the same as that for a simple 1:1 complex may be an oversimplification. Comparison of Figure 5 with inhibition curves for ovomucoid and soybean trypsin inhibitor (Green, 1953) suggests that these values for ovoinhibitor are reasonable estimates.

Physical Characterization. Purified ovoinhibitor sedimented as a single boundary (Figure 6). In 0.01 M phosphate at pH 6.5, $s_{20,w} = 3.31 \pm 0.03$ S. Plots of ln A_{280} *vs.* x^2 for low-speed, short-column sedimentation equilibrium in H_2O and in D_2O (Edelstein and Schachman, 1967) were linear (Figure 7). From the data given in the legend to Figure 7, $\bar{v}$ was calculated to be 0.707 ± 0.011 and the molecular weight, 48,700 ± 2500. Most of the uncertainty in the calculation of the molecular weight was produced by the uncertainty in $\bar{v}$. Calculation of $\bar{v}$ from the amino acid and carbohydrate composition by using the tables of Schachman (1957) and Gibbons (1966) gave a $\bar{v}$ of 0.712 for those ovoinhibitors containing the minimum amount of carbohydrate, 0.708 for those containing the maximum amount of carbohydrate.

In 6 M guanidinium chloride plus 0.002 M dithiothreitol (pH 8), a plot of ln A_{280} *vs.* x^2 for the mixture of ovoinhibitors was slightly concave upward, suggestive of heterogeneity. The value of $\bar{v}$ in this solvent was assumed to be 0.02 less than in water (Hade and Tanford, 1967). With this assumption, the weight-average molecular weight of ovoinhibitor was calculated to be 48,600 in this solvent.

The absorptivity of the ovoinhibitors at 278 mμ (λ_{max}) was determined to be $E^{1\%}_{1cm}$ 6.5 to 6.9.

Discussion

It is apparent from the starch gel electrophoresis pattern shown by Tomimatsu *et al.* (1966) and from the results of disc electrophoresis (Figure 3) and ion-exchange chromatography (Figure 1) that chicken ovoinhibitor is heterogeneous with respect to net charge. The differences in charge among the five ovoinhibitor fractions do not appear to be due to differences in amino acid composition, although the results shown in Table I are not sufficiently precise to completely preclude this. It is possible that the different carbohydrate contents of the fractions indicate differing numbers of carbohydrate side chains. If it is assumed that different numbers of these side chains are attached

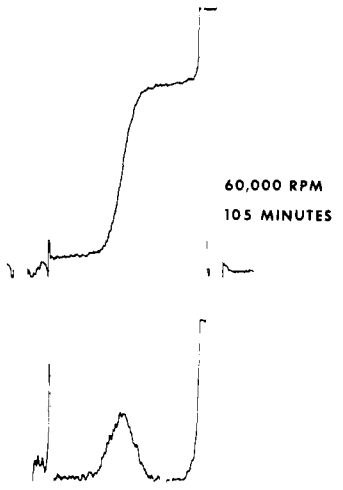

FIGURE 6: Sedimentation velocity of ovoinhibitor C in 0.01 M phosphate (pH 6.5). Ovoinhibitor concentration, 0.54 mg/ml; temperature, 24.2°. Scan at 280 mμ taken after 105 min at 60,000 rpm.

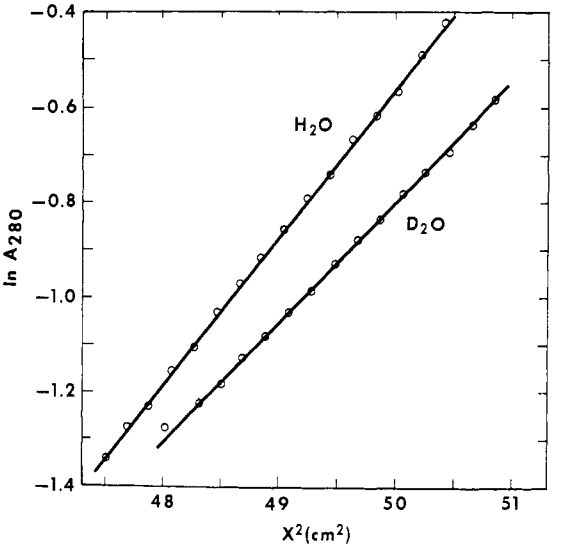

FIGURE 7: Sedimentation equilibrium of ovoinhibitor C in H_2O and in D_2O at 21° and 10,000 rpm. Density of solvents (containing 0.1 M KCl–1.5% sucrose): H_2O solvent, 1.0056; D_2O solvent (89.7% D_2O), 1.1022. Slope of line for H_2O: 0.312 ± 0.003; for D_2O, 0.253 ± 0.001. Errors stated are standard deviations of the slope determined for four and three replicate scans, respectively; 12-mm cells; ovoinhibitor concentration 0.48 mg/ml.

to the protein portion of ovoinhibitor through the β-carboxyl group of aspartic acid residues, the observed charge differences would result from differences in number of free carboxyl groups. This mode of attachment of carbohydrate side chains has been demonstrated for the chicken egg-white proteins: ovalbumin (Neuberger and Marshall, 1966), ovomucoid (Melamed, 1966), and conalbumin (Williams, 1968).

Comparison of Figures 1 and 3 indicates that the charge differences do not have the same effect on electrophoretic mobility as they do on the elution from DEAE-cellulose. Fractions A and B, for instance, are well separated on elution but are barely distinguishable on disc electrophoresis. The same also appears true of fractions D and E, although the elution separation is not so clear because of the small amount of E present. Since charge configuration would affect elution behavior under elution conditions which approach maximum resolution more than it would affect electrophoretic mobility (Sober and Peterson, 1960), the heterogeneity of ovoinhibitor is due to differences in both number and location of carbohydrate side chains.

The attainment of single symmetrical peaks by rechromatography of fractions such as those discussed here is sometimes considered to be evidence of homogeneity. The central portions of the five ovoinhibitor fractions (Figure 1) were rechromatographed until single symmetrical peaks were obtained. When two of these (A and D) were subdivided and again rechromatographed, it was evident that they were not homogeneous (Figure 2). It is not clear whether the inhomogeneity is due to differences in number or location of charges or simply to cross-contamination between fractions. It is clear, however, that the attainment of a single symmetrical peak does not denote homogeneity.

Binding of either trypsin or chymotrypsin by unfractionated ovoinhibitor does not interfere with the binding of the other enzyme; hence the binding sites are independent of each other (Tomimatsu *et al.*, 1966).

When it was shown that ovoinhibitor consisted of several components (Tomimatsu *et al.*, 1966), two possibilities arose. (1) The ovoinhibitor mixture might consist of proteins with the same inhibitory activity toward both enzymes (but with slightly different net charge); (2) the separate inhibitors were specific for either trypsin or chymotrypsin. Since separated ovoinhibitors do not differ appreciably in their specificity or in their complexing ratio with bovine trypsin or chymotrypsin (Tables II–IV), binding sites for trypsin and chymotrypsin are at different loci on the *same* molecule.

Since the ovoinhibitor molecule can bind two molecules of trypsin and two molecules of chymotrypsin (see also Feeney *et al.*, 1963a; Tomimatsu *et al.*, 1966), it might consist of two subunits. However, the average molecular weight obtained from sedimentation equilibrium measurements in a denaturing, reducing solvent was essentially the same as that obtained for the native molecule. This suggests that the molecule consists of one polypeptide chain which has more than one binding site for each enzyme.

The molecular weight of the protein moiety calculated from the composition is 44,000 g/mole with 800–1800 g of hexose/mole and 1500–3000 g of glucosamine/mole. The total molecular weight of approximately 46,000–49,000 g/mole agrees closely with the value obtained by sedimentation equilibrium. Recovery of nitrogen, determined by comparing nitrogen from amino acid analyses with micro-Kjeldahl determinations on the hydrolysates used for amino acid analysis, was between 96.6 and 99.8%, except for one sample. The ovoinhibitors contain 16.3–16.8% (w/w) nitrogen.

Previous work (Matsushima, 1958a; Rhodes *et al.*,

1960; Ryan and Clary, 1964; Ryan *et al.*, 1965; Tomimatsu *et al.*, 1966; Haynes and Feeney, 1967) has shown that chicken ovoinhibitor is comparatively nonspecific for proteolytic enzymes, as are some ovomucoids. Chicken ovomucoid, on the other hand, is specific for trypsin (Feeney *et al.*, 1963a). Furthermore, binding affinities of chicken ovoinhibitors for bovine trypsin and chymotrypsin are lower than affinities of chicken ovomucoid and soybean trypsin inhibitor for trypsin (Green and Neurath, 1954; Lebowitz and Laskowski, 1962). This is indicated by (1) higher K_i values with ovoinhibitor, (2) slower attainment of equilibrium between ovoinhibitor and enzyme, and (3) greater deviation of the inhibition curve for ovoinhibitor from linearity at high inhibitor:enzyme ratios (Laskowski and Laskowski, 1954).

Several naturally occurring proteinase inhibitors exist in a number of different forms, which can be distinguished electrophoretically (Laskowski and Laskowski, 1954). In the case of soybean trypsin inhibitor (Eldridge *et al.*, 1966; Rackis and Anderson, 1964; Frattali and Steiner, 1968) and lima bean trypsin inhibitor (Jones *et al.*, 1963; Haynes and Feeney, 1967), different forms may have both different chemical composition and inhibitory specificity. Chicken ovoinhibitor consists of glycoproteins which differ in carbohydrate content and probably not in amino acid composition but have the same specificity. Correspondence of the bands in electrophoretic patterns of ovoinhibitors separated by DEAE-cellulose chromatography with the bands of the unfractionated material supports the hypothesis that the ovoinhibitors are not artifacts of an associating system.

The inhibition of *microbial* proteases may be the natural function of ovoinhibitors. If this is so then the animal proteases, trypsin and chymotrypsin, may be inhibited only incidentally, because they are similar in structure to some microbial proteases. For example, chymotrypsin and subtilisin apparently compete for the same sites on ovoinhibitor (Tomimatsu *et al.*, 1966). In order to utilize egg-white proteins as a source of nutrient amino acids, microorganisms would have to degrade egg proteins by means of proteases secreted into the surrounding medium. Breakdown of egg protein and multiplication of the invading organisms would in turn result in spoilage of the egg.

Added in Proof

The tryptophan content of the mixture of ovoinhibitors was also determined by the method of Spies and Chambers (1949). Only 0.2–0.4 mole of tryptophan/mole of inhibitor was calculated from the absorption at 600 mμ, but the spectrum did not show a pronounced peak between 550 and 650 mμ because of background absorption.

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Nuclear Magnetic Resonance Studies of the Structure and Binding Sites of Enzymes. VII. Solvent and Temperature Effects on the Ionization of Histidine Residues of Ribonuclease*

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ABSTRACT: The chemical shift of the nuclear magnetic resonance absorption of the C_2 hydrogen of histidine is a measure of the protonation of the imidazole ring. By this means the titration curves of the four histidine residues of ribonuclease have been obtained under a variety of conditions. Comparisons are made between the titration curves obtained in H_2O or D_2O and 0.2 M sodium acetate or 0.2 M sodium chloride, and thermodynamic data are reported for the ionization of histidine residues 12, 119, and 105. Evidence for an isomerization of the enzyme involving histidine residue 48 is discussed.

The resolution of the nuclear magnetic resonance absorptions of the imidazole C_2 hydrogens of the four histidine residues in RNase[1] (Meadows *et al.*, 1967) and their assignment to specific residues in the amino acid sequence of the enzyme (Meadows *et al.*, 1968) have been reported. These histidine C_2–H peaks shift position as a function of pH, reflecting the protonation state of each imidazole ring (Meadows *et al.*, 1967, 1968). Thus, it is possible for the first time to determine the microscopic hydrogen ion dissociation constants of individual amino acids in a protein and to study the effects of solvent and temperature upon them.

Materials and Methods

Lyophilized, phosphate-free RNase (Worthington) was used without further treatment. Solutions of 0.2 M sodium chloride or sodium acetate were made up in 99.85% D_2O (Bio-Rad) unless otherwise stated, using NaCl (Merck reagent grade) or CD_3COOD and NaOD (Merck of Canada). H_2O solutions were made up in distilled, deionized water. RNase was dissolved in these solutions to give a final concentration of 0.0065 M

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[1] Abbreviation used: RNase, bovine pancreatic ribonuclease A (EC 2.7.7.16).