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Characterization of an Avian Protease Inhibitor

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Research Article

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ABSTRACT

Background: Ovomucoid is a serine proteinase inhibitor in the egg whites of all avian species at a concentration of about 10 mg/ml. The involvement of proteinases in a multitude of control functions in an organism has created an interest in their physiological inhibitors. Regulation of proteolytic activity in tissues is a critical requirement in the maintenance of homeostasis. Egg white proteins possess ACE-inhibitory activity, & also high radical-scavenging activity. The combined antioxidant and ACE-inhibitory properties of egg white hydrolysates, or the corresponding peptides, would make a useful multifunctional preparation for the control of cardiovascular diseases. Proteases play key roles in several physiological processes, including intracellular protein degradation, bone remodeling, and antigen presentation, and their activities are increased in pathophysiological conditions such as cancer metastasis and inflammation. They are also required for invasion by microorganism. Four protease inhibitors have been identified in egg white: cystatin, ovomucoid, ovomacroglobulin (also known as ovostatin), and ovoinhibitor. Use of proteinase inhibitors in the treatment of certain diseases has renewed interest in their specificity and stability, both of which in turn depend on the tertiary structure of the inhibitor. Structural alteration to obtain molecules of desired properties requires knowledge of relationship between structure, function and stability.
Aims: In view of its importance, in the present study duck ovomucoid was isolated and characterized for its physicochemical properties.
Methodology: Duck ovomucoid was isolated and characterized for its physicochemical properties. Analytical gel filtration (Sephacryl S-100 HR column) was used for purification, determination of molecular weight (MW), carbohydrate content and Stokes radius.

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Results & Conclusion: The fluorescence emission spectrum was 302 nm, comparable to that reported earlier. Stokes radius was found to be 2.91nm the value was comparable with white leghorn hen (stokes' radius 3.15nm). The extraordinary large value of stokes radius can be attributed to its high carbohydrate content which increases the hydration of the molecule. The inhibitor had the molecular weight of 29,300 and the carbohydrate content was 22%, the specific extinction coefficient of duck ovomucoid was found to be 5.82 at 279 nm and the stokes radius was 2.91nm.

Keywords: Avian proteinase inhibitor; ovomucoid; physicochemical properties;

1. INTRODUCTION

Ovomucoid is a serine proteinase inhibitor in the egg whites of all avian species at a concentration of about 10 mg/ml which accounts for 10% of the protein produced by the tubular gland cells of the oviduct in laying birds (Lineweaver and Murray, 1947; Palmiter, 1972; Davis et al., 1971; Leach et al., 1980; Melamed, 1967; Beeley, 1971; Katob et al., 1987). The involvement of proteinases in a multitude of control functions in an organism has created an interest in their physiological inhibitors. Regulation of proteolytic activity in tissues is a critical requirement in the maintenance of homeostasis (Mine 2002; Brady et al., 2003; Li and Nakai 1989; Davalos et al., 2004; Fujita et al., 1995; Matoba et al., 1999). Egg white proteins possess ACE-inhibitory activity and also high radical-scavenging activity. The combined antioxidant and ACE-inhibitory properties of egg white hydrolysates, or the corresponding peptides, would make a useful multifunctional preparation for the control of cardiovascular diseases (Fujita et al., 1995; Matoba et al., 2001; Yamada et al., 2002; Yoshikawa and Fujita, 1994; Davalos et al., 2004; Ames et al., 1993; Majumder and Jianping, 2009). Egg proteins are rich source of biologically active peptides. Besides having protease inhibitory activity, several other biological activities are associated with egg components, including novel antimicrobial activities, anti adhesive properties, immunomodulatory, anticancer, antioxidant, and antihypertensive properties (Yoshinori and Marie, 2008; Marie et al., 2009; Jennifer et al., 2005; Li and Nakai, 1989; Abrahamson et al., 2003). Proteases play key roles in several physiological processes, including intracellular protein degradation, bone remodeling, and antigen presentation, and their activities are increased in pathophysiological conditions such as cancer metastasis and inflammation. They are also required for invasion by microorganism. Four protease inhibitors have been identified in egg white: cystatin, ovomucoid, ovomacroglobulin (also known as ovostatin), and ovoinhibitor (Kato et al., 1987; Shah and Khan, 2004; Agarwal et al., 2000). Ovomucoid- a serine proteinase inhibitor has been found to be useful for oral delivery of protein/peptide therapeutics (Agarwal et al., 2001; Agarwal et al., 2001; Hilpert et al., 2003; Davis,1964; Adams et al., 1970; Laemelli, 1970).

In view of its importance, duck ovomucoid was isolated and characterized for its physico chemical properties.

2. MATERIALS AND METHODS

Column Chromatographic Media: Sephacryl S-100 HR and blue dextran-2000 were obtain from sigma chemical company. ovalbumin, carbonic anhydrase, trypsinogen, trypsin, B-

lactoglobulin, lysozyme, ribonuclease A and cytochrome **C** were also purchased from Sigma chemical Company USA.

2.1 Polyacrylamide Gel Electrophoresis

Vertical slab gel electrophoresis was performed (Davis, 1964) except that instead of tubes glass plates were used as gel support (Adams, 1970) using non dissociating discontinuous buffer systems.

The method of Laemmelli (1970) was used for performing electrophoresis using dissociating buffer system.

2.2 Gel-Filtration

Sephacryl S-100HR column (1.938x1.83.4cm) was used for the purification and estimation of molecular weight and stokes' radius of ovomucoid Sephacryl S-100HR column was calibrated by elution of marker proteins of known hydrodynamic properties. Fractions eluted were monitored by at 280 nm.

The equation given below was employed for the estimation of molecular weights of the sample proteins:

$V_e/V_o = m \log M + c$ (Whittaker 1963) (1)

Where V_e and V_o are the elution and void volume of the column respectively, m is the slope, c the intercept and M is the molecular weight.

The value of K_d and K_{av} (distribution coefficient and available distribution coefficient were calculated from the equation:

$$K_d = (V_e-V_o) / V_i \quad (2)$$

$$K_{av} = (V_e-V_o)/ (V_t-V_o) \quad (3)$$

Where V_t is the total volume of the column used for the calculation of Stokes' radii of the sample proteins employing the method of Laurent and Killander (1964) and Ackers (1967).

$$(-\log K_{av})^{1/2}=A) + rB \quad (4)$$

$$r = a_o + b_o \, \text{erfc}^{-1} K^d \quad (5)$$

where a, b, a_o and b_o are the constants, r is the stokes's radius and erfc^{-1} is the inverse error function complement of the distribution coefficient.

2.3 Fluorescence Spectroscopy

Emission spectra of the proteins were obtained in 0.06 M sodium phosphate buffer, pH 7.0, in the wavelength range 300-400 nm using scan speed of 240 nm per minute and a band pass width of 10 nm. The wavelength of maximum excitation was determined by prescanning the samples in the wavelength range of 200-500 nm.

2.4 Determination of Neutral Hexose Content

The neutral hexose content of protein was preparations was estimated by the method of Dubois (41) The hexose content of the protein was determined by comparing the absorbance of the sample with the standard plot for glucose (varying the amount of glucose between 0.1 and 1.0 mg) obeying the straight line equation:

$$Y=0.979x + 0.016 \quad (6)$$

2.5 UV-absorption Spectroscopy and Determination Specific Extinction Coefficient

UV absorption spectrum of the protein in 0.06 M sodium phosphate buffer, pH 7,0, was taken to determine the wavelength of maximum absorption. The absorbance of varying amounts of the protein at this wavelength was plotted against protein concentrations (gms/100ml) obtained by Lowry's method. The slope of this plot is equal to the specific extinction coefficient of the protein in the given solvent.

3. RESULTS AND DISCUSSION

Ovomucoid was isolated from duck egg white using Sephacryl S-100 HR column (Figure 1). The antitryptic and antichymotryptic activity was checked. The eluted protein showed specific inhibition of 39.76 per mg against trypsin and 20.14 per mg inhibitory protein against chymotrypsin.

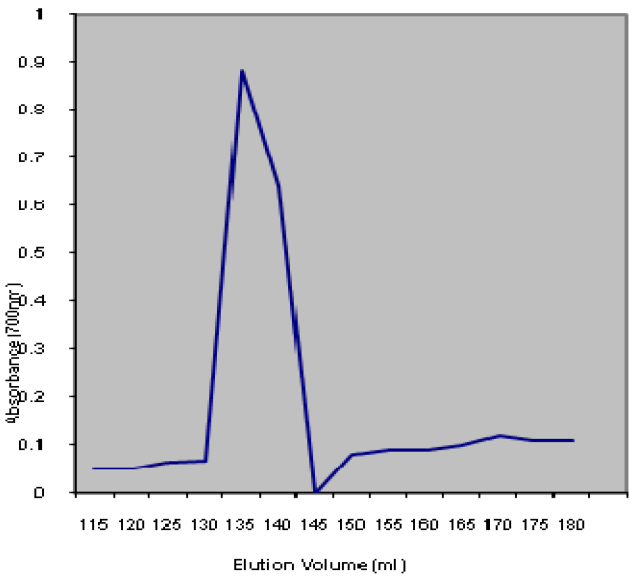

Fig. 1. Elution profile of crude duck ovomucoid on Sephacryl S 200 HR column

Size homogeneity of ovomucoid was obtained by rechromatography on the same column. This showed single symmetrical peak (Figure 2).

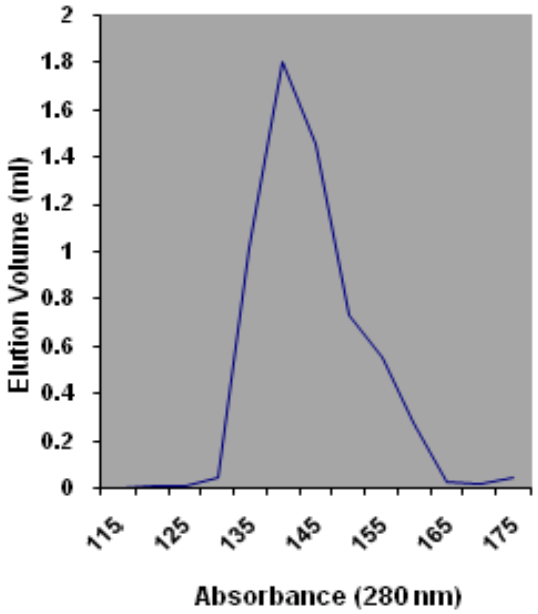

Fig. 2. Elution Profile of gel filtered ovomucoid on Sepahcryl S 100 HR clolumn

The fluorescence emission maximum was obtained at 302 nm. The determination of stoichiometry of binding of the inhibitor with the serine proteinases trypsin and chymotrypsin showed two reactive sites for trypsin and one for chymotrypsin (Figure 3 (A) and (B)).

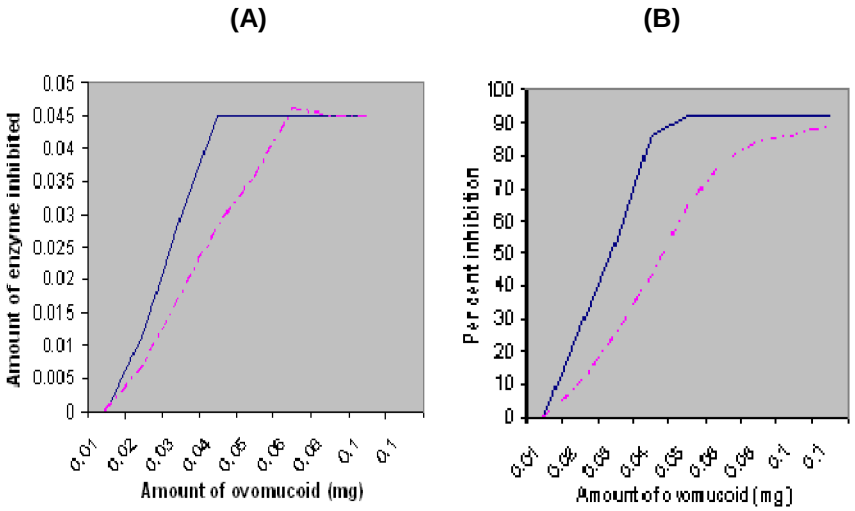

Fig. 3 (A) and (B). Amount of enzyme trypsin (solid line) and chymotrypsin (broken pink line) inhibited by increasing amount of duck ovomucoid

The Rm values of marker proteins when plotted against the molecular weights given in Table I. and fits (figure. 4) the following straight line equation:

Log M = -**R**m + 4.955 (7)

Table 1. Estimation of Molecular weight of duck ovomucoid by SDS- PAGE

Property	Mol. Wt.	Log M	Rm
BSA(monomer)	66000	4.82	0.177
ovalbumin	45000	4.65	0.294
Carbonic anhydrase	29000	4.46	0.480
Trypsinogen	24200	4.38	0.520
Trypsin	24000	4.38	0.549
Beta- lactoglobulin	18400	4.26	0.745
Lysozyme	14300	4.16	0.796
Ribonuclease A	13700	4.17	0.823
Cytochrome c	12300	4.09	0.851
Ovomucoid			0.486

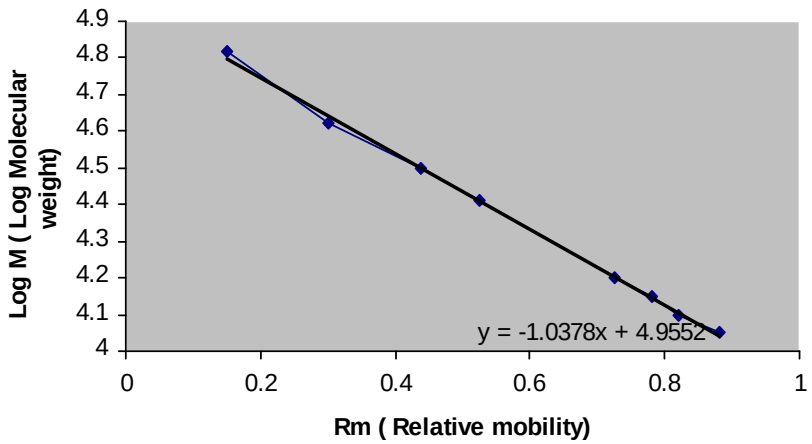

Fig. 4. Plot of Rm versus Log M of marker proteins performed according to Laemmelli (1970)

From the above equation the molecular weight of ovomucoid was found to be 29, 000. The analytical gel filtration data was analysed according to the method of Laurent and Killander (1964) and Ackers (1967) to fit the following straight line equations (Table 2).

$(-\log K_{av})^{1/2} = 0.298 + 0.159\ r$ (8)

$r = 0.0205 + 3.9129\ \text{erfc}^{-1}\ Kd$ (9)

Table 2. Treatment of gel filtration data according to the method of Laurent and Killander (1964) and Ackers (1967)

Proteins	Stokes radius (nm)	V_e (ml)	K_{av}	$(-\log k_{av})^{1/2}$	k_d	$Erfc^{-1}K_d$
BSA	3.55	129.5	0.168	0.880	0.191	0.925
Ovalbumin	2.73	150	0.314	0.709	0.358	0.652
α-chymotrpysinogen	2.24	160	0.386	0.643	0.439	0.543
Ribonuclease A	1.92	164.5	0.418	0.616	0.476	0.499
Cytochrome c	1.64	171	0.464	0.577	0.529	0.447
Ovomucoid		142	0.261	0.764	0.297	0.730

The calibration plot is shown in Figure 5. A mean value of 2.91nm for stokes' radius of ovomucoid was determined.

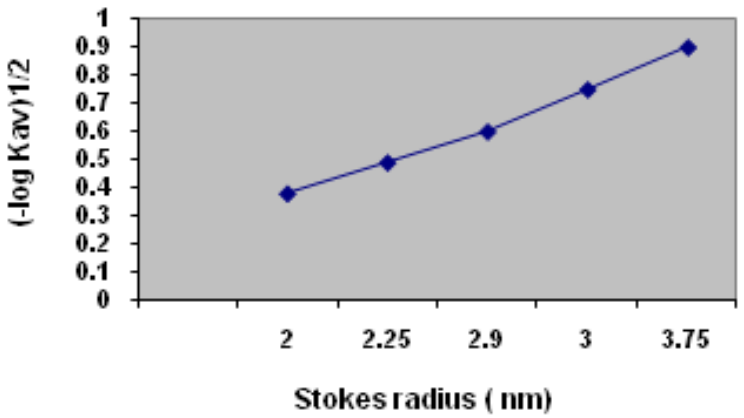

Fig. 5.Treatment of gel filtration data on Sephacryl S200 HR according to the methods of Laurent and Kilander (1964) and Ackers (1967)

Neutral hexose content of isolated ovomucoid was found to be 22% as determined by the method of Dubois et al. (1956). The ultraviolet spectrum of ovomucoid showed single peak at 279 nm (Figure 6).

The specific extinction coefficient of ovomucoid was calculated to be 5.82. The different physicochemical properties of duck ovomucoid as determined in this study are listed in Table 3.

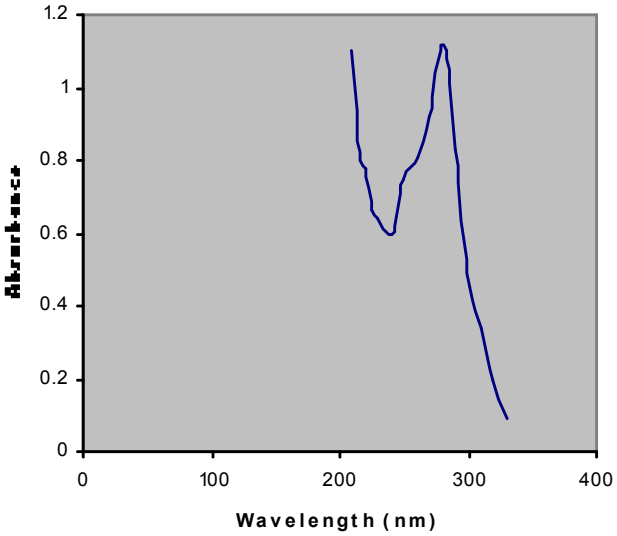

Fig. 6. UV absorption spectra of duck ovomucoid in .06 M sodium phosphate buffer, pH 7.0

Table 3. Physicochemical properties of duck ovomucoid determined in this study

Property	Value obtained in this study	Reported for avian ovomucoid
Molecular Weight by SDS-PAGE	29,300	27000-32600[a.b]
Stokes radius	2.91 nm	3.15 nm[c]
Carbohydrate content	22.2%	25-30% [d]
$E_{1cm}^{1\%}$	5.818 (at 279 nm)	4.13-6.1 (at 280 nm)[a,b,c]
E_{max}	302 nm	306 nm[b]

a. Lineweaver and Murray (1947), Leach et al., 1980; b. Davis e. al., (1971)
c. Waheed and Salahuudin (1975) for chicken ovomucoid; d. Melamed (1967)

4. CONCLUSION

The values of molecular weight (29, 300) determined by SDS –PAGE and neutral hexose content (22%) were found to lie in the range of 27,000- 32, 000 and 20-25%, respectively, reported earlier for avian ovomucoids (Lineweaver and Murray, 1947; Leachet et al., 1980; Melamed, 1967; Waheed and Salahuddin, 1975). However exact molecular weight may be less than 29,300 as reported in this study since glycoproteins show anomalous behavior on SDS-PAGE.

Stokes' radius of 2.91 nm as determined by gel filtration was found to be comparable to the value of 3.15 nm for ovomucoid from leghorn hen (Waheed and Salahuudin, 1975). The extraordinary large value of stokes' radius of duck ovomucoid can be attributed to its high

carbohydrate content which increases the hydration of the molecule resulting in large stokes' radius. The specific extinction coefficient of duck ovomucoid was found to be 5.82 at 279 nm which again lies in the range reported for avian ovomucoids, i.e., 4.13 – 6.1 at 280 nm (Davis et al., 1971, Melamed, 1966; Waheed and Salahuddin, 1975).

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