

## **Inter-protein bonding and other molecular interactions in hen egg white**

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The analysis of the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) data, combined with the comparison of viscosity data corresponding to the thin fraction of hen egg white and 2 mM purified ovalbumin solution, showed that the thin fraction is a protein solution. Dissolution of the thick fraction of hen egg white in SDS and urea solutions followed by SDS-PAGE, in the presence or absence of  $\beta$ -mercaptoethanol, revealed that it is a protein gel. It was also found that the gel structure consists of the three-dimensional ovomucine-ovomucoid network (matrix) held together by S-S bridges which captures the rest of the proteins (ovalbumin, conalbumin, *etc*). The captured proteins can also be interconnected by S-S bridges thus forming agglomerates or conglomerates, but they are predominantly held by weak electrostatic interactions, as demonstrated by the washing out and dissolving experiments. The matrix structure does not prevent denaturation of the captured proteins as indicated by the 50% decrease in turbidity following gel swelling by the addition of 1 part of an 8 M urea solution to 9 parts of the gel.

**Keywords:** protein, gel, disulfide bond, bound water, denaturation.

### INTRODUCTION

A hen egg is one of the most extensively investigated biological objects; the pioneering work in biochemistry of embryo development was performed using this inexpensive, easy-to-handle, and in many other ways convenient system<sup>1</sup>. Hen egg consists of an outer and inner thin albumen separated by a firm (thick) albumen, of a chelazae and a chelaziferous layer around the yolk, of yolk and egg shell. From the point of view of protein chemistry, hen albumen, *i.e.*, egg white, possesses unique properties. It was proclaimed to be a thixotropic material, but no explanation was given concerning the molecular forces that would be responsible for such a feature<sup>2</sup>. In an earlier study, we showed that a hen egg white is one order of magnitude more resistant to radiation induced structural damages, compared to an aqueous solution of its major purified constituent, ovalbumin<sup>3</sup>. Also, scissoring of egg white proteins

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was observed in the absence of oxygen, in contrast to the radiolysis of purified ovalbumin <sup>4</sup>. In order to explain completely the observed phenomenon, it is necessary to gain more insight into the protein conformation, inter-protein and water-protein interactions of egg white.

In this paper, the results of our investigation on the nature of the chemical bonds between the protein molecules of hen egg white, as well as on water-protein interactions, are presented. Precise knowledge on water-protein interactions was necessary for the understanding of the mechanisms of egg white radiolysis. Namely, egg white is comprised of 88% of water so that the majority of the radiolytic damage to the proteins occurs indirectly, *i.e.*, through the reactions with free radicals generated in the radiolysis of water. The complete understanding of the mechanism of radiation induced egg white damage would also enable insight into the radiation induced damage of similar proteinaceous structures, *e.g.*, various extracellular matrixes of the human body.

#### EXPERIMENTAL

The thin and the thick fraction of egg white were separated by filtration on a Buchner funnel. The thin fraction samples were boiled for 2 min at 100 °C in an equal volume of 125 mM Tris-HCl (pH 6.8) buffer containing 2% SDS, either with or without 5%  $\beta$ -mercaptoethanol, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli <sup>5</sup>. The protein bands were visualized by Coomassie Brilliant Blue staining and scanned using an UltroScan XL scanning densitometer. The gel scans were processed using PC UltroScan and Microcal Origin 4.0 software. The thick fraction samples were carefully placed in laboratory glasses containing an equal mass of (a) distilled water, (b) 8 M urea solution in water, (c) 2% SDS solution in water, (d) both agents concomitantly. The mixtures were incubated for 3 hours at room temperature and then heated for 5 minutes at 80 °C. The protein coagula were separated by filtration and the proteins dissolved in the filtrates were analyzed by SDS-PAGE, either with or without 5%  $\beta$ -mercaptoethanol in the sample buffer. The viscosities of the albumen samples and of a 2 mM purified ovalbumin (Sigma, purity grade V) solution were measured at 20.0 °C using an Ostwald viscometer.

#### RESULTS AND DISCUSSION

In order to gain more insight into the protein interactions of the thin fraction of egg white, SDS-PAGE analysis of the samples that had been previously boiled in the denaturing buffer, both with and without 5%  $\beta$ -mercaptoethanol, was performed and the resulting electrophoretograms are compared in Fig. 1.

On the electrophoretogram corresponding to the experiment performed with 5%  $\beta$ -mercaptoethanol, it can be observed that the thin fraction consists of several proteins whose mass % (*i.e.*, mass of specific protein vs. total protein mass) is given in Table I.

From the data presented in Table I marked differences in mass % of the major protein constituents of both albumen fractions obtained by SDS-PAGE analysis compared to the values reported by Romanoff <sup>1</sup> may be noticed. However, their results were obtained on the albumen as a whole and, consequently, reflects the

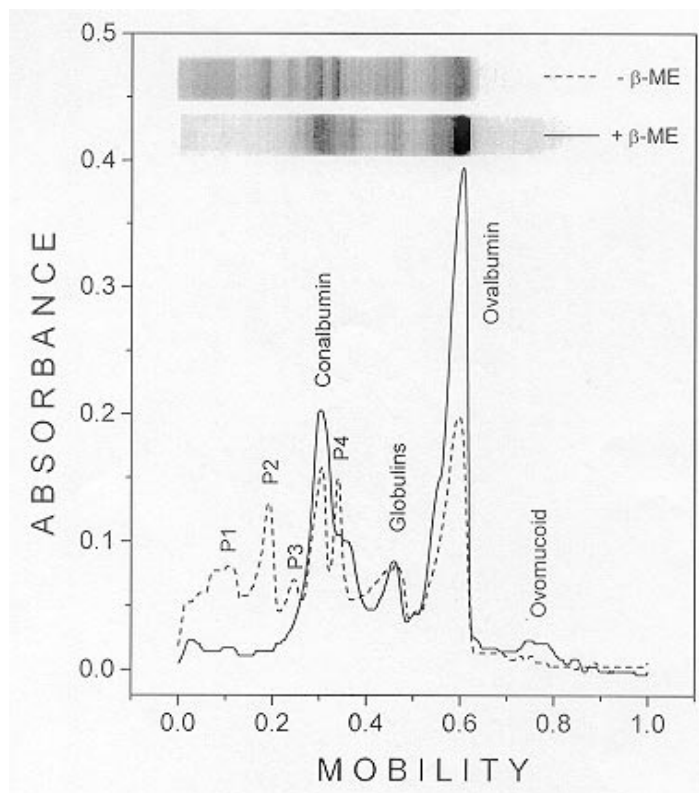


Fig 1. The thin fraction of hen egg white; electrophoretograms of SDS-PAGE performed with and without 5%  $\beta$ -mercaptoethanol and the corresponding densitograms.

average mass % distribution of proteins of the mixture of the thick and thin fraction. Nevertheless, this can hardly be the complete explanation for the observed discrepancies, as the SDS-PAGE values for conalbumin in both the thin and thick fractions are 2–3 times higher, the values for ovalbumin are 26% and 9% lower for the thin and thick fraction, respectively, and the values for ovomucoid are more than 2 times lower compared to the reported values<sup>1</sup>. In addition, in all our experiments a protein of 60 kDa was observed, as one of the major albumen constituents. This protein was not previously reported by Romanoff. The discrepancies between the results could be explained by both individual and inbred genetic differences between the eggs. In all our experiment, 24 hour old eggs obtained from Brown Leghorn farm hens were used, while the exact breed of *Gallus gallus* was not previously specified<sup>1</sup>. The other explanation emerges from the different analytical technique used to obtain the mass % distribution of the albumen proteins. Namely, the data of Romanoff<sup>1</sup> are mainly based on their early work when the most powerful separation technique was paper chromatography. We believe that data obtained by the much more powerful separation technique of SDS-PAGE followed by an accurate scanning technique are more precise.

TABLE I. Molecular mass and mass % distribution of the major protein constituents of the thin and the thick fraction of egg albumen.

Protein	<i>Mr</i> (kDa)	mass % thin fraction	mass % thick fraction	mass % from reference <sup>1</sup>
Conalbumin	80	34.4 (29.8–37.9) – 12%	25.1 (19.1–29.8) – 22%	9.6,
Ovalbumin	44	50.2 (44.9–58.4) – 13.5	61.9 (52.7–69.6) – 13.5%	67.7,
Ovomucoid	28	3.6 (2.6–4.4) – 25%	4.9 (3.9–6.1) – 23%	9.9,
Globulins	60	8.1 (5.9–10.5) – 29%	7.3 (5.4–9.4) – 23%	n.d.

However, the electrophoretogram of the thin fraction obtained without 5%  $\beta$ -mercaptoethanol showed that conalbumin and ovalbumin are partially present in the form of agglomerates and conglomerates, as shown in Table II.

TABLE II. Molecular mass and mass % distribution of the major protein bands of the fraction obtained by SDS-PAGE without 5 %  $\beta$ -mercaptoethanol.

Protein	<i>Mr</i> (kDa)	mass % thin fraction	Possible constituents of agglomerate/conglomerate
Conalbumin (CON)	80	12.2,	
Ovalbumin (OVA)	44	26.1,	
Globulins (GLO)	60	15.3	2 · OVOMUCOID,
P-1	(112)	14.6	1 · CON + 1 · OVA,
P-2	(93)	12.6	1 · OVA + 1 · GLO,
P-3	(85)	5.3	2 · OVA
P-4	(71)	8.9	1 · OVA + 1 · OVOMUCOID

The only difference between these two experiments was the presence or absence of 5%  $\beta$ -mercaptoethanol, an agent that cleaves S–S bonds. Consequently, it was concluded that the protein agglomerates and conglomerates obtained in the absence of  $\beta$ -mercaptoethanol were bonded by the inter-protein S–S bridges. The cleavage of these S–S bridges in the presence of  $\beta$ -mercaptoethanol led to disaggregation of the protein agglomerates and conglomerates to their basic constituents.

The thin fraction of egg white could also be dissolved in 0.1 M Na/K phosphate buffer, which indicated that the protein agglomerates and conglomerates, as well as free protein monomers mutually interact only by weak interactions. Taken together with the molecular mass distribution, this observation suggested that the thin fraction of egg white is a true protein solution.

The assumption was further corroborated by viscosity measurements. Namely, the viscosity of the thin fraction is typically around 2 mPa s, while the viscosity of a 2 mM buffered solution of its major constituent ovalbumin (2 mM is the approximate protein concentration of albumen) is equal to 1.53 mPa s at 20.0 °C. The similarity of these two values proves that the thin albumen fraction is a protein

solution. The small difference in viscosity ( $\approx 30\%$ ) could be attributed to agglomeration and conglomeration of the thin albumen constituents. Namely, the previous polymer cross-linking experiments showed that the viscosity increased with increasing average molecular mass of the polymers, although the mass concentration of the polymers was constant<sup>6</sup>.

However, the viscosity of the thick fraction of albumen is significantly higher compared to the viscosity of a 2 mM ovalbumin solution. In fact, the viscosity of the thick fraction could not be measured with a capillary viscometer, because it does not even flow through a tube of 10 mm diameter. In addition to this, the thick fraction could not be dissolved in 0.1 M Na/K phosphate buffer and prolonged (3 h) incubation in the buffer caused only swelling. According to this observation, it might be concluded that the thick fraction is a protein gel. Figure 2 shows the result of SDS-PAGE analysis of the protein components of the thick fraction of albumen performed after thermal denaturation for 2 min at 100 °C in the presence of 5%  $\beta$ -mercaptoethanol, 125 mM Tris and 2% SDS.

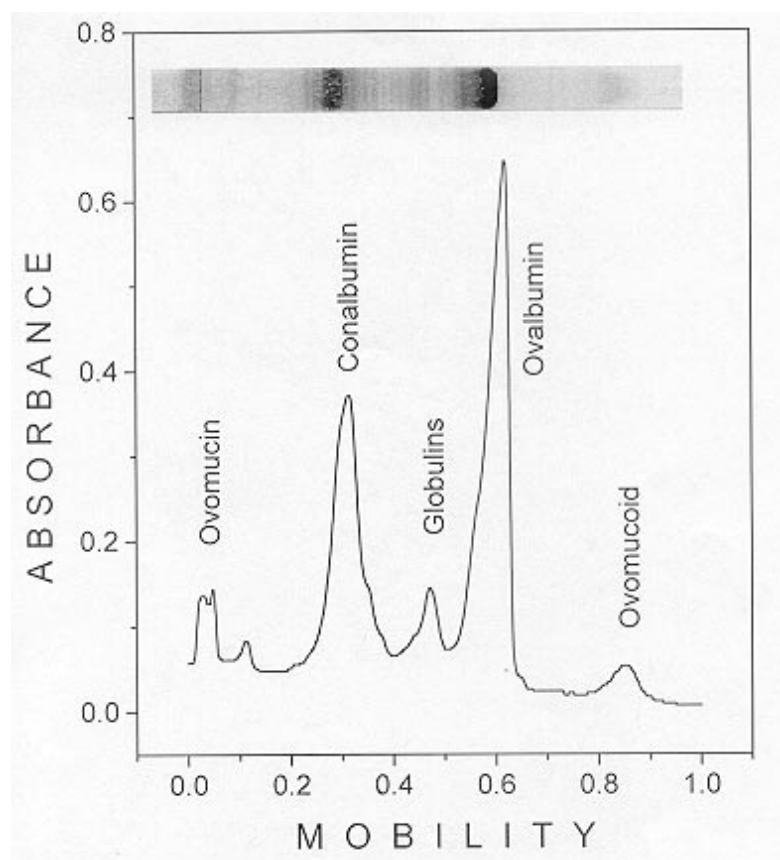


Fig 2. The thick fraction of hen egg white; electrophoretogram of SDS-PAGE performed with 5%  $\beta$ -mercaptoethanol and its densitogram.

The obtained protein profile is almost identical to the profile of the thin fraction analyzed in the presence of 5%  $\beta$ -mercaptoethanol, with exception of ovomucin which is a constituent of the thick fraction only. Namely, no protein band with molecular mass higher than ovomucin (*i.e.*, above 250 kDa) was observed, indicating that thermal denaturation in the presence of the chemical denaturants, led to the complete disruption of all bonds that hold the gelatinous structure of the thick fraction together. Taking into account the chemical characteristics of the proteins of which the gel is composed, it was concluded that they do not mutually interact either by strong ionic or by coordinative bonds. However, S–S bridges and weak electrostatic interactions remained the possible types of bonding holding the protein gel together.

In order to elucidate further the inter-protein bonding of thick albumen, dissolution experiments were performed. The experiments were designed on the bases of the following rationale: if the proteins inside the gel are predominantly held by polar bonds, such as H-bonds, the gel would be soluble in urea solutions; if the dominant type of interactions are nonpolar bonds, *i.e.*, van der Waals forces, then the gel would be soluble in SDS solutions; if the gel structure is held by both polar and nonpolar interactions, then the protein constituents would be soluble in solutions containing both urea and SDS; if S–S bridges participate in the formation of the gel, then  $\beta$ -mercaptoethanol must be added in order to dissolve the gel proteins.

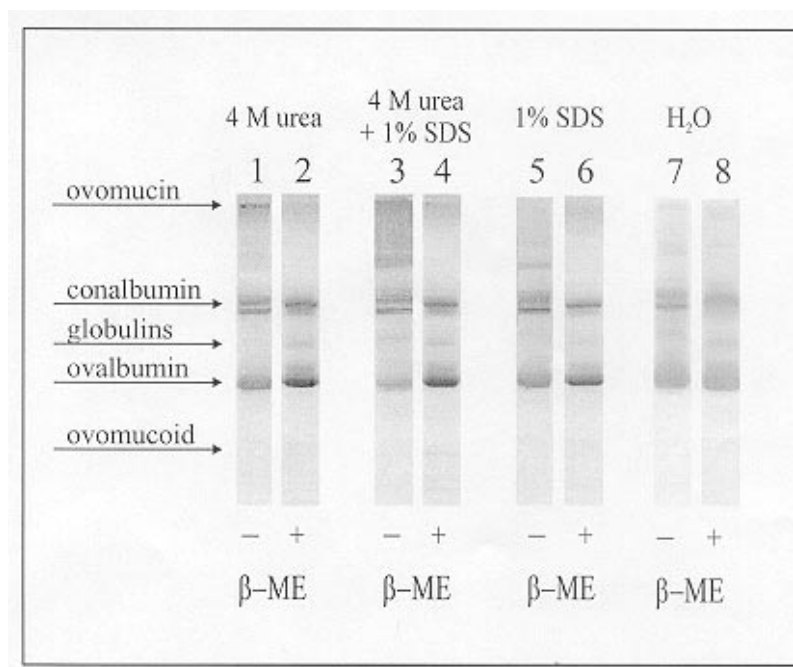


Fig. 3. Proteins washed out of the thick fraction of hen egg white by various solvents; chromatograms of SDS-PAGE performed with and without 5%  $\beta$ -mercaptoethanol, see text for details.

TABLE III

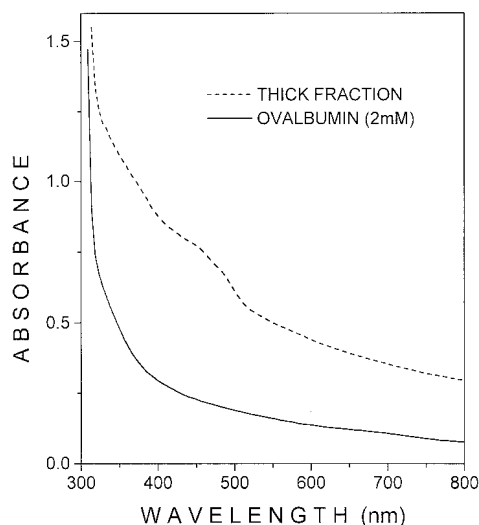


Fig 4. Optical absorption spectra of the thick fraction of egg white and 2 mM buffered solution of ovalbumin, grade V.

From the results of these experiments, presented in Fig. 3 and Table III, the following observations were made:

The thick fraction is neither dissolved nor swollen by water; the water phase remains well separated from the thick fraction and the thick fraction does not change its volume. The corresponding lanes (lanes 7 and 8) on the electrophoretogram (Fig. 3) show only traces of proteins from the thin fraction and a small amount of proteins from the thick fraction, mainly ovalbumin, conalbumin, as well as a small amount of higher molecular mass agglomerates and conglomerates, probably originating from the surface of the thick fraction.

Ovomucin and ovomucoid were not observed in these samples. The obtained result clearly indicates the low trans-gel mobility of particles of molecules whose Stock's radius exceeds approximately 10 nm. The observed feature of the thick fraction of egg white elucidates at least one part of the mechanism by which antiviral and bacterial protection of the chick embryo is achieved.

In the case when 1% SDS was used as the solvent, weak gel swelling was observed, but none of the proteins from the thick fraction were dissolved and found in the filtrate (Fig. 3, lanes 5 and 6). Only the remainders of the thin fraction and some lower molecular mass agglomerates and conglomerates were observed. The presence of small traces of ovomucin indicated that the gel swelled only partially and that its mechanical rupture was negligible under these conditions. The conglomerate of 100 kDa is predominantly hydrophobic as it dissolved better in the SDS solution than in the urea solution.

Further, the thick fraction gel swelled significantly but did not dissolve in 4 M urea solution. In this case, two times more protein agglomerates and conglomerate of higher molecular mass were extracted from the gel compared to the extraction with water (Fig. 3, lanes 1 and 2).



When 4 M urea and 1% SDS were concomitantly used as solvents, the matrix proteins of the thick fraction were not dissolved although the gel swelled significantly. However, the total quantity of extracted proteins from the gel was the greatest in this case (Fig. 3, lanes 3 and 4). As the solution of 4 M urea and 1 % SDS is an appropriate solvent for the proteins that have both hydrophobic and hydrophilic surface regions in the native conformation, this observation suggests the existence of hydrogen bondings and hydrophobic interactions among the captured proteins both mutually and with the matrix of the thick fraction.

However, for the complete desolution of the thick egg white fraction, the presence of 5%  $\beta$ -mercaptoethanol in the solvent is a prerequisite. Only such a solvent was capable of disrupting the matrix structure of the gel and releasing both its components and the captured proteins in the gel infrastructure. This demonstrates that interprotein S–S bridges are the main structural motif responsible for the jelly structure of the thick egg white.

On the basis of these results, it might be concluded that the thick fraction of albumen is a protein gel comprised of a three-dimensional ovomucin-ovomucoid network (matrix) that captures the other proteins.

The UV-VIS spectrum of the thick egg white fraction and the spectrum of a 2 mM purified ovalbumin solution are illustrated in Fig. 4. The spectrum of thick egg white showed an abrupt decrease in absorbance in the wavelength interval from 300 nm to 320 nm due to aromatic amino acids; in the interval from 400 to 500 nm a moderate absorption peak due to the coordinately bound  $\text{Fe}^{2+}$  of conalbumin may be observed. In the wavelength interval from 550 to 800 nm, in which chromophores are absent, a significantly higher turbidity (3.7 times) compared to the 2 mM ovalbumin solution can be seen. The size and shape of the molecules are the primary parameters that control the intensity of scattered light, *i.e.*, turbidity. From the results it might be concluded that the native proteins in the thick fraction of egg white are in different conformations to those in the purified form. However, it is not apparent which forces hold the proteins in their native conformations and how these interactions influence the protein mobility inside the gel. In order to elucidate one aspect of this subject we measured the UV-VIS spectrum of the thick egg white sample swelled for 2 h by addition of 1 part of 8 M urea and 2% SDS solution to 9 parts of the sample. As previously described, under these chosen swelling conditions the jelly (matrix) structure of the thick egg white remained intact. The UV-VIS spectrum of such a sample showed a 50% decrease in turbidity compared to the native thick egg white fraction. As the dilution of the native thick egg white was 10%, *i.e.*, the concentration of proteins that scattered the light was diminished by 10%, a 10% decrease in the turbidity would be expected. The observed difference between the expected turbidity value and the measured one indicates that the captured proteins inside the ovomucin-ovomucoid network might be denatured *in situ*, *i.e.*, their conformations could be changed without the breakdown of the gel matrix.

## CONCLUSIONS

The results presented in this paper indicated that the thin fraction of egg white is a true solution of the proteins. The proteins of the thin fraction are present either in the form of the agglomerates and conglomerates (about 60%) or as monomers (about 40%). The thick fraction of egg white is a protein gel. The gel is composed of an ovomucin-ovomucoid network which is connected by disulphide bonds. The interior the network encompasses the other proteins which exist either in the form of monomers, or in the form of agglomerates and conglomerates.

It is concluded that the inter-protein S-S bonding may be a structural motif giving rise to the specific functional characteristics of jelly-structures of vertebrate extracellular compartments.

## ИЗВОД

## ИНТЕР-ПРОТЕИНСКЕ ВЕЗЕ И ДРУГЕ МОЛЕКУЛСКЕ ИНТЕРАКЦИЈЕ У КОКОШИЈЕМ БЕЛАНЦУ

МАРИЈА ВУЧКОВИЋ, МАРИЈА РАДОЈЧИЋ И БРАТОЉУБ Х. МИЛОСАВЉЕВИЋ

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Анализа танке фракције беланца и 2 mM раствора пречишћеног овалбумина електрофорезом на SDS-полиакриламидном гелу (SDS-PAGE) и мерење њихове вискозности, показали су да је танка фракција беланца протеински раствор. Растварање густе фракције беланца у 1 % SDS-у и 4 М уреи и анализа раствореног узорка методом SDS-PAGE у присуству и одсуству β-меркаптоетанола, показало је да је густа фракција беланца протеински гел. Такође је нађено да се структура гела састоји од тродимензионалне овомуцин-овомукоид мреже (матрикса), која је повезана S-S мостовима и у којој су заробљени остали протеини (овалбумин, коналбумин, итд). Заробљени протеини могу, такође, бити повезани S-S мостовима, формирајући агломерате и конгломерате, али су они претежно везани slabим електростатичким интеракцијама, као што је показано у експериментима раставарања. Структура матрикса не спречава денатурацију заробљених протеина, што се види по паду турбидности за 50% при растваранју гела у 8 М уреи.

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