

Relationship Between Functional Properties and Structure of Ovalbumin

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The effects of ovalbumin (OVA) denaturation using urea, guanidinium chloride (GdnHCl), sodium dodecyl sulphate (SDS), cetylpyridinium chloride (CPC), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 5 different cationic detergents with various side chains, HCl, and CH₃COOH were observed. Progressive unfolding in ovalbumin was measured as a function of fluorescent light intensity, peak response and shift in the maximum of emission. Kinetic measurements demonstrated that the rate of denaturation usually followed a double exponential decay pattern, but at small concentrations of urea and acids first-order reaction was indicated. The reversibility of the unfolding-folding transitions was confirmed from tryptophan fluorescence and circular dichroism (CD) measurements. Differences in secondary structure were observed and changes of α -helical content were calculated. Polyacrylamide gel electrophoresis (PAGE) with and without sodium dodecyl sulphate (SDS-PAGE) showed differences in the structure of native and denatured ovalbumin. Native protein samples in PAGE demonstrated smaller number and larger mobilities of subunits than denatured ones with different reductants, such as SDS and 2-mercaptoethanol (2 ME). Scanning of SDS protein patterns showed the appearance of aggregated forms in region of 45 kD.

KEY WORDS: Conformation; denaturation-renaturation; ovalbumin; secondary structure.

1. INTRODUCTION

Ovalbumin is widely used as a matrix for sustained drug release, as well as food product, rich in amino acids (Stein *et al.*, 1991; Imai *et al.*, 1991; Torrado-Duran *et al.*, 1991).

Changes in the matrix-drug mixture can be induced by high temperatures and also by the addition of different detergents and acids. The

quantification of these changes is important for understanding of the mechanisms which affect the formation of three-dimensional protein structures. This matter has drawn the attention of many researchers (Gruen *et al.*, 1987; Miyazawa *et al.*, 1984; Rosato *et al.*, 1990; Walbridge *et al.*, 1987; Flanagan and Hesketh, 1974).

Ovalbumin has been the object of physicochemical studies for over 40 years. In spite of this, information about its secondary and tertiary structures was quantitatively limited till recent time. Several studies on the conformational changes in ovalbumin induced by certain denaturing agents have been reported (Gorbunoff 1969; Koseki *et al.*, 1990; Batra *et al.*, 1989b).

In the present study measurements of fluorescence intensity and wavelength of the peak output response, CD measurements of backbone structure of the protein, kinetic studies, and electropho-

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resis under denaturing and nondenaturing conditions were used to monitor the unfolding of ovalbumin from the native structure to the random coil.

2. MATERIALS AND METHODS

2.1. Materials

Ovalbumin (standard), guanidinium chloride (GdnHCl), urea, sodium dodecyl sulphate (SDS), cetylpyridinium chloride (CPC), and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were reagent grade chemicals from BDH, Merck, and Sigma Chemical Co. Cationic detergents with different side chains, hexadecyldimethyl-(N-methylhydroxamic acid)-ammonium bromide (cationic I), hexadecyldimethyl-3-carboethoxypropyl-ammonium bromide (cationic II), and hexadecyldimethyl-2-hydroxyethyl-ammonium bromide (cationic III) were synthesized. Trade OVA was purchased from the Trima Pharmaceutical Industry. Deionized distilled water was used throughout.

2.2. Sample Preparation

All solutions were prepared in 0.01 M phosphate buffer, pH 7.2. The denaturant concentrations (M) were the following: urea—0, 2, 4, 6, and 8; GdnHCl—0, 1, 3, 5, and 6; SDS—0, 0.001, 0.01, 0.1, 0.4, 0.6; CPC—0, 0.00001, 0.00003, 0.00005, 0.00008, 0.0001; CHAPS—0, 0.1, 0.01, 0.001; cationics I, II, and III—0, 0.001, 0.0001, and 0.00001; values of pH 0.1, 1.0, 2.0, 3.0, 4.0, and 7.2 (CH₃COOH and HCl). Protein assays were performed by the Lowry method (Lowry *et al.*, 1951). Also solid OVA was denatured at 100°C for 12 hr in hermetically closed ampoules. OVA was prepared in granules with approximately 20% of water. After denaturation OVA was dissolved in phosphate buffer and filtered from undissolved material. Protein concentration was adjusted and emission was measured.

2.3. Fluorescence, UV Absorbance, and Circular Dichroism

Fluorescence measurements were performed using Model FP-770, Jasco-Spectrofluorometer. The temperature of the samples was maintained at 30°C using a thermostatically controlled circulating

water bath. A series of fluorescent emission spectra were recorded for protein solution with concentration of approximately 0.015%, which corresponds to the absorbance less than 0.1 in a 1 cm path length to guarantee a linear increase in the relative fluorescence intensity. For the temperature studies, the sample was brought to an equilibrium state in 10 min at the desired temperature. Absorbance values were measured using Uvikon 930 spectrophotometer. Fluorescence emission spectra for all samples were determined at excitation wavelengths (nm) of 274 and 295 and recorded over the frequency range from the excitation wavelength to a wavelength of 450 nm.

The magnitude of protein denaturation was calculated using the following equation:

$$\text{Percent denaturation (\%D)} = [(I_0 - I_1)/I_0] \times 100$$

where, I_0 and I_1 are the fluorescence intensities of protein in the absence and in the presence of denaturants, respectively. The percent of denaturation was determined after incubation of OVA with denaturants for 1 hr. All data were determined in triplicate for all experimental conditions.

Rate constants for denaturation were calculated using least-square analysis.

Circular dichroism (CD) measurements were made on a Jasco J-500c spectropolarimeter using a 1 cm quartz cell at room temperature.

The absorbances of all solutions were kept below 1.0. CD results are reported in terms of mean residue ellipticity $[\Theta]$, based upon a molecular weight of 45,000 and 385 amino acid residues per molecule of OVA. Helical content of globular proteins was obtained from the relationship:

$$[\Theta]_{222} = -30300f_h - 2340$$

where $[\Theta]_{222}$ is residue ellipticity at 222 nm and f_h is fraction of α -helix in protein.

Denaturation of OVA was performed with 6 M GdnHCl, 0.1% SDS, 0.025% SDS, and with HCl to pH 2.0. Guanidinium chloride- and sodium dodecyl sulphate-denatured samples were renatured by 48 hr dialysis against 0.01 M phosphate buffer (pH 7.2) at 4°C. Samples were diluted to the same concentration of protein (Ward and Bokman, 1982). Following neutralization to pH 7.2, acid-denatured samples became renatured upon storage for 48 hr at 4°C.

2.4. Amino Acid Analysis

Analysis of OVA was done by the procedure of Spackman *et al.* (1958). Freeze-dried samples were hydrolyzed with 6N HCl in sealed tubes for 22–44–66 hr at 110°C with and without previous oxidation with performic acid. The vacuum-dried hydrolysate was analyzed and applied on a Beckman 120 C automatic amino acid analyzer. For tryptophan determination, samples were hydrolyzed with 4 N LiOH for 20–24–28–36–40 hr at 110°C followed by treatment with 6 N HCl for 22 hr at the same temperature (Lucas and Sotelo, 1980).

2.5. Polyacrylamide Gel Electrophoresis

PAGE was done both in native and in denatured forms in 15% gel. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure of Laemmli (1970). Fifty micrograms of protein was applied to sample slots. Gels were stained with 0.25% Coomassie Brilliant Blue R 250 in methanol/water/acetic acid and destained in the same solvent. Molecular weight standards were obtained from Sigma Chemical Co.

Nondenaturing gradient polyacrylamide gel electrophoresis (ND-PAGE) was done using 8–25% acrylamide gradients on Phast System (Pharmacia). ND-PAGE conditions were applied according to Pharmacia technical file 120. For 8–25% ND-PAGE running conditions were: prerun (400 v; 10 mA; 2.5 w; 15°C; 10 vh), sample application (400 v; 1 mA; 2.5 w; 15°C; 2 vh), run (400 v; 10 mA; 2.5 w; 15°C; 270 vh). Molecular weight (MW) markers (Sigma) ranged between 14,200 D and 545,000 D. The sample buffer was of 10 mM Tris-HCl (pH 8.0); 1 mM ethylenediaminetetraacetic acid (EDTA); 0.01% bromophenol blue. A prefocusing step of 30 min was carried out before applying the protein samples of 30 ng/ μ l and ND-MW markers. The total electrophoresis and staining time with Coomassie Brilliant Blue R2 50 was about 2 hr, similar to conditions of Van-Seuningen and Davril (1992).

Densitometry of Coomassie Brilliant Blue stained spots on the slab gel was carried out with a Scan Maker 600Z Microtek at sample wavelength of 580 nm (reference wavelength 750 nm) by measuring of transmission.

3. RESULTS AND DISCUSSION

3.1. Amino Acid Composition of Ovalbumin

Table I contains results of amino acid analysis of trade ovalbumin and literature data. Special treatment was used to prevent the destruction of tryptophan (Lucas and Sotelo, 1980) in comparison with others (Smith and Back, 1970). The values given by Smith and Back (1970) for chicken ovalbumin are in reasonable agreement with our results. The main differences in the analysis of ovalbumin were in the values for histidine, tryptophan, threonine, proline, and aspartic acid. It can be explained by different methods, which were used during hydrolysis of amino acids. Differences in the amounts of the individual ovalbumin also have been found between various strains of chickens (Feeney *et al.*, 1960). However, data in Table I show quite similar amounts of the most important amino acids such as 2.41 μ mol% for tyrosine and 2.33 for tryptophan, which are used in fluorescence measurements.

3.2. Intrinsic Fluorescence Properties of Ovalbumin

One could expect to receive two emission peaks, considering amounts of tyrosine and tryptophan. However, Khan *et al.* (1980) indicate that tyrosine has a lower extinction coefficient than

Table I. Amino Acid Composition of Ovalbumin (μ mol%)

	Ovalbumin	Ovalbumin ^a
Lysine	5.92	5.14
Histidine	2.53	1.74
Arginine	4.86	3.88
Tryptophan	2.33	0.90
Aspartic acid	10.30	8.13
Threonine	5.34	3.83
Serine	7.99	9.73
Glutamic acid	11.65	13.38
Proline	2.87	3.89
Glycine	5.90	4.89
Alanine	8.21	9.10
1/2 Cystine	2.48	1.48
Valine	7.32	8.24
Methionine	3.56	3.95
Isoleucine	4.79	6.16
Leucine	7.07	8.02
Tyrosine	2.41	2.60
Phenylalanine	4.46	5.05

^a Smith and Back (1970).

tryptophan. In addition, an energy transfer from tyrosine to tryptophan residues may result in the dominance of the tryptophan fluorescence response. The presence of even one tryptophan residue in protein gives spectrum with single peak of tryptophan emission in the interval 331–342 nm at both excitation wavelengths at 295 nm and 274 nm (Arntfield *et al.*, 1987).

In accordance with this postulation, excitation at either 274 nm or 295 nm produced the emission spectra of native ovalbumin with a single emission peak with maximum at (nm) 335.0 and 337.5, respectively (not shown). Replacement of the excitation wavelength of 274 nm to 295 nm resulted in decrease of relative fluorescence intensity that connected with decrease in tryptophan emission and almost complete absence of tyrosine fluorescence (Khan *et al.*, 1980). Tryptophan appears to be the only aromatic amino acid that absorbs light at this radiation wavelength.

It was shown by x-ray crystallographic studies (Wright *et al.*, 1990) that all three tryptophan residues in ovalbumin, Trp¹⁴⁸, Trp¹⁸⁴, and Trp²⁶⁷ are situated in the interior of molecule. Trp¹⁴⁸ and Trp²⁶⁷ are included in helices F and H, Trp¹⁸⁴ is located in the loop between strand 3 in β -sheet A and helix F₁. With the changes of tertiary structure they become accessible to the external quenchers and display altered fluorescence.

3.3. Urea-Induced Denaturation

Denaturation of ovalbumin with urea was examined at both excitation wavelengths 295 nm (Fig 1) and 274 nm (Fig. 1A). Increasing levels of urea showed a gradual decrease in fluorescence intensity at 274 nm up to urea concentration 3 M, at which significant decrease in the fluorescence intensity and small shift in the wavelength appeared. Above concentration of 6 M urea shoulder at approximately 307 nm appeared due to tyrosine fluorescence. Simultaneously, emission peak (tryptophan fluorescence) shifted from 337.5 nm to 352.5 nm. These values continued to change up to the addition of 8 M urea, where they were maximal (emission maximum shifted from 337.5 nm to 355.0 nm). The appearance of shoulder and shift in the maximum of emission were the results of protein unfolding. Distinct shoulder appears as a result of ceasing of energy transfer in albumin from excited tyrosine to tryptophan residues with unfolding, when the distance between

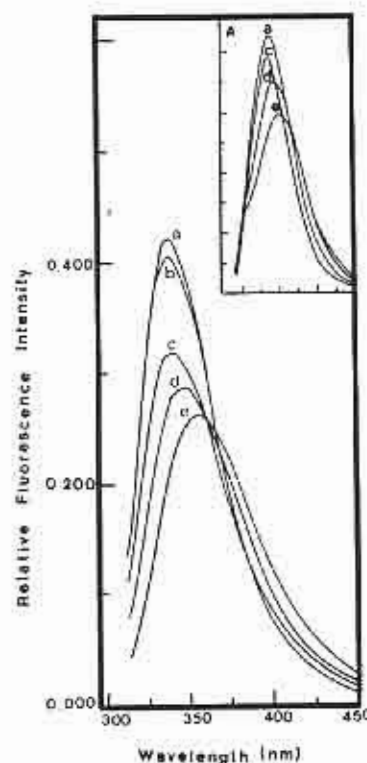


Fig. 1. Dependence of the fluorescence emission of OVA on urea concentrations. Excitation at 295 and 274 nm are shown on the graph and insert A, respectively. a, b, c, d and e correspond to the intensity at urea concentrations 0 M, 2 M, 4 M, 6 M, and 8 M.

the tryptophan and tyrosine residues increases (Khan *et al.*, 1980). On the other hand, shift of the tryptophan residues to a more polar environment upon unfolding causes a shift to the lower energy in the maximum of emission and quenching of fluorescence intensity.

The changes in tryptophan fluorescence in the presence of urea were determined by monitoring emission spectra at excitation wavelength at 295 nm. At this wavelength, there is no contribution from tyrosine fluorescence, and fluorescence intensity at a fixed wavelength can be used as a measure of the extent of denaturation (Permyakov and Burstein, 1984).

Denaturation of ovalbumin from Trimu Pharmaceutical Industry and standard ovalbumin were carried out at the same conditions and comparative results were received (data not shown).

3.4. GdnHCl-Induced Denaturation

The denaturation of ovalbumin in the presence of GdnHCl was similar to that of urea, but showed

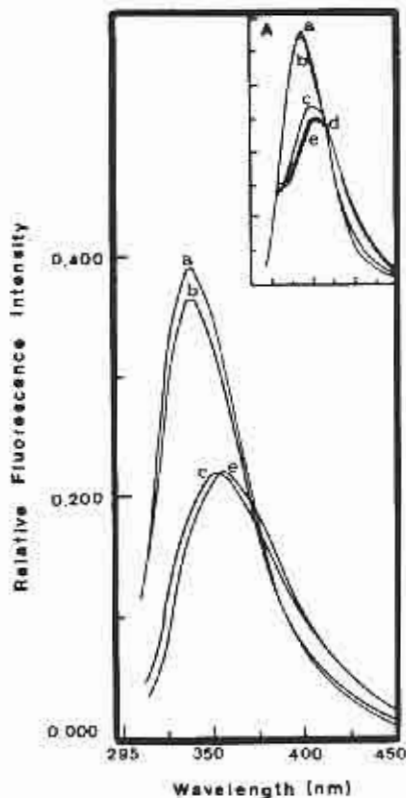


Fig. 2. Dependence of the fluorescence emission of OVA on GdnHCl concentrations. Excitations (nm) at 295 and 274 on the graph and insert A, respectively. a, b, c, d, and e refer to the intensity at GdnHCl concentrations 0 M, 1 M, 3 M, 5 M, and 6 M. All samples were dissolved in 0.01 M phosphate buffer at pH 7.2. The protein concentration was 0.15 mg/ml at 30°C.

more dramatic changes. The relative fluorescence intensity decreased with addition of GdnHCl at both wavelengths of excitation 295 nm and 274 nm (Fig. 2 and 2A). Denaturation achieved 49% at maximal concentration of 6 M GdnHCl and 39% at 8 M urea. At concentrations of GdnHCl above 3 M further decrease in fluorescence intensity was not observed, but only difference in the shift was established. It may be explained in terms of the solvent perturbation effect of GdnHCl on the fluorescence. Shift in the maximum of emission achieved 357 nm at 6 M GdnHCl. Minimal shift was observed to 341 nm at 2 M GdnHCl, demonstrating the lower stability of the protein toward GdnHCl than toward urea. Also shoulder corresponding to fluorescence of tyrosine at wavelength excitation 274 nm appeared at 3 M GdnHCl and transformed in a distinct peak centered at approximately 309 nm at the highest concentrations of GdnHCl (5 M and 6 M).

These changes in the tertiary structure of ovalbumin with urea and GdnHCl are consistent with the results of Takeda *et al.* (1992) on the reactivity of free sulphydryl groups in ovalbumin. Above concentrations of 5 M GdnHCl all four SH groups of ovalbumin reacted, (i.e., the protein was unfolded to a cross linked random coil). However, unfolding of ovalbumin by urea is not sufficient to expose free SH groups buried in the interior of the protein, its helices resist the disruption by urea.

3.5. Influence of Other Denaturing Reagents on the Unfolding of Ovalbumin

The effect of sodium dodecyl sulphate (SDS) on the ovalbumin differed from effects exhibited by previous denaturants. Addition of detergent also produced a decrease of the intrinsic fluorescence. But the percentage of denaturation was larger with SDS than with previous substances and reached 58% (Fig. 3). Much lower concentrations of

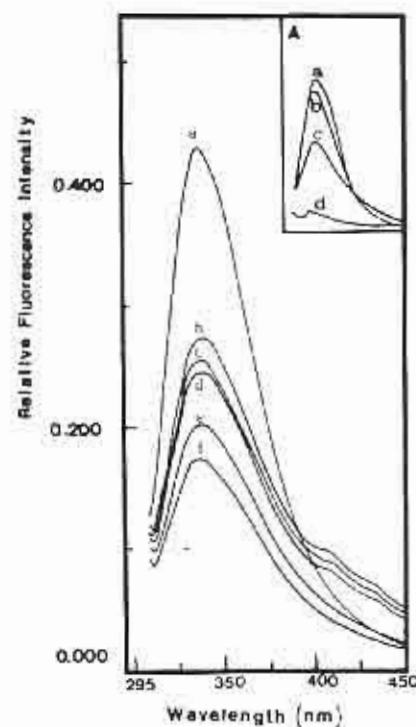


Fig. 3. Dependence of the fluorescence emission of OVA on SDS concentrations. Excitation at 295 nm. a, b, c, d, e, and f refer to the intensity at SDS concentrations 0 M, 0.6 M, 0.4 M, 0.001 M, 0.01 M, and 0.1 M. (A) Effect of different detergents on the fluorescence emission of OVA. a, b, c, and d correspond to the intensity of native OVA and OVA treated with CHAPS, SDS, and CPC, respectively.

detergent were necessary to initiate decrease in intensity. On the other hand, at concentrations of SDS above 0.1 M an increase of fluorescence intensity was noted. Such behavior may be attributed to decrease in SDS-monomers, because formation of SDS-protein complexes does not connected with micellar form. Shift in the maximum of wavelength corresponding to tryptophan fluorescence was only to 338.5 nm and 338 nm at concentrations of SDS 0.6 M and 0.4 M. There was no shift at concentration of 0.2 M SDS; but at concentrations of 0.1 M and 0.01 M, the peak moved in the opposite side to 336 nm. At wavelength of excitation 274 nm significant blue-shift in the emission peak was observed to 320 nm at the concentration of 0.1 M SDS.

Guanidine and urea induce transition by interfering with the hydrophobic regions in the interior as well as in the hydrogen-bonding pattern involved in the polar regions of the peptide chain in a protein molecule. In contrast, SDS interacts with the hydrophobic side chains of a protein, disrupts hydrophobic bonds, and therefore permits an increased development of helix (Meyer and Kauzmann, 1962). These different mechanisms are reflected also in fluorescence behavior.

Small concentrations of cetylpyridinium chloride (CPC) bring momentary full irreversible denaturation. Therefore, concentrations of 0.00001 M, 0.00003 M, 0.00005 M, 0.00008 M, and 0.0001 M were checked. Decrease in the relative fluorescence intensity consisted with increasing in the concentration of CPC. Excitation at 274 nm and 295 nm exhibited shift in the maximum wavelength to the higher energy, similar to that observed with SDS (328.5 nm and 331.5 nm, respectively, at maximal CPC concentration of 0.0001 M). Shoulder did not appear. Its behavior was similar to the case of SDS-denaturation, but the effect was stronger (data not shown).

Cationic detergents with various side chains (cationic I, cationic II, and cationic III) did not differ in their fluorescence spectrum (not shown). It may be suggested, that carboxy and alcohol functional groups have not sufficient influence on the denaturing properties of detergents. The length of the main hydrocarbon chain and halogenic substituent were the same in all three cases. Concentrations of cationic detergents—0.00001 M, 0.00005 M, 0.0001 M, 0.0005 M, and 0.001 M—were checked. Concentration of detergents 0.00001 M did not cause changes of emission

spectrum. Concentration of 0.0001 M showed decrease in fluorescence intensity and small shift to the longer wavelength (to 338.5 nm). Concentration of 0.001 M produced increase of fluorescence intensity and maximal shift to 342 nm. Percent of denaturation achieved maximally 17%. Apparently, these detergents don't propagate helical structure and change only tertiary conformation of ovalbumin molecule.

Effects of different types of surfactants [amphoteric (CHAPS), anionic (SDS), and cationic (CPC)] on the emission spectrum of OVA were compared (Fig. 3A). Even maximal concentrations of CHAPS show no essential changes in the fluorescence intensity and only a small shift to the wavelength of 338 nm. Such different effects of detergents can be explained on the basis of the earlier work of Nakamura (1972). Since interaction between protein and detergent arises from the electrostatic interaction between oppositely charged groups and hydrophobic interactions, basic amino acid residues of protein interact with SDS and carboxylic amino acid residues with CPC. These interactions bring on the change of protein conformation. From this point of view, slight influence of CHAPS became clear.

Ovalbumin treated with HCl and CH_3COOH showed similar behavior which differed only in the extent of denaturation. Intensity decreased with increasing concentration of HCl, but shift to the lower wavelength was observed only at pH 1.0 (not shown). It might be related to the fact that the globular conformation of ovalbumin at acidic pH is similar to that at neutral pH, but that molecules become more flexible as the pH is lowered. CD study also supports the conclusion that unfolding does not take place in the case of acid denaturation and agrees with the concept of the molten-globule state (Koseki *et al.*, 1988). The alterations in the fluorescence emission are the result of direct proton quenching of tryptophan fluorescence induced by low pH and connected with protonation of α -carboxyl group in amino acids, that are in close to tryptophan residues (Uehara *et al.*, 1976).

Also, viscosity measurements indicated that the globular conformation of OVA did not change at acid pH (Bull, 1940).

The temperature dependence of the fluorescence of OVA was investigated at 295 nm and 274 nm excitation wavelengths. Effects similar to those resulting from denaturants but less marked were observed (Fig. 4, solid curve). The shift of the

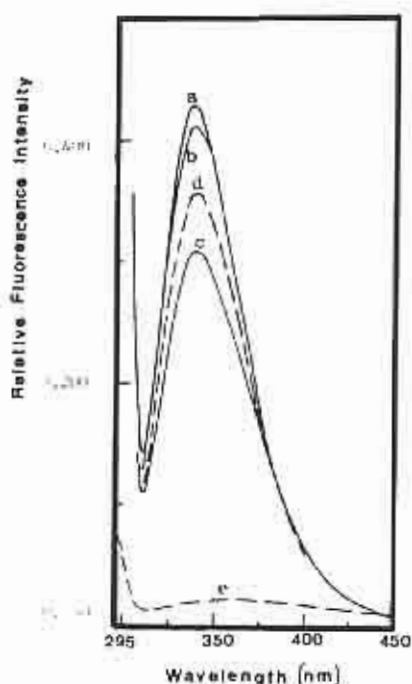


Fig. 4. Temperature dependence of the fluorescence emission of OVA. Excitation at 295 nm. a, b, and c correspond to the intensity of protein solution at 40°C, 60°C, and 95°C. Dotted curves d and e correspond to the intensity of protein heated in the solid state in hermetically sealed conditions and in non-sealed ones, respectively.

response was smaller (to wavelength of 340 nm at temperature 95°C), and the fluorescence was reduced by 27%. The shoulder, which corresponds to breaking of energy transfer from exciting tyrosine to tryptophan residues was not observed. The shift in the wavelength of maximum emission was a result of unfolding of protein with temperature and exposing of amino acids residues from the hydrophobic interior to collisional quenching by the solvent (Arntfield *et al.*, 1987).

OVA, treated in hermetically closed conditions, showed large shift of the maximum of emission to the longer wavelength of 364 nm and denaturation achieved 78% (Fig. 4, dotted curve). OVA, treated in opened pans, gave small shift to 338.5 nm and decrease in fluorescence intensity to 10%.

3.6. Kinetic Studies of Denaturation

A number of researchers employed kinetic studies for examination of changes in protein structure during denaturation (Gryczynski *et al.*, 1988; Flangman and Hesketh, 1974). In the present work, rate constants for OVA denaturation with

different substances were measured fluorimetrically, following the decrease in fluorescence intensity at fixed wavelength of maximal shift, then calculated and included in Table II. These data demonstrate dependence of relative fluorescence intensities in OVA upon conformational state. Increase of urea concentration up to 6 M results in a monotonic decrease in intensity (Table II). Also, emission maxima indicate a nonpolar environment for the tryptophan residues. Above 6 M concentration, the emission maximum shifts to 355 nm, which characterizes tryptophan in an aqueous environment. This increase in emission maximum is accompanied by a rapid decrease in the fluorescence intensity (Fig. 5), (i.e., strictly first-order intensity decay of the native OVA became obviously second-order in conditions which result in protein unfolding). The same transitions were observed for HCl and CH₃COOH. The progressive decrease of fluorescence intensity which is consistent with unfolding was more intensive with guanidine hydrochloride and detergents than with previous substances (Fig. 6). Even at minimal concentrations of the last ones, transition from the native to the denatured state occurred in two separate stages, corresponding to the three-state mechanism of unfolding. Similar biphasic features were observed earlier (Uchida *et al.*, 1983) and confirmed the existence of the stable intermediate conformation. According to the two-state model, through which small globular proteins undergo (Chmelik, 1989), after the rapid disappearance of ordered structures (mostly α -helical), there still remain large parts of folded protein molecule. These molecules become unfolded during the subsequent denaturation (Gryczynski *et al.*, 1988).

Figures 5 and 6 demonstrate different velocity of the fluorescence transitions of egg albumin with different surfactants. High concentrations of GdnHCl, SDS, CPC, HCl, and CH₃COOH which were used for denaturation show a very rapid and significant decrease in the rate of their fluorescence transition. For CPC, the first stage contributed about 87% of the total fluorescence change and was completed in 2 min. Rate constants confirm the stronger effect of detergents than of denaturants (urea and GdnHCl) (Table II).

3.7. CD Studies

The far-UV CD spectra for different states of OVA (the native, denatured and renatured) are

Table II. Fluorescent Properties^a of Ovalbumin^b in Different Denaturants

Concentration of denaturant	% of denaturation	Time of denaturation (min)	Shift (nm)	K_1^c (min ⁻¹)	K_2^d (min ⁻¹)
Urea (M)					
1.0	6	60	—	1.154×10^{-3}	—
3.0	12	60	339.0	1.728×10^{-3}	—
4.0	25	60	339.5	5.250×10^{-3}	—
6.0	32	60	345.0	6.993×10^{-3}	—
8.0	39	60	355.0	1.490×10^{-1}	2.71×10^{-3}
Gdn HCl ^e (M)					
2.0	27	60	341.0	1.78×10^{-2}	6.30×10^{-4}
3.0	46	15	351.5	4.54×10^{-1}	2.93×10^{-2}
5.0	46	15	356.0	2.84	7.97×10^{-1}
6.0	49	15	357.0	5.86	1.72×10^{-2}
SDS ^f (M)					
0.001	43	60	340.0	8.2×10^{-2}	2.95×10^{-3}
0.010	53	7	336.0	8.56	1.23×10^{-1}
0.100	56	7	—	9.33	1.45×10^{-2}
0.200	54	7	—	9.98	7.69×10^{-3}
0.400	46	7	338.0	8.68	9.02×10^{-3}
0.600	40	7	338.5	5.93	7.31×10^{-4}
CPC ^g (M)					
0.00001	34	60	336.5	2.89×10^{-1}	3.074×10^{-3}
0.00003	53	60	335.0	1.47	6.130×10^{-3}
0.00005	67	60	334.5	2.28	9.350×10^{-3}
0.00008	88	60	331.5	4.12	1.260×10^{-2}
0.00010	90	60	331.5	4.44	1.270×10^{-2}
HCl (pH)					
0.1	45	60	331.5	2.34	2.40×10^{-1}
1.0	35	60	—	2.83	3.06×10^{-1}
2.0	30	60	339.0	3.54	2.34×10^{-1}
3.0	14	60	—	2.45×10^{-3}	—
4.0	5	60	—	—	—
CH ₃ COOH (pH)					
0.1	69	—	319.0	—	—
1.0	40	10	333.5	3.50	2.150×10^{-2}
2.0	31	60	—	7.40×10^{-1}	2.480×10^{-3}
3.0	16	60	—	1.22×10^{-1}	1.798×10^{-3}
4.0	9	60	—	1.45×10^{-1}	—

^a Each value represents the mean of data from triplicate experiments; $P \leq 0.05$.

^b Emission spectrum of native albumin has maximum at 337.5 nm.

^c Rate constant of first phase of denaturation.

^d Rate constant of second phase of denaturation.

^e Guanidinium chloride.

^f Sodium dodecyl sulphate.

^g Cetylpyridinium chloride.

shown in Fig. 7. Optical activity of α -helix in far-UV permits the use of CD studies for investigations of conformational changes in protein solutions (Chen *et al.*, 1972). Received values of helical content for different states of ovalbumin are reported (Table III).

The CD of native ovalbumin spectrum (Fig. 7, curve *b*) shows a large negative peak centered around 222 nm. This band demonstrates a sig-

nificant α -helical content of protein. Another smaller band at 213 nm is characteristic for proteins with significant quantities of β structure. Takeda and Moriyama (1990) reported for intact ovalbumin 33% α -helical, 7% β -structure, and 60% random coil content. Influence of GdnHCl on the secondary structure of OVA is shown by curve *d* on Fig. 7. Negative maximum around 202 nm is typical for a protein with a disordered random coil structure.

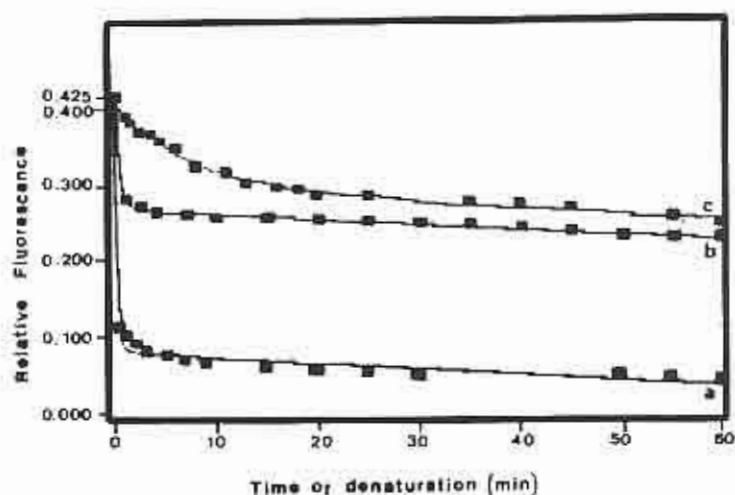


Fig. 5. The time-course at the fluorescence transitions of OVA in 0.0001 M CPC (a); HCl pH 0.1 (b); 8 M urea (c) at 30°C. The fluorescence was measured at 331.5 nm, 331.5 nm, and 355.0 nm. Excitation at 295 nm.

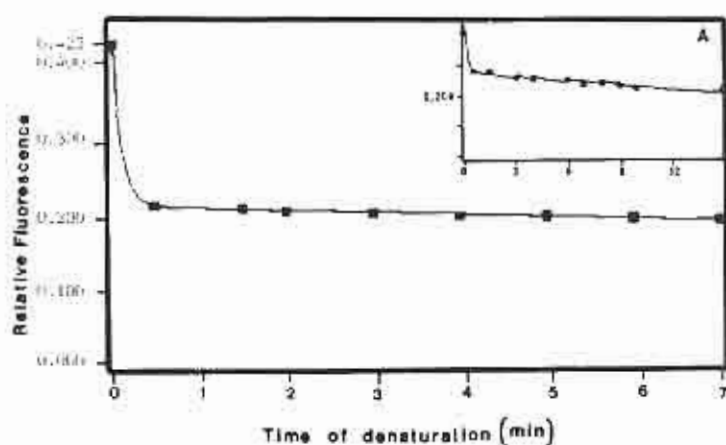


Fig. 6. The time-course of the fluorescence transition of OVA in 0.1 M SDS at 30°C. The fluorescence was measured at 337 nm. Excitation at 295 nm. (A) The time-course of the fluorescence transition of OVA in 6 M GdnHCl at 30°C. The fluorescence was measured at 357 nm.

GdnHCl causes the protein to undergo a disorganization of its secondary structure involving a complete helix-to-coil transition. These data agree with others, such as contents of α -helix, β -structure, and random coil in ovalbumin with 6.4 M GdnHCl as 6%, 13%, and 81%, respectively (Batra *et al.*, 1989a). SDS-denaturation of the protein (0.1% SDS) leads to increase in α -helical content (40% compared to 36% in the native state) (Fig 7, curve a). The spectrum does not closely resemble that of the native state and demonstrates the much more pronounced negative maxima at 222

and 208 nm typical of α -helix. However, at low concentrations of SDS (0.025%), a decrease in α -helical content in comparison to native state was observed (Table III). At the same time, spectrum shape was similar to the high-binding protein state. It is obvious that SDS does not unfold the protein molecule at concentrations of 0.001 M. Random coil undergoes transition in α -helix and β -form. These data agree with results of Takeda and Moriyama (1990) who described an increase in helicity for ovalbumin-sodium dodecyl sulfate complexes: 35% of α -helix (increase from 33%).

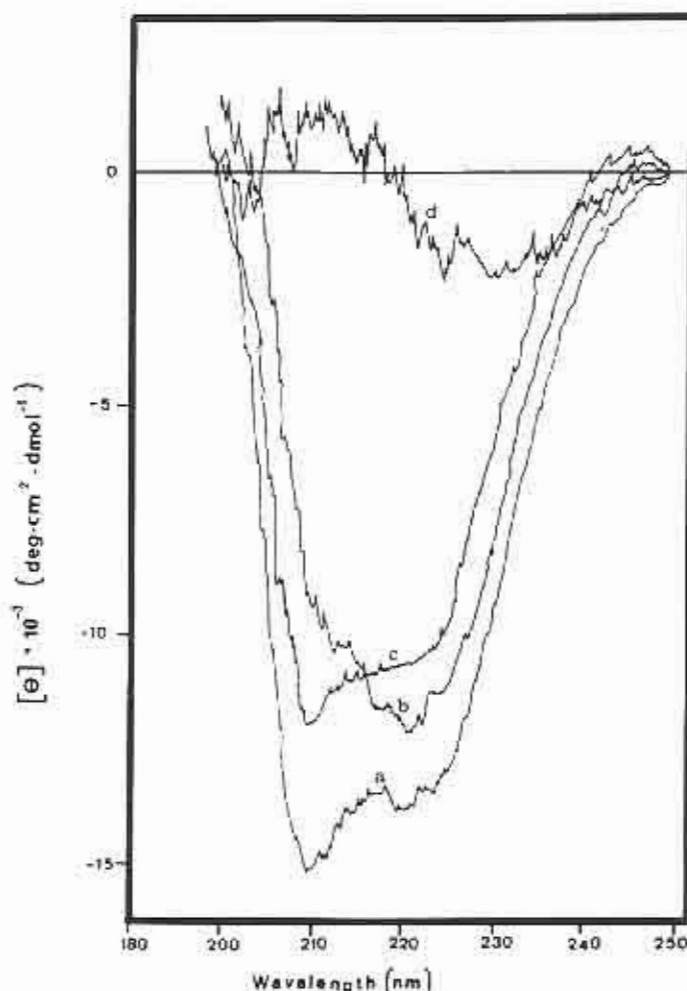


Fig. 7. Circular dichroism spectra of native (b); denatured with GdnHCl (d); renatured (c) and denatured with SDS (a) OVA. Samples in concentration 0.0375 mg/ml were dissolved in 0.01 M phosphate buffer. Each spectrum was run at room temperature.

Table III. α -Helix Estimation of Various States of OVA

Samples	% of α -helix
Native protein (0.01 M phosphate buffer, pH 7.2)	~36
Denatured proteins:	
1. Random-coiled protein (6 M GdnHCl)	~7
2. Acid-denatured (0.01 M phosphate buffer, pH 2)	~36
3. Detergent-denatured (0.1% SDS)	~40
4. Detergent-denatured (0.025% SDS)	~33
Renatured proteins:	
1. From 0.1% SDS	~27
From random-coiled state:	
2. After 1 hr of incubation	~33
3. After 24 hr of incubation	~32

13% of β -structure (increase from 7%), and 52% of random coil (decrease from 60%). In contrast

with this, Batra *et al.* (1989a) pointed out that SDS, even at a concentration of 10 mM, has no effect on the conformation of ovalbumin.

The far-UV spectrum of the acid denatured protein (not shown) shows spectrum similar to the native protein.

3.8. Regeneration of the Native State

Renaturation of denatured egg albumin from the random-coiled state by dialysis showed partial recovery of CD structure of native ovalbumin (curve c) with clear expressed features of α -helical proteins (presence of two large negative bands centered around 209 nm and 220 nm). Calculations indicate 33% of α helix (Table III).

Renaturation of ovalbumin also showed partial recovery of fluorescence properties. Maximum of

emission spectrum shifted from 357 nm in denatured protein to 339 nm in renatured one. Shoulder at wavelength excitation of 274 nm also disappeared. Fluorescence intensity returned up to 51% of the original fluorescence.

The protein is fully unfolded by the action of 6 M GdnHCl to yield a random coil and refolds upon removal of the denaturing conditions to reform the native structure. However, differences in far-UV CD and fluorescence spectra indicate that the renatured protein has not fully regained its native secondary structure.

Since SDS-binding is very strong, direct renaturation by dialysis does not lead to the complete removing of the detergent. However, significant influence even of minimal amounts of bound SDS on the conformational changes has previously been reported by Tanford (1968). Therefore, decrease in α -helical content similarly to the low-binding state was observed after renaturation of high-binding protein form (Table III).

Fluorescence spectral renaturation up to 30% can be possible following the treatment with 0.1% SDS.

3.9. Electrophoretic Analysis

Electrophoresis under native conditions does not reveal sharp bands (Fig. 8A); therefore, its schematic presentation was done for visuality. There was no difference in distribution of protein bands in denatured samples with SDS and with 2-ME. Samples 1, 2, and 5 (native and boiled ovalbumin in SDS buffer and boiled ovalbumin in native buffer) showed similar distribution of bands. Only protein in native sample buffer (line 6) formed smaller quantities of bands (2 instead of 6 and illegible slur in the top) and their mobility was larger than mobility of denatured ones. Under denaturing conditions, when unfolding takes place, the proteins differ in shape and size. Folded proteins are remarkably compact in comparison with a random polypeptide, and the shape of a protein affects the rate at which it migrates in polyacrylamide gels.

Phast System gradient electrophoresis in native conditions showed better resolution than homogeneous electrophoresis (Fig. 8B). Ovalbumin in native and SDS sample buffer differs in distribution of electrophoretic bands. Lane 1 is similar to lane 3 on gel C. Five bands can be distinguished in this

sample. Bands in the native samples (3,4) have larger mobilities than in 1, but the band at the top of the lane became resolved in 4 thin bands. Granulated OVA after heating in ampule shows very illegible behavior, but two bands in the region of MW 45 and 55 can be distinguished.

PAGE was performed in SDS-conditions (Fig. 8C). However, ovalbumin in sample buffer without reducing agent differed from others. It had larger quantity of subunits (5 instead of 3) than protein, denatured by boiling in SDS and ME. All these results are consistent with data appearing in the literature (Chmelic *et al.*, 1988; Henkens *et al.*, 1982; Chang and Hosey, 1990).

A scan of SDS protein patterns demonstrates the differences between lines 2, 3, 5, and 6 (Fig. 9). After treatment with ME, which breaks disulphide bonds, new interchain disulphide bonds are formed. As a result, aggregated forms were received in the bands at a position corresponding to molecular mass of 45 kD. These results are consistent with the data of Greighton (1979), who described the tendency of the unfolded hen ovalbumin to aggregate. Smaller quantities of spots but broader ones with larger molecular weight of patterns than in nonreduced protein are shown. Aggregation was approximately as much as twice in major bands than in minor ones (Fig. 9). Minor fractions (Fig. 9) were not discussed, because the remarkable changes were observed only for major protein bands.

4. CONCLUSIONS

Conformational changes in ovalbumin resulted from the addition of anionic (SDS), cationic (CPC), cationic I, cationic II, and cationic III with different side chains, and amphoteric (CHAPS) detergents, denaturants (urea and GdnHCl), organic and inorganic acids (CH_3COOH and HCl) were examined using the fluorescence of the tryptophan residues at 295 nm. The relative order in which the detergents affected the extent of conformational changes followed the sequence:



Rate of denaturation followed double exponential decay in most cases. At small concentrations of urea, HCl and CH_3COOH rate of denaturation followed single phase first-order reaction.

Decreasing in fluorescence intensity and shift

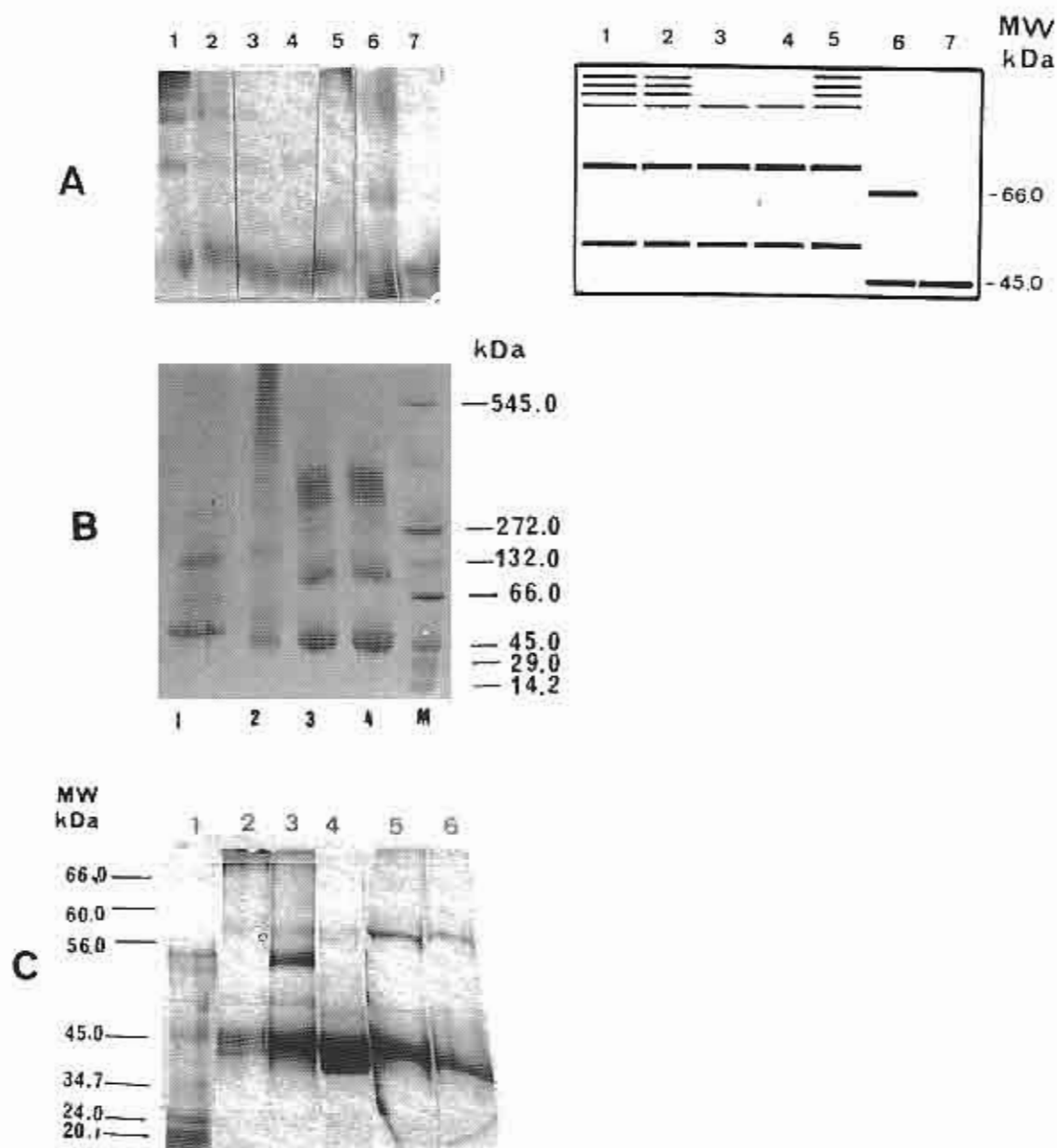


Fig. 8. Native gel in 15% PAAG and its schematic presentation (A), native gradient gel 8–25% (B), and SDS-PAGE of OVA in 15% PAAG (C). (A) 1, Boiled OVA in sample buffer (S.B) with SDS; 2, native OVA in S.B. with SDS; 3, boiled OVA in S.B. with ME; 4, native OVA in S.B. with ME; 5, boiled OVA in native S.B.; 6, native OVA in native S.B.; 7, standard OVA in native S.B. (B) 1, Native OVA in S.B. with SDS; 2, granulated OVA after heating at 100°C in native S.B.; 3, native granulated OVA in native S.B.; 4, native powder OVA in native S.B.; 5, molecular weight marker. (C) 1, Molecular weight marker; 2, boiled OVA in S.B. with SDS; 3, native OVA in S.B. with SDS; 4, standard OVA in S.B. with SDS; 5, boiled OVA with S.B. with ME; 6, native OVA with S.B. with ME.

in the maximum of emission which were the result of unfolding of the coiled polypeptide chain were observed.

CD spectra for native, denatured, and renatured ovalbumin showed changes in its

secondary structure under denaturation.

Dialysis of denatured ovalbumin with 6 M GdnHCl against 0.01 M phosphate buffer gave partial recovery of CD spectra and fluorescence properties.

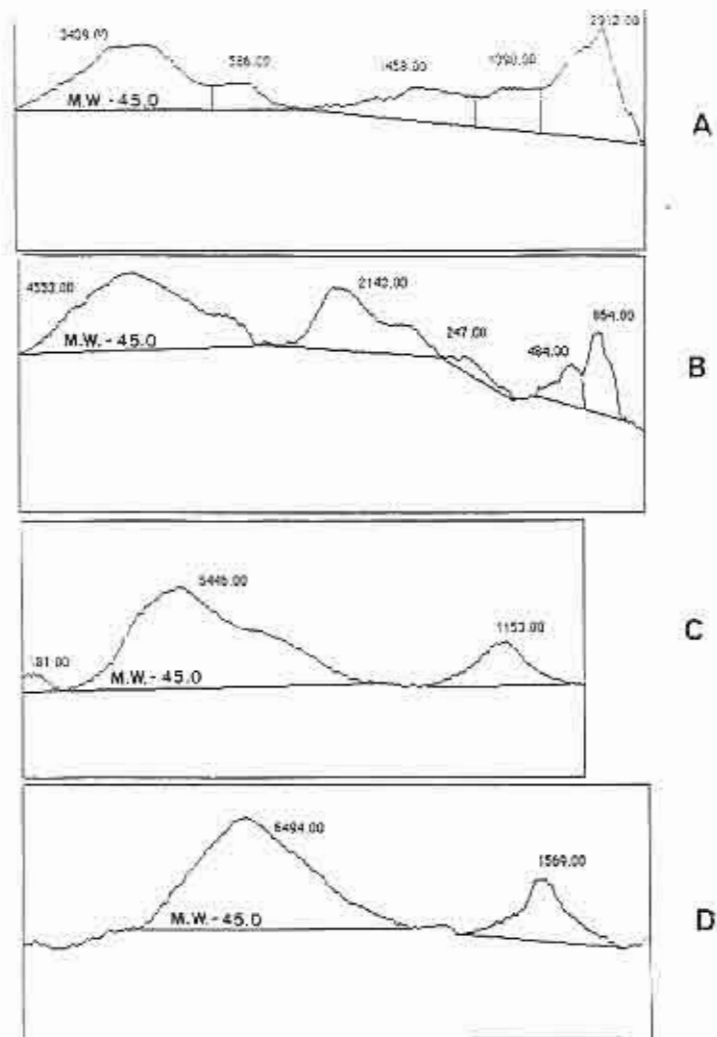


Fig. 9. Densitometric quantitation of protein bands of OVA on polyacrylamide slab gels. (A) Boiled OVA in sample buffer (S.B.) with SDS, corresponding to Fig. 11B, band 2; (B) native OVA in S.B. with SDS, corresponding to Fig. 11B, band 3; (C) boiled OVA in S.B. with ME, corresponding to Fig. 11B, band 5; (D) native OVA in S.B. with ME, corresponding to Fig. 11B, band 6.

The unfolding of ovalbumin was shown by electrophoresis. Denaturation was marked by reduction of mobility and differences in molecular weights of subunits. Effects of ME resulted in formation of aggregated subunits as it is seen from densitometric quantitation.

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