



pH and heat induced structural changes of chicken ovalbumin in relation with antigenic properties



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ABSTRACT

Ovalbumin is the major egg white protein known to induce allergic reactions in humans. A comprehensive evaluation of the structural and antigenicity features of ovalbumin subjected to different pH and heat treatments was performed by combining fluorescence spectroscopic measurements, ELISA and *in silico* prediction. The intrinsic fluorescence spectra indicated modification of the ovalbumin tertiary structure depending on pH and applied temperature. The heat treatment caused the alteration of ovalbumin structure, which exhibited gradual hydrophobic exposure. The in depths check of ovalbumin molecular model, after performing molecular dynamics simulations, indicated the slight transition toward a typical β -strand dominant structure with the temperature increase. Moreover the immunoenzymatic test was employed to estimate the effect of the pH and thermal treatment on the stability of ovalbumin epitopes. Only a 5.5% reduction of the residual antigenicity was observed when heat treating the ovalbumin samples at pH 7.0, whereas a significant reduction (over 82%) of the antigenicity was obtained at pH 9.5 and temperatures over 80 °C. Both pH and thermal treatment affected the conformation of ovalbumin. The reduced recognition of the modified native ovalbumin by specific antibodies at alkaline pH is most probably a consequence of significant changes in the local conformation of the epitopes.

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1. Introduction

The presence of even small amounts of egg in food might cause allergic reaction, because about 80% of the egg white protein exhibits allergenic potential. The main proteins responsible for inducing allergic reactions in humans are ovalbumin (54%), ovomucoid (12%), ovomucoid (11%) and lysozyme (3.5%) [1–3]. A great diversity of allergic symptoms was reported for different egg allergic individuals, as well as varying threshold levels (0.2–200 mg protein) for inducing the adverse reactions [2,4,5].

Despite the large number of studies focused on antigenicity and allergenicity of egg white proteins, there is a still running debate on the most dominant egg allergen. According to Mine and Zhang [1], ovalbumin (OVA) and ovomucoid are generally considered to be the most antigenic and allergenic proteins.

As there is no cure reported for food allergies, the avoidance of food allergens is currently the only way of preventing the health

consequences in case of allergic consumers. Anyway, since the efficient avoidance of food allergens is not always possible, is highly desired to identify suitable processing techniques for reducing or even eliminating the allergenic potential of certain products. Depending on the intensity, both thermal and non-thermal processing of foods might alter protein structure, therefore causing potential modifications of the epitopes. It is generally accepted that the biological activity of a protein is related to its structural features [5]. There are several papers reporting on the effects of thermal and non-thermal processing on the IgE binding properties and allergenicity of egg in food products [1,5]. Concerning thermal processing, although the heat treatment is usually applied for microbiological safety purpose or for enhancing texture and flavor properties, *in vitro* and clinical studies indicate no reaction to the thermally treated egg white for egg-allergic patients [2,6–8]. Anyway, there is limited information on the molecular behavior of individual proteins under thermal treatment at different pH values. The knowledge of the particularities of each allergen in model system might provide a better understanding of the ways to overcome the factors that currently compromise the safe use of eggs in the diet.

The present study is focused on OVA, the major protein in egg white. OVA is a globular phosphoglycoprotein, consisting on 385 aminoacids, with a molecular weight of 45 kDa [9,10]. OVA belongs

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to the serine proteinase inhibitor (serpin) superfamily, although has no protease inhibitory activity [2,11]. Even if OVA served as antigen model in many immunology studies, limited information regarding the effect of different environmental conditions on protein properties is available. Therefore, the aim of the present study was to investigate the impact of combined pH and thermal treatment on structural properties and antigenic character of chicken OVA. The experimental and *in silico* approaches were combined such as to provide a quantitative description on the relation between OVA structure under different pH-temperature processing conditions and the allergenic potential.

2. Materials and methods

2.1. Materials

Ovalbumin (purity over 90%, by agarose gel electrophoresis) and 8-anilinoanthracene-sulfonic acid (ANS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Unless otherwise stated, all other reagents were of analytical grade.

2.2. Heat treatment

Plastic tubes (1 cm diameter) were filled with 0.35 mL OVA solutions of 1 mg/mL concentration. In order to study the influence of pH on the structural properties of OVA, three different 20 mM Tris-HCl buffers of pH 4.5, 7.0 and 9.5 were used for preparing the protein solutions. The samples were heated at different temperatures ranging from 25 to 100 °C for 20 min using a thermostatic water bath (Digibath-2 BAD 4, Raypa Trade, Barcelona, Spain). The samples were afterwards cooled by placing the tubes in ice water to avoid further thermal denaturation of the protein.

2.3. Fluorescence spectroscopy

Fluorescence measurements were performed on a LS-55 luminescence spectrometer (Perkin Elmer Life Sciences, Shelton, CT, USA) equipped with the software Perkin Elmer FL Winlab. All measurements were made at 25 °C.

2.3.1. Intrinsic fluorescence spectra

A volume of 25 µL of (un)-thermally treated protein solution was suspended in 3.0 mL Tris buffer of appropriate pH. The excitation wavelength was subsequently set at 280 and 292 nm, while the emission spectra were collected from 310 nm to 400 nm, with increments of 0.5 nm. Both the excitation and emission slit widths were set at 10 nm.

2.3.2. Phase diagram

The *phase diagram* method described by Kuznetsova et al. [12] is a sensitive method suitable for detecting structural transformation of proteins. The *phase diagrams* were built as previously reported by Stănciuc et al. [13], by representing the I_{λ_1} versus I_{λ_2} dependence, where I_{λ_1} and I_{λ_2} are the spectral intensity values measured on wavelengths λ_1 and λ_2 .

2.3.3. Synchronous fluorescence spectra

The synchronous fluorescence spectra were recorded from 240 to 340 nm by scanning simultaneously the excitation and emission monochromator. In order to follow the individual spectroscopic behavior of Tyr and Trp residues, the wavelength interval ($\Delta\lambda$) was fixed at 15 and 60 nm, respectively. Appropriate blanks corresponding to the buffers used for sample preparation were subtracted to correct the fluorescence background.

2.3.4. ANS fluorescence intensity

The ANS binding studies were performed by incubating the thermally (un)-treated protein samples for 15 min at 25 °C in the dark with 10 µL of 8 mM ANS in 10 mM Tris-HCl buffer (pH 7.5). The fluorescence of ANS was excited at 365 nm and emission was collected between 400 and 660 nm. Assays in the absence and in the presence of the protein were performed in order to correct for unbound ANS emission fluorescence intensities.

2.3.5. Fluorescence quenching experiments

Fluorescence quenching experiments were performed with acrylamide and KI. Fresh 8 M acrylamide and 5 M KI solutions, prepared in 10 mM Tris-HCl buffer at pH 7.7, were used for the quenching experiments. Quenching titrations were performed by sequentially adding aliquots of the quencher stock solutions to the heat-treated samples and gently stirring. The excitation wavelength was set at 292 nm, and the fluorescence emission spectra were recorded from 310 to 420 nm. The fluorescence quenching data were analyzed by fitting to the Stern-Volmer equation as described by Ioniță et al. [14].

In case of nonlinear correlations the data were fitted to the modified Stern-Volmer equation (Eq. (1)):

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K_a [Q]} + \frac{1}{f_a} \quad (1)$$

where F_0 and F are the fluorescence intensities of OVA in the absence and presence of quencher, $[Q]$ is the concentration of the quenchers, f_a is the fraction of accessibility to the quencher, and K_a is the effective quenching constant for the accessible fluorophore.

In order to estimate the equilibrium between free and bound molecules, the Eq. (2) was fitted to calculate the binding constant (K_b), and the number of binding sites (n) between OVA and the quenchers [15]:

$$\log \frac{F_0 - F}{F_0} = \log K_b + n \log [Q] \quad (2)$$

where K_b and n were determined from the slope and intercept of double logarithm regression curve of $\log[(F_0 - F)/F]$ versus $\log[Q]$ based on Eq. (2).

Thermodynamic parameters were calculated at different temperatures using the van't Hoff equation (Eq. (3)):

$$\ln K_b = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

where K_b is the binding constant at the corresponding temperature, T is the experimental temperature, R is the gas constant. ΔH and ΔS are enthalpy and entropy change and were derived from the slope and intercept of the regression line obtained by plotting the natural logarithm of K_b versus $1/T$. Gibbs free energy (ΔG) of the reaction was calculated using the Eq. (4):

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

2.4. Enzyme-linked immunosorbent assay

The commercially available enzyme-linked immunosorbent assay (ELISA) for the rapid detection of egg white proteins Ridascreen FAST egg ELISA (Biopharm AG, Darmstadt, Germany) was used to detect OVA. Samples preparation was performed according to the specifications provided by the manufacturer (Biopharm AG, Darmstadt, Germany). The OVA detection through sandwich ELISA involved binding of the allergen to the specific antibodies which are immobilized on the wells of the microtiter plate, followed by detection by a second specific antibody conjugated to peroxidase. Finally the substrate was converted by the enzyme conjugate and after stopping the reaction with 1N H₂SO₄,

the absorbance was measured at 450 nm with a microplate spectrophotometer (Infinite 200 PRO NanoQuant, Tecan, Switzerland). Before measurements, each sample was appropriately diluted. Calibration was performed using the protein standard included in the assay.

The residual antigenicity after subjecting OVA solutions of pH 4.5, 7.0 and 9.5 to different thermal treatments, was expressed as percentage of allergen protein recognized by the specific antibodies with respect to the corresponding sample at 25 °C.

2.5. Molecular modelling

The atomic structure of ovalbumin, PDB ID: 1OVA [16], available in the RSCB Protein Data Bank was used to simulate the thermal induced behavior of the protein. Energy minimizations and molecular dynamics steps were performed with Gromos 43a1.ff force field and Gromacs 4.6.1 package [17]. All simulations were performed in parallelization conditions on an Intel(R) Core(TM)2 CPU 6300 1.86 GHz processor-based machine running Linux.

In order to be prepared for the heating tests, the protein model was centered in a rectangular box with periodic boundary condition, and was minimized first in vacuum and after solvation using Single Point Charge water molecules. No pH variation was considered in the *in silico* experiment.

The complex was then heated at 25 °C, 60 °C and 90 °C by using a weak coupling Berendsen thermostat [18] and finally was equilibrated such as to stabilize the protein in terms of temperature and energy oscillations. Three different configurations extracted from the last 100 ps of each equilibration trajectory were compared based on the C α atoms positions to ensure the statistical independence of the *in silico* results.

Detailed analysis of the protein models equilibrated at different temperatures was performed using dedicated Gromacs (Groningen University, Netherlands) [17] and PDBsum (The European Bioinformatics Institute, Cambridge, UK) tools [19].

3. Results and discussion

3.1. Intrinsic fluorescence spectra

The structural characterization of OVA was performed by collecting fluorescence spectra after the heat treating the protein in the temperature range of 25–100 °C at different pH values. The changes in protein fluorescence in different conditions are due to the environment dependent emission properties of Trp and Tyr residues, which give indications about protein tertiary structure alteration [20,21]. Therefore, spectra obtained under different experimental conditions will describe the contribution of these residues on the protein structural changes.

The intrinsic fluorescence of OVA was examined by exciting the protein solutions at 280 nm to follow the microenvironmental changes around Tyr and Trp residues, and at 292 nm to monitor the Trp residues (Fig. 1a and b).

The fluorescence of OVA depended both on pH and temperature values. When excited at 280 nm the highest value of the maximum fluorescence intensity of OVA at 25 °C was observed at pH 7.0, and decreased by 29% at pH 4.5 and by 36% at pH 9.5. Moreover, the wavelength corresponding to the maximum of fluorescence emission varied with the pH value as follows: 339 nm at pH 7.0, 340 nm at pH 4.5 and 342 nm at pH 9.5 (Fig. 1a). Xiong et al. [22] reported also a maximum emission at 338 nm for OVA at pH 7.0. The thermal treatment up to 80 °C caused the decrease of fluorescence intensity of the protein at pH 7.0, followed by an increase in fluorescence intensity at even higher temperatures. When heating the protein solution of pH 4.5, the fluorescence intensity increased

up to 80 °C, followed by a decrease at higher temperatures. A significant increase in fluorescence intensity at 60 °C was observed when the heat-treatment was performed at pH 9.5, followed by a decrease at higher temperatures. Xiong et al. [22] explained these variations of fluorescence as the result of different energy transfer efficiency between Trp and Tyr, but they also considered that the chromophores of OVA might become more exposed to solvent due to heat treatment. When compared to OVA incubated at 25 °C, at neutral pH, a 2 nm red-shift was registered when heating was performed at 80 °C, whereas at pH 4.5, a 2 nm blue-shift was obtained at 90 °C. A significant 8–9 nm red-shift was registered at temperatures higher than 80 °C when heating at pH 9.5. Heating promotes protein unfolding, in which hydrophobic aminoacids are exposed conferring an increase in protein surface hydrophobicity [23,24]. The changes in the tertiary structure of OVA at thermal treatment varied with the pH value; the partial unfolding was observed at pH 7.0 and 9.5, whereas at pH 4.5 protein folding and aggregation were the most significant events highlighted by the fluorescence measurements.

In Fig. 1(b) shows the influence of the pH values on the intrinsic fluorescence spectra obtained when exciting the OVA solutions at 292 nm. The intensity of OVA fluorescence at 25 °C decreased with the pH values. In agreement with the observations of Wang et al. [25], a detailed check of the crystallographic model used for carrying out molecular dynamics simulation indicated the OVA contains three Trp residues: Trp¹⁴⁸ in helix F, Trp¹⁸⁴ as the nearest neighbor residue of the COOH terminus of strand 3A, and Trp²⁶⁷ in helix H. In the native protein structure, Trp¹⁴⁸ residue is partially exposed to the solvent. Anyway, about 88.65% of the solvent accessible surface of 47.39 Å² gets buried in monomers interaction. In agreement with Wright et al. [26], Trp¹⁸⁴ and Trp²⁶⁷ residues are located toward in the interior of OVA molecule, having solvent accessible surface of 7.34 Å² and 7.84 Å², respectively with no evident burial tendency when protein aggregates are formed. Upon heat treatment, variations in protein emission fluorescence intensity usually consist on shifts in the wavelength that corresponds to the maximum fluorescence intensity. The wavelength corresponding to the maximum of fluorescence emission was 335 nm for OVA at pH values of 7.0 and 9.5, and 333 nm at pH 4.5. The thermal treatment of the protein at pH 7.0 did not produce significant changes in $\lambda_{\max}$ up to 70 °C, indicating that the microenvironment of Trp residues is not substantially altered. Further protein heating up 80 °C and 90 °C caused a 3 nm blue-shift, followed by a 3 nm red-shift at even higher temperature, indicating slight exposure of the Trp residues to a more polar environment. Moreover, the temperature increase up to 80 °C caused the fluorescence intensity decrease, followed by an increase at higher temperatures. Sponton et al. [27] explained the decrease of OVA fluorescence when heating at 70 and 80 °C for 10 and 5 min by the initiation of the protein aggregation, involving new hydrophobic interactions and burial of the Trp with the polypeptides core.

When testing the behavior of OVA at pH 4.5, heating caused a small 3 nm blue-shift in $\lambda_{\max}$ over the whole temperature range studied, and fluorescence intensity decrease at temperatures over 70 °C. Significant 11–15 nm red-shifts in $\lambda_{\max}$ were registered in the temperature range of 60–100 °C, suggesting a more flexible conformation of OVA at pH 9.5. In addition an increase in fluorescence intensity at 60 °C, followed by a significant decrease at higher temperatures was also registered. Hu and Du [9] suggested that heat or alkali treatment can trigger significant structural transformation of α -helix to β -sheet of OVA.

3.2. Phase diagram

It is known that globular proteins may exist in at least four different conformations: native (ordered), molten globule, pre-

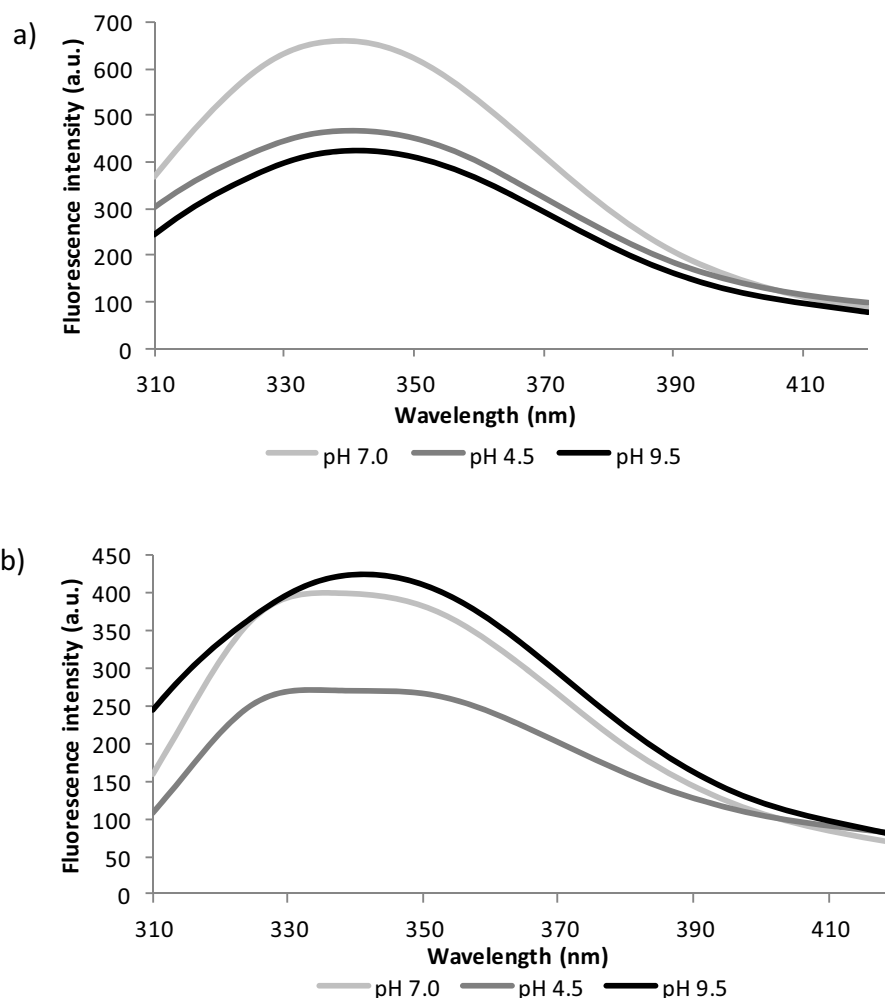


Fig. 1. pH-induced structural changes of OVA monitored by emission spectrum. The excitation wavelength was 280 nm (a) and 292 nm (b) and the spectrums were collected between 310 and 420 nm. Three independent tests were carried out in each case and SD was lower than 3.5%.

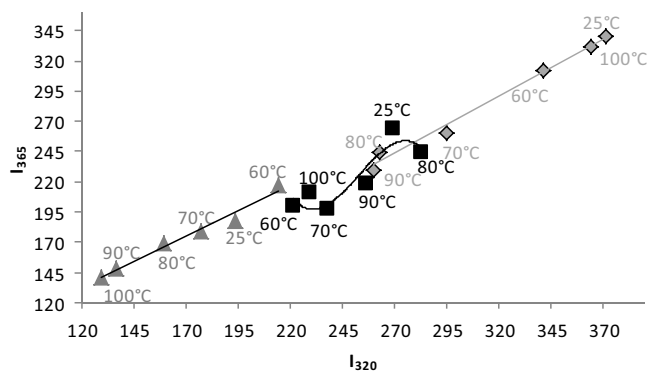


Fig. 2. Phase diagram analysis of heat-induced conformational changes of OVA at pH 7.0 (grey diamonds), pH 4.5 (black squares) and pH 9.5 (grey triangles) based on intrinsic fluorescence intensity values measured at wavelengths 320 and 365 nm. The temperature values are indicated in the vicinity of the corresponding symbol. Three independent tests were carried out in each case and SD was lower than 2.5%.

molten globule, and unfolded [28]. These protein conformations may be easily discriminated using a multiparametric approach, as described by Kuznetsova et al. [12]. The *phase diagram* describing the heat-induced structural changes of OVA at different pH is given in Fig. 2. When plotting I_{320} versus I_{365} , the linear correlation suggests an *all-or none transition*, whereas the non-linearity of this function reflects the sequential character of structural transforma-

tions [29]. As it can be seen from Fig. 2, at pH 7.0 and 9.5, the *phase diagrams* are linear, indicating the presence of two structurally distinct species of OVA in the temperature range 25–100 °C. On the other hand, when heating OVA solutions with pH 4.5, a non-linear correlation was observed, indicating sequential transition between different conformations.

Given the values of fluorescence intensity, it seems that thermal treatment in the temperature range of 25–90 °C for pH 7.0, and between 25 and 80 °C for pH 4.5, favors the protein folding. This phenomenon is driven by molecular interactions between exposed hydrophobic patches and disulphide bonds formation [30]. Heating at higher temperatures promotes protein unfolding, when hydrophobic aminoacids are partially exposed to the solvent, generating the increase of protein surface hydrophobicity [23,27]. When heating the protein at pH 9.5, the unfolding events were observed up to 60 °C, followed by polypeptide chains folding at higher temperatures most probably on a different pattern with respect to the native protein structure.

The physicochemical properties of the protein dictates the interaction with the solvent as well as contacts established within and between polypeptides [31]. Changes in pH affect the charge distribution on protein surface and subsequently the attraction between side chains of the amino acids, which are essential for defining the molecule shape and stability. The salt bridges and hydrogen bonding are mainly perturbed by the variation of the pH value. When raising the pH from 7.0 to 9.5, the positively charged amino acids

turn neutral therefore decreasing the ionic attraction. Although the strength of the noncovalent interactions is rather low compared to the covalent bonds, these atomic events might cause the partial loss of the protein conformation especially in the areas where the salt bridges are involved in the stabilization of the OVA structure. Moreover, the repulsion among OVA molecules occurs at high pH value, as a consequence of the excess of negative charges, preventing protein aggregation [31]. Concerning the S-OVA, a heat stable form of the protein formed at elevated pH values both in developing fertilized or stored unfertilized eggs and *in vitro* conditions, Yamasaki et al. [11] reported that, except for the significant motion of the loop preceding the strand 1A, the overall structure resemble that of native OVA. Due to conformational changes of the side chains, important alteration of the solvent accessibility to the hydrophobic core has been also reported, suggesting the improvement of the thermodynamics and structural stability. On the other hand, Hu and Du [9] suggested that when subjecting the OVA to alkali, an important increase of the β -sheet content was obtained.

The salt bridge formation is impeded with pH decrease, when the negatively charged amino acids convert to neutral. In particular, at pH 4.5 which is the isoelectric point of OVA, the protein has zero liquid charge and protein-protein interactions are favored along with the reduction of the electrostatic force acting between water and protein [31]. Hu and Du [9] studied the effect of heat and pH on OVA behavior, and they report structural transformations of the protein associated with aggregation events through the cross- β -sheet-dominant link of the interfaces.

3.3. Synchronous fluorescence spectra

Compared to the conventional fluorescence spectroscopy, the synchronous fluorescence spectroscopy offers the advantage of spectral simplification, light scattering reduction, and selectivity improvement, and plays an important role in the simultaneous analysis of multicomponent samples [32]. Vekshin [33] suggested that synchronous fluorescence spectroscopy is a very useful method for studying amino acid residues microenvironment due to shifts in $\lambda_{\max}$, corresponding to the changes of the polarity around the chromophores molecule. Figs. 3 and 4 shows the synchronous fluorescence spectroscopy spectra for Trp residues at $\Delta\lambda$ of 60 nm, and for Tyr residues at $\Delta\lambda$ of 15 nm.

When heating the OVA sample of pH 7.0, a decrease in fluorescence intensity was observed up to 80 °C, followed by an increase at higher temperature (Fig. 3a). At pH 4.5, the fluorescence intensity of OVA decreased in the whole tested temperature range studied, except for 80 °C (Fig. 3b). On the other hand, when testing thermal behavior of the protein at pH 9.5, a significant decrease in fluorescence intensity was observed up to 90 °C, followed by an increase at 100 °C (Fig. 3c). Regardless of the pH value and thermal treatment applied to OVA, the maximum fluorescence intensity was obtained at constant $\lambda_{\max}$ of 277 nm, indicating no influence of the environmental conditions on the polarity of Trp residues (Fig. 3a–c).

Further examination of OVA behavior based on Tyr residues showed that heating the protein solution of pH 7.0 caused a significant 8 nm blue-shift in $\lambda_{\max}$ at 100 °C (Fig. 4a). As indicated by the small 2–3 nm red-shift OVA, unfolding occurred in the temperature range of 70–90 °C at acidic pH, followed by folding events at even higher temperature when a blue-shift of 2 nm was observed (Fig. 4b). A completely different behavior of the protein was observed at alkaline pH value. The heat treatment of OVA up to 90 °C caused a blue-shift of the maximum fluorescence intensity of 6 nm, followed by a red-shift of 4 nm at 100 °C (Fig. 4c).

Although the polarity around the Trp residues was well conserved, the synchronous fluorescence spectra following particularities of the Tyr residues microenvironment indicate the occurrence of important folding and unfolding events depending

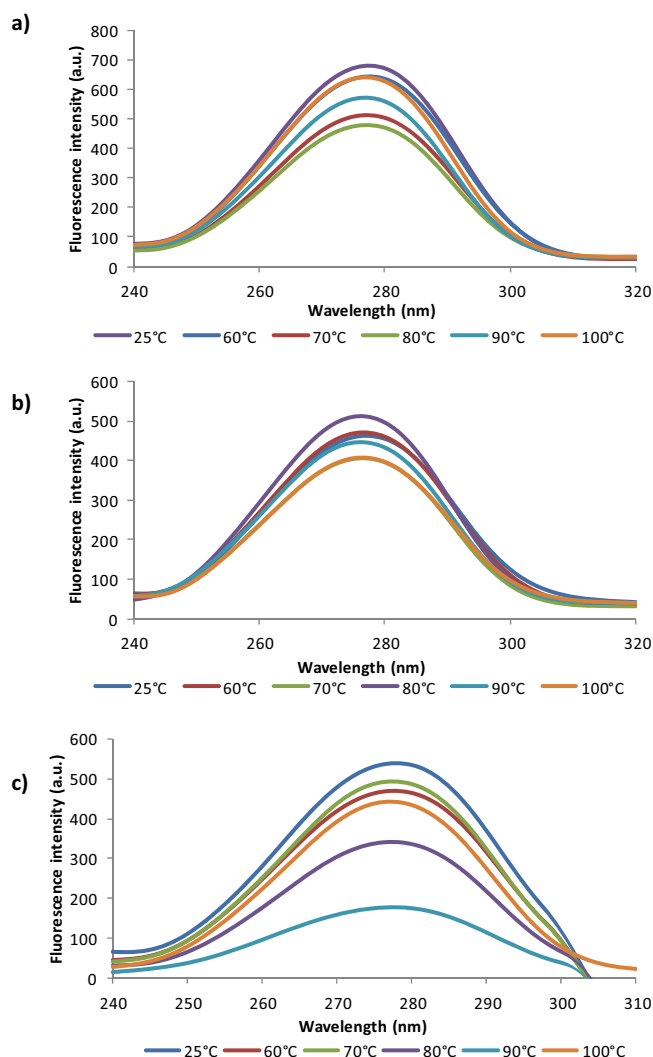


Fig. 3. Synchronous fluorescence spectra of OVA at $\Delta\lambda = 60$ nm after heat-treatment at different temperature: (a) pH 7.0, (b) pH 4.5 and (c) pH 9.5. Three independent tests were carried out in each case and SD was lower than 2.5%.

of the pH and temperature condition. Based on our results, it can be stated that the polarity around the Trp residues was conserved, while the polarity around the Tyr residues changed with an increase and sequential decrease in hydrophobicity.

3.4. ANS fluorescence intensity

Extensive literature indicates ANS as an efficient fluorescent probe for investigating the existence of folding intermediates and characterization of protein binding sites. The restricted mobility of ANS as a consequence of binding to the hydrophobic patches of protein exposed to the solvent is indicated by blue shifts of the fluorescence emission maxima and the increase of fluorescence intensity [34]. The thermal induced changes in fluorescence spectra of OVA-ANS at tested pH values are given in Fig. 5. It has been reported that $\lambda_{\max}$ for free ANS is 510 nm, whereas the wavelength specific to the ANS bound to protein is 470 nm [35]. The wavelength corresponding to the maximum fluorescence intensity emission in case of the protein solutions incubated at room temperature varied with the pH values; $\lambda_{\max}$ was 515 nm at pH 7.0, 518 nm at pH 4.5 and 522 nm at pH 9.5. Binding of ANS to the OVA in such a way that the $-\text{NH}$ group of the fluorophore is located near a positively charged amino acid induces a blue shift in $\lambda_{\max}$ due to the changes

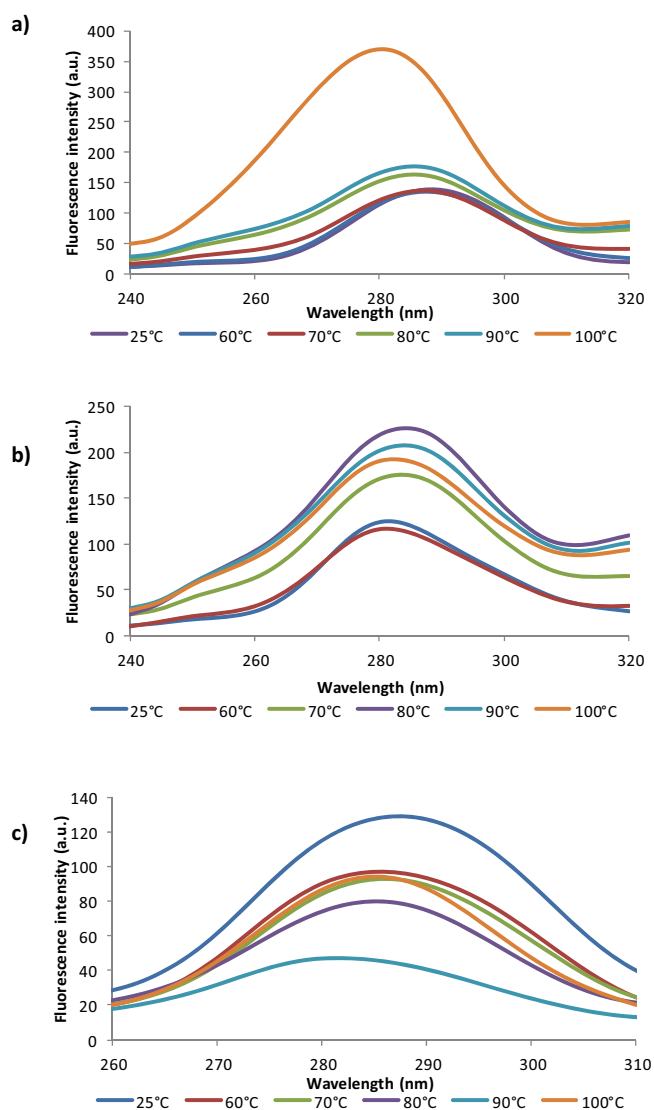


Fig. 4. Synchronous fluorescence spectra of OVA at $\Delta\lambda = 15$ nm after heat-treatment at different temperature: (a) pH 7.0, (b) pH 4.5 and (c) pH 9.5. Three independent tests were carried out in each case and SD was lower than 2.5%.

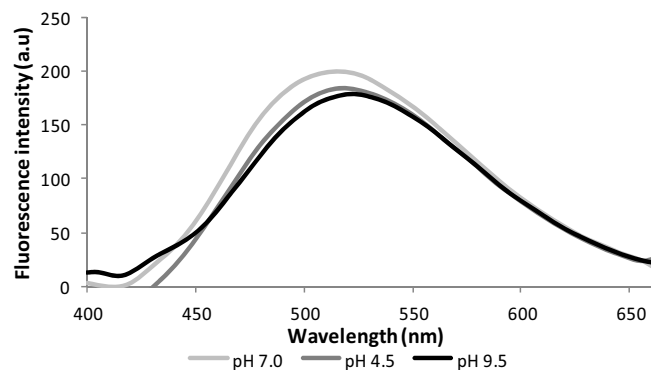


Fig. 5. pH-induced structural changes of OVA monitored by ANS fluorescence intensity. The excitation wavelength was 356 nm and the spectra were collected between 400 and 660 nm. Three independent tests were carried out in each case and SD was lower than 3.5%.

in the intramolecular charge transfer process. The heat treatment of OVA caused the overall increase in ANS fluorescence intensity, with a maximum value recorded at 90 °C. Sequential red- and blue-shifts were found when the heat treatment was performed at pH 7.0. Compared to the protein tempered at 25 °C, a 8 nm red-shift was registered by heating at 60 °C, followed by 18 nm blue-shift at 80 °C and 7 and 15 nm red-shifts at 90 and at 100 °C, respectively. Charge redistribution on protein surface at pH values of 4.5 and 9.5 caused important changes in the events governing the thermal induced behavior of the protein. In case of OVA samples of pH 4.5 and 9.5, large initial blue-shifts were registered when heating up to 80 °C (a 25 nm blue-shift registered by heating the protein solutions up to 60 °C, followed by 16–17 nm blue-shift at 70–80 °C) and 90 °C (blue-shifts of 10 nm), respectively, followed by red-shifts (of 5–9 nm for pH 4.5 and of 5 nm for pH 9.5) at even higher temperatures. In agreement to Sharma and Kishore [36], ANS has higher affinity for partially unfolded state compared to native and denatured states of the protein. It can be concluded that the thermal denaturation mechanism of OVA highly depends on acidity of the solution, and involves unfolding of polypeptides chains at pH 7.0 and 4.5 up to 60 °C, and folding at temperatures up to 80 °C. When heating the OVA solution of pH 9.5, the polypeptide chain folding was favored up to 90 °C, followed by limited unfolding at 100 °C. At higher temperatures some hydrophobic and other aminoacids buried within the core of OVA molecule become exposed, thus favoring the molecular aggregation.

3.5. Quenching experiments

Quenching of Trp fluorescence by adding small solutes has been widely employed in structural studies because can provide useful information on the exposure of Trp residues to the aqueous environment, and on the permeability of proteins to solutes of various molecular size [37]. In order to monitor the Trp microenvironment from OVA, two different quenching experiments were employed in our study, namely by using a polar neutral (acrylamide) and an anionic (iodide) quencher. Acrylamide can quench the fluorescence of the Trp residues exposed on protein surface or partially buried, but not those buried in the hydrophobic core of a protein. Moreover, quenching with KI was employed to provide additional information regarding the accessibility of the Trp residues.

When quenching was performed at pH 7.0, the values of the quenching constant for the untreated protein were $2.81 \pm 0.08 \times 10^{-3} \text{ mol}^{-1} \text{ L}$ for acrylamide, and $2.97 \pm 0.10 \times 10^{-3} \text{ mol}^{-1} \text{ L}$ for KI (Table 1). In case of quenching with acrylamide the heat treatment up at 90 °C produced the decrease of K_{SV} values, suggesting Trp residues distancing from the quenchers, compared to the structures tempered at 25 °C. The increase of the acrylamide K_{SV} value at 100 °C is an indicative of the local structural rearrangements and partial additional exposure of Trp residues within the protein molecules. On the

Table 1

Stern Volmer quenching constant (K_{SV}) of the heat treated OVA at different pH and temperatures.

T(°C)	$K_{SV} (10^{-3} \text{ L mol}^{-1})$					
	pH 7.0		pH 4.5		pH 9.5	
	Acrylamide	KI	Acrylamide	KI	Acrylamide	
25	2.81 ± 0.08^a	2.97 ± 0.10	1.32 ± 0.0	0.61 ± 0.11	3.27 ± 0.09	
60	2.83 ± 0.21	5.22 ± 0.09	1.12 ± 0.19	1.23 ± 0.12	3.56 ± 0.44	
70	2.29 ± 0.07	3.77 ± 0.05	1.31 ± 0.46	1.66 ± 0.35	3.22 ± 0.09	
80	2.40 ± 0.33	1.29 ± 0.08	1.76 ± 0.65	1.50 ± 0.14	3.28 ± 0.15	
90	2.27 ± 0.12	0.88 ± 0.02	1.50 ± 0.48	0.98 ± 0.08	1.95 ± 0.05	
100	2.91 ± 0.14	1.25 ± 0.11	1.49 ± 0.33	1.24 ± 0.23	1.99 ± 0.47	

^a Standard deviation.

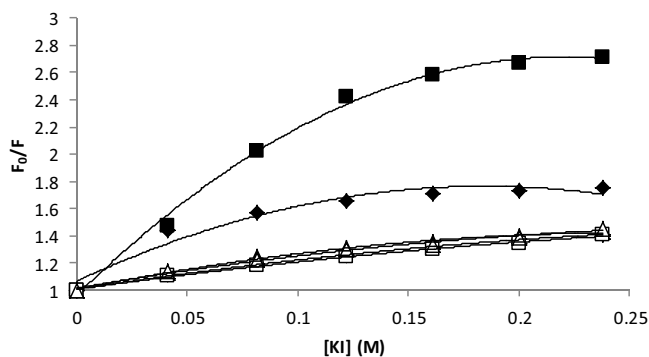


Fig. 6. The Stern – Volmer plots for the quenching of OVA with KI at pH 9.5 at different temperatures: (◆) 25 °C, (■) 60 °C, (▲) 70 °C, (◇) 80 °C, (□) 90 °C, (○) 100 °C.

other hand, higher KI quenching constants were obtained for OVA treated at 60 °C (1.76 times higher) and 70 °C (1.26 times higher). At even higher temperatures, the KI quenching constant significantly decreased (Table 1), indicating that the accessibility of Trp is affected by the heat induced protein folding. When static quenching is prevalent, the Stern-Volmer constant decreases as function of temperature, as the decreased collision frequency is responsible for the static quenching mechanism.

A more rigid conformation was evidenced at pH 4.5, K_{SV} values being significantly lower when compared with the corresponding values at pH 7.0. When performing quenching experiments with acrylamide, the highest values of the quenching constants were measured at 80 °C ($1.76 \pm 0.65 \times 10^{-3} \text{ mol}^{-1} \text{ L}$), whereas the lowest values were calculated for the quenching experiments performed after heat treatment at 60 °C ($1.12 \pm 0.19 \times 10^{-3} \text{ mol}^{-1} \text{ L}$). However, no significant differences in the protein behavior can be considered because of the variations within standard deviation values.

Koseki et al. [38] suggested that the globular conformation and secondary structure of OVA are almost the same in acid as at neutral pH. However, the side-chains of the molecule (or at least part of the chains) are more flexible at low pH than in neutral conditions, and the whole molecule is very susceptible to denaturation.

When performing the quenching experiments on OVA at pH 9.5, no significant differences in the acrylamide quenching constant values were observed between 25 and 80 °C, whereas a significant decrease (~40%) was obtained at higher temperature (Table 1).

Lakowicz [39] explained that the fluorescence quenching mechanisms highly depend on temperature, and are usually classified as dynamic quenching or static quenching. In the dynamic quenching process the fluorophore is in contact with the quencher during the lifetime of the excited state, whereas in static quenching the quencher and fluorophore molecules form a complex in the ground state [40]. A high linearity was obtained in the whole temperature range indicating that quenching of OVA fluorescence emission by acrylamide and KI is static. However, in case of quenching with KI at pH 9.5, a positive deviation from linearity was observed (Fig. 6), most probably due to the combination of static and dynamic quenching mechanisms [41]. Therefore, the experimental data were fitted to equation 1. From Table 2 it can be noticed that K_a values presented a high sensitivity to temperature. Thus, K_a values increases from $1.96 \pm 0.15 \times 10^{-3} \text{ mol}^{-1} \text{ L}$ at 25 °C to $6.13 \pm 0.58 \times 10^{-3} \text{ mol}^{-1} \text{ L}$ at 60 °C, suggesting that in this temperature range the quenching process is dynamic. Further temperature increase up to 70 °C caused the K_a decrease to $1.31 \pm 0.60 \times 10^{-3} \text{ mol}^{-1} \text{ L}$, suggesting that the static quenching mechanism is predominant. Higher temperatures led to higher energy and stronger collision, with consequences on the K_a values, which were found to increase [42].

Table 2

The effective quenching constant for the accessible fluorophore (K_a) and the fraction of accessibility to the quencher (f_a) of the heat treated OVA at pH 9.5 and different temperatures.

T (°C)	K_a ($10^{-3} \text{ L mol}^{-1}$)	f_a
25	1.96 ± 0.15^a	0.41 ± 0.11
60	6.13 ± 0.58	1.00 ± 0.42
70	1.31 ± 0.60	0.73 ± 0.19
80	3.16 ± 0.52	0.45 ± 0.01
90	4.00 ± 0.96	0.36 ± 0.05
100	4.47 ± 0.88	0.52 ± 0.04

^a Standard deviation.

In general, the interaction between quenchers and proteins can take several forms, i.e., electrostatic interactions, hydrophobic attraction, hydrogen bond formation, van der Waals attraction, and even covalent binding [43]. The temperature dependence of the binding constant (K_b) provided information on the sign and magnitude of the enthalpy change (ΔH), entropy change (ΔS) and change in free energy (ΔG) of the OVA upon addition of acrylamide and KI (Table 3). For quenching with acrylamide at pH 7.0 in the temperature range of 60–80 °C, the positive ΔH indicated that the interaction between acrylamide and OVA is endothermic, whereas at higher temperature the interaction was exothermic. For acrylamide, in the lower temperature range, ΔG was negative suggesting that binding occurred spontaneously. The positive entropy value (ΔS) between 60 and 80 °C suggested that the solvent surrounding molecules arranged themselves in a less orderly fashion when the interaction took place. The values for ΔH and ΔS suggested that the interaction between acrylamide and OVA at pH 7.0 is determined by electrostatic interactions. At higher temperature, the interaction seemed to be unfavorable from a thermodynamic point of view due to the folding, which normally promotes more ordered configuration of the molecules.

Based on thermodynamic parameters presented in Table 3(a) the interaction between OVA and acrylamide between 70 and 100 °C at pH 4.5 is also unfavorable due to folding of polypeptide chains. When the heat treatment was performed on protein solution of pH 9.5, the interaction between OVA and acrylamide was exothermic in the whole studied temperature range. The negative value of ΔH , correlated with positive value of ΔS suggested that the contacts established were mainly electrostatic, whereas the negative value of ΔG was an indicative of the spontaneous nature of the interaction.

The same trend was observed in case of quenching with KI, except for the sample at pH 7.0, when the quenching was favored at temperatures higher than 70 °C, the interaction being mainly driven by the electrostatic forces acting between the two types of compounds.

3.6. pH and heat induced changes in OVA antigenicity

ELISA, which is an easy-to-use and rapid analytical technique widely used for the detection of allergens in food, was further employed to quantify the potential of pH and thermally treated OVA to bind to the specific antibodies.

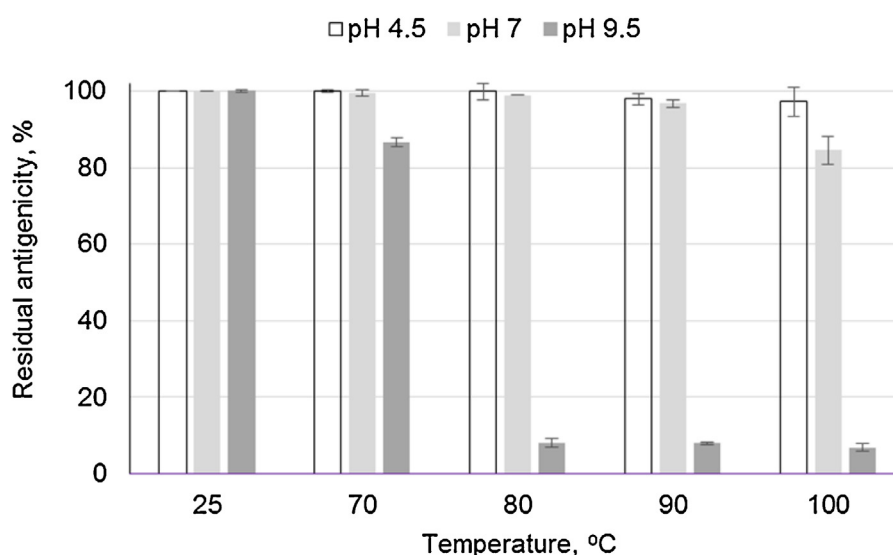
Analyzing the results presented in Fig. 7 one can see that heat treatment up to 100 °C of OVA at pH 4.5 and 7.0 had no significant influence on the capacity of the antibodies to recognize the protein. These results suggest that even at high temperatures the OVA preserves the immunoreactive epitopes. In case of the heat treatment at 100 °C, the residual antigenicity of OVA samples is $84.56 \pm 3.66\%$ at pH 7.0 and $95.37 \pm 3.89\%$ at pH 4.5. Anyway, the overestimation of the antigenicity should be taken into account, because of the contamination of the commercially purified OVA by ovomucoid [1]. Due to important modification of protein conformation at alkaline

Table 3

The thermodynamic parameters for the association of heat treated OVA with acrylamide (a) and KI (b).

a)									
T(K)	pH 7.0			pH 4.5			pH 9.5		
	ΔH (KJ mol ⁻¹)	ΔS (J mol ⁻¹ K ⁻¹)	ΔG (KJ mol ⁻¹)	ΔH (KJ mol ⁻¹)	ΔS (J mol ⁻¹ K ⁻¹)	ΔG (KJ mol ⁻¹)	ΔH (J mol ⁻¹)	ΔS (J mol ⁻¹ K ⁻¹)	ΔG (KJ mol ⁻¹)
298			-12.72 ± 0.95						-42.89 ± 1.58
333			-14.12 ± 0.78			43.66 ± 1.23			-46.32 ± 1.47
343	-11.45 ± 3.75 ^a	39.93 ± 0.90 ^a	-14.52 ± 0.68	23.82 ± 1.33 ^c	-59.56 ± 0.30 ^c	44.25 ± 2.63	-13.72 ± 0.38	9.79 ± 0.01	-47.30 ± 1.89
353	16.62 ± 4.29 ^b	-39.57 ± 0.10 ^b	19.51 ± 0.74			44.85 ± 2.98			-48.28 ± 2.01
363			19.91 ± 1.25						-49.26 ± 1.25
373			20.30 ± 1.36						-50.24 ± 2.13

b)									
T(K)	pH 7.0			pH 4.5			pH 9.5		
	ΔH (KJ mol ⁻¹)	ΔS (J mol ⁻¹ K ⁻¹)	ΔG (KJ mol ⁻¹)	ΔH (KJ mol ⁻¹)	ΔS (J mol ⁻¹ K ⁻¹)	ΔG (KJ mol ⁻¹)	ΔH (J mol ⁻¹)	ΔS (J mol ⁻¹ K ⁻¹)	ΔG (KJ mol ⁻¹)
298			82.48 ± 3.56						-12.40 ± 1.07
333			88.64 ± 2.56			20.94 ± 0.78			-13.16 ± 0.48
343	80.82 ± 3.04 ^d	-17.58 ± 0.01 ^d	-13.52 ± 1.24	11.52 ± 3.10 ^f	-28.30 ± 0.08 ^f	21.23 ± 0.95	-59.60 ± 0.45	21.93 ± 0.01	-13.38 ± 1.74
353	-10.69 ± 1.66 ^e	36.46 ± 0.03 ^e	-13.88 ± 1.45			21.51 ± 0.74			-13.59 ± 0.87
363			-14.24 ± 0.98			21.79 ± 0.63			-13.81 ± 0.89
373			-14.61 ± 1.02						-14.03 ± 1.05

^a Temperature range 60–80 °C.^b Temperature range 80–100 °C.^c Temperature range 60–80 °C.^d Temperature range 25–70 °C.^e Temperature range 70–100 °C.^f Temperature range 60–90 °C.**Fig. 7.** Residual antigenicity of OVA solutions at pH 4.5, 7.0 and 9.5 after thermal treatment.

pH, a significant decrease of over 82% of the residual antigenicity of OVA was observed for temperatures higher than 80 °C (Fig. 7).

When subjecting the protein to denaturing agents, the conformational epitopes are prone to changes or even destruction with significant alteration of the clinical importance, whereas the linear epitopes are usually rather well conserved. On the other hand, the thermal processing in different environmental condition might even favor the formation of new conformational epitopes. The recovery in case of the egg-allergic children appears to be more difficult when developing IgE antibodies against linear epitopes compared to cases when the IgE antibodies recognized conformational epitopes [5,44,45]. Moreover, there are some reports on the existence of egg-allergic individuals who tolerate thermally treated eggs but react to raw eggs [8,46].

3.7. *In silico* characterization of IgE epitopes of OVA

The *in silico* approach was used to gather information on the atomic events responsible for the thermal induced behavior of OVA. The protein model was minimized, solvated and equilibrated at 25, 60 and 90 °C, temperatures indicated by the experimental techniques as being relevant for the thermal behavior of OVA. Detailed analysis of OVA at single molecule level allowed establishing the motifs of the secondary structure responsible for protein behavior at tested temperatures. Regardless of the simulated temperature, at neutral pH there is an equilibrium in terms of contents of amino acids involved in helices and strands based secondary structure elements. Although the root means square deviation obtained by superimposing the backbones of OVA models equilibrated at 25 and

Table 4
Secondary structure and surface descriptors of OVA under thermal treatment.

Descriptors	Temperature, °C		
	25	60	90
Structure descriptors			
Strands, %	28.2	29.8	28.3
α helix, %	28.9	28.5	28.5
3–10 helix, %	1.0	0.2	0.4
H bonds within the protein	1132 $\pm$ 15	1134 $\pm$ 17	1119 $\pm$ 16
H bonds between protein and water	2528 $\pm$ 24	2338 $\pm$ 29	2255 $\pm$ 29
Surface descriptors			
Total protein surface, nm ²	173.54 $\pm$ 1.69	171.91 $\pm$ 1.77	173.43 $\pm$ 1.97
Hydrophobic surface, nm ²	88.43 $\pm$ 1.30	88.84 $\pm$ 1.23	89.15 $\pm$ 1.48
Volume, nm ³	63.78 $\pm$ 2.37	64.13 $\pm$ 2.16	63.92 $\pm$ 2.42

90 °C was 2.03 Å, no important variation of strands and helical contents could be observed (Table 4). Anyway, in agreement with the observations of Hu and Du [9], the thermal treatment caused the increase of the total number of β -sheets from 11 to 15.

The protein models equilibrated at different temperatures were further used for identifying the main changes induced in the conformation of the IgE-binding epitopes. A complete fine mapping of the IgE-binding epitopes of chicken egg OVA molecule was reported by Mine and Rupa [10] after performing in human study involving egg-allergic patients. They provided indication regarding the existence of five different immunodominant IgE allergenic epitopes, namely Leu³⁸-Thr⁴⁹, Asp⁹⁵-Ala¹⁰², Glu¹⁹¹-Val²⁰⁰, Val²⁴³-Glu²⁴⁸ and Gly²⁵¹-Asn²⁶⁰. The sequence of amino acids in the first four epitopes appears to be critical, as substitution of a single amino acid (except for Ala¹⁰²) led to the complete loss of IgE binding activity. In case of the ten amino acids long Gly²⁵¹-Asn²⁶⁰ epitope, the substitution of only three residues (Gly²⁵¹-Glu²⁵³) caused the alteration of the IgE binding [10]. These epitopes are located in hydrophobic regions of the proteins. In fact, out of the 46 amino acids of OVA epitopes, over 54% of the residues are hydrophobic, whereas the rest are polar (11) and charged residues (10). In agreement with Mine and Rupa [10] the close analysis of the models equilibrated at different temperatures indicated that, except for the Glu¹⁹¹-Val²⁰⁰, all other epitopes are located on protein surface and mainly consist on strand and turn motifs. Only small changes in the conformation of the OVA epitopes were observed when increasing the temperature up to 90 °C. The structure of the Leu³⁸-Thr⁴⁹ epitope, consisting on a pair of α -helix (Pro³¹-Tyr⁴² and Asp⁴⁷-Val⁵⁷) connected through the Tyr⁴²-Ala⁴⁵ β -turn, was well conserved at thermal treatment. Although the total number of hydrogen bonds involving amino acids of the Asp⁹⁵-Ala¹⁰² epitope decrease from 17 to 12 when heating the protein from 25 °C to 90 °C, its overall conformation, containing a mixture of beta turn and beta-alpha-beta motifs, was not affected. The most important changes were observed in case of the Glu¹⁹¹-Val²⁰⁰ epitope, where the hydrogen bonding pattern varied significantly with the temperature. The increase of both hydrogen bond donors and acceptors caused the loss of the G helical organization of Asp¹⁹⁰-Asp¹⁹² sequence, which suffered essential repositioning of the side-chains. Finally, the beta hairpin conformation of the Val²⁴³-Glu²⁴⁸ epitope and the mixture of helix and beta turn of the Gly²⁵¹-Asn²⁶⁰ epitope appeared to be stable against temperature. Based on the sequence of amino acids of OVA epitopes, the highest instability is expected in case of Gly²⁵¹-Asn²⁶⁰ sequence which contained two glutamic acid residues prone to be involved in establishing salt bridges that could stabilize unfavorable folded conformations. On the other hand, due to the existence of the positively charged Lys and Glu residues in two different epitopes of OVA, significant conformational changes are expected at alkaline pH due to the decrease of ionic attraction, therefore explaining the significant reduction of the antigenicity obtained at pH 9.5 and

temperatures over 80 °C. This detailed characterization of the IgE-binding epitopes of OVA in different environmental condition is crucial because they play a critical role in the aetiology of allergic disease [10].

4. Conclusions

The fluorescence measurements indicated important folding and/or aggregation events when heat treating the OVA solution of pH 4.5, whereas the thermally induced changes in the tertiary structure of OVA at pH 7.0 and 9.5 mainly consists on partial unfolding. Although the fluorescence measurement suggested the alteration of OVA conformation at high temperatures, the protein at pH values of 7.0 and 4.5 retains most of the allergenic epitopes even at 100 °C. Low levels of residual allergenic components were observed in case of heating at temperatures higher than 80 °C the OVA at alkaline pH value. Further tests will be performed to determine the influence of pH and thermal treatment on the resistance of ovalbumin to proteolysis by digestive enzymes such as to estimate the changes in overall allergenicity of the protein.

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References

- [1] Y. Mine, J.W. Zhang, Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins, *J. Agric. Food Chem.* 50 (2002) 2679–2683.
- [2] Y. Mine, M. Yang, Recent advances in the understanding of egg allergens: basic, industrial, and clinical perspectives, *J. Agric. Food Chem.* 56 (2008) 4874–4900.
- [3] B. Kerkaert, F. Mestdagh, B. De Meulenaer, Detection of hen's egg white lysozyme in food: comparison between a sensitive HPLC and a commercial ELISA method, *Food Chem.* 120 (2010) 580–584.
- [4] S. Baumgartner, P. Schubert-Ullrich, Egg allergens, in: L. Jedrychowski, H.J. Wichers (Eds.), *Chemical and Biological Properties of Food Allergens*, CRC Press, 2009, pp. 213–222.
- [5] EFSA Panel on Dietetic Products, Nutrition and Allergies, Scientific opinion on the evaluation of allergenic foods and food ingredients for labelling purposes, *EFSA J.* 12 (2014) 3894.
- [6] A. Urisu, H. Ando, Y. Morita, E. Wada, T. Yasaki, K. Yamada, K. Komada, S. Torii, M. Goto, T. Wakamatsu, Allergenic activity of heated and ovomucoid-depleted egg white, *J. Allergy Clin. Immunol.* 100 (1997) 171–176.
- [7] P.A. Eigenmann, Anaphylactic reactions to raw eggs after negative challenges with cooked eggs, *J. Allergy Clin. Immunol.* 105 (2000) 587–588.
- [8] A.S. Kemp, Egg allergy, *Pediatr. Allergy. Immunol.* 18 (2007) 696–702.
- [9] H.Y. Hu, H.N. Du, α -to- β Structural transformation of ovalbumin: heat and pH effects, *J. Protein Chem.* 19 (2000) 177–183.
- [10] Y. Mine, P. Rupa, Fine mapping and structural analysis of immunodominant IgE allergenic epitopes in chicken egg ovalbumin, *Protein Eng.* 16 (2003) 747–752.
- [11] M. Yamasaki, N. Takahashi, M. Hirose, Crystal structure of S-ovalbumin as a non-loop-inserted thermostabilized serpin form, *J. Biol. Chem.* 278 (2003) 35524–35530.
- [12] M. Kuznetsova, K.K. Turoverov, V.N. Uversky, Use of the phase diagram method to analyze the protein unfolding–refolding reactions: fishing out the invisible intermediates, *J. Proteome Res.* 3 (2004) 485–494.
- [13] N. Stănciuc, I. Aprodu, G. Răpeanu, G. Bahrim, Fluorescence spectroscopy and molecular modelling investigations on the thermally induced structural changes of bovine β -lactoglobulin, *Innovative Food Sci. Emerg. Technol.* 15 (2012) 50–56.
- [14] E. Ioniță, N. Stănciuc, I. Aprodu, G. Răpeanu, G. Bahrim, pH-induced structural changes of tyrosinase from *Agaricus bisporus* using fluorescence and *in silico* methods, *J. Sci. Food. Agric.* 94 (2014) 338–344.
- [15] D. Li, T. Zhang, B. Ji, Influences of pH, urea and metal ions on the interaction of sinomenine with lysozyme by steady state fluorescence spectroscopy, *Spectrochim. Acta A* 130 (2014) 40–446.
- [16] P.E. Stein, A.G. Leslie, J.T. Finch, R.W. Carrell, Crystal structure of uncleaved ovalbumin at 1.95 Å resolution, *J. Mol. Biol.* 221 (1991) 941–959.
- [17] B. Hess, C. Kutzner, D. van der Spoel, E. Lindahl, GROMACS 4: algorithms for highly efficient, load-balanced, and scalable molecular simulation, *J. Chem. Theory Comput.* 4 (2008) 435–447.

- [18] H.J.C. Berendsen, J.P.M. Postma, W.F. Van Gunsteren, A. DiNola, J.R. Haak, Molecular dynamics with coupling to an external bath, *J. Chem. Phys.* 81 (1984) 3684–3690.
- [19] R.A. Laskowski, PDB sum new things, *Nucleic Acids. Res.* 37 (2009) D355–D359.
- [20] A.A. Perez, R.B. Andermatten, A.C. Rubiolo, L.G. Santiago, β -Lactoglobulin heat-induced aggregates as carriers of polyunsaturated fatty acids, *Food Chem.* 158 (2014) 66–72.
- [21] A. Shpigelman, G. Israeli, Y. Livney, Thermally-induced protein polyphenol co-assemblies: beta lactoglobulin-based nanocomplexes as protective nanovehicles for EGCG, *Food Hydrocolloids* 24 (2010) 735–743.
- [22] W. Xiong, Y. Wang, C. Zhang, J. Wan, B.R. Shah, Y. Pei, B. Zhou, J. Li, B. Li, High intensity ultrasound modified ovalbumin: structure, interface and gelation properties, *Ultrason. Sonochem.* 31 (2016) 302–309.
- [23] T. Croguennec, A. Renault, S. Beaufils, J. Dubois, S. Pezennec, Interfacial properties of heat-treated ovalbumin, *J. Colloid Interface Sci.* 315 (2007) 627–636.
- [24] M. Weijers, R.W. Visschers, T. Nicolai, Influence of the ionic strength on the structure of heat-set globular protein gels at pH 7. Ovalbumin, *Macromolecules* 37 (2004) 8709–8714.
- [25] R. Wang, Y. Yin, H. Li, Y. Wang, J. Pu, R. Wang, et al., Comparative study of the interactions between ovalbumin and three alkaloids by spectrofluorimetry, *Mol. Biol. Rep.* 40 (2013) 3409–3418.
- [26] T. Wright, H. Ouian, R. Hubert, Crystal structure of plakalabumin. A proteolytically nicked form of ovalbumin, *J. Mol. Biol.* 213 (1990) 513–528.
- [27] O.E. Sponton, A.A. Perez, C.R. Carrara, L.G. Santiago, Impact of environment conditions on physicochemical characteristics of ovalbumin heat-induced nanoparticles and on their ability to bind PUFAs, *Food Hydrocolloids* 48 (2015) 165–173.
- [28] O.B. Ptitsyn, Molten globule and protein folding, *Adv. Protein Chem.* 47 (1995) 83–229.
- [29] I. Aprodu, N. Stanciuc, I. Banu, G. Bahrim, Probing thermal behavior of microbial transglutaminase with fluorescence and *in silico* methods, *J. Sci. Food Agric.* 15 (2013) 794–802.
- [30] A.C. Sánchez-Gimeno, A.V.P. López-Buesa, Studies of ovalbumin gelation in the presence of carrageenans and after manothermosonication treatments, *Innovative Food Sci. Emerg. Technol.* 7 (2006) 70–274.
- [31] F.F. Machado, J.S. Coimbra, E.E.G. Rojas, L.A. Minim, F.C. Oliveira, S.S. Rita de Cássia, Solubility and density of egg white proteins: effect of pH and saline concentration, *LWT: Food Sci. Technol.* 40 (2007) 1304–1307.
- [32] Y.Q. Li, X.Y. Li, A.A. Falih Shindi, Z.X. Zou, Q. Liu, L.R. Lin, N. Li, Synchronous fluorescence spectroscopy and its applications in clinical analysis and food safety evaluation, in: C.D. Geddes (Ed.), *Reviews in Fluorescence*, vol. 10, Springer, New York, 2010, pp. 95–117.
- [33] N.L. Vekshin, Division of tyrosine and tryptophan fluorescent components by synchronous scanning method, *Biofizika* 41 (1996) 1176–1179.
- [34] O.K. Gasymov, B.J. Glasgow, ANS fluorescence: potential to augment the identification of the external binding sites of proteins, *Biochim. Biophys. Acta* 1774 (2007) 403–411.
- [35] R. Sharma, N. Kishore, Isothermal titration calorimetric and spectroscopic studies on (alcohol + salt) induced partially folded state of α -lactalbumin and its binding with 8-anilino-1 naphthalenesulfonic acid, *J. Chem. Thermodyn.* 40 (2008) 1141–1151.
- [36] R. Sharma, N. Kishore, Thermodynamic insight into the binding of ANS with salt induced molten globule state of cytochrome c, *J. Chem. Thermodyn.* 41 (2009) 342–348.
- [37] G.B. Strambini, M. Gonnelli, Fluorescence quenching of buried Trp residues by acrylamide does not require penetration of the protein fold, *J. Phys. Chem.: B* 114 (2010) 1089–1093.
- [38] T. Koseki, N. Kitabatake, E. Doi, Conformational changes in ovalbumin at acid pH, *J. Biochem.* 103 (1988) 425–430.
- [39] J. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3rd edn., Springer Publications, New York, 2006, pp. 237–265.
- [40] L. Dumitrascu, N. Stănciuc, G.E. Bahrim, I. Aprodu, Insights into the binding of ferulic acid to the thermally treated xanthine oxidase, *Luminescence* (2016), <http://dx.doi.org/10.1002/bio.3099>.
- [41] B. Chakraborty, S. Basu, Interaction of BSA with proflavin: a spectroscopic approach, *J. Lumin.* 129 (2009) 134–139.
- [42] X. Zhao, R. Liu, Y. Teng, X. Liu, The interaction between Ag^+ and bovine serum albumin: a spectroscopic investigation, *Sci. Total Environ.* 409 (2011) 892–897.
- [43] N. Bordenave, B.R. Hamaker, M.G. Ferruzzi, Nature and consequences of non-covalent interactions between flavonoids and macronutrients in foods, *Food Funct.* 5 (2014) 18–34.
- [44] K.M. Jarvinen, K. Beyer, L. Vila, L. Bardina, M. Mishoe, H.A. Sampson, Specificity of IgE antibodies to sequential epitopes of hen's egg ovomucoid as a marker for persistence of egg allergy, *Allergy* 62 (2007) 758–765.
- [45] S.A. Leonard, G. Martos, W. Wang, A. Nowak-Węgrzyn, M.C. Berin, Oral immunotherapy induces local protective mechanisms in the gastrointestinal mucosa, *J. Allergy Clin. Immun.* 129 (2012) 1579–1587.
- [46] A.W. Burks, S.M. Jones, R.A. Wood, D.M. Fleischer, S.H. Sicherer, et al., Oral immunotherapy for treatment of egg allergy in children, *New Engl. J. Med.* 367 (2012) 233–243.