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Abstract

BACKGROUND: Ovomucoid (OVM) is the dominant allergen found in egg white. The heat induced changes on chicken OVM structure and antigenic properties were assessed at acidic, neutral and alkaline pH values.

RESULTS: The fluorescence spectroscopy measurements indicated changes in the conformation of OVM caused by both pH and thermal treatment. The OVM molecule exhibited higher exposure of hydrophobic residues at 7.0, as indicated by the synchronous spectra, intrinsic fluorescence and

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quenching experiments. When heating the protein at pH 9.5, the molecular structure appeared more compact. The antigenic properties of OVM, estimated through the enzyme-linked immunosorbent assay, appeared not to be sensitive to heat at pH 7.0 and 4.5. Single molecule level investigations indicated that the secondary and tertiary structure of OVM was affected by the thermal treatment.

CONCLUSIONS: Experimental results indicated over 90% reduction of the antigenicity at pH 9.5 and temperature of 100°C. Significant changes of the linear epitopes exposure and location of the conformational epitopes were highlighted after performing heating molecular dynamics simulations of OVM from 25°C to 100°C.

Keywords: ovomucoid, epitopes, structural changes, antigenic properties

Introduction

The most common foods reported worldwide for inducing allergy among infants, school-aged children and adults are chicken eggs, cow's milk, wheat, fish and shellfish, peanuts and tree nuts, fruits and soy.¹⁻³ Eggs were reported as the second most frequent source of food allergy, affecting up to 1.6% of children.⁴ Eggs may cause a variety of symptoms from atopic dermatitis to systemic anaphylaxis.⁵ Egg allergy is mainly due to four allergens found in egg white, namely ovomucoid (OVM), ovalbumin (OVA), ovotransferrin and lysozyme.⁶ Even if OVA is the most abundant

protein in egg white (54%), OVM is the major player in inducing allergic reactions.⁶⁻⁸ OVM is a glycoprotein with molecular weight of 28kDa and accounts for 11% of proteins found in egg white.⁵ OVM molecule consists of 186 amino acids organised into three well separated domains (I – Gal d 1.1, residues 1-64; II – Gal d 1.2, residues 65-130; III – Gal d 1.3, residues 131-186), and is highly glycosylated, with carbohydrate moieties representing about 25% of the molecule.⁶ The carbohydrate moieties (N-acetyl-glucoseamine, hexose and N-acetylneuraminic acid) exert no influence on OVM binding by immunoglobulin E (IgE).^{9,10}

Transglutaminase mediated cross-linking and different conventional methods of food processing performed for improving functional, nutritional or sensory attributes might affect digestibility, antigenicity and allergenicity of several proteins.^{3,11,12} Several processing techniques have even been proposed for designing allergoids which are allergen derivatives with sufficient immunogenic but reduced allergenic activity, suitable to be used in allergen-specific immunotherapy.¹³ In particular thermal treatment, which is the most widely used processing technique in food industry, has been reported to alter proteins structure through unfolding and aggregation events, affecting mainly the conformational epitopes. On the other hand, when discussing the antigenic or allergenic properties of a protein which was subjected to thermal processing within a complex food matrix, the epitopes burial as a result of the interaction with other food components should be also considered.¹⁴

Test carried out to investigate the influence of different types of thermal treatments on the allergenicity of egg white proteins indicated the highest efficiency of boiling for 30 min over frying or baking.¹⁵ Egg white boiling resulted in significant reduction of the IgE reactivity against OVA and ovotransferrin antigenic activity, but no effect on the IgE reactivity against OVM was obtained, regardless of the temperature or duration of the thermal treatment.¹⁵ On the other hand,

Mine and Zhang ⁸ indicated that heat treatment caused a dramatic decrease of the antigenicity of ovotransferin and OVM, whereas the human IgG binding activity of OVA remained unaffected. The measurements performed with IgE antibody in human sera from egg allergic patients indicated lower antigenicity of OVA and OVM caused by heat treatment, and no significant effect on lysozyme. In 50-70% of children exhibiting egg allergy, continued ingestion of heat treated eggs resulted in increase of the tolerance, being associated with lower levels of OVA specific IgE, but increased levels OVA and OVM-specific IgG4.¹⁵

Even if the individual contribution of each allergen protein to the overall allergenicity of egg white was investigated, only limited information about the molecular behaviour of the allergen proteins at thermal processing is available. In a previous study we showed that chicken OVA is rather stable when heat treated in model system of pH 7.0, but a significant reduction (over 82%) of the residual antigenicity was obtained at pH 9.5 and temperatures higher than 80°C.¹⁶ Although the OVM from chicken egg white has been used as model for biochemical and physico-chemical studies because of the trypsin inhibitory activity, the information regarding the effect of different kind of processing on conformational and potential allergenic properties is limited. The aim of the present study was to investigate the effect of thermal treatment on structural and antigenic properties of chicken OVM under different pH conditions. Fluorescence spectroscopy techniques and enzyme-linked immunosorbent assay (ELISA) were used to quantify the changes in the structure and antigenic character of OVM, when processing the protein in model system at different pH-temperature conditions. Atomic level details on the linear and conformational epitopes of OVM model were further provided after performing molecular modeling simulations to resemble the experimental thermal treatment.

Materials and methods

Materials

Ovomucoid was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and was used in the fluorescence spectroscopy and antigenicity tests with no further purification. All other reagents used in the experiments were of analytical grade.

Heat treatment

Plastic tubes (1 cm diameter) were filled with 0.15 mL of OVM solutions of 1 mg/mL concentration, prepared in 20 mM Tris-HCl buffers of pH 4.5, 7.0 and 9.5. The samples were heated for 20 minutes at two different temperatures, namely at 80°C and 100°C using a thermostatic water bath (Digibath-2 BAD 4, Raypa Trade, Barcelona, Spain). After the thermal treatment samples were immediately cooled by placing the tubes in ice water to avoid any further thermal denaturation. In order to perform the fluorescence measurements, a volume of 100 µL thermally treated OVM solutions was further suspended in 3.0 mL 20 mM Tris buffer at appropriate pH.

Fluorescence spectroscopy measurements

All fluorescence spectra were recorded on a LS-55 luminescence spectrometer (Perkin Elmer Life Sciences, Shelton, CT, USA) equipped with the software Perkin Elmer FL Winlab. For measuring the intrinsic fluorescence intensity of tyrosine (Tyr) residues, the excitation wavelength was set at 274 nm and 280 nm, while the width of both excitation and emission slits was set to 10 nm. The synchronous scans were collected between 240-320 nm with intervals of 15 nm. Phase diagram,

three dimensional spectra and quenching experiments were performed as previously reported by Stănciuc et al.¹⁷

Enzyme-linked immunosorbent assay

The effect of thermal treatment on the antigenicity of OMV at pH 4.5, 7.0 and 9.5 was estimated by means of ELISA. The Ridascreen FAST egg ELISA (Biopharm AG, Darmstadt, Germany) kit was used for rapid detection of OVM, and the absorbance was measured with a microplate spectrophotometer (Stat Fax 4200 microplate reader, Awareness Technology, USA).

The residual antigenicity of OVM solutions of pH 4.5, 7.0 and 9.5 after heat treatment for 20 min at 80°C and 100°C, was estimated as indicated by Stănciuc et al.¹⁷, as percentage of allergen protein recognized by the specific antibodies with respect to the corresponding sample at 25°C.

Single molecule level investigations on ovomucoid particularities

The amino acids sequences of OVM from *Gallus gallus* was taken from UniProtKB database (entry P01005¹⁸) and used for predicting the three dimensional (3D) model using I-TASSER algorithm.^{19,20} The OVM model with the highest confidence was further used for the *in silico* investigations. The molecular mechanics calculations to optimize the 3D model *in vacuum* and in explicit solvent environment, and molecular dynamics steps to heat and equilibrate the solvated protein were performed using Gromacs 5.1.1 (Groningen University, Netherlands) package running in Linux.²¹ All molecular dynamics simulations for equilibrating the system at 25°C, 80°C and 100°C were performed in parallel conditions on the High Performance Computer System (HPC) from *Dunarea de Jos* University using Intel E5 2680 v3, 12-cores, 2.5 GHz. The set-up used for carrying out the molecular dynamics simulations was considered as in Stănciuc et al.¹⁷

The OVM models equilibrated at different temperatures were analyzed using dedicated Gromacs, Visual Molecular Dynamics (VMD) and PDBsum tools.^{22,23}

Results and discussion

Intrinsic fluorescence intensity

Because of the specific fluorescence properties, tryptophan (Trp) is a convenient natural probe usually exploited to study protein conformation as influenced by the environment.²⁴ Anyway, there are proteins, such as OVM, which lack Trp residues, case in which the fluorescence of Tyr residues is significant and can be successfully used for studying the behavior of the macromolecules.²⁵ Chicken OVM has six Tyr residues per molecule, evenly distributed within the protein domains (Tyr³⁶ and Tyr⁴⁶ in domain I; Tyr⁷³ and Tyr¹⁰² in domain II; Tyr¹⁴¹ and Tyr¹⁶¹ in domain III). The analysis of the protein model used for the *in silico* study indicated that no Tyr residue is buried within the protein core, the surfaces exposed to the solvent ranging from 7.76 Å² to 198.06 Å² at 25°C.

Figure 1 shows an overlay of the fluorescence spectra due to the six Tyr residues of OVM solutions of different pH at 25°C by excitation of absorption at 280 nm. The λ_{max} corresponding to the highest intensity of fluorescence emission varied with the pH of the solution. At pH 4.5 the protein was characterized by λ_{max} of 306 nm, whereas at pH 7.0 and 9.5, the maximum emission was registered at 342 nm and 350 nm, respectively (**Figure 1**).

Depending on the pH value different protons and protons acceptors can be formed in a protein. In particular, Tyr has three dissociable protons, attached to the ammonium, carboxyl, and aromatic hydroxyl groups, which can be either in the proton on (acidic) or proton off (basic) states, depending on the pH of the environment. Therefore, the pH dependent changes in the Tyr

fluorescence, are directly related to the 3D protein structure, being caused by energy transfer from Tyr to other residues and dynamics in the hydrogen bonds involving the hydroxyl group of Tyr residues.²⁵ Because of the distribution of the charges, even isometric forms of Tyr having the same net charge behave different in terms of established electrostatic interactions. At pH values higher than those considered in the present study dissociation of the phenolic hydroxyl group occurs, but these ionised species are non-fluorescent.

As indicated by Maeno et al.²⁶ when excited at 280 nm Tyr residues give maximum intensity of the fluorescence emission at λ_{max} of 306 nm, as in the case of OVM at pH 4.5. The maximum emission at λ_{max} of 342 nm registered for the OVM sample at pH 7.0 could be ascribed to the presence of tyrosinate. Our results are in good agreement with Szabo et al.²⁷ who reported fluorescence maxima at 345 nm for two different peptides lacking Trp residues, at pH 7.0. Anyway, according to Szabo et al.²⁷ it is unlikely to have any tyrosinate present in the protein ground state at pH 7.0. Therefore, the maximum fluorescence emission was attributed to the intramolecular excited states proton transfer from the hydroxyl of Tyr side chains to different acceptors from protein molecule such as glutamic and aspartic acids.²⁷ At pH values approaching the pKa (~10.4) the Tyr turns into tyrosinate with emission at λ_{max} of 340 nm. Therefore, in case of the OVM sample of pH 9.5 the presence of the Tyr – tyrosinate mixture should be considered with specific contributions to the overall fluorescence of the protein (**Figure 1**). Anyway, the λ_{max} of 350 nm might be also due to the polarization effect on phenolic hydroxyl group of Tyr residues because of the salt bridges and involvement in hydrogen bonding.²⁶

In general, heating caused the decrease of fluorescence intensity of the protein solutions, except for OVM sample of pH 9.5. However, the spectrum obtained at pH 4.5 and 9.5 have no specific structure. In addition to the changes in the charge distribution on the surface of the protein, the pH

change might also alter the globularity of the protein. In this condition, the structure of OVM can be linked to *molten globule* state, characterized by the loss of the tertiary structure, while retaining the secondary structure. Therefore, the *molten globule* form corresponds to a partially folded conformation that can be distinguished from either the native or the fully denatured forms. *Globule* refers to the native compactness, and *molten* refers to the increased enthalpy and the entropy on transition from the native structure to the new state.²⁸

Spectra with specific structure were obtained for OVM at pH 7.0. The heat-induced changes in OVM structure at pH 7.0 are depicted in **Figure S1** (in the **Supplementary material**). Heating caused significant 5 nm and 7 nm red-shifts in λ_{max} at 80°C and 100°C when excited at 274 nm. In agreement with the intrinsic fluorescence results, the detailed analysis at single molecule level indicated significant increase of the surface available to the solvent (Δ SAS) with increasing temperature from 25°C to 100°C, in case of Tyr³⁷ (Δ SAS 19.79 Å²), Tyr⁴⁶ (Δ SAS 20.21 Å²), Tyr¹⁴¹ (Δ SAS 56.05 Å²) and Tyr¹⁶¹ (Δ SAS 18.77 Å²) residues. On the other hand, partial burial of the Tyr residues located in domain II was observed at 100°C. Considering both fluorescence spectroscopy results and *in silico* observations one may consider that domains I and III, which appear to unfold with increasing temperature, play the leading role in defining the thermal dependent behavior of OVM molecule.

Similarly, Maeno et al.²⁶ reported the decrease of fluorescence intensity with increasing pressure, accompanied by red-shifts of λ_{max} . The result strongly suggests a two-state transition taking place, involving changes in the exposure of the tyrosine (R-OH) and tyrosinate (R-O⁻) forms of Tyr residues of OVM at 80°C and 100°C. No significant heat-induced structural changes were recorded when excited at 280 nm.

In agreement with the observations of Szabo et al.²⁷, the differences registered in the trend of fluorescence spectra obtained through excitation at different wavelengths might be explained through the presence of at least two different types of Tyr residues in the OVM molecules. The atomic level contacts within proteins which favor the rapid proton transfer may account for the fluorescence spectral variations. Perturbation of the intrinsic fluorescence of Tyr can arise as a consequence of hydrogen bonding with glutamic acid (Glu) and aspartic acid (Asp) residues.²⁷ Indeed, close analysis of the chicken OVM models indicated the existence of two hydrogen bonds (Hb) in the protein structure equilibrated at 25°C involving the side chains of Tyr³⁷ and Asp³³ (length 2.5Å) and the backbones of Glu⁴⁹ and Tyr⁴⁶ (length 2.5Å), and three Hb at 100°C between the side chains of Tyr⁷³ - Glu⁵⁷ (length 2.7Å) and Tyr³⁷ - Asp³³ (length 2.6Å) pairs and the backbones of Tyr¹⁴¹ - Asp¹²⁰ (length 3.1Å).

Phase diagrams

The *phase diagrams* describing the heat-induced structural changes of OVM solutions of different pH values are given in **Figure 2**. Considering the plot of I_{365} versus I_{320} , it can be seen that at pH 4.5 and 7.0, OVM unfolds in an *all or none* manner, as the temperature increased from 25°C to 100°C. The non-linearity of the plot obtained at pH 9.5 reflected the sequential character of structural transformations, involving a three-state thermal transition.²⁹

The two-state models are typical for thermal denaturation of small compact globular proteins, involving the transition between two molecular states, usually from native to the unfolded one. However, Matsuda et al.³⁰ suggested that the simple two-state assumption for the thermal transition of OVM must be treated with caution. The protein structural changes were correlated with the decrease of the β -structure content as the temperature increased up to 60°C, followed by the

decrease in α -helix content by further increasing the temperature up to 90°C. These authors suggested that the thermal denaturation process of OVM does not involve major unfolding events between 60 and 90°C.

In an earlier study, when performing urea titration experiments on isolated domains or domain combinations of chicken OVM at pH 7.0, Das et al.³¹ suggested that the domain 3 has by far the highest stability when compared to other two domains. Maeno et al.²⁶ suggested also that the single domain remaining intact at extreme condition of pH 12.0 and 700 MPa was domain 3, which is responsible for the strong allergenicity of this protein.

Excitation–emission matrix fluorescence spectra

Three-dimensional excitation–emission matrix (EEM) fluorescence spectroscopy is a rapid, selective and sensitive technique. The outstanding advantage of EEM fluorescence spectroscopy is that information regarding the fluorescence characteristics can be entirely acquired by changing the excitation wavelength and emission wavelength simultaneously. The EEM spectra of OVM in solution of different pH values, heat treated at 25, 80 and 100°C are illustrated as the 3D spectra and elliptical shape of contours in **Figure 3**, and **Figures S2-4 in Supplementary material**. The X-axis represents the emission wavelength from 200 to 500 nm, whereas the Y-axis is the excitation wavelength from 200 to 500 nm. Twenty contour lines, as the third dimension, are shown for each EEM spectra to represent the fluorescence intensity at an interval of 10. Regardless of the temperature and pH values, four peaks were readily identified in the EEM fluorescence spectra of OVM (**Figure 3 and Figures S2-4 in Supplementary material**).

When analyzing the pH dependent behavior of OVM at 25°C, one can see that the location and intensity of peaks 1 and 2 varied with pH value (**Table 1**). The peak 1 was identified at

excitation/emission wavelengths of 280/309 nm at pH 4.5 (**Figure 3a**), 280/338 nm at pH 7.0 (**Figure 3b**), and at 280/356 nm at pH 9.5 (**Figure 3c**). The second peak (peak 2) was identified at excitation/emission wavelengths of 230/304 nm at pH 4.5 (**Figure 3a**), at 230/333 nm at pH 7.0 (**Figure 3b**) and at 280/349 nm at pH 9.5 (**Figure 3c**). The two peaks have been described as protein-like peaks, in which the fluorescence is associated with the aromatic amino acids.³² Bortolotti et al.³³ clearly demonstrated that the observed emission upon excitation at 220-230 nm is due to the excitation of Tyr and/or Trp, with subsequent emission from the lowest excited state (i.e. the same as obtained with 280 nm excitation), in agreement with Kasha's rule. Therefore, the fluorescence of both peaks provides information on the local environment around the aromatic side chain of Tyr residues in the OVM molecule.

For all tested heating temperatures, the position of peaks 1 and 2 of the EEM fluorescence spectra varied significantly with pH value (**Table 1**). The locations of both peaks for OVM at pH 7.0 (**Figure S3 in Supplementary material**) and 9.5 (**Figure S4 in Supplementary material**) were significantly red shifted to longer wavelengths than those at pH 4.5 (**Figure S2 in Supplementary material**). These differences imply that the main contribution of the fluorophores at different pH come from different domains.

Significant heat-induced changes in terms of position and fluorescence intensity of peaks 1 and 2 were found at pH 9.5 (**Table 1** and **Figure S4 in Supplementary material**). For example, heating at 80°C-100°C caused 44-46 nm blue-shifts in emission maxima, suggesting a more compact molecular structure of OVM in these conditions.

Synchronous spectra

Dubayová et al.³⁴ suggested that the synchronous spectrum can be considered as characteristic “fingerprint”, because it is unique for a given mixture and characterizes it graphically as one unit. In our study, the synchronous spectra of OVM at different pH were registered by scanning simultaneously the excitation and emission monochromators, while maintaining a fixed wavelength difference $\Delta\lambda$ between them at 15 nm, with the aim to study the microenvironment in the vicinity of the fluorophore groups. The synchronous fluorescence spectra of OVM solutions at $\Delta\lambda$ of 15 nm and different pH values are given in **Figure 4**. At temperature of 25°C, significant higher fluorescence intensity was found for OVM solutions at pH 4.5, with a maximum wavelength (λ_{max}) position at 274 nm. The wavelengths corresponding to the maximum fluorescence at pH 7.0 and 9.5 were 283 nm and 278 nm, respectively. Therefore, it can be concluded that the Tyr residues of OVM molecules in solution of pH 7.0 are more exposed to the hydrophilic environment.

Measurement performed at $\Delta\lambda$ of 15 nm indicated that heating the OVM samples of pH 4.5 and 7.0 resulted in fluorescence intensity decrease, whereas an increase was observed in case of the samples of pH 9.5 (**Figure 4**). In case of thermally treating the samples, the most significant changes in λ_{max} , compared to the sample at 25°C, consisted in a 2 nm red-shift at 80°C and a 2 nm blue-shift at 100°C, suggesting sequential translocation of Tyr residues toward more polar and further apolar environment.

Quenching experiments

The accessibility and polarity of the hydrophobic regions of OVM in different environmental conditions was further monitored by means of the quenching experiments, using acrylamide as external quencher. The main purpose considered the fact that fluorescence quenching is an

important tool to measure the extent and accessibility of protein binding sites to small molecules.³⁵

The acrylamide is often used because of its ability to quench both exposed and buried hydrophobic residues.³⁶

The values of the quenching constant (K_{SV}) with acrylamide at different temperature and pH values are given in **Table 2**. In case of the OVM not subjected to thermal treatment, quenching at pH 7.0 with acrylamide generated the highest K_{SV} value of $8.50 \pm 0.58 \text{ mol}^{-1}\text{L}$, whereas the lowest was obtained at pH 9.5 ($5.34 \pm 0.06 \text{ mol}^{-1}\text{L}$). In agreement with the synchronous spectra and intrinsic fluorescence results, the K_{SV} values indicate that in neutral conditions, the hydrophobic residues of OVM are more exposed to solvent. The higher accessibility of acrylamide in case of the heat-treated OVM solutions was ascribed to the increased exposure of hydrophobic residues as a consequence of denaturation and rearrangements of the side chains of the polypeptide. Regardless of the pH value, increasing K_{SV} values were obtained at thermal treatment, especially when heating the protein up to 100°C (**Table 2**). These results suggest that heating caused local unfolding events in the polypeptide chain, allowing increased accessibility of acrylamide to hydrophobic residues.

Heat induced changes in ovomucoid antigenicity

The Ridascreen FAST egg ELISA (Biopharm AG, Germany) was used to estimate the impact of the thermal treatment on OVM antigenicity as a function of pH. Thermal processing for 20 min at temperatures up to 100°C had no significant influence on the antigenic properties of OVM at pH of 7.0 and 4.5 (**Figure 5**). Our results comply with Shin et al.¹⁵ who studied the allergenicity of egg white proteins as a function of heat treatment intensity, and reported no changes in the IgE reactivity against OVM, regardless of tested processing type (egg white boiling for 10 min and 30 min, frying for 3 min or baking at 170°C for 20 min). An important decrease (by 91.1%) of the

residual antigenicity was obtained in case of treating the OVM samples of pH 9.5 at 100°C (**Figure 5**). Higher sensitivity in similar processing conditions was obtained for chicken OVA in solution of pH 9.5 when the antigenicity decreased by 82% at 80°C.¹⁶

The reduction of antigenic properties of a protein at thermal treatment is usually the result of the alteration of conformational epitopes, while the linear epitopes are more resistant. Anyway, the contribution of the neoepitopes formed at high temperature to the increase of protein allergenicity should be considered.¹⁵ Detailed epitope mapping of whole OVM molecule has been performed previously by Mine and Zhang¹⁰ who reported the existence of eight IgG and nine IgE overlapping linear epitopes, mainly incriminated for high residual antigenicity and allergenicity after thermally treating the protein. IgE is responsible for mediating the most common types of food allergies (type I), whereas IgG is involved in mediating both the cytotoxic hypersensitivity induced by antibody binding to the cell-bound antigen, which further leads to phagocytosis (type II), and type III hypersensitivity when the large quantities of antibody-antigen complexes cause tissue injuries.⁸

In silico characterization of ovomucoid at thermal treatment

Solving the three-dimensional structures of allergen proteins is an effective step for providing detailed information at single molecule level, useful both for understanding the food allergy pathogenesis, and for identifying processing techniques which might allow reducing the antigenicity. The characteristics of the 3D model of chicken OVM predicted through I-TASSER algorithm^{19,20} comply with experimental observations, mainly based on circular dichroism, difference-spectral and fluorescence methods, showing that the protein has a rather ordered structure which is not very compact or symmetric.⁹ The OVM model has been used here for estimating the thermal dependent behaviour of the molecule as well as the exposure and location

of linear and conformational epitopes, after performing molecular dynamics simulations in aqueous environment at 25°C, 80°C and 100°C. Concerning the OVM structure descriptors presented in **Table 3**, one can see that, regardless of the simulated temperature, all kinds of structural elements were identified in the protein models. A significant variation of the total number of amino acids involved in defining α -helices and strands based secondary structure motifs was observed at temperature increase. For instance, the amount of amino acids involved in defining strand motifs decreased from 16.7% to 12.9%, whereas the amount of amino acids organized as helices increased from 15.1% to 19.9% when raising the temperature from 25°C to 100°C (**Table 3**). The main secondary structure elements of the third domain of the protein seemed better preserved compared to domains I and II. Two new helices were observed in the second domain of OVM at high temperature. The increase of the helical content with the temperature is a consequence of important reorganization of the side chains of Asn⁶⁹-Ser⁷², Cys⁸⁷-Ala⁹⁰ and Glu⁶⁴-Asn⁶⁹ sequences, organised as β and γ turns, respectively at 25°C. Regardless of the simulated temperature, four antiparallel β sheets were observed in the OVM structure, with varying irregularity regions caused by disruptions in the specific hydrogen bonding pattern. Out of the three antiparallel β bulges located in each domain of the protein model at 25°C, the one involving Cys⁹⁵ residue (domain II) disappeared when treating the protein at 80°C, and only the one located in domain III was identified at 100°C. The OVM model used for the *in silico* investigations contains five disulphide bridges, two in each of the domains I (Cys²²-Cys⁴¹ and Cys³⁰-Cys⁶²) and III (Cys¹⁴⁶-Cys¹⁶⁵ and Cys¹⁵⁴-Cys¹⁸⁶), and one in domain II (Cys⁹⁵-Cys¹²⁷), contributing in a cooperative manner to the high stability of the protein structure at thermal treatment. The root mean square deviation (RMSD) values computed after superimposing the models treated at different temperatures were used to estimate the extent to which the stability of each domain of

OVM molecule was affected. Thus, when comparing the models equilibrated at 25°C and 100°C, significantly lower RMSD values were obtained for domains I (5.24Å) and III (4.15Å) compared to domain II (8.52Å), most probably as a consequence of higher number of disulphide bridges. In addition, other eight free Cys residues are found in OVM molecule, with surfaces exposed to the solvent ranging from 9.16 to 63.32Å², therefore prone to establish intermolecular disulphide bridges. The protein structural rearrangements at temperature of 100°C resulted in significant burial of over 79% and 47% of the surface of Cys residues from domains I and II, respectively. On the other hand, the Cys residues located in domain III became more available for intermolecular disulphide bridges because of the increased exposure to the solvent (from 52.86Å² at 25°C to 111.42Å² at 100°C). Furthermore, potential degradation of the disulphide bonds at thermal treatment under neutral and basic conditions should be also considered, as might lead to local destabilization of the protein structure, in addition to the new Cys residues prone for establishing new intra- or intermolecular interactions.³⁷ OVM cross-linking through intermolecular disulfide bonds might result in the burial of the epitopes located on protein surface.

The total number of hydrophobic bonds, which are the major force driving protein folding, decreased from 71 to 55 with the temperature increase from 25°C to 100°C. The reduction of the hydrophobic contact involvement in stabilising the specific structure of the protein is compensated by the increase of the number of ionic bonds from 13 to 20, with strength approaching the covalent bonds. Moreover, as a measure of significant changes occurring in the protein structure at thermal treatment, a 17% reduction of the hydrogen bonds established between protein and water molecules (Hb protein-water) was observed (**Table 3**).

The IgE and IgG epitopes determined by Mine and Zhang¹⁰ within the primary structure of the OVM were further identified in the molecular model used for the *in silico* investigations. Thus,

domain I contains three linear regions (amino acids 32–42, 40–50 and 56–66) recognised by IgE and three (amino acids 6–15, 31–35, and 46–50) by IgG, domain II has five IgE binding epitopes (amino acids 71–75, 80–90, 101–105, and 121–130) and two IgG binding epitopes (amino acids 71–75 and 101–105), and domain III has two linear epitopes (amino acids 159–174 and 179–186) recognized by IgE and three (amino acids 160–167, 165–174 and 177–183) by IgG.¹⁰ These amino acid sequences are organized as strands, helices or turns exposed on the protein surface, and displayed rather good stability at thermal treatment. The high residual antigenicity at thermal treatment, as indicated by the ELISA tests, might be due to the fact that three of the OVM linear epitopes include in their sequences Cys⁴¹, Cys¹²⁷ and Cys¹⁶⁵ residues which are involved in establishing intramolecular disulfide bridges, therefore ensuring local conformational rigidity. In order to estimate the chance of OVM epitopes to be available for the antigen-antibody interaction in the ELISA test after subjecting the protein to the thermal treatment, the exposure to the solvent of the amino acids sequences incriminated for inducing allergenic response has been checked on the models equilibrated at different temperatures. Due to the changes in the relative position of amino acids and reorientation of their side chains, the solvent exposed surface of the linear epitopes varied with the temperature. The most important alteration of the linear epitopes observed when heating the OVM model at 100°C regarded the IgE binding sequences from domain I, where complete burial of Gly³⁴, Cys⁴¹, Leu⁴³, Cys⁴⁴ and Cys⁶² was observed. Concerning domain III, only the Ser¹⁸¹ residue shared by the IgE and IgG binding epitopes was found to migrate toward protein core at high temperature. Finally, the epitopes from domain II appeared the most stable against temperature.

The EpiSearch method³⁸ based on phage display technology, was further used to identify the potential thermal dependent conformational epitopes on OVM surface. The default values of the

EpiSearch tool, which ensure the best precision and sensitivity of the method, were used for mapping the conformational epitopes on OVM models equilibrated at 25°C, 80°C and 100°C. Temperature increase resulted in significant changes in the location of conformational epitopes of OVM model, represented with magenta (25°C), orange (80°C) and blue (100°C) in Surf style in **Figure 6a-c**. The predicted top scoring patches included mainly amino acids from the first and the third domains. In case of the OVM at 25°C (**Figure 6a**), the highest scoring patch was centered on residue H58 (score 0.889), and predicted residues ranged from R27 to D80. A total of 22 amino acids predicted in this patch was also reported by Mine and Zhang¹⁰ to belong to four linear IgG and IgE epitopes, out of which 8 residues (T32, G34, V35, T36, C62, K63, E64, T65) are considered critical, because their substitution allowed significant decrease of antibody binding. The second highest scoring patch (score 0.818) predicted on OVM model equilibrated at 25°C was centered on Y102 (predicted residues in the range V83 – C127) and shares no residues with the first one.

Due to the significant changes in the conformation of OVM molecules when heated at 80°C, the most probable conformational epitope mapped onto protein structure appeared to be located on domain I (**Figure 6b**), being centered on L42 (score 0.901; predicted residues in the range C5 – K56). This patch includes 17 amino acids listed by Mine and Zhang¹⁰ in the linear epitopes recognized by IgG (sequence S6-E15) and IgE (sequences T32-L42 and D40-F50), among which four are critical for antigen-antibody binding (P9, I48, E49 and F50). In addition, there are two predicted patches centered on C44 and Y46 (scores of 0.676 and 0.827, respectively), which share most of the amino acids with the highest scoring one. Other five patches, including the second highest scoring one (centered on R122, score 0.835) share amino acids in the range V94 - L178.

Finally, in case of OVM model equilibrated at 100°C the highest scoring patch was centered on L180 (score 0.931) and included mainly residues from the third domain of the protein (A132 – C186) (**Figure 6c**). The critical residues identified in this patch are Y161, G162, N163, F167, C168, K185 and C186 and belong to the linear IgE epitopes K159-S174 and T179-C186. Although significant changes were observed in the OVM structure when increasing the temperature from 80°C to 100°C (RMSD of domain III was 4.77Å), it appears that protein motifs with antigenic features are rather conserved. The second highest scoring patch identified at 100°C centered on residue A45 (score 0.874) overlap to high extent (71%) with the most probable conformational epitope predicted on the model equilibrated at 80°C.

Conclusions

The experimental and *in silico* tests showed that molecular behavior and antigenic properties of OVM vary with the thermal treatment. Lower exposure of the hydrophobic residues on OVM surface was observed at thermal treatment and at acidic or alkaline pH compared to the native protein. The lowest level of residual antigenicity was obtained when heating the OVM at pH 9.5. The exposure of the IgE and IgG linear epitopes and the location of conformational ones varied with the temperature. Taking into account the high importance of the matrix within which the allergen proteins reside during the processing, further tests will be performed to determine the influence of pH and thermal treatment on the stability and antigenic properties of OVM in systems with increasing complexity.

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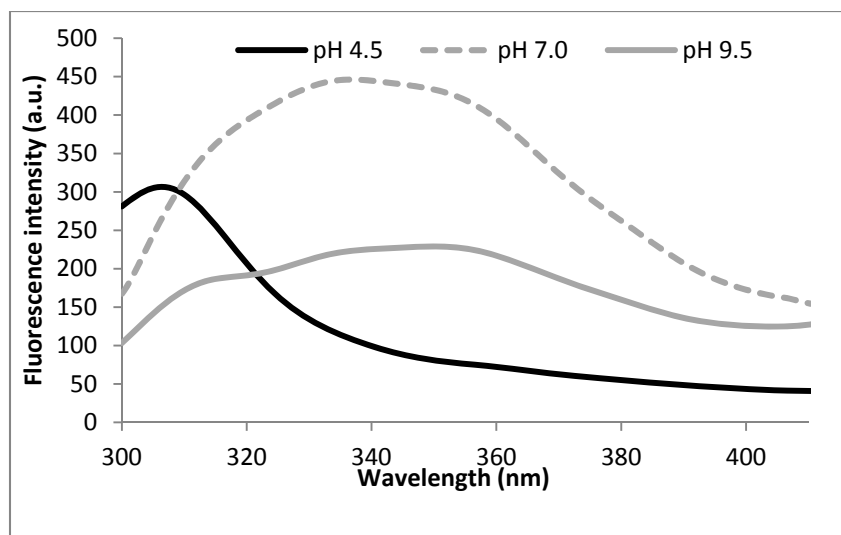


Figure 1. The emissions spectra of the ovomucoid solutions at 25°C and different pH values. The excitation wavelength was 280 nm and the spectrums were collected between 300 and 420 nm. Three independent tests were carried out in each case and SD was lower than 10%.

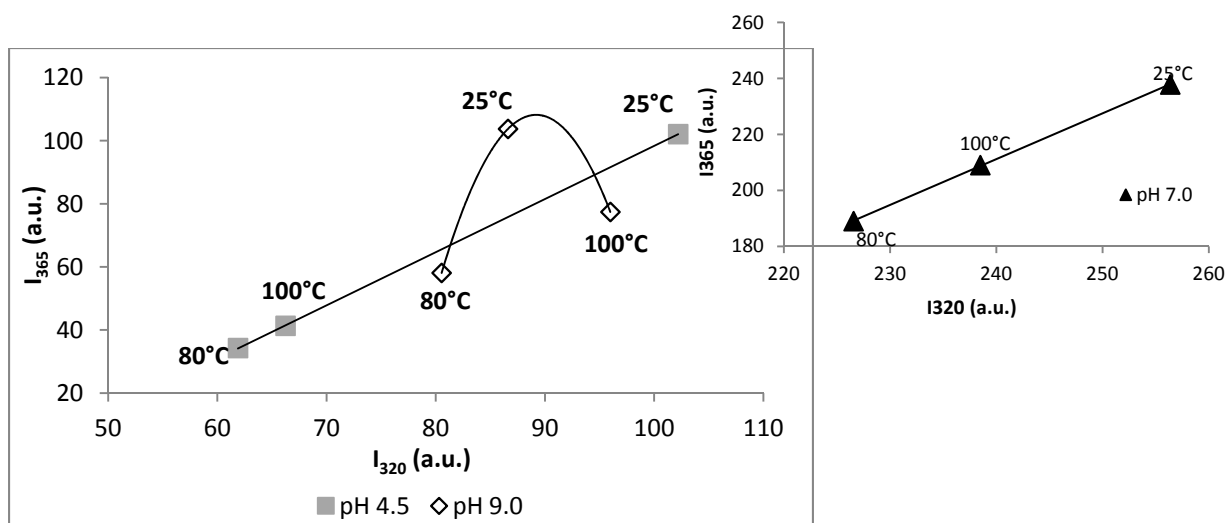
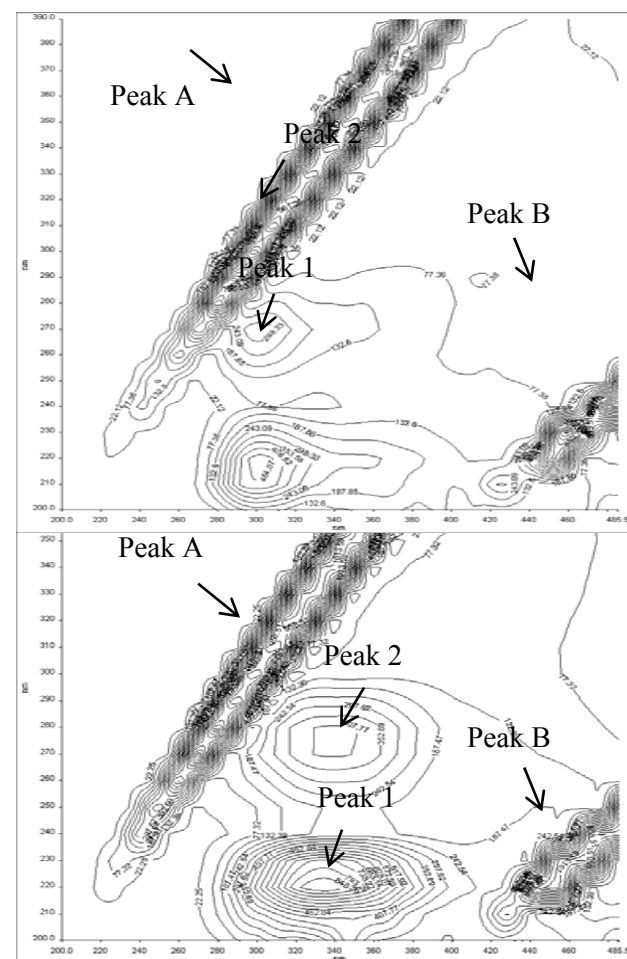
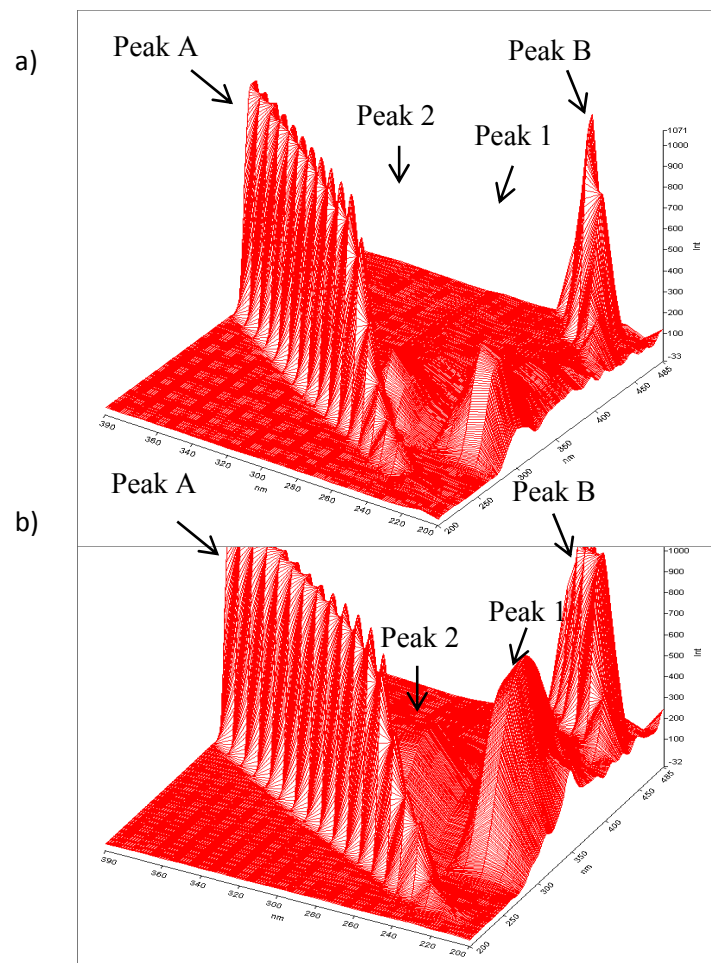


Figure 2. Phase diagram analysis of pH and heat-induced conformational changes of the ovomucoid solutions based on intrinsic fluorescence intensity values measured at wavelengths 320 and 365 nm. The temperatures values are indicated in the vicinity of the corresponding symbol. Three independent tests were carried out in each case and SD was lower than 2.5%.



c)

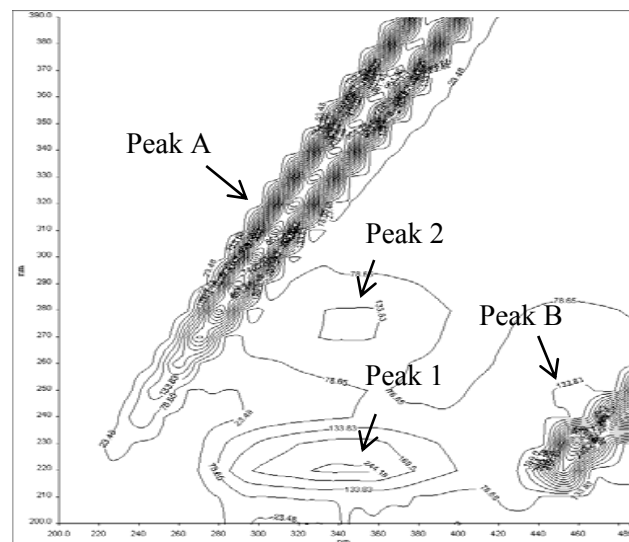
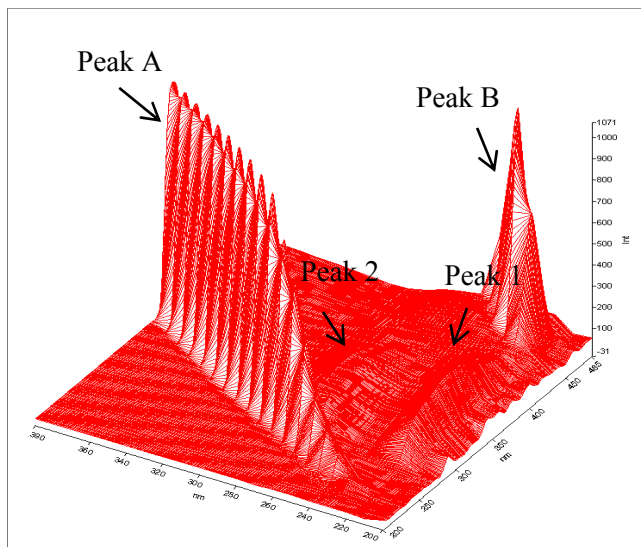


Figure 3. The EEMs spectra of ovalbumin solutions at 25°C: (a) pH 4.5, (b) pH 7.0 and (c) pH 9.5. Three independent tests were carried out in each case and SD was lower than 3.5%.

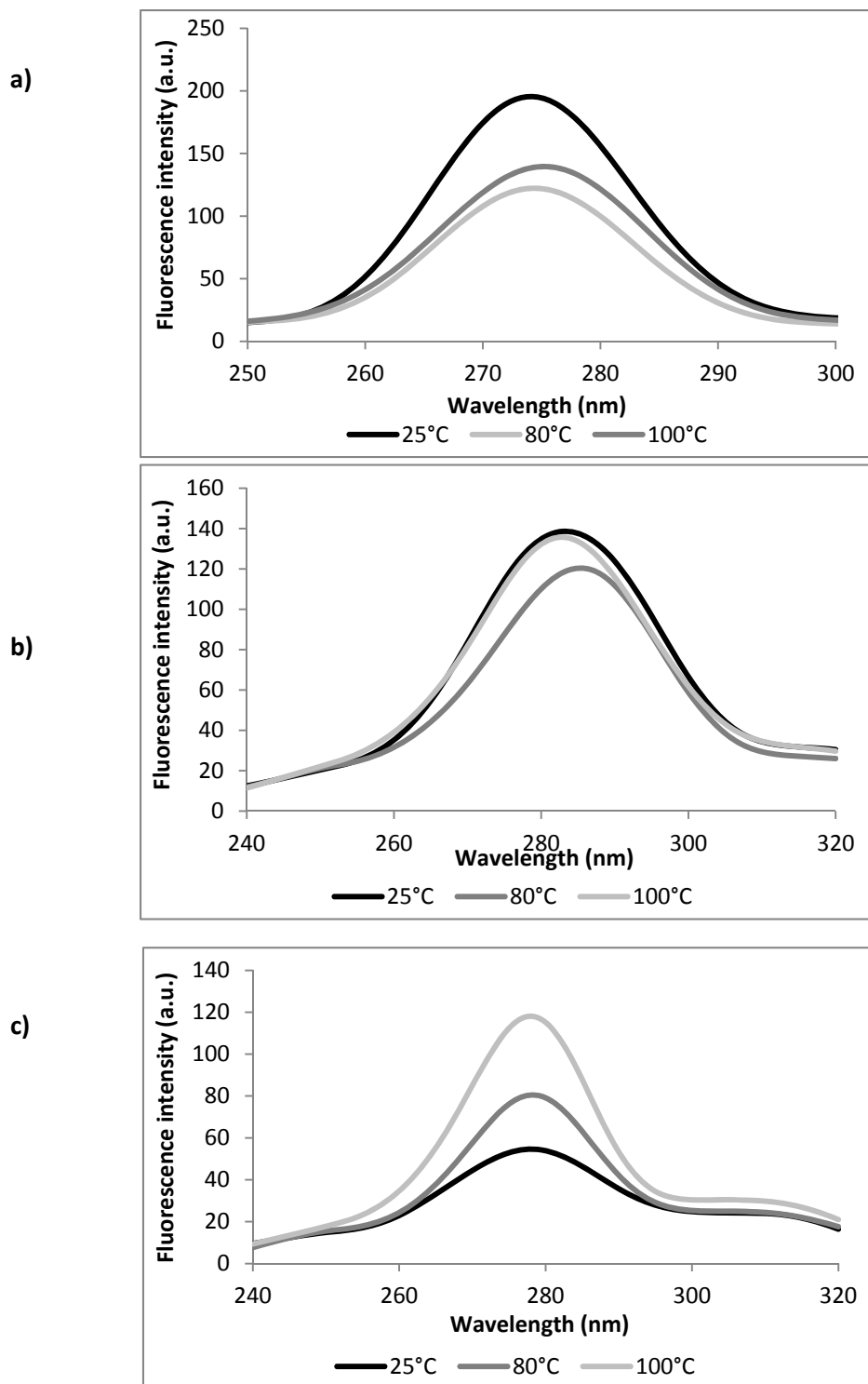


Figure 4. Synchronous fluorescence spectra ($\Delta\lambda = 15$ nm) of ovomucoid solutions at different temperatures and pH of 4.5 (a), 7.0 (b) and 9.5 (c). Three independent tests were carried out in each case and SD was lower than 3.5%.

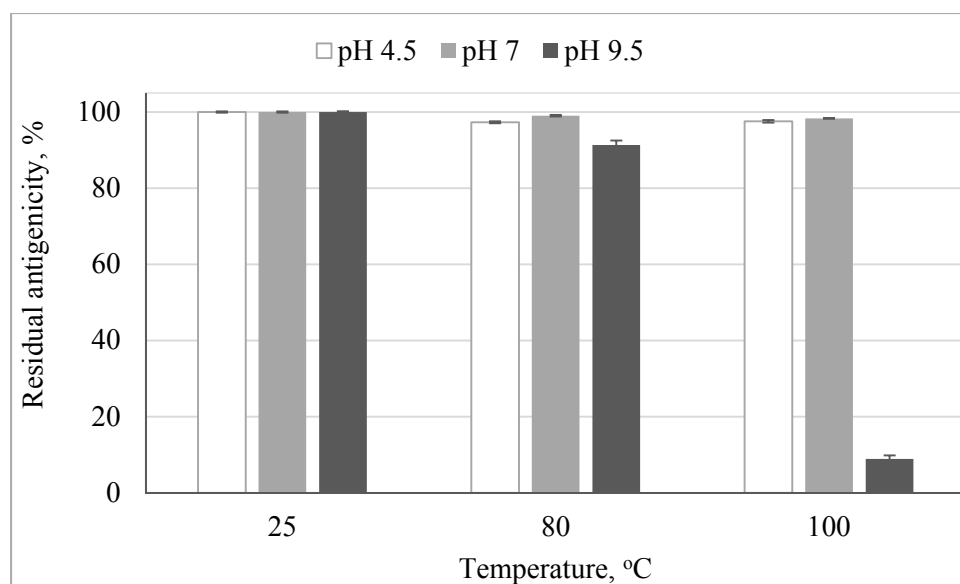


Figure 5. Residual antigenicity of ovomucoid solutions of pH 4.5, 7.0 and 9.5 after thermal treatment.

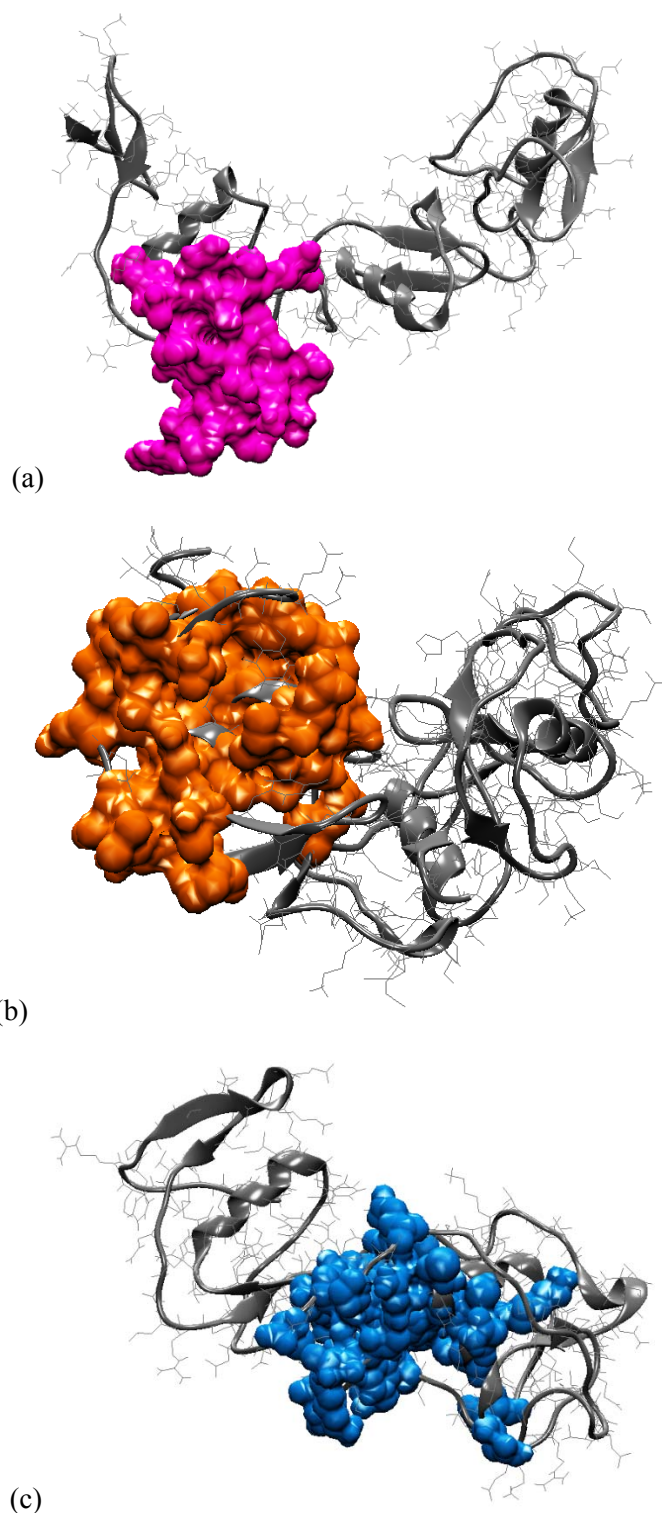


Figure 6. Comparison of the overall folding (represented in grey in New Ribbons Catmull-Rom style) and location of the conformational epitopes (highlighted using Surf style in magenta, orange and blue) on the surface of ovomucoid models equilibrated at 25°C (a) 80°C (b) and 100°C (c). Only the conformational epitopes with the highest scoring predicted with EpiSearch algorithm were considered. Images were prepared with VMD software.

Table 1. Characteristic parameters of the three-dimensional fluorescence spectra of ovomucoid at different temperature and pH values

Temperature, °C	pH 4.5				pH 7.0				pH 9.5			
	Peak 1		Peak 2		Peak 1		Peak 2		Peak 1		Peak 2	
	Position	Intensity (a.u.)	Position	Intensity (a.u.)	Position	Intensity (a.u.)	Position	Intensity (a.u.)	Position	Intensity (a.u.)	Position	Intensity (a.u.)
	$\lambda_{\text{exc}}/\lambda_{\text{em}}$		$\lambda_{\text{exc}}/\lambda_{\text{em}}$		$\lambda_{\text{exc}}/\lambda_{\text{em}}$		$\lambda_{\text{exc}}/\lambda_{\text{em}}$		$\lambda_{\text{exc}}/\lambda_{\text{em}}$		$\lambda_{\text{exc}}/\lambda_{\text{em}}$	
25	280/309	201.86 ±16.85	230/304	294.71 ±27.95	280/338	383.44 ±50.33	230/333	666.51 ±12.65	280/356	140.75 ±29.77	230/349	173.71 ±44.19
	80	280/308	157.95 ±1.22	230/302	245.40 ±9.76	280/336	405.08 ±33.65	230/333	740.24 ±9.71	280/311	132.03 ±34.38	230/305
100	280/308	150.57 ±38.28	230/304	227.94 ±7.74	280/335	395.60 ±58.60	230/333	687.94 ±9.48	280/310	176.76 ±16.54	230/304	324.65 ±40.20

Table 2. Stern–Volmer quenching constants ($10^{-3} \text{ mol}^{-1} \text{ L}$) corresponding to hydrophobic residues of ovomucoid at different temperatures and pH values

Temperature, °C	pH 4.5	pH 7.0	pH 9.5
25	7.81±0.15	8.50±0.58	5.34±0.06
80	6.38±0.55	8.99±1.33	5.61±0.45
100	8.11±1.54	9.48±0.62	6.95±0.39

Table 3. Single molecular level descriptors on energy and structure of ovomucoid under thermal treatment

	Temperature		
	25°C	80°C	100°C
Energy descriptors			
Total energy, kJ mol ⁻¹	$(-1195.55 \pm 1.55) \cdot 10^3$	$(-976.62 \pm 0.79) \cdot 10^3$	$(-901.91 \pm 0.22) \cdot 10^3$
Potential energy, kJ mol ⁻¹	$(-1619.53 \pm 1.29) \cdot 10^3$	$(-1479.54 \pm 0.70) \cdot 10^3$	$(-1432.85 \pm 1.04) \cdot 10^3$
Structure descriptors			
Main secondary structure elements, %	Strand - 16.7; α -helix – 15.1	Strand - 11.3; α -helix – 14.0	Strand - 12.9; α -helix – 19.9
No of strands	11	9	8
No of β hairpins	5	4	4
No of β bulges	3	1	1
No of helices	3	3	5
No of β turns	24	26	17
No of γ turns	11	11	9
Hb ¹ within protein	107 $\pm$ 4	112 $\pm$ 6	110 $\pm$ 3
Hb protein-water	435 $\pm$ 6	359 $\pm$ 17	361 $\pm$ 10

¹Hb – hydrogen bonds

Supplementary material

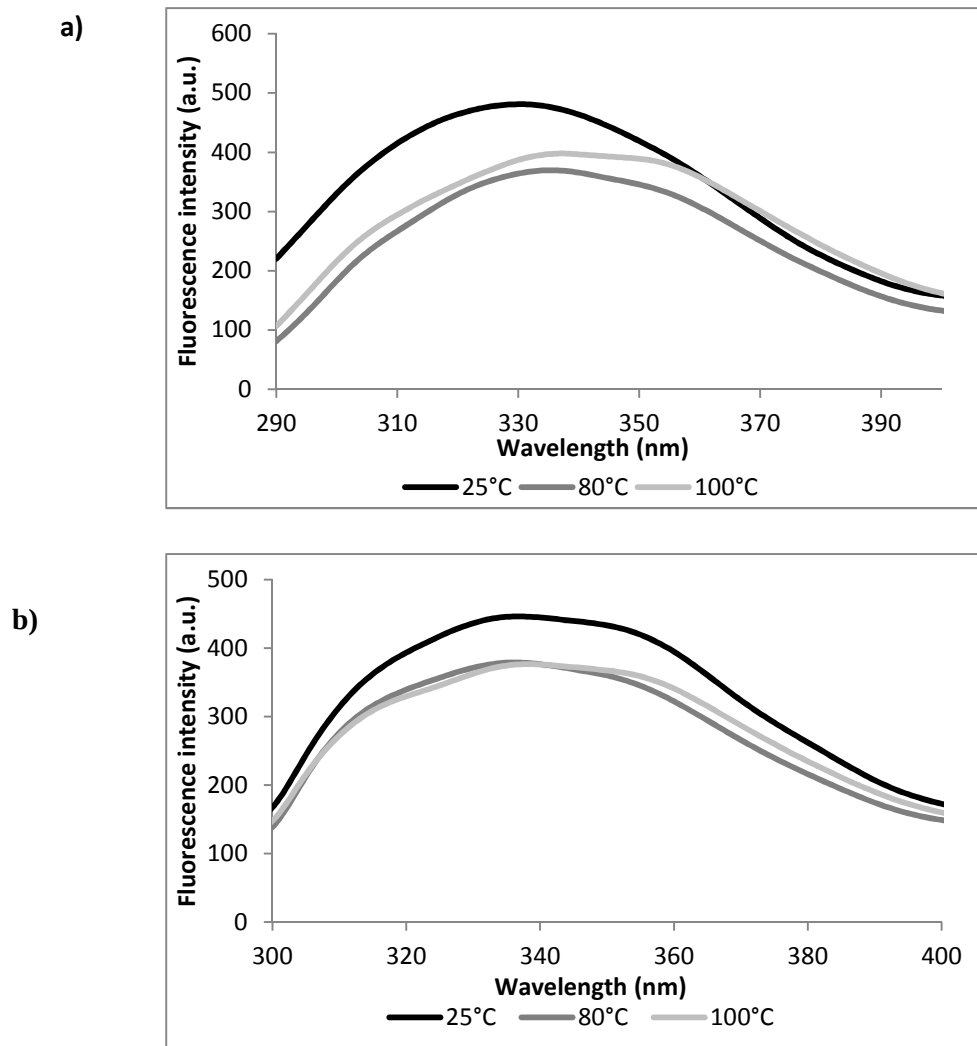


Figure S1. Heat induced structural changes of the ovomucoid solutions at pH 7.0 monitored as emissions spectra. The excitation wavelength was 274 nm (**a**) and 280 nm (**b**) and the spectrums were collected between 300 and 420 nm. Three independent tests were carried out in each case and SD was lower than 10%.

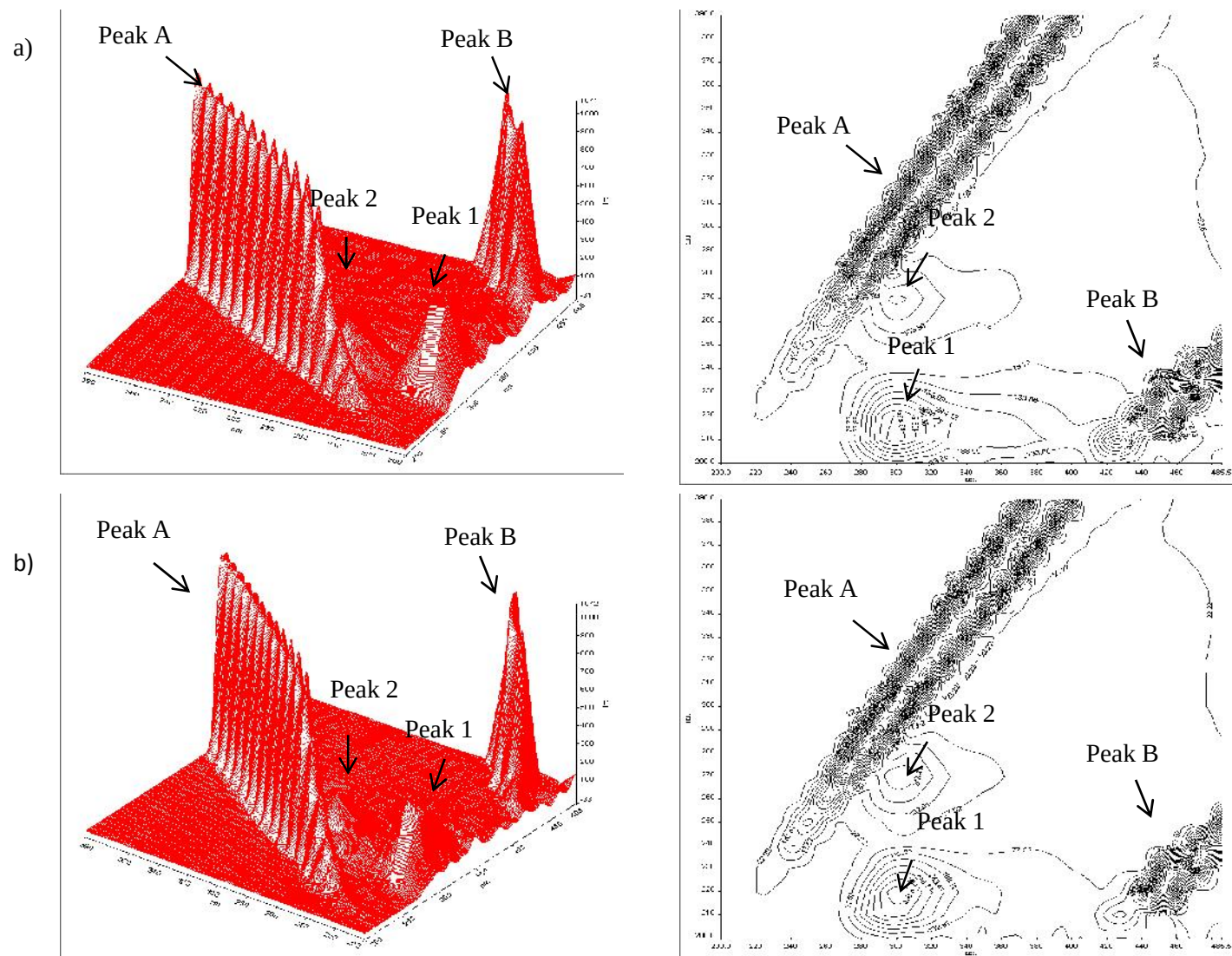


Figure S2. The EEMs spectra of ovalbumin solutions at pH 4.5 and selected temperatures: (a) 80°C, and (b) 100°C. Three independent tests were carried out in each case and SD was lower than 3.5%.

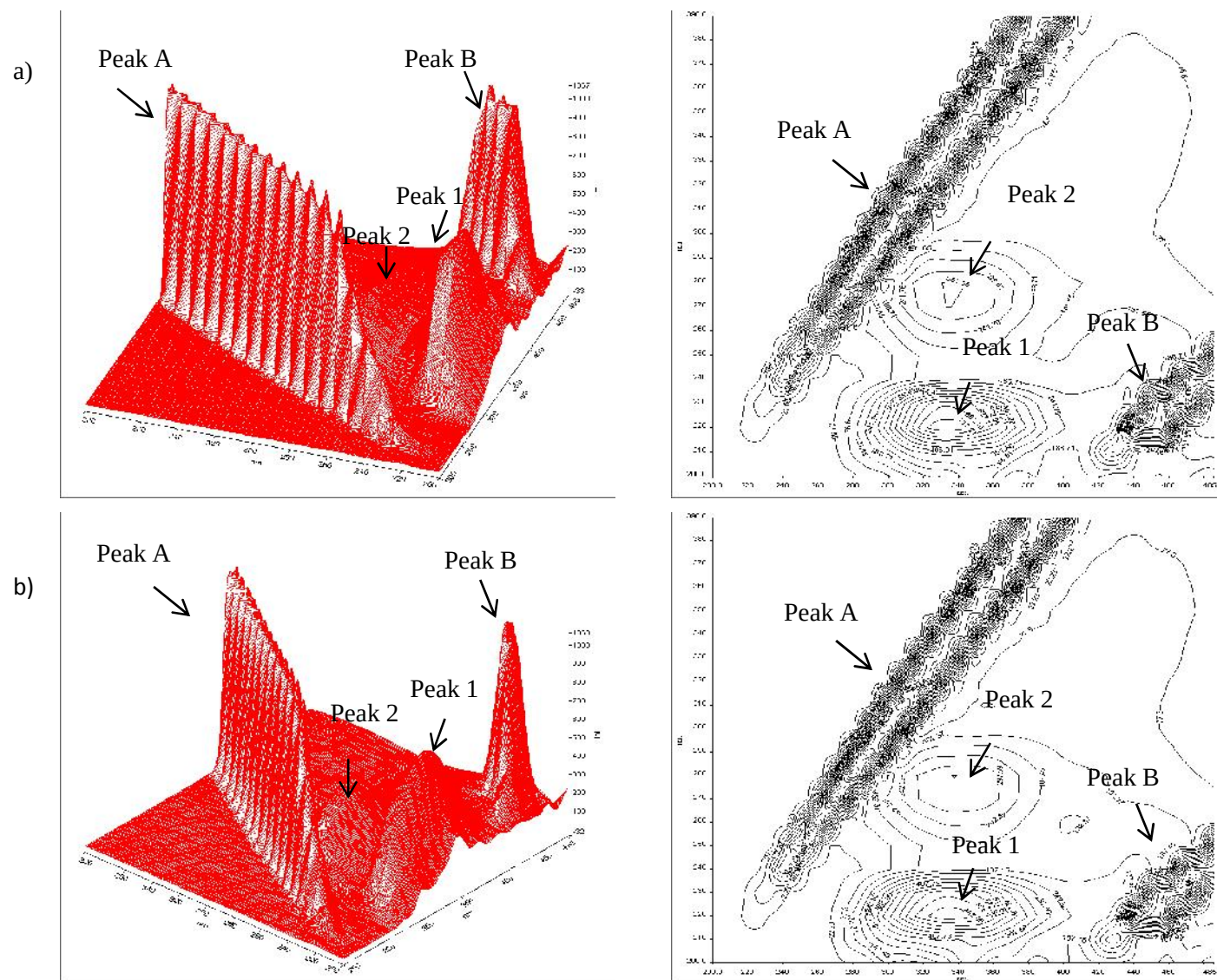


Figure S3. The EEMs spectra of ovalbumin solutions at pH 7.0 and selected temperatures: (a) 80°C, and (b) 100°C. Three independent tests were carried out in each case and SD was lower than 3.5%.

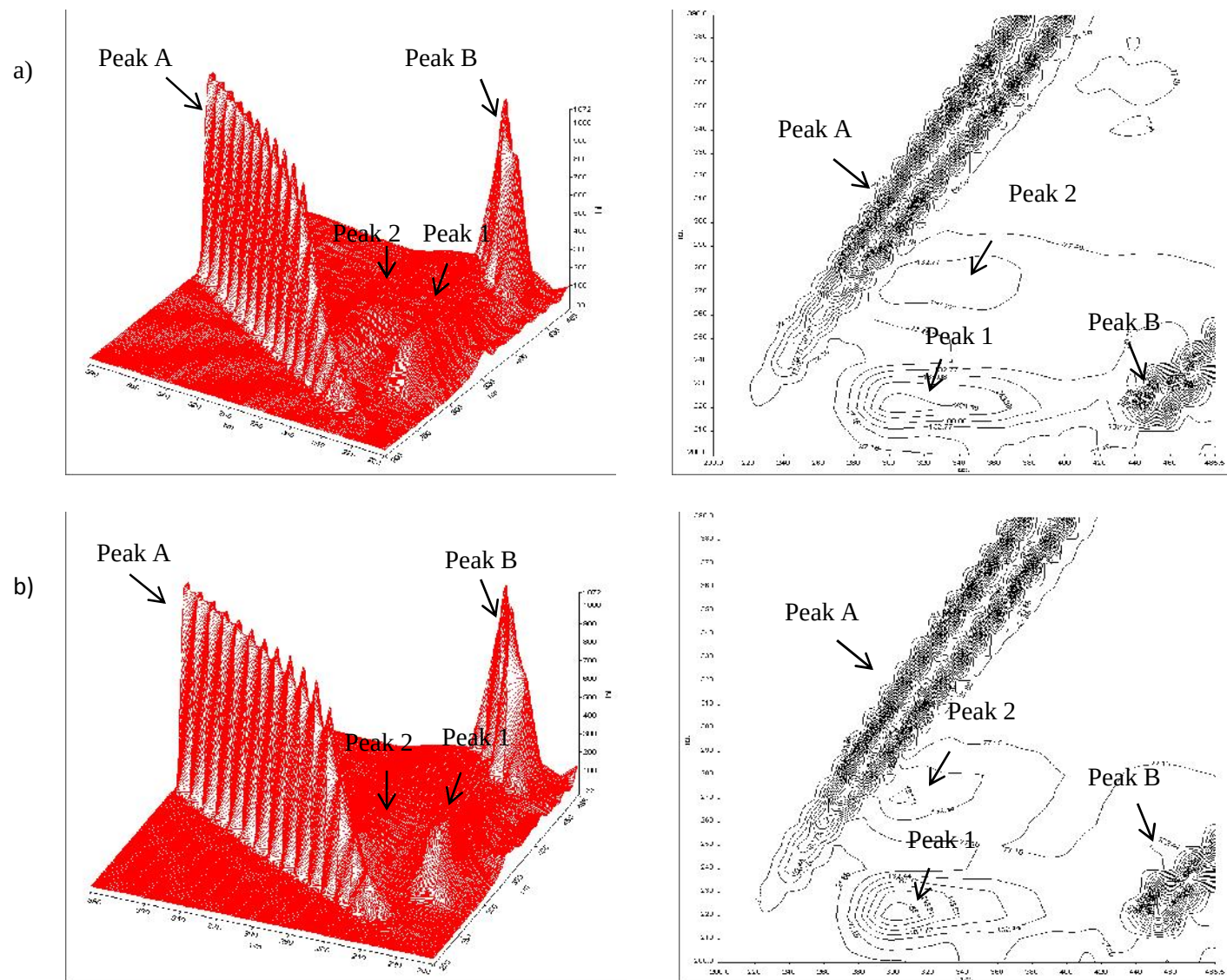


Figure S4. The EEMs spectra of ovalbumin solutions at pH 7.0 and selected temperatures: (a) 80°C, and (b) 100°C. Three independent tests were carried out in each case and SD was lower than 3.