

Effect of heat treatment on hen's egg ovomucoid: An immunochemical and calorimetric study

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Abstract

Egg causes a great number of allergies, being the allergenic proteins mainly contained in the white fraction. Among white proteins, ovomucoid is one of the most allergenic and can be considered as a good marker to detect egg in foods due to its high thermostability. We have studied the effect of heat treatment on ovomucoid immunoreactivity by ELISA and its denaturation in different media by differential scanning calorimetry (DSC). Ovomucoid was subjected to heat treatment in a range from 85 to 95 °C in buffer at pH 7.4. *D* values were obtained for the process of loss of immunoreactivity at the different temperatures and the *Z* value was of 15.6 °C. DSC of ovomucoid has been performed in two different buffers, at neutral or basic pH, and also in egg white. It has been found that ovomucoid is more thermoresistant at basic than at neutral pH. Furthermore, the resistance of ovomucoid in egg white is different to that observed in buffer at the same pH.

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1. Introduction

The frequency of food allergy has increased during the last decades and nowadays affects up to 2% of adult population and up to 8% of children (Poms, Klein, & Anklam, 2004). Different proteins derived from plants, milk and egg are used as ingredients in food technology, constituting a potential risk for allergic individuals who are not aware of ingesting them. Manifestation of allergic symptoms has been associated in some cases with contamination of food products with mere traces of the allergenic component. Therefore, the best solution for allergic patients is to avoid completely the ingestion of allergens (Taylor, 1985).

Egg constitutes one of the most common causes of food allergy, being probably the second cause in infant popula-

tion (Taylor & Nordlee, 1996). White egg is the main fraction responsible for the allergenicity of this food, ovomucoid, ovalbumin and ovotransferrin having been identified as the main allergenic white proteins (Cooke & Sampson, 1997; Hoffman, 1983; Langeland, 1982), while lysozyme and ovomucin seem to be the cause of egg allergies in a lower extent (Holen & Elsayed, 1990). In one study made on egg allergic persons, it was concluded that ovomucoid was the component which caused the greatest number of reactions as higher levels of serum IgG and IgE were found against ovomucoid than against any other white protein (Bernhisel-Broadbent, Dintzis, Dintzis, & Sampson, 1994). Some yolk proteins, such as apovitelin I and IV, have also been identified as egg allergens (Walsh et al., 1988).

Ovomucoid, which comprises 11% of egg white proteins, is a highly glycosylated protein containing 20–25% of carbohydrates (Besler, Steinhart, & Paschke, 1997). It has a molecular weight of 28.0 kDa, an acidic isoelectric point

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of 4.1 (Kato, Schrode, Kohr, & Laskowski, 1987) and it presents trypsin inhibitory activity (Rhodes, Bennett, & Feeney, 1959). Ovomucoid consists of three tandem domains clearly defined, each domain being internally linked by disulfide bonds (Kato et al., 1987), which makes ovomucoid so resistant to heat denaturation and proteolytic digestion (Matsuda, Watanabe, & Nakamura, 1982). Ovomucoid may cause allergies even when present in very small amount and it maintains the allergenic capacity though having been subjected to long heat treatments and to hydrolysis with digestive enzymes (Gu, Matsuda, & Nakamura, 1986; Matsuda, Watanabe, & Nakamura, 1983; Matsuda & Nakamura, 1993; Urisu et al., 1997). It has been observed that fragments of ovomucoid may be transferred through the epithelia of the intestinal membrane interacting with T cells that stimulate B cells to produce IgE against this protein (Kovacs-Nolan, Zhang, Hayakawa, & Mine, 2000). The high resistance to heat and proteolysis makes ovomucoid a good marker for the presence of egg in food products even in those heat-treated. Some assays based on the ELISA technique have been proposed to detect the presence of egg proteins, including ovomucoid, in manufactured products (Poms et al., 2004). It is important to know the degree of loss of immunoreactivity that ovomucoid may undergo with certain heat treatments to take it into account when analysing processed products containing egg.

The objective of our work was to determine the loss of ovomucoid immunoreactivity when subjected to heat treatment at different temperatures using an indirect competitive ELISA. The thermoresistance of ovomucoid was also studied by differential scanning calorimetry in order to investigate the effect of different conditions like pH, or the effect of other white proteins by heating ovomucoid in egg white.

2. Materials and methods

2.1. Ovomucoid isolation

2.1.1. TCA/acetone precipitation procedure

Ovomucoid was isolated from hen's egg white according to Lineaweaver and Murray (1947). White from hen eggs was homogenized by gentle agitation at 25–30 °C and the pH was lowered to 3.5 by adding slowly a solution of 0.5 M trichloroacetic acid (TCA)/acetone (1:2 v/v). The precipitated proteins were separated by sedimentation at 4 °C overnight and the supernatant after filtration was added with 2.5 vol. of 99% acetone. The mixture was left for 30 min to achieve precipitation of ovomucoid. The supernatant was added with one volume of acetone and the two precipitates obtained were mixed. Another volume of acetone was added to the precipitates and the supernatant eliminated. The precipitate dried with vacuum was resuspended in distilled water and its pH was adjusted to 4.5 with 0.1 N HCl. The solution of ovomucoid was dialyzed against distilled water to eliminate TCA. Two vol-

umes of acetone were added to the dialyzed solution, the ovomucoid was recovered with acetone and ether (1:1 v/v) and the precipitate dried with nitrogen. The powder was dissolved in 14 mM NaCl, 1 mM monopotassium phosphate, 8 mM sodium phosphate dibasic and 2 mM potassium chloride buffer, pH 7.4 (PBS). The isolated ovomucoid was used to immunize several rabbits in order to obtain specific antisera.

2.1.2. TCA/ammonium sulphate precipitation procedure

A second method was carried out to isolate ovomucoid according to Waheed and Salahuddin (1975). This procedure is gentler than that of Lineaweaver and Murray (1947) and therefore was more adequate to obtain ovomucoid for the purpose of studying its heat resistance. To a certain volume of hen's egg white the same volume of 10% TCA was added, the mixture homogenized by agitation and the pH adjusted to 3.5 with 1 M NaOH. The mixture was left overnight without agitation and a precipitate was obtained. This precipitate consisted of the majority of white proteins, except ovomucoid which does not precipitate in such conditions. Afterwards, the mixture was centrifuged during 15 min at 1400g at 10 °C and the supernatant was recovered. The supernatant was brought to 90% (w/v) ammonium sulphate and adjusted to pH 4.6. After settling overnight, the mixture was centrifuged during 15 min at 1400g and the precipitate was recovered, dissolved in distilled water and dialyzed against distilled water.

2.1.3. Gel filtration chromatography

The chromatography used to purify the TCA/acetone precipitated ovomucoid was performed in a Sephadex G-100 column (2 cm diameter × 90 cm length) (Sigma, USA). The column was equilibrated in 0.15 M NaCl, 0.1 M potassium phosphate buffer, pH 7.4 (phosphate buffer), and 5.8 ml of a 14 mg/ml solution of ovomucoid were applied. The column flow was 0.5 ml/min and the fractions collected were of 2 ml. The absorbance of the fractions was determined at 280 nm and the purity checked by electrophoresis. The concentration of ovomucoid in the isolated fraction was determined by the BCA protein assay (Pierce, USA). This purified ovomucoid was used to immunize the rabbits.

2.2. Preparation of antisera against ovomucoid

Antisera against ovomucoid were raised in rabbits by injection of the protein isolated by the described TCA–acetone method. In the first immunization, 1 ml of an emulsion consisting of 0.5 ml of complete Freund adjuvant (Sigma, USA) and 0.5 ml of 2 mg/ml ovomucoid solution were administered in several subcutaneous injections in the back. After 20 days of the first injection, a second injection was administered with the same amount of protein emulsified with incomplete Freund adjuvant (Sigma, USA). Ten days after the second injection, a test bleeding was done to check the presence of antibodies against

ovomucoid by immunodotting, as described below. Positive rabbits were bled and new immunizations were administered to the same animals to maintain the level of antibodies in the serum during a certain period of time.

2.3. Immunodotting

This technique was performed to determine the presence of antibodies against ovomucoid in the sera of immunized rabbits according to Aranda, Oria, and Calvo (1988). Samples (1 µl) containing ovomucoid as 1:2 diluted egg white and 1 mg/ml ovomucoid in phosphate buffer were applied onto a nitrocellulose membrane Trans-Blot Transfer Medium (Bio-Rad, USA) and left to dry. A sample containing bovine β-lactoglobulin was applied as negative control. The membrane was saturated with 3% (w/v) skimmed milk powder in phosphate buffer incubating it for 1 h at 37 °C with gentle agitation. Afterwards, the nitrocellulose was incubated with a dilution of the tested antisera in phosphate buffer containing 1% of skimmed milk powder during 1 h at 37 °C. The nitrocellulose was exhaustively washed with phosphate buffer (10 times with agitation). Then, it was incubated for 1 h at 37 °C with antibodies against rabbit IgG conjugated with peroxidase (Sigma, USA) 1:2000 diluted in phosphate buffer containing 1% of skimmed milk powder. Finally, after 10 washings, the nitrocellulose was incubated with a peroxidase substrate: 6 mg of 4-chloro-1-naphthol (Sigma, USA) diluted in 2 ml of ethanol, 8 ml of phosphate buffer and 10 µl of hydrogen peroxide (30%). The development of a greyish blue spot indicated a positive result.

2.4. Indirect competitive enzyme-linked immunosorbent assay (ELISA)

ELISA applied to determine ovomucoid concentration was an indirect competitive assay according to the following procedure. 96-well Maxisorp microtitre plates (Nunc, Denmark) were coated with 300 µl/well of 1 µg/ml native ovomucoid solution, isolated by the TCA/ammonium sulphate method, in 15 mM sodium carbonate, 35 mM sodium bicarbonate buffer, pH 9.6, overnight at 4 °C. Afterwards, the plate was washed five times with 300 µl/well of PBS containing 0.05% Tween 20 (PBST) using a Multiwash microtitre plate automatic washer (Labsystems, Finland). After the washings, 100 µl of the samples was added per well: those consisting of ovomucoid standards (200 µg/ml, 100 µg/ml, 20 µg/ml, 2 µg/ml, 1 µg/ml and 20 ng/ml) and ovomucoid samples after heat treatment at different temperatures and times. Immediately after adding the samples to the wells, 100 µl of 1:5000 dilution of the anti-ovomucoid antiserum in PBST was added and the plate was incubated during 45 min at room temperature. The plate was washed five times and 200 µl of 1:4000 dilution in PBST of the goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma, USA) was added per well being incubated for 45 min. Five more washings were done

and then, 100 µl of 3,3'-5,5'-tetramethylbenzidine Peroxidase substrate Immunopure R TMB Kit (Pierce, USA) was added. After 15 min of incubation with the substrate at room temperature, the reaction was stopped by adding 100 µl of 2 M sulphuric acid to each well and the absorbance was read at 450 nm in a Multiskan MS microtitre photometer (Labsystems, Finland).

2.5. Heat treatment by capillary method

Samples of isolated ovomucoid were subjected to heat treatment following the capillary method as previously described (Sánchez et al., 1992). A volume of 20 µl of a 0.5 mg/ml ovomucoid solution in phosphate buffer, pH 7.4, was introduced into glass capillary tubes (1.5–1.6 mm outer diameter, 1.1–1.2 mm inner diameter) which were sealed with a microflame and immersed in tepid water to check hermetic closure. Three capillaries for each temperature and time were prepared and immersed in a temperature-controlled water bath at different temperatures: 85, 87, 90, 92, and 95 °C (± 0.1 °C). Heated capillaries were removed at different times and immediately cooled in an ice-water bath. Samples from the capillary tubes were extracted with a micropipette, diluted to the adequate dilution in phosphate buffer and their ovomucoid concentration was determined by the indirect competitive ELISA as described. Heat treatments at each temperature studied were performed at least twice, in independent experiments. The advantage of using the capillary method for heating the samples of ovomucoid instead of using any other method is that it is easy to heat many samples at different times of treatment by triplicate, heat transfer of the sample is fast through the wall of the capillary and it requires a very small amount of protein.

2.6. Calculation of *D*- and *Z*-values

D-values, the time required to decrease immunoreactive protein by 90%, were calculated by regression analysis of the lines obtained by plotting the logarithm of ovomucoid concentration against time. *D*-values correspond to the reciprocal of the slope of those lines.

Z-value, degrees necessary to reduce *D*-value in one logarithmic cycle, was calculated by regression analysis of the line obtained by plotting the logarithm of *D*-values against the corresponding temperatures. *Z*-value corresponds to the reciprocal of the slope of that line.

2.7. Differential scanning calorimetry (DSC) analysis

Thermal analysis was performed in a DSC 10 Differential Scanning Calorimeter (Du Pont Instruments, USA). Ovomucoid obtained by the TCA/ammonium sulphate isolation as described or commercial ovomucoid (Sigma, USA) was treated in different media. A solution of 300 mg/ml ovomucoid in phosphate buffer, pH 7.4, and a solution of 211 mg/ml ovomucoid in 0.1 M sodium

carbonate buffer, pH 9.1 or pH 9.6, or in homogenized egg white (pH 9.1) were prepared. About 3 mg of protein, in a volume of 10 μ l for the solutions of 300 mg/ml and in 15 μ l for the solutions of 211 mg/ml, was introduced in aluminium pans (TA Instruments, USA) which were sealed and weighed. In a reference capsule, 10–15 μ l of the corresponding medium was introduced. The initial heating temperature was 35 °C, the heating rate was 10 °C/min and the final temperature was 110 °C. Once the aluminium pans had been heated and cooled, they were weighed again to check that no leak of solution had been produced during the heating process.

2.8. Statistical analysis

Data obtained were statistically evaluated by analysis of variance (ANOVA) by the Tukey test using the SPSS 13.0 package for Windows.

3. Results and discussion

3.1. Ovomuroid isolation and obtention of specific antisera

The fractions enriched in ovomucoid obtained by the precipitation methods were subjected to chromatography on Sephadex G-100 to eliminate contaminant proteins from egg white, mainly lysozyme. The profile of one chromatography is shown in Fig. 1, the main peak of which consists of ovomucoid. The fraction of purified ovomucoid was used for rabbit immunization. The presence of specific antibodies against ovomucoid was detected in the sera of the four immunized rabbits by immunodotting (results not shown).

3.2. Thermoresistance of ovomucoid: determination of immunoreactivity

The study of ovomucoid thermoresistance has been performed on the protein isolated by the TCA/ammonium sulphate precipitation as this isolation is less harmful for the protein integrity. However, ovomucoid isolated by the TCA/acetone precipitation has been used for rabbit immunization. Although it was slightly more denatured than ovomucoid isolated by TCA/ammonium sulphate precipitation, as the DSC studies showed, this was not a problem to obtain the antibodies against it.

The concentration of immunoreactive ovomucoid subjected to different heat treatments by the capillary method was determined by an indirect competitive ELISA technique. After heat treatment, ovomucoid solution was extracted from the capillaries, diluted 1:12 in PBST and analysed by ELISA. The graphical representations of the effect of different heat treatments on ovomucoid immunoreactivity are shown in Fig. 2a–e. Immunoreactivity of ovomucoid decreases with the increase in temperature and time of treatment. *D* values (*D*_t) shown in Table 1 have been calculated as the mean of *D* values obtained from the graphical representation of every experiment, 2–4 independent experiments were performed for each temperature. It has been observed that *D*-values decrease with temperature and that this decrease is more rapid in the temperature range from 85 to 90 °C than in that from 92 to 95 °C. Graphical representation of the effect of temperature on *D* value is shown in Fig. 3, where *Z* value is calculated from the slope of the regression line obtained, this value being of 15.6 °C.

In previous works, the effect of heat on antigenic reactivity had been studied together with the effect on the trypsin inhibitory activity of ovomucoid. In the work of Matsuda et al. (1982), a treatment of 90 °C for 120 min diminished trypsin inhibitory activity of ovomucoid to around 15% of the initial value, whereas with the same treatment 40% of the immunoreactivity was maintained. Gu et al. (1989) also found a high decrease in the trypsin inhibitory activity of ovomucoid, to 8% of the initial activity, after treatment at 90 °C for 100 min; however, they found that ovomucoid immunoreactivity remained very high, 84% of the initial value, after the same treatment. The treatment we have applied in the present work at 90 °C has been only up to 30 min, therefore, we cannot compare our results of immunoreactivity with those at a longer period of treatment. However, we can consider the times of heat treatment needed for 50% loss of immunoreactivity which were of 8 min in our work and 75 min in the work of Matsuda et al. (1982). According to the study performed by Gu et al. (1989), it is not possible to determine such value because there is no appreciable decrease of immunoreactivity after 100 min of treatment, the maximum time they applied. These discrepancies between results of loss of ovomucoid immunoreactivity with heat treatment are probably due to the different source of antibodies and techniques

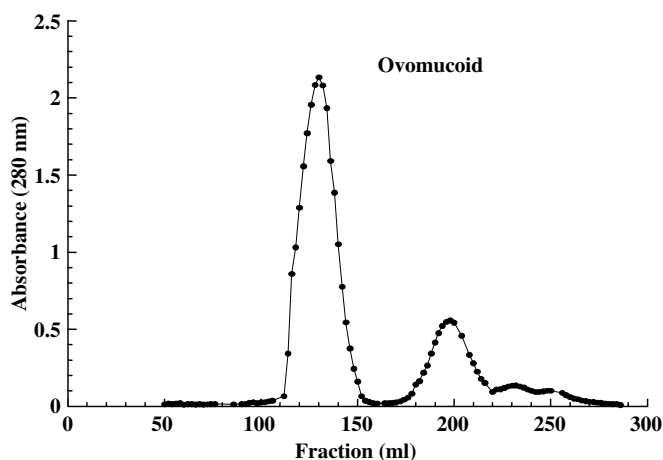


Fig. 1. Gel filtration chromatography in Sephadex G-100 (2 cm diameter $\times$ 90 cm) of ovomucoid fraction. The sample applied was 5.8 ml of a 14 mg/ml ovomucoid isolated by precipitation with TCA/acetone. The fractions collected were of 2 ml and the flow of 0.5 ml/min.

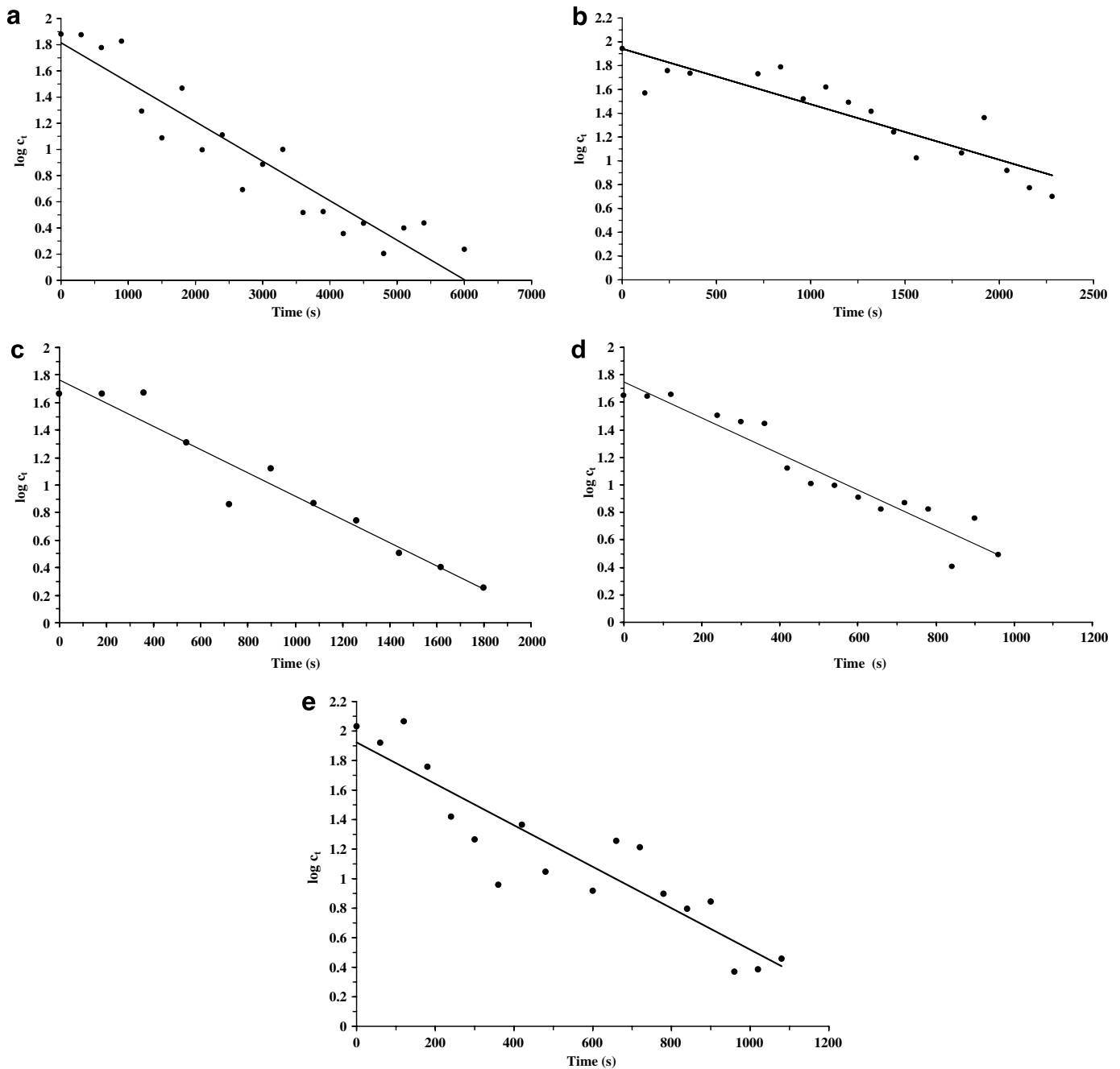


Fig. 2. Effect of heat treatment on immunoreactivity of ovomucoid in PBS (pH 7.4) measured by competitive ELISA at different temperatures: 85 °C (a), 87 °C (b), 90 °C (c), 92 °C (d) and 95 °C (e). c_i is immunoreactive ovomucoid at each holding time.

Table 1

D and Z values corresponding to the process of loss of immunoreactivity of ovomucoid heated in PBS (pH 7.4) determined by ELISA

	D_t values (s)
D_{85}	2.98×10^3
D_{87}	2.22×10^3
D_{90}	1.06×10^3
D_{92}	9.03×10^2
D_{95}	7.40×10^2
Z value (°C) = 15.63	

used. Gu et al. (1989) used monoclonal antibodies against domain III, the most thermoresistant of the three domains of ovomucoid (Biplap, Sdhir, Arwaral, & Khan, 1990), whereas in our work and in that of Matsuda et al. (1982) polyclonal antibodies directed against the whole protein have been employed. In fact, it has been reported that specific IgG against ovomucoid obtained in rabbits are directed towards antigenic regions present in the three domains (Cooke & Sampson, 1997). Furthermore, different techniques have been used to estimate loss of ovomucoid

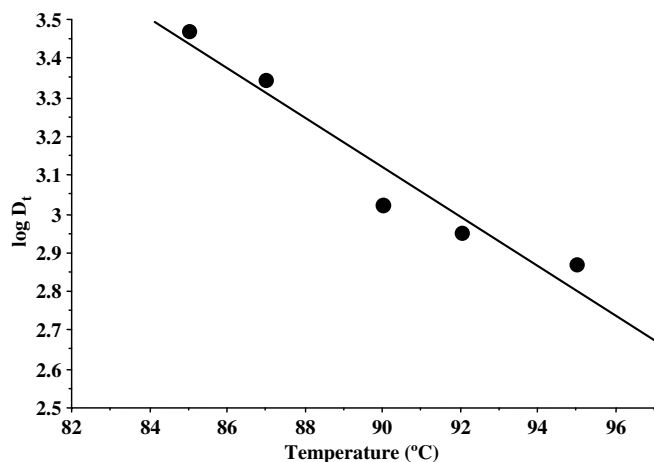


Fig. 3. Effect of temperature on D_t values corresponding to the process of loss of immunoreactivity of ovomucoid heated in PBS (pH 7.4) determined by indirect competitive ELISA.

immunoreactivity. In the study done by Matsuda et al. (1982), a rather rudimentary technique based on immunoprecipitation and colorimetric determination of precipitates after centrifugation was applied, whereas in our work and in that of Gu et al. (1989) more sensitive and precise techniques were used, an indirect competitive ELISA and a direct ELISA, respectively.

Moreover, it has been shown that ovomucoid immunoreactivity is dependent on the type of immunoglobulins used. Actually, ovomucoid has been even detected after very high treatments (100 °C for 45 min) using human specific IgE, while in the same study rabbit specific antibodies were not able to detect it (Gu et al., 1986). This finding indicates that products containing egg treated at a very high temperature could not be completely safe for allergic individuals to ovomucoid, as it has been stated in some works (Urisu et al., 1997).

In the analysis we have made of the effect of heat on ovomucoid immunoreactivity, the adjustment of the regression lines obtained when plotting the values of concentration along the time of treatment was not as good as expected. Although the standard curves of ELISA were linear in the range of concentration of the standards used, with high regression coefficients ($r^2 = 0.92$ – 0.99), the relationship between the immunoreactivity of ovomucoid and the time of treatment was not as linear as we had found in similar studies (Mainer, Sánchez, Ena, & Calvo, 1997; Sánchez et al., 1992). This fact can be due to the different immunoreactivity that ovomucoid molecules can display after heat treatment as it has been previously reported. Matsuda et al. (1982) found that heated ovomucoid subjected to affinity chromatography gave two different fractions and suggested that those fractions were composed by molecules denatured in different degree with different affinity for specific antibodies. Hirose, Kitabatake, Kimura, and Narita (2004) also reported the existence of two immunologically distinct forms of ovomucoid, which reacted with different monoclonal antibodies. Further-

more, the heterogeneity of heated ovomucoid immunoreactivity could affect in a different way depending on the format of immunoassay performed, as it has been observed in our laboratory for ovomucoid and for other proteins (results not shown).

It has to be pointed out that ovomucoid experiments an important refolding while cooling after heat treatment, as it has been observed in the DSC study of the present work and in previous studies (Matsuda, Watanabe, & Sato, 1981). The partial restoration of ovomucoid while the samples are cooling in the capillary tubes could have a strong contribution to the nonlinear behaviour shown by the process of loss of ovomucoid immunoreactivity. Despite the heterogeneity of ovomucoid, the results obtained in our work permit to estimate the loss of immunoreactivity of this protein at different heat treatments in the range of temperatures studied, from 85 to 95 °C, and compare it with the kinetic values obtained for loss of trypsin inhibitory activity of ovomucoid reported in a wide and well documented study by Van der Plancken, Van Remoortere, Van Loey, and Hendrickx (2004). The D values obtained in that work at the same temperatures we have used for heat treatments were very close to ours, though slightly higher, and the Z value was of 17.51 °C in their work and of 15.63 °C in ours, which is also very similar. This similarity between sensitivity of immunoreactivity and trypsin inhibitory activity of ovomucoid to heat treatment had also been previously reported by Konishi, Kurisaki, Kaminogawa, and Yamauchi (1985).

3.3. Thermoresistance of ovomucoid: DSC analysis

Samples of ovomucoid isolated by precipitation with TCA/ammonium sulphate and from commercial origin were analysed by differential scanning calorimetry in different conditions. Thermograms, which are the recording of rate of heat flow as a function of temperature, are shown in Figs. 4–6 and the thermal parameters are shown in Table 2. The maximum temperature of denaturation is considered as the temperature of maximum rate of heat flow in the thermogram. The enthalpy of denaturation is calculated by integrating the rate of heat flow into the sample over the time interval of the denaturation and dividing the total heat absorbed by the total moles of protein analysed. The thermogram corresponding to commercial ovomucoid heated in pH 7.4 buffer presented one single peak with a maximum temperature of 76.54 °C and a denaturation enthalpy of 460.04 kJ/mol (Fig. 4a). The thermogram corresponding to DSC of ovomucoid isolated by TCA/ammonium sulphate and treated in a pH 7.4 buffer (Fig. 4b) also showed one single peak with a maximum temperature of denaturation of 78.26 °C and a denaturation enthalpy of 397.01 kJ/mol. Comparing the values of ovomucoid from two origins by ANOVA, it was found that only maximum temperatures of denaturation are significantly different. This could be explained by slight differences in the structure of ovomucoid due to the different method of isolation.

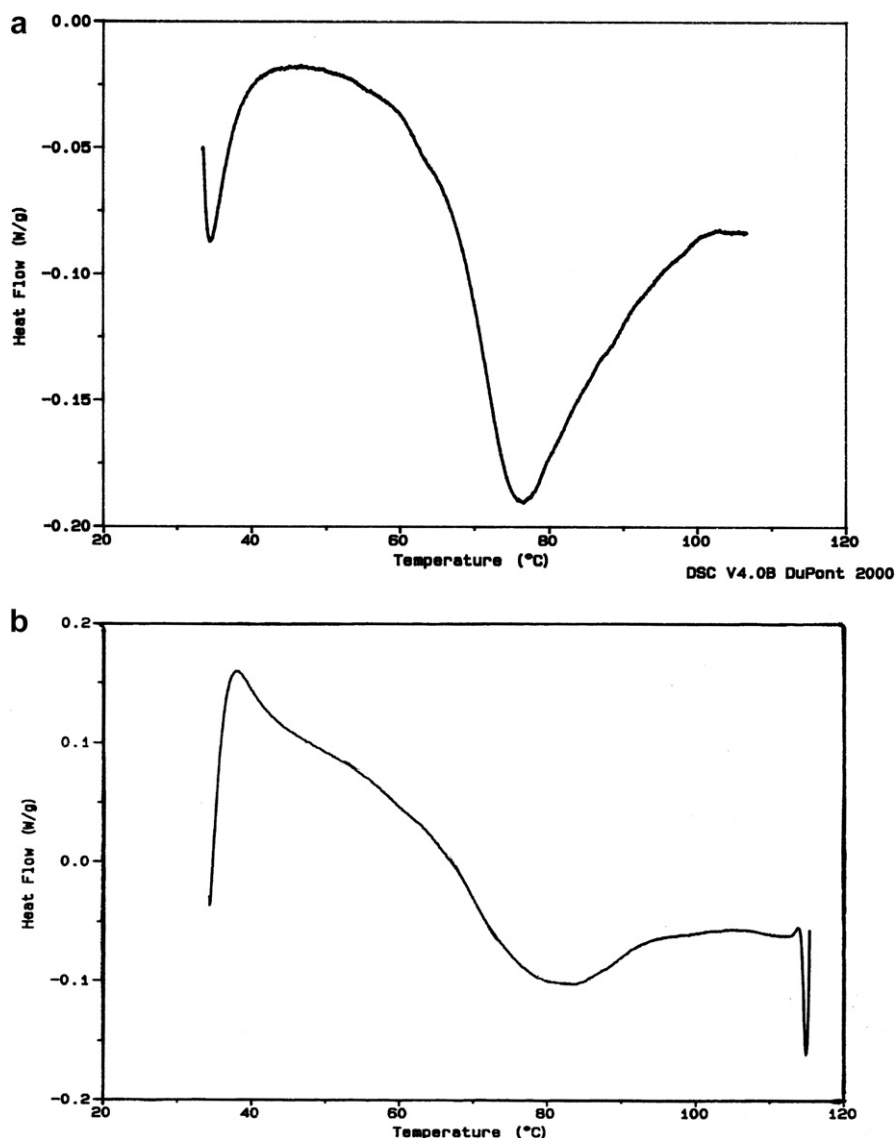


Fig. 4. Differential scanning calorimetry thermograms of commercial ovomucoid (a) and isolated ovomucoid by TCA/ammonium sulphate precipitation (b) in PBS (pH 7.4). Heating rate 10 °C/min.

The shape of both peaks is markedly different, being that obtained for commercial ovomucoid sharper than that obtained for the isolated protein. In the work of [Donovan and Beardslee \(1975\)](#) in which ovomucoid was subjected to DSC at neutral pH, they found very close values of maximum temperature of denaturation to ours, though the thermogram showed a more extended peak than that obtained in our work, and consequently a higher value of enthalpy (636 kJ/mol). [Donovan and Beardslee \(1975\)](#) indicated that this kind of shape reflects a complex denaturation in which ovomucoid experiments predenaturational conformational alterations. These alterations appearing with increase in temperature could produce new forms of ovomucoid which are more stable to heat denaturation than the normal form found at room temperature.

We observed that the samples of ovomucoid prepared in alkaline buffer had to be stabilized before being subjected

to DSC. When samples of ovomucoid were analysed immediately after being dissolved, the thermogram obtained was very broad and extended to 100 °C in some of the replicates. However, after 2 h of stabilization of the solution at room temperature, the thermograms did not extend to 100 °C in any of the replicates analysed, and their shape was more similar among replicates. The influence of the stabilization time for ovomucoid solutions on DSC thermograms was not observed for solutions of neutral pH.

The endotherm obtained when ovomucoid was heated in pH 9.1 buffer presented two maximum temperatures, 74.34 and 82.31 °C ([Fig. 5a](#)), and 73.16 and 81.97 °C when heated in pH 9.6 ([Fig. 5b](#)). Variations of enthalpy were 732.61 and 609.97 kJ/mol for ovomucoid in pH 9.1 and pH 9.6, respectively. None of the parameters for ovomucoid treated in pH 9.1 were significantly different from those corresponding to the ovomucoid treated in pH 9.6.

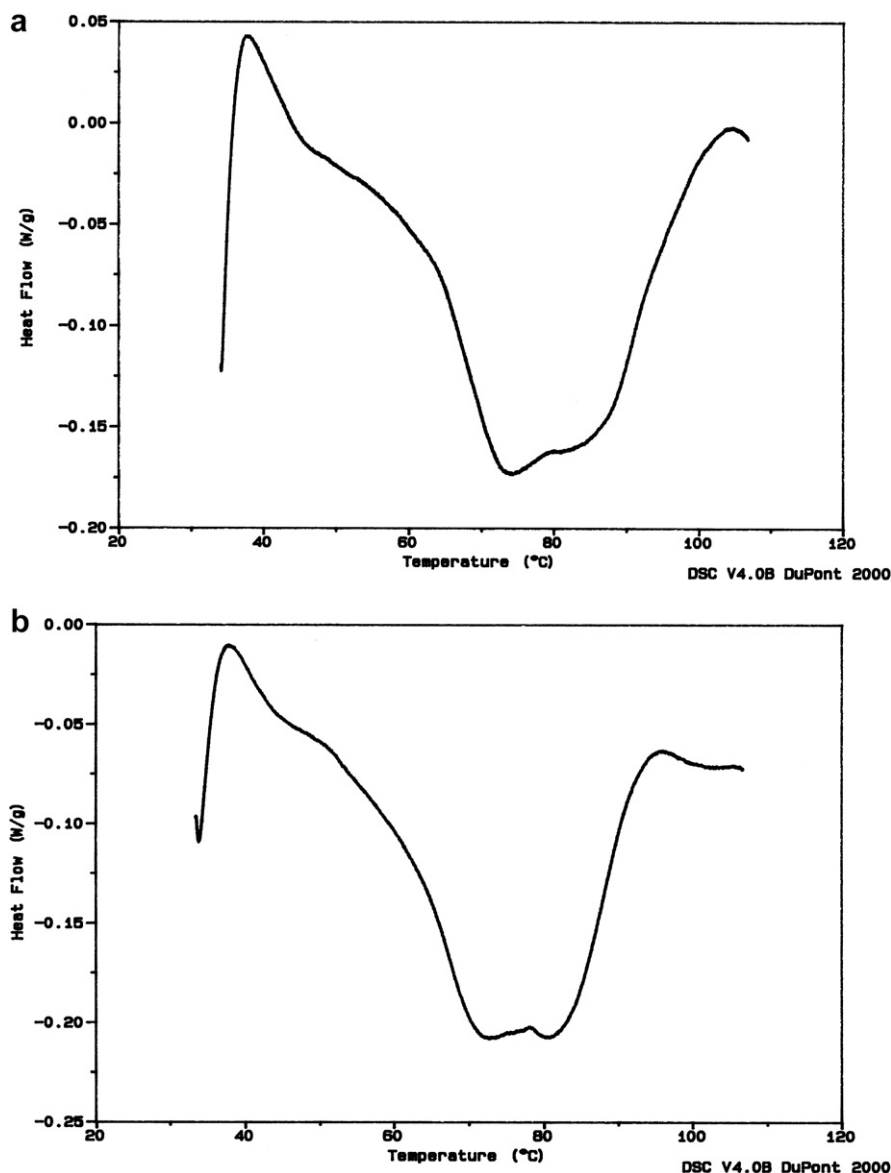


Fig. 5. Differential scanning calorimetry thermograms of commercial ovomucoid in 0.1 M sodium carbonate pH 9.1 (a) and pH 9.6 (b). Heating rate 10 °C/min.

Commercial ovomucoid was also treated in egg white and a thermogram with a single peak was obtained (Fig. 6) with a maximum temperature of denaturation of 79.75 °C and an enthalpy of denaturation of 527.39 kJ/mol. Although it has to be considered that the egg white subjected to DSC gives an endotherm with two peaks, as shown in the inset of Fig. 6, the thermogram obtained for DSC of ovomucoid in egg white did not show the double maxima observed in carbonate buffer of pH 9.1, though the two media presented the same pH. This is probably due to the influence of the other white proteins on ovomucoid heat resistance.

Studies have shown that the thermal transitions of the different domains of ovomucoid, though independent, are all produced in a very short range of temperature at neutral pH (Matsuda et al., 1981). It is possible that at another pH there is a shift in the transition of the different domains of

ovomucoid, which could explain that the peak observed at basic pH showed two maxima. The high enthalpy of denaturation that presented the endotherm of ovomucoid treated at basic pH could also reflect a change in the conformation of the protein. However, when ovomucoid was heated in egg white the appearance of the peak was different from the peaks corresponding to samples in basic buffers. The maximum temperature and enthalpy of denaturation were lower when compared with the parameters given by ovomucoid heated in carbonate buffer of pH 9.1. If the value of enthalpy of denaturation of the endotherm given by egg white alone (214 kJ/mol is the mean of three replicates) is subtracted from the value of enthalpy for ovomucoid heated in egg white, the value obtained is of 313 kJ/mol, clearly lower than that obtained for ovomucoid heated in basic buffers. This indicates that

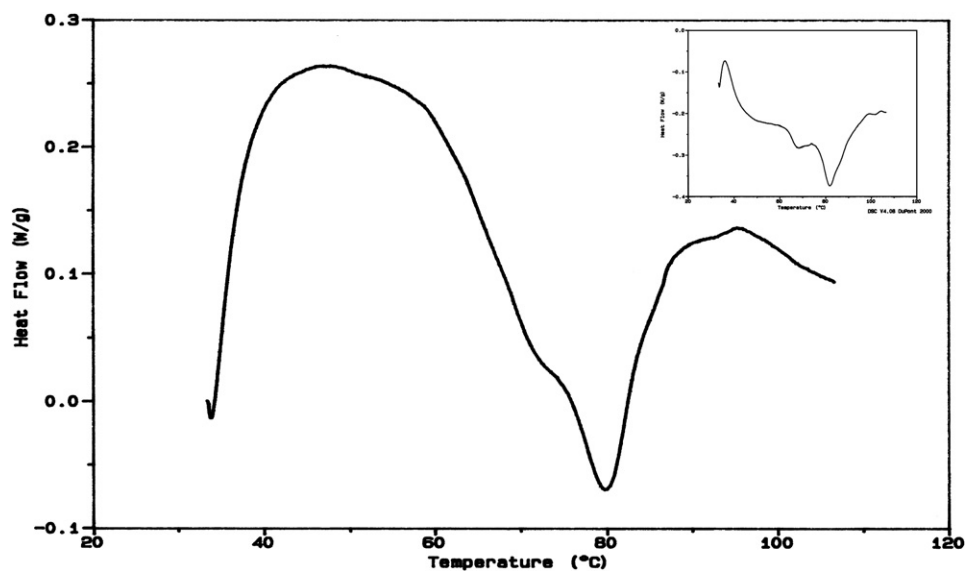


Fig. 6. Differential scanning calorimetry thermograms of commercial ovomucoid in hen egg white (pH 9.1). Inset corresponds with differential scanning calorimetry thermogram of egg white. Heating rate 10 °C/min.

Table 2

Thermal parameters for denaturation of ovomucoid in buffers of pH 7.4, 9.1 and 9.6, and in egg white (pH 9.2) by differential scanning calorimetry

	T_s (°C)	T_{max} (°C)	ΔH (kJ mol ⁻¹)
Commercial ovomucoid in phosphate buffer (pH 7.4) $n = 6$	66.37 ± 1.15a	76.54 ± 0.24a	460.04 ± 97.41ace
Isolated ovomucoid in phosphate buffer (pH 7.4) $n = 6$	62.75 ± 1.56bc	78.26 ± 0.57b	397.01 ± 85.17ad
Commercial ovomucoid in carbonate buffer (pH 9.1) $n = 5$	60.97 ± 1.73b	74.34 ± 0.31c 82.31 ± 1.48	732.61 ± 94.46b
Commercial ovomucoid in carbonate buffer (pH 9.6) $n = 5$	60.69 ± 1.17b	73.16 ± 1.71c 81.97 ± 0.94	609.97 ± 60.34bc
Commercial ovomucoid in hen's egg white (pH 9.1) $n = 5$	65.49 ± 2.19ac	79.75 ± 0.36d	527.39 ± 130.11cde

Values represent the mean and the standard deviation of the values obtained in 5–6 independent experiments. Same letters (a, b, c, d) in the same column indicate no significant differences between means ($P < 0.05$).

T_s , onset temperature; T_{max} , peak maximum temperature; ΔH , denaturation enthalpy.

the other white proteins exert a certain influence on ovomucoid when subjected to heat treatment decreasing its thermal stability. These results would be in agreement with those of Van der Plancken et al. (2004) who found that the decrease of trypsin inhibitory activity of ovomucoid was faster when heated in egg white than in citrate buffer (pH 7.6), even if they had used egg white at pH 7.6 as for the buffer. They explained this finding by the previously reported interaction of ovomucoid with lysozyme (Matsuda, Watanabe, & Sato, 1982).

Some studies have been performed on the effect of pH on heat denaturation of ovomucoid and the results are contradictory. Gu et al. (1989) found that the immunoreactivity of ovomucoid heated at 90 °C measured by direct ELISA decreased more rapidly at pH 9.5 than at pH 7.5. Likewise, Lee et al. (2002) found a higher decrease of immunoreactivity at basic pH than at physiological conditions measuring it by a competitive indirect ELISA using IgE. On the contrary, Van der Plancken et al. (2004) found lower thermal stability of ovomucoid at pH 7.6 than at 9.0 in terms of trypsin inhibitory activity. On the other hand, some authors did not find differences at different pHs as Konishi et al. (1985) who reported the same kinetics for

denaturation of ovomucoid at 100 °C, when measuring antigenicity and trypsin inhibitory activity at pH 9.0 or 7.0. It can be concluded that there is an influence of pH on ovomucoid thermoresistance in a different degree depending on measured parameters and method of evaluation, but in any case, it has to be taken into account as pH increases in the storage of eggs, from pH 7.6 in fresh eggs to 9.2 in eggs stored for 12 days (Gu et al., 1986).

4. Conclusions

In this work we have obtained the kinetic parameters, D and Z values, for the process of loss of ovomucoid immunoreactivity with heat treatment in a temperature range from 85 to 95 °C. The knowledge of those parameters is of relevance to estimate the remaining immunoreactive protein after a certain treatment. The high thermoresistance of ovomucoid makes this protein especially suitable to detect egg presence in products that have been subjected to heat treatment. Ovomucoid denaturation has also been studied by calorimetry at different pHs: 7.4, 9.1 and 9.6, and also in egg white. We have observed that ovomucoid denatures following different patterns at basic or neutral

pH and that white proteins exert a certain influence on ovomucoid denaturation.

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