Analysis of Structural Properties and Immunochemical Reactivity of Heat-Treated Ovalbumin

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Protein detection to certify foodstuff composition is frequently accomplished by immunochemical methods. Product processing (heating, pH change, etc.) usually alters protein structure and can modify immunochemical reactivity of the protein. We studied the structural changes caused by heating ovalbumin under different time and temperature conditions and the influence of heat on the immunochemical reactivity of ovalbumin. Differential scanning calorimetry (DSC), exposed sulfhydryl groups, and surface hydrophobicity (H_o) were used to evaluate structural changes. The immunochemical reactivity was measured by an inhibition ELISA. By DSC we obtained an equation that can predict the denatured fraction after a heat treatment. We found a great increase of exposed sulfhydryl groups and surface H_o after denaturing treatments, with a good correlation between the theoretically calculated denaturation degree and the measured values. Using ELISA we observed that only denaturing treatments have an influence on immunochemical reactivity. Heating up to 65 °C does not introduce any change in reactivity. At higher temperatures, different behavior was observed. Mild denaturing treatments cause an important rise in reactivity, whereas a loss of reactivity is produced by total denaturing treatments. Consequently, when dealing with heat-processed samples, immunochemical methods could lead to an over- or underestimation of the actual protein level.

Keywords: *Ovalbumin; heat treatment; immunochemical reactivity; denaturation*

INTRODUCTION

Ovalbumin (OVA) has a widespread use in food industry because its properties contribute to increase product quality (Powrie and Nakai, 1987: Horn, 1980). It is important to study the processes that affect protein structure because many functional properties, such as emulsifying capacity (Kato et al., 1981) or gelling capacity (Arntfield et al., 1991), depend on protein structure. Doi et al. (1987) described the effect of proteolisis on OVA functional properties. Several studies have been developed regarding the influence of pH and thermal treatments on conformation in different salt solutions (Koseki et al., 1988; Hegg et al., 1979; Hayakawa and Nakai, 1985).

Immunological methods have become important as analytical tools for studying food samples. These methods are the best means to detect a single component in a complex matrix, such as in food products. Consequently, these methods have been applied to solve analytical problems in different systems (Hemmen et al., 1993; Skerrit and Hill, 1990). Food proteins are often altered by the foodstuff production processes (Hurrel, 1984). The use of pH changes, enzymatic reactions, or thermal treatments can change protein conformation and alter the immunochemical reactivity of the protein (Cheftel et al., 1987). Among these treatments, heating is by far the most used and the one that has proved to have drastic effects on protein structure (Nakai et al., 1989; Neucere and Cherry, 1982).

The aim of this study was to describe the changes caused by heating proteins, under several time and temperature conditions, in the immunochemical reactivity and to correlate them with conformational changes. OVA was used as a model system because it is a monomeric protein, has good solubility, and its conformational changes can be followed by several simple analytical methods, such as measures of surface hydrophobicity or exposed sulfhydryls. Differential scanning calorimetry (DSC) analysis was used to calculate the denaturated fraction caused by heating. Changes in immunochemical reactivity produced by those treatments were assessed by a competitive ELISA with polyclonal antibodies.

The effects of heat treatments which introduced different degrees of structural changes in protein, were analyzed. A correlation between the changes in immunochemical reactivity and the conformation was observed. An enhancement of immunochemical reactivity for mild denaturing treatments followed by a drop in the reactivity for completely denaturing treatments was found.

MATERIALS AND METHODS

Thermal Treatments. OVA (Sigma Chemical Co.) was dispersed in phosphate buffer (PBS; NaCl, 0.03 mM; KCl, 0.54 mM; KPO_4H_2, 0.30 mM; $Na_2PO_4H{\cdot}12H_2O$, 1.62 mM; pH 7.4) at appropriate concentrations for each experience (from 1 μg/mL to 8 mg/mL). PBS provides a neutral pH and low ionic strength and is usually employed as a solvent in many immunochemical detection procedures. Samples were heated

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S0021-8561(95)00470-5 CCC: $12.00

at the desired temperature in a Haake 2000 water bath for different periods of time. Sample temperature was recorded with a constantan/copper thermocouple. The time−temperature curve was plotted, and the area under the curve was measured. The time of treatment employed in the results expression was obtained as the ratio between the measured area and the bath temperature.

Differential Scanning Calorimetry. The dynamic method (Ozawa, 1970) was used to determine the denaturation rate constants. OVA dispersions (33% w/v) in PBS were hermetically sealed in aluminum DuPont capsules. Five to seven milligrams of protein were tested in each assay. After calorimetric analysis, the capsules were punctured and their dry weight was determined after heating overnight in an oven at 105 °C. An empty aluminum double capsule was used as a reference. Samples were analyzed at different heating rates (2, 4, 7, 10, 15, and 20 °C/min) in a DuPont thermal analyzer (cell model 910; recorder Hewlett-Packard 7046B). Each condition was tested at least three times. To corroborate the values obtained by the dynamic method, OVA samples in hermetically sealed aluminum capsules were isothermally treated at 75, 77, 80, and 84 °C for 5, 10, and 20 min. After being quickly cooled in a water-ice bath, samples were tested in the thermal analyzer between 20 and 120 °C at 10 °C/min. After the DSC run, the capsules were punctured and the weight of the dry matter was determined after drying overnight at 105 °C.

From thermograms, the peak area, onset transition temperature (T_o) and peak temperature (T_d) were measured. Areas were measured with a Jandel Scientific scanner with an area reader program.

Calibration parameters were obtained from pure indium thermograms (15.00 mg, using an empty capsule as a reference) at the same heating rate used to test the samples (ASTM E 698-79).

Exposed Sulfhydryl Groups. Determinations were done in triplicate, following the method described by Ellman (1959), employing 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at 4 mg/mL. OVA (1 mg/mL in PBS) was heat treated and cooled in a water−ice mixture. DTNB was added to each sample immediately after room temperature was reached. The absorbance at 412 nm was measured in a UV−vis Shimadzu UV 150-02 spectrophotometer. Absorbance at 412 nm of heat-treated OVA and of DTNB in PBS were used as sample and reagent blank values, respectively, and subtracted from the measured values. To calculate the number of exposed sulfhydryls per molecule a molar absorbance of thionitrobenzoic acid (TNB) of 13 900 M^{-1} cm^{-1} was used. This value was determined with cysteineclorhydrate as described by Riddles et al. (1979). The obtained value is in acordance with values reported by others (Riddles et al., 1979; Degani and Patchornik, 1971). Protein concentration was calculated by measuring the absorbance at 280 nm using the OVA extinction coefficient (ϵ_{280}) of 26 900 M^{-1} cm^{-1} (Lalignat et al., 1991). A control experiment was performed with OVA at 1 mg/mL in 8 M urea and 0.1% SDS, processed as the rest of the samples. Sample and reagent blanks were included for each determination.

Surface Hydrophobicity (H_o). Surface H_o was measured by the method described by Kato and Nakai (1980). Anilinonaphthalenesulfonic acid (ANS) from Aldrich Chemical Co. was used as the fluorescent probe. Three-milliliter aliquots of OVA at different concentrations (0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/mL in PBS) were added with 50 μL of 8 mM ANS in PBS. The mixture was excited at 364 nm, and the relative fluorescence intensity at 480 nm was measured in a Perkin-Elmer spectrofluorometer (model 2000). As sample and reagent blanks, different heat-treated OVA solutions and 130 μM ANS in PBS were used, respectively. For each determination, a 13.6 μM ANS methanolic solution was used to adjust the relative fluorescence intensity (FI). This ANS solution was assigned a value of 900 fluorescence units (UF) emission. Protein concentration was determined as described before.

The initial slope (H_o) of the FI versus protein concentration plot (calculated by linear regression analysis) was used as an index of protein surface hydrophobicity. Results are expressed in hydrophobicity units (HU). One HU is one FI unit measured in the stated condition per milligram of protein.

Antiserum Preparation. Anti-OVA serum was obtained by hyperimmunization of New Zealand White rabbits as follows. One milliliter of native OVA (Sigma, St. Louis, MO) emulsified with complete Freund's adjyuvant (1 mg/mL) was injected intradermally in four different sites. Four weeks later, the procedure was repeated, injecting 200 μg of OVA emulsified in incomplete Freund's adjuvant. Rabbits were boosted monthly until the desired seric anti-OVA titer was reached. Serum was titrated 5, 10, and 15 days after each injection.

Sequential Competitive ELISA. *Coating.* Polystyrene strips (Maxisorp, Nunc) were coated for 16 h at 4 °C with 100 μL/well of OVA standard solution (1 μg/mL in PBS). Wells were washed with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Nonspecific binding sites were blocked by incubating with 200 μL/well of 3% (w/v) dry skimmed milk in PBS for 2 h at 37 °C. After each incubation step, wells were washed three times with PBS-T solution.

Interaction between Antigens and Antibodies in Liquid Phase (Preincubation). OVA standard solutions, ranging from 0.64 ng/mL to 10 μg/mL, and antibodies (dilution 1:100 000 of the rabbit anti-OVA serum) were dissolved in 1% PBS-T (w/v) dry skimmed milk (diluent solution). Equal volumes of antigen and antibody solutions were mixed and incubated for 1 h at 37 °C.

Competition with Antigens in Solid Phase. A 100-μL/well aliquot of each sample from the previous step was incubated at 37 °C for 30 min in wells coated with OVA (1 μg/mL) as described.

Incubation with Peroxidase Conjugate and Color Reaction. Goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad), diluted 1:1500 in diluent solution, was incubated (100 μL/well) for 1 h at 37 °C. The color reaction was developed by adding a solution containing *o*-phenylendiamine (1 mg/mL, Merck) and 30% H_2O_2 (1 μL/mL) in 0.1 M citrate phosphate buffer (pH 5.0). The enzymatic reaction was stopped after 20 min by addition of 40 μL/well of 4 N H_2SO_4. Optical density was determined at 490 nm. A linear plot was obtained from absorbance values transformed by logit function (Chirdo et al., 1995).

OVA samples at 1 μg/mL in PBS were heated at different time and temperature conditions and quantified by the competitive ELISA. An unheated sample control was processed simultaneously and used as a reference. Results are expressed as the ratio between measured value and the control sample value. Each sample was analyzed in duplicate, and each experiment was repeated at least four times.

RESULTS

Differential Scanning Calorimetry (DSC). An endotherm with a $T_d = 89.7 \pm 0.2$ °C (maximum peak temperature) and a $T_o = 75.6 \pm 0.6$ °C (onset peak temperature) for native OVA was observed (Figure 1A). The denaturing enthalpy measured was 18.4 ± 1.7 J/g. When heat-treated samples were analyzed, a progressive drop in the peak size that correlated with the denaturation degree was observed. Completely denaturing heat treatments (e.g., 90 °C for 5 min) produced a flat thermogram, where no peak can be observed (Figure 1A).

By applying the kinetic method (Ozawa, 1970), the plot depicted in Figure 1B was obtained. The parameters of the Arrhenius equation were calculated from the slope and *x*-axis intercept. The obtained values were as follows: activation energy (E_a) = 376.6 kJ/mol; Arrhenius preexponential factor (Z) = 1.4×10^{56} min^{-1}. The native remaining fraction after different time and temperature treatments were calculated as described by Bombara et al. (1994). The behavior of the native fraction versus time of treatment at different temperatures is shown in Figure 2A. These data can be used to estimate the native fraction after any heating pro-

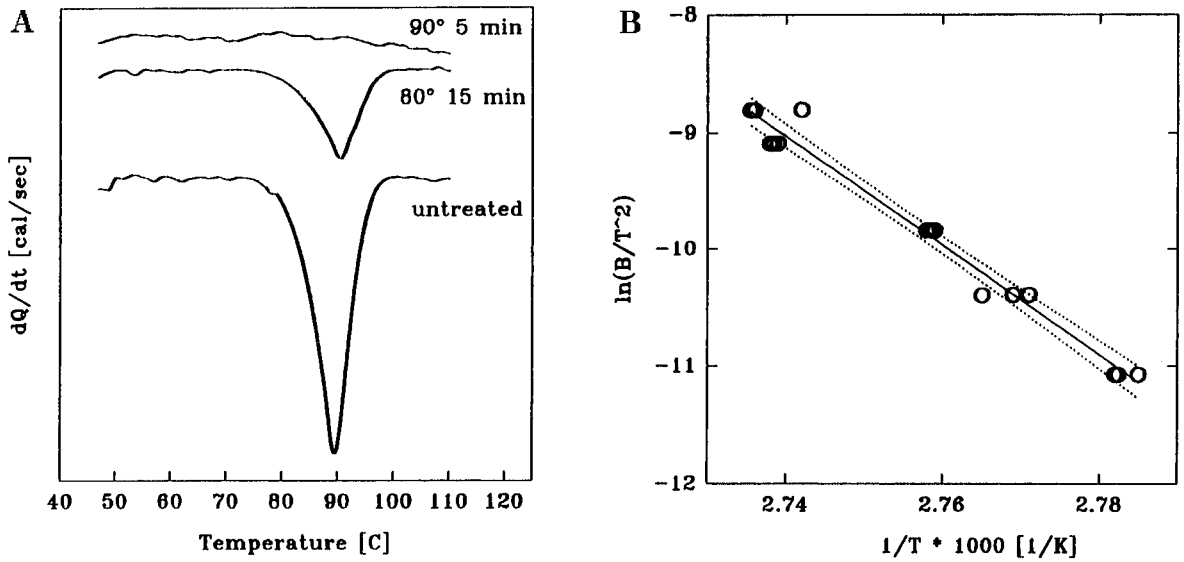

Figure 1. (A) Thermograms of OVA treated with three different conditions (untreated, 80 °C for 15 min, and 90 °C for 5 min). Samples (33% w/v) dissolved in PBS (pH 7.4) were analyzed. (B) DSC kinetic method. The data represent the following equation: $\ln(B/T^2) = \ln(Z^*R/E_a) - E_a/(R^*T)$; where B = heating speed (°C/min); T = denaturation temperature (T_d) (K); E_a = activation energy; Z = Arrhenius preexponential factor; and R = gas constant. The obtained regression parameters are: $a = 119.1$; $b = -46.71$; $r = 0.990$. From the slope and the origin ordinate, the Arrhenius equation parameters can be calculated. Triplicate experiments were performed at five different heating rates.

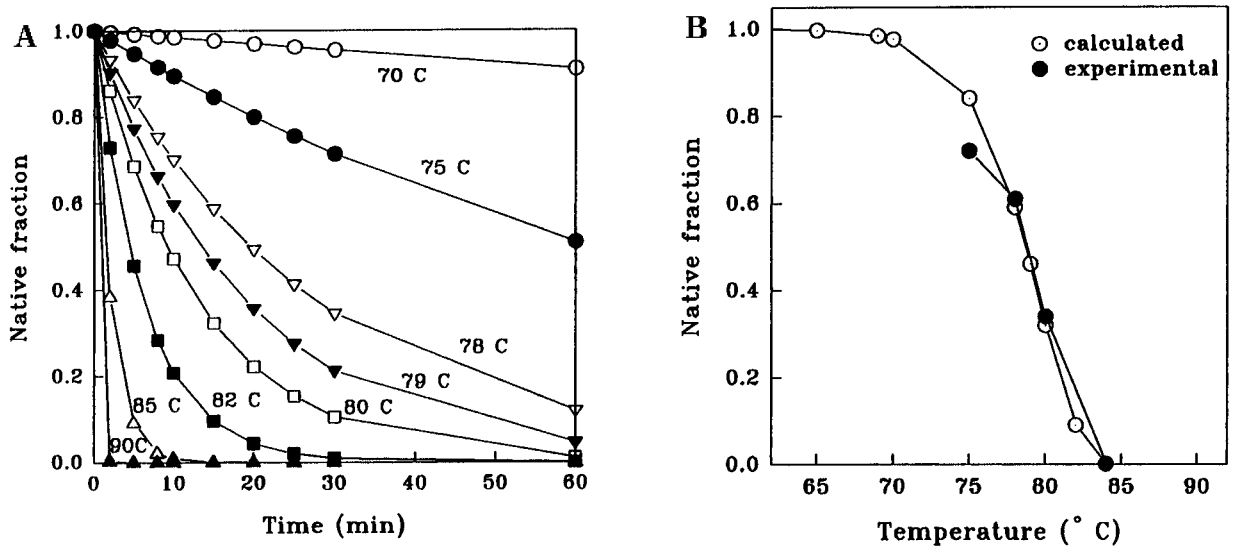

Figure 2. (A) Changes in native remaining fraction with increasing the time of treatment at different temperatures. The native fractions were calculated with the parameters obtained from the results of the DSC kinetic analysis. (B) Native remaining fraction versus temperature of treatment. (○) Calculated using the denaturation parameters; (●) measured from thermograms of heated samples. Results of 20 min of heat treatment are depicted.

cedure. To corroborate this procedure, isothermally preheated samples were analyzed. The heated sample endotherm area was measured, and the native remaining fraction was calculated as the ratio of this measure and the untreated OVA endotherm area. Results of 20-min treatments are shown in Figure 2B. A high concordance between the measured native remaining fraction and the calculed one can be observed. Similar results were obtained when 5- and 10-min treatments were analyzed (not shown).

Thermodynamic parameters of activation were calculated employing the Wynne-Jones-Eyring equation (Laidler and Bunting, 1973). The obtained values were: $\Delta S^* = 0.82$ kJ/mol; $\Delta H^* = 373.1$ kJ/mol; $\Delta G^* = 74.3$ kJ/mol.

Surface Hydrophobicity (H_o). Results of measuring H_o are shown in Figure 3. Very low scatter of experimental data was observed. The coefficient of variation (CV) was always <10% for the different conditions tested. Untreated OVA has a surface hydrophobicity of 32 HU. Treatments at temperatures of <70 °C for times of <15 min do not introduce significant changes in H_o. Also, H_o suffers an important rise after treatments at temperatures of >80 °C. Under these experimental conditions, the higher the temperature of treatment, the greater the rise in H_o. Nevertheless, every treatment seems to lead, at the longest time of treatment, to a maximum of H_o corresponding to the complete denaturation of the sample.

Exposed Sulfhydryl Groups. Results of determining exposed sulfhydryl groups are summarized in Figure 4, where the mean values of three determinations are shown. The CV of the different experimental measures was always <20%. The variation of exposed sulfhydryl groups in OVA treated with different time and temperature conditions can be observed in the figure. As

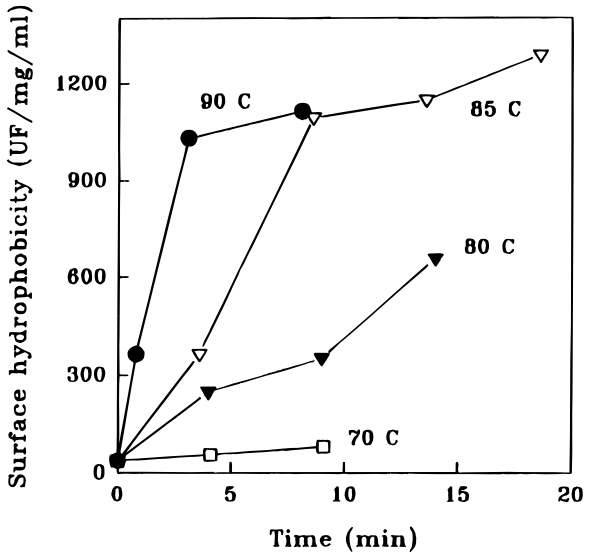

Figure 3. Surface hydrophobicity versus time of treatment at different temperatures. Surface hydrophobicity was calculated as the slope of the fluorescence intensity versus protein concentration plot (not shown). It is expressed as UF/mg/mL, where UF are units of relative fluorescence intensity and mg/mL are protein concentration units. The determinations were performed by triplicate. The CV (CV = 100 × SD/mean value) for each condition was always <10%.

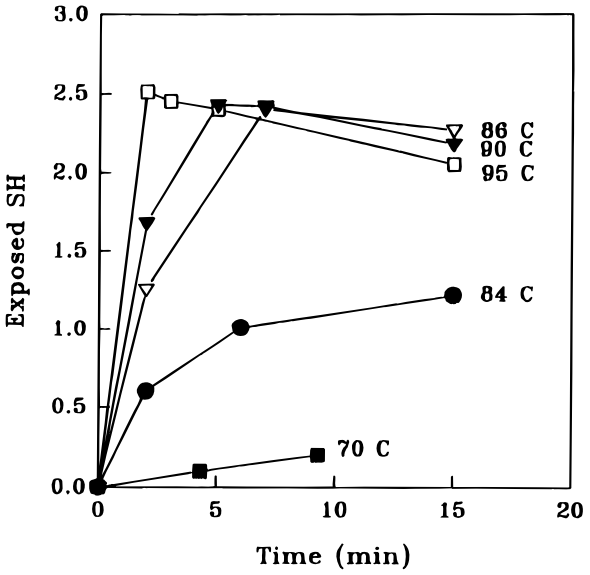

Figure 4. Exposed sulfhydryls, expressed as sulfhydryl (SH) groups exposed per molecule versus time of treatment at different temperatures. Each measure was performed in triplicate, and the CV was always <20%.

shown by others (Takeda et al., 1992), under the experimental conditions used, native OVA does not have any exposed sulfhydryl group. A low fraction of sulfhydryls is exposed after treatment at temperatures of <70 °C. The use of higher treatment temperatures caused an increase of the number of exposed sulfhydryls. This behavior was observed by both employing a fixed time of treatment and heating to different temperatures or vice versa. Temperatures >85 °C produced the highest exposition; that is, almost three exposed sulfhydryl groups per molecule. When controls of OVA in 8 M urea and 0.1% SDS were analyzed, 4.1 ± 0.5 sulfhydryl groups per molecule were exposed.

Immunochemical Reactivity. The mean values of at least four determinations of immunochemical reactivity are shown in Figure 5. Reactivity is expressed as the ratio between quantified OVA in treated samples and an untreated control sample. Each sample was tested in duplicate, and each experiment was performed at least four times. The highest scatter of data was observed for treatments causing the higher rises in reactivity. In these cases, the CV was always <40%. As observed in Figure 5, treatments at temperatures of >70 °C have a significative influence on the immunochemical reactivity measured by ELISA. An increase in temperature of treatment produced a progressive rise in reactivity. For every temperature analyzed, there was a different time of treatment that caused the maximum increase in reactivity. Finally, a drop in reactivity for longer times is observed in every case.

Heat treatments at temperatures between 70 and 80 °C show rises of 3−7-fold in reactivity when compared with the native protein. The time of treatment that produced the maximum reactivity was ∼30 min for 70 and 75 °C treatments and 10 min for the 80 °C treatment. The lower the temperature of treatment, the slower the subsequent drop in reactivity (Figure 5A). Treatment at 85 °C produced a 20-fold increment in the immunochemical reactivity in 4.5 min and a deep drop to <5% of the untreated sample reactivity for treatments of >14.5 min. Treatments at 90 °C or higher had very quick effects on reactivity, producing the highest reactivity in very short times: 1.5 min for a 90 °C treatment and <1 min for higher temperature treatments (Figure 5B). In addition, the drop in reactivity was sharper as temperature of treatment was increased, falling to values of 50-fold smaller than the native protein reactivity.

DISCUSSION

A model system of heating pure OVA in a salt solution was chosen instead of a protein in a food matrix because it allows the use of simple determinations such as surface hydrophobicity and exposed sulfhydryl groups, that give information about conformational aspects of OVA samples heated under different time and temperature conditions. The study of structural changes that occur when OVA is thermally treated is important because of the wide variety of uses given to this protein in foodstuffs. Conformational changes affect surface properties and intermolecular interactions. OVA gelling and aggregating capacity are influenced by heating (Kato and Tagaki, 1987; Nakamura et al., 1978). Thermal treatment also affects the emulsifying capacity of OVA (Mine et al., 1991). OVA has also been characterized as being responsible for food hypersensitivity reactions (Langland, 1982). The conformation of the antigen can influence the intensity of the immune reaction or can even trigger a pathological reaction (Kondo et al., 1993).

The denaturation parameters E_a and Z reported in this work are similar to the ones described for other proteins (Martens et al., 1982; Lupano and Añón, 1986; Wagner and Añón, 1985). The T_d and ΔH observed are slightly higher than the ones described by Donovan and Mapes (1976) for ovalbumin. This difference may be because these authors worked under different conditions of pH 9, and OVA is more stable at pH 7.4, the pH used in this work. The thermodynamic activation parameters obtained are in accordance with those reported by Grinberg et al. (1993), if it is assumed that these authors calculated the parameters at 60 °C. The agreement between the measured native remaining fraction and the value estimated with the denaturing parameters (Figure 2) confirms the validity of the applied procedure.

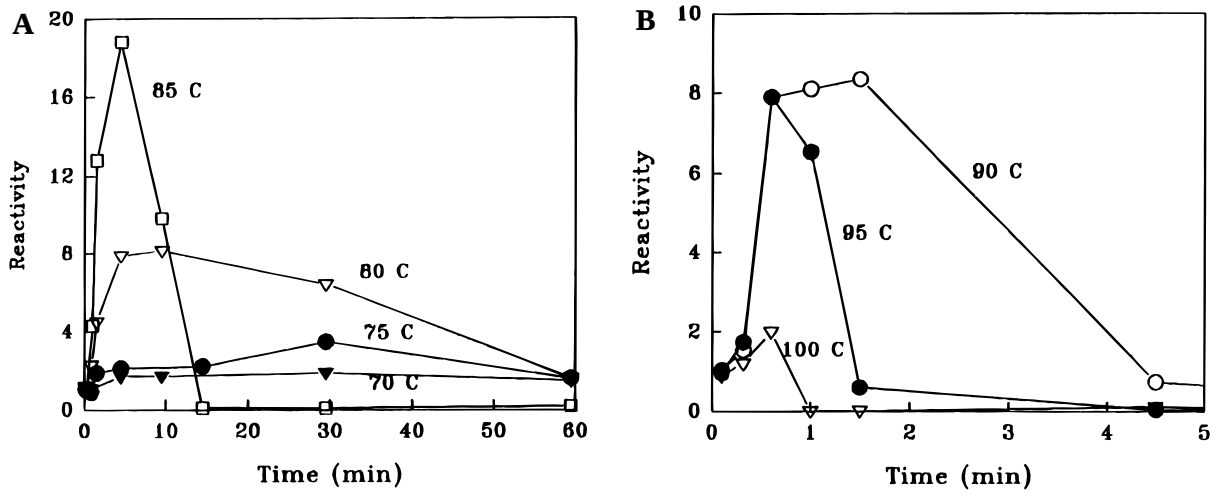

Figure 5. Immunochemical reactivity measured by ELISA versus time of treatment at different temperatures. Reactivity is expressed as the ratio between quantified OVA in treated samples and the untreated control sample. Each sample was tested in duplicate, and each experiment was performed at least four times. Treatments causing the highest rise in reactivity showed the highest scatter of data (CV was always <40%). (A) Representation of long time treatments. (B) Representation of short time treatments.

Thermal denaturation leads to a partial unfolding of the protein, exposing side chains hidden in the native structure. During the heating process, OVA undergoes different conformations, suffering changes even in its secondary structure (Kato and Tagaki, 1988). This report shows that a rise in surface hydrophobicity and exposed sulfhydryl groups is produced by partially denaturing treatments. Totally denaturing treatments produce the highest levels of these two parameters under the experimental conditions used.

The exposition of three sulfhydryl groups per molecule as a result of a thermal treatment is described in the literature (Doi et al., 1989). Slightly smaller values were reported in this work. This difference can be due to the oxidation of some of the sulfhydryl groups at the temperature of treatment, to a partial unavailability of sulfhydryl groups hidden by protein aggregation, or to the combined effect of both factors. The measure of exposed sulfhydryl groups under strong denaturing conditions (0.1% SDS and 8 M urea) is in accord with the results of Beveridge et al. (1974). With these conditions, all the sulfhydryl groups are exposed as the protein is completely unfolded.

To our knowledge, a study of the effect of as many different time and temperature conditions as those here reported on OVA immunochemical reactivity has not yet been described. Breton et al. (1988) analyzed the reactivity of both native OVA and OVA heated at 100 °C for 30 min. These authors proposed an ELISA to detect OVA in specific products (Breton et al., 1988) or to control heating processes (Varshney and Paraf, 1990) based on heat-induced changes in reactivity to different fractions of an anti-OVA serum. Sajdok et al. (1989) reported a drop in immunochemical reactivity caused by heat treatment, measured by rocket immunoelectrophoresis, although they did not observe the rise in immunochemical reactivity at mild denaturing conditions reported here. Paraf et al. (1991) studied the differences in immunochemical reactivity of OVA against a panel of monoclonal antibodies. They included in their work samples of heated OVA, with little degree of denaturation (50 or 60 °C for 30 min) or completely denatured (85 or 100 °C for 30 min). They found that some monoclonal antibodies preferentially recognized the native or slightly denatured forms of the protein, whereas another group had a higher reactivity against the completely denatured protein.

In this work, many thermal treatments were studied. These treatments produced very different denaturation effects, from slight alterations to complete denaturation. Partially denaturating treatments cause an increase in immunochemical reactivity. If the treatment goes on, reactivity drops almost to zero. Mild denaturing treatments lead to the exposure of hidden conformational epitopes that are recognized by the antiserum, causing an increment of the immunochemical reactivity. Stronger treatments would destroy the conformational epitopes, producing the observed drop in reactivity. Aggregation of denatured OVA may take place depending on the protein concentration. Aggregation could also produce a drop in reactivity, due to the partial hiding of epitopes inside the aggregates. Similar behavior has been described by Wolberg et al. (1970) for bovine serum albumin and by Plumb et al. (1994) for soybean glycinin, and may extend to other systems.

The use of immunochemical methods to detect food proteins faces the problem of the changes introduced in protein structure during processing (Stevenson et al., 1994). The results shown here demonstrate that heat treatment strongly affects immunochemical reactivity. Immunochemical quantification can lead to an under- or overestimation of the actual protein content depending on the thermal history of the sample. A correlation was also described between alteration in immunochemical reactivity and structural modifications (calculated as the native remaining fraction using the parameters obtained by DSC) after heat treatment.

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Received for review July 24, 1995. Revised manuscript received December 27, 1995. Accepted June 6, 1996.[⊗] This work was financed with grants from Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC) and National Reserch Council (CONICET).

JF950470Q

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1996.