Macroscopic and Structural Consequences of High-Pressure Treatment of Ovalbumin Solutions

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The effects of high pressure on the solubility and on some structural features of ovalbumin were investigated on protein solutions at neutral pH, containing different protein concentrations and treated at 400−800 MPa for different times. Ovalbumin solutions were prepared in the presence and in the absence of common food ingredients, such as sucrose and NaCl, that could act as "protectants" against treatment-induced modification. Protein insolubilization occurred in the absence of protectants as a function of the protein concentration and the treatment intensity; it appeared to be a consequence of structural modifications involving in particular the tertiary structure of the protein, and it was completely prevented by blocking the free −SH groups in the protein. Several of the structural features of the protein were modified also when it was treated in the presence of protectants. Modifications depended on the treatment pressure and time and on the protectant used. All of the treated proteins showed an increased susceptibility to proteolysis by trypsin and a decrease in the recognizability by specific antibodies. These effects were particularly evident when treatments were performed in the presence of sucrose, which was less effective than NaCl in preventing structural modifications upon treatment.

Keywords: *Ovalbumin; high pressure; protein structure; digestibility; food allergens*

INTRODUCTION

High-pressure processing is gaining popularity in the food industry, because it can be used to obtain stable products with minimal effects on flavor, color, and nutritional value (Cheftel, 1992; Tauscher, 1995) or to create products with novel texture and taste.

The effects of high pressures on proteins have been the subject of many reviews (Balny and Masson, 1993; Silva and Weber, 1993; Gross and Jaenicke, 1994). Pressure treatment can cause different effects on proteins, inducing reversible or irreversible structural modifications leading to protein denaturation, aggregation, or gelation. All of these effects depend on the nature of the protein, as well as on the applied pressure, the temperature, and the treatment time. As pointed out in most studies, the physicochemical state of solvent and the characteristic of hydration water are of paramount importance in establishing the effects of high pressures on protein structure. In this context, the effects of compounds capable of affecting the water activity (such as carbohydrates and polyols) or the solvation of surface protein charges (such as neutral salts) have been investigated both theoretically and

practically (Silva and Weber, 1993; Smeller and Heremans, 1997; Dumay and Cheftel, 1997). The relevance of these observations stems from the effects of these "protectants" on the survival figures for microorganisms when food systems are processed.

Studies on the effects of physical treatments on protein structure usually have been undertaken with globular proteins, including ovalbumin. Ovalbumin is a monomeric glycophosphoprotein, representing >50% (w/w) of the egg white proteins. The crystal structure of ovalbumin is known (Stein et al., 1991), and the presence of a single disulfide bond and of four −SH groups has been shown. The −SH are inaccessible in the native protein and were shown to play a prominent role in the refolding of ovalbumin from the unfolded form obtained upon denaturation with chaotropes (Creigthon, 1986; Takahashi et al., 1992). The −SH groups are relevant for the formation of protein aggregates and for the stabilization of a gel structure upon heat treatment, which likely occur through a disulfide exchange mechanism (Yoshinori, 1996).

Ovalbumin represents one of the best studied models for understanding the functionality of food proteins in molecular terms (Yoshinori, 1995, 1996). There have been many studies on the heat denaturation of ovalbumin (Kato et al., 1987) and on the physical properties of gels formed by ovalbumin as a function of temperature and of the presence of other compounds (Kato et al., 1988; Yoshinori, 1996). The effects of pressure on the stability of network bonds in heat-induced ovalbumin gels (Doi et al., 1991) and on some functional properties of the protein, such as its emulsifying properties, were also studied (Denda et al., 1992). The

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conformation of ovalbumin was reported to remain fairly stable when the protein was treated at 400 MPa (Haykawa et al., 1992), but the effects of pressure on ovalbumin itself and on the products of subsequent processing for food never have been analyzed in detail.

Ovalbumin is also one of the major food allergenes (Tsukasa et al., 1993), and it can cause several forms of more or less severe food intolerance in humans. In this context, an allergenic and antigenic epitope of ovalbumin has been identified (Johnsen and Elsayed, 1990; Kahlert et al., 1992).

In this study we report on the structural modification induced by pressure treatment of ovalbumin solutions in a pressure range relevant to microbiological food sanitation (Miglioli et al., 1997). The consequences of treatment-induced modifications on the digestibility of the treated protein by trypsin and on its recognition by specific antibodies were studied along with the effect of some common food ingredients (sucrose and NaCl).

MATERIALS AND METHODS

Reagents and Chemicals. Ovalbumin (grade III), trypsin (type XIII), and peroxidase-labeled anti-rabbit immunoglobulins were from Sigma. Chemicals were of analytical or HPLC grade. Unless otherwise specified, buffer was 50 mM sodium phosphate, pH 6.8.

Methods. High-pressure treatments were performed on ovalbumin solutions in buffer at protein concentrations of 0.5, 2, or 10 mg/mL in the absence or presence of 10% (w/w) sucrose or 10% NaCl. For each treatment, 10 mL of an ovalbumin solution was sealed in a plastic bag. Pressure treatment was carried out at a nominal temperature of 25 °C in an isostatic high-pressure pilot instrument (ABB model QFP-6, ABB, Sweden), at 450, 600, and 800 MPa. Under our conditions, the temperature increase was 2 °C/100 MPa during the pressurization step (60–90 s). During the holding time (which is referred to as the actual treatment time), and the following instantaneous decompression, the temperature of the samples decreased to 25 ± 2 °C. Immediately after the pressure treatment, the treated solutions were refrigerated and kept at 0–4 °C until further analysis. Under these conditions, a reversible pH change occurs as a consequence of a pK_a shift of phosphate, which could be as high as −0.4 pH unit/100 MPa (Van Eldik et al., 1989). However, even the lowest pH value theoretically attained in our treatment conditions (≈4) is by far higher than that required to observe significant structural changes in ovalbumin, which retains a highly ordered molten globule-like state at pH 2.2 (Tatsumi and Hirose, 1997).

Ovalbumin concentration in the soluble fraction of treated samples was determined spectrophotometrically by using A_{280} = 32000.

Intrinsic fluorescence measurements were taken at room temperature in a Perkin-Elmer LS 50 spectrofluorometer. Emission spectra were recorded by using λ_{ex} = 298 nm.

CD spectra were recorded at 25 °C in thermostated cells of 1 or 0.2 cm path length (for near- and far-UV measurements, respectively), on a Jasco 500A spectropolarimeter, and analyzed by means of the Jasco J700 software.

When required, the sulfhydryl reagent, dithiobis(2,4-nitrobenzoate) (DTNB), was added prior to the pressure treatment. Reacted SH groups were determined spectrophotometrically by using A_{412} = 13600 for the nitrobenzoate thiolate anion (Ellman, 1959).

In direct, noncompetitive ELISA tests, polystyrene microtiter plates (Nunc-Immuno Plate Maxi Sorp) were coated overnight at 4 °C with 0.1 mL of ovalbumin solutions in 0.1 M sodium bicarbonate, pH 9.6, having a protein concentration covering the range 0.1–1 or 0.0001–0.001 mg/mL according to the sample to be analyzed. Coated plates were washed with buffer (phosphate buffer saline/Tween, PBST: 0.015 M potassium phosphate, 0.15 M NaCl, pH 7.4, containing 0.5 g/L Tween 20). The remaining protein binding sites were blocked
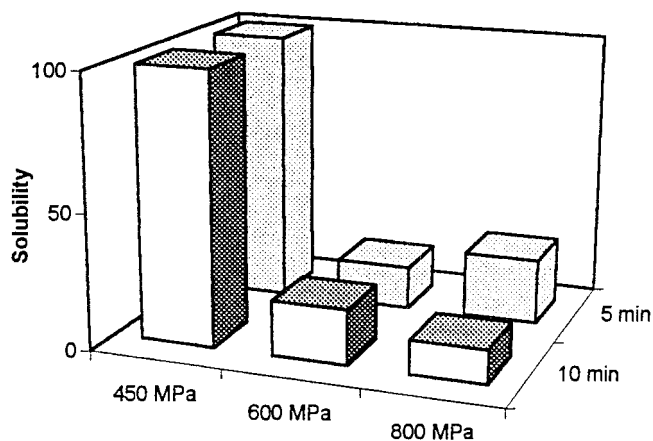
Figure 1. Solubility of pressure-treated ovalbumin: dependence on treatment conditions. Ovalbumin (2 mg/mL) was dissolved in 50 mM phosphate, pH 6.8, and treated at the given pressure for the times given. The residual solubility is given as percent of the initial protein.

by incubation with 30 g/L gelatin (EIA grade reagent gelatin, Bio-Rad) in PBS (0.015 M potassium phosphate, 0.15 M NaCl, pH 7.4) for 90 min at 37 °C. After extensive washing with PBST, plates were incubated for 2 h at 37 °C with 0.1 mL of rabbit anti-ovalbumin serum, diluted 1/20000 (v/v) in PBST buffer containing 1% (w/v) gelatin. Wells were washed again with PBST and rinsed with 0.1 mL of peroxidase-labeled goat anti-rabbit immunoglobulins diluted 1/2500 in PBST containing 1% (w/v) gelatin, followed by incubation for 1 h at 37 °C in PBST containing 1% (w/v) gelatin. After unbound reagents were removed by washing with PBST and PBS, antigen−antibody reaction was revealed by addition of a solution containing 0.3 mg/mL *o*-phenylenediamine in 0.5 M citrate buffer, pH 5.0, containing 0.03% (v/v) H_2O_2. The reaction was stopped by addition of 0.05 mL of 4 N sulfuric acid. The optical density of each well was measured at 490 nm in a Bio-Rad 3550 microplate reader. Absorbance figures were corrected for average blank readings, and Bio-Rad software was used to calculate absorbance values and to correlate them with the antigen concentration.

Direct competitive ELISA test was carried out as described above except for the following modifications. The plates were coated with 0.1 mL of an untreated ovalbumin standard solution with a protein concentration of 0.005 mg/mL. The interaction between rabbit anti-ovalbumin serum [diluted 1/20000 (v/v) with PBST] and coating ovalbumin was performed in the presence of different concentrations of treated ovalbumin samples (competitor). For all of the ELISA tests, data are reported as percent residual antibody recognition, where the antibody recognition of untreated ovalbumin was considered equal to 100%.

Susceptibility of ovalbumin samples to trypsin was detected by incubation of 0.4 mL of a 1 mg/mL solution of treated ovalbumin in 50 mM phosphate buffer, pH 6.8, with 0.5 mg of trypsin at 37 °C. The proteolytic reaction was stopped by adding to the test tube 0.5 mL of 20% (w/v) trichloroacetic acid, and the protein precipitate was removed by centrifugation at 13000*g* for 15 min. The absorbance at 280 nm of the clear supernatant phase was measured, and the hydrolyzed substrate was expressed as the amount of tyrosine released in the supernatant, determined by using A_{280} = 1.25 for tyrosine.

RESULTS AND DISCUSSION

Changes in the Macroscopic Properties of High-Pressure-Treated Sample. As shown in Figure 1, pressure-dependent formation of insoluble aggregates occurred upon high-pressure treatment of ovalbumin samples dissolved in plain buffer. Residual soluble ovalbumin was ≈10% of the total protein after treat-

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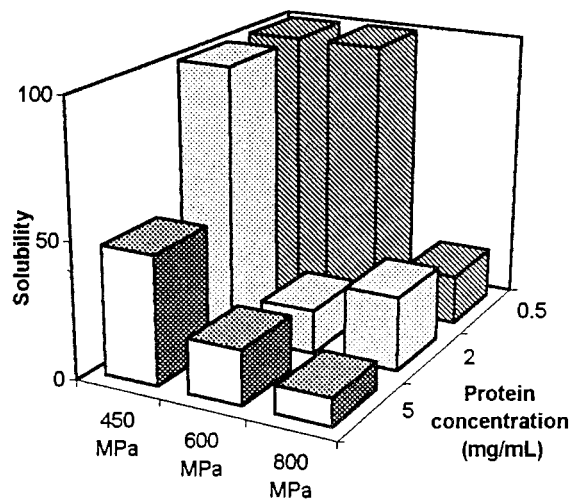
Figure 2. Solubility of pressure-treated ovalbumin: dependence on the protein concentration and on the treatment pressure. Ovalbumin was dissolved in 50 mM phosphate, pH 6.8, at the given concentration, and was treated for 5 min as indicated. The residual solubility is given as percent of the initial protein.

Table 1. Solubility of Ovalbumin Treated in the Presence of Protectants[a]

protectant	pressure, MPa	solubility, %
none	600	16
10% sucrose	600	100
10% NaCl	600	100
none	800	27
10% sucrose	800	100
10% NaCl	800	100

[a] Ovalbumin (2 mg/mL in 50 mM phosphate, pH 6.8) was treated at the given pressure for 5 min. Residual soluble ovalbumin after centrifugation at 30000*g* for 10 min is given as percent of total ovalbumin.

ment at 800 MPa for 10 min. Formation of insoluble aggregates was independent of the treatment time over the 2–10 min range.

Formation of insoluble aggregates also depended on the ovalbumin concentration. Figure 2 shows the residual solubility in ovalbumin solutions of different concentrations as a function of pressure. At 450 MPa, a decrease in solubility was observed only at 5 mg/mL, while at 600 MPa some loss of solubility was evident already at 2 mg/mL, where the insoluble protein was 80% of the total, a value not modified by increasing the protein concentration. No correlation between residual soluble protein and protein concentration was found at 800 MPa.

The observation that protein insolubilization depended more on protein concentration than on treatment time indicates that insolubilization occurred through formation of large aggregates of denatured protein. Thus, as for other proteins and treatments (Iametti et al., 1995), formation of aggregates of denatured protein is favored by high protein concentrations.

Formation of insoluble aggregates upon high-pressure treatment could be completely prevented by treating the protein in the presence of 10% sucrose or 10% NaCl. Ovalbumin (0.5–2 mg/mL) treated at 600 or 800 MPa for 10 min in the presence of these "protectants" remained completely soluble, as shown in Table 1. A minor formation of insoluble aggregates (made evident by some haze that was not removable by centrifugation) was observed only when protein solutions at 10 mg/mL were treated at 800 MPa for 5–10 min.

To understand the nature of the structural modifications leading to the effects on solubility, a characterization of the structural changes ensuing from high-pressure treatment of ovalbumin was necessary. In the case of treatments producing insoluble aggregates, such a characterization was feasible only on the protein left in solution. This fraction of the treated protein likely contained modified protein species that were representative of intermediates formed in the course of the denaturation and aggregation process. Therefore, structural modifications in pressure-treated ovalbumin were

studied on the residual soluble protein by combining different approaches.
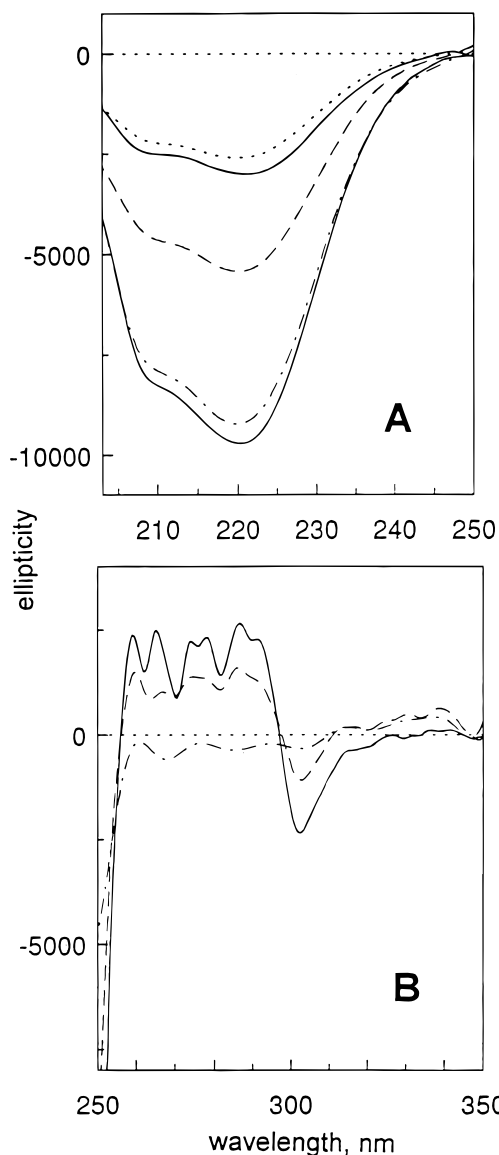
Modifications of Protein Folding As Detected by CD Spectroscopy. As shown in Figure 3A, modifications of the far-UV CD spectra of treated ovalbumin were pressure-dependent. The features in the far-UV CD spectra of samples treated at 450 MPa were very similar to those of native ovalbumin, whereas treatment at 600–800 MPa gave increased modification of the native secondary structure with increasing treatment pressure. In particular, the absolute ellipticity at 222 nm decreased as a function of pressure.

Even in the harshest treatment conditions (800 MPa, 10 min), the soluble fraction of treated ovalbumin did not show conversion of secondary structure to an unfolded random coil, as observed when ovalbumin was denatured with guanidine hydrochloride (Tani et al., 1997).

Pressure treatments of increased intensity also increasingly affected the tertiary structure of the protein, as seen in the near-UV CD spectra in Figure 3B. Whereas the protein treated at 450 MPa had near-UV CD features identical to those of the untreated protein, raising the pressure at 600 MPa led to a partial loss of typical ellipticities, which disappeared completely after treatment at 800 MPa.

Contrarily to what was reported in the previous section for the formation of insoluble aggregates, the structural modifications observed for the soluble fraction of ovalbumin were correlated with the treatment time (Figure 3A). This is consistent with the residual, partly unfolded form in solution being an intermediate of the denaturation and aggregation of ovalbumin. This hypothesis is supported by the concentration dependence of pressure-induced structural modifications. Indeed, at the same treatment pressure, the higher the protein concentration, the more intense the structural modifications observed by far-UV CD (Figure 3A). That is, concentrations favoring protein insolubilization (Figure 2) also favored structural modifications, suggesting that high protein concentrations did not protect the soluble protein fraction from pressure-induced structural modifications.
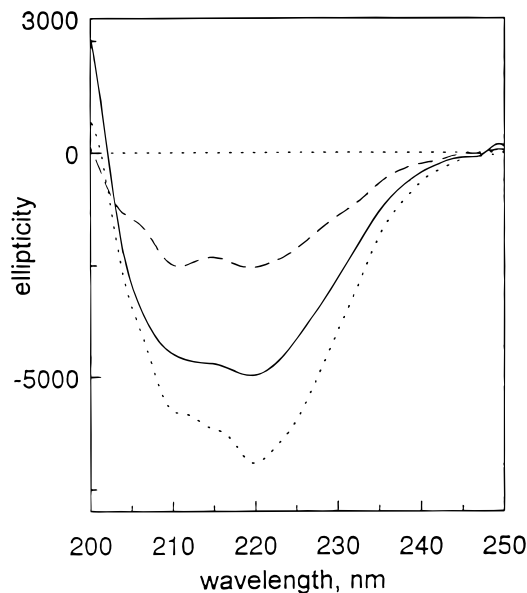
The extent of the modifications discussed above was much reduced in the samples that were treated in the presence of "protectants", where no protein precipitation occurred. Elements of secondary structure typical of native ovalbumin were better conserved when ovalbumin was treated in the presence of NaCl than in the presence of sucrose. This suggests that a combination of increasing ionic strength and decreasing water activity, as provided by NaCl, could prevent protein denaturation more effectively than just the decrease in water activity provided by the addition of sucrose.

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Figure 4. Far-UV CD spectra of ovalbumin treated in the presence of 10% sucrose: effect of protein concentration. The spectra are those of ovalbumin solutions in 10% sucrose after treatment at 600 MPa for 5 min (0.5 mg/mL, dashes; 2 mg/mL, solid line). The spectrum of untreated ovalbumin in 10% sucrose is given as the dotted line. The treated ovalbumin samples were diluted to 0.25 mg/mL with buffer containing 10% sucrose.

Figure 3. CD spectra in the far-UV (A) and near-UV (B) regions of ovalbumin treated at different pressures: (A) Far-UV CD spectra of ovalbumin (0.5 mg/mL) before treatment (bottom-most solid line) or after treatment at 600 MPa for 2 min (dots and dashes) or 10 min (dashes) or for 2 min at 800 MPa (topmost solid line). The spectrum of ovalbumin (2 mg/mL) treated for 10 min at 600 MPa is given as the dotted line. (B) Near-UV CD spectra of ovalbumin (0.5 mg/mL) before treatment (solid line) or after treatment at 600 MPa for 2 min (dashes) or at 800 MPa for 2 min (dashes and dots).

The effect of NaCl or sucrose in preventing pressure-induced structural modifications of the protein also depended on the protein concentration, as shown for sucrose in Figure 4. Under comparable treatment conditions, the far-UV CD features of native ovalbumin were conserved best in the samples at the highest protein concentration. Thus, in conditions where aggregation of a vastly denatured protein to give an insoluble form did not occur (and did not alter the reaction equilibrium), an increase in protein concentration cooperated with the effect of protectants against extensive denaturation upon treatment.

Modification of the Enviroment of Tryptophan Residues As Detected by Intrinsic Fluorescence. As shown in Figure 5A, pressure treatment did not modify the emission maximum in tryptophan fluores-

cence spectra. Increased treatment pressure resulted in a decrease in the fluorescence intensity to 50 and 20% of the original intensity after treatment at 600 and 800 MPa, respectively. A similar insensitivity of the position of the fluorescence maximum to the treatment and a marked decrease in the intensity of fluorescence were reported for heat-treated ovalbumin (Tani et al., 1997).

As observed for CD spectra, changes in intrinsic fluorescence were dependent on protein concentration, because treatment of the most diluted solutions gave the greatest decrease in fluorescence, as shown in Figure 5B. Thus, also the hydrophobic regions in the soluble fraction of the treated protein were modified more extensively in conditions minimizing the formation of aggregates.

The fluorescence spectra of ovalbumin treated at 600 MPa in the presence of sucrose or NaCl were identical to those of the untreated protein. This suggests that both compounds exerted their protecting action also against modification of localized hydrophobic regions of the protein structure.

Disulfide Bonds and Free Thiols in Treated Ovalbumin. Native ovalbumin contains one disulfide bridge, formed by cysteine residues at positions 73 and 120, and four free −SH groups (Cys11, 30, 367, 382). All of these groups are relevant to thermal aggregation of the protein, and exposure of both −S−S and −SH groups in this protein has been considered an index of protein denaturation (Tani et al., 1997).

We found that in the absence of protectants, formation of insoluble aggregates upon high-pressure treatment was completely prevented by blocking the accessible free −SH groups in the protein with a thiol reagent added before high-pressure treatment. In these experiments, consistent amounts of the yellow anion derived from the reaction of thiols with DTNB were present at the end of the reaction, suggesting a reaction of DTNB with thiols exposed during the treatment itself.

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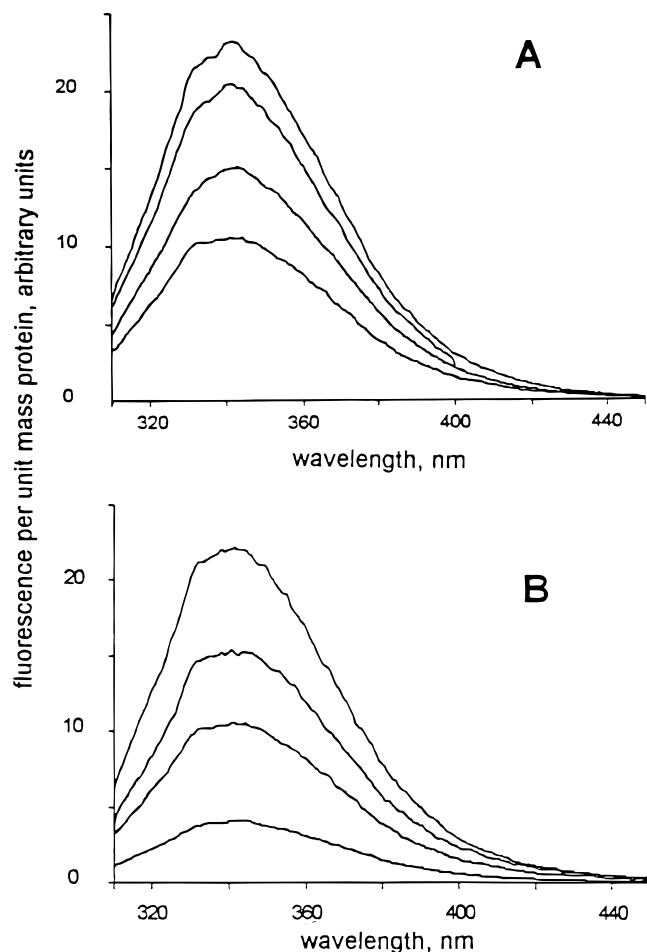
Figure 5. Tryptophan fluorescence spectra of ovalbumin treated at different pressures: (A) Ovalbumin (2 mg/mL) was dissolved in 50 mM phosphate, pH 6.8, and spectra were recorded on the untreated protein (topmost line) or on the soluble fraction after treatment for 5 min at 450, 600, or 800 MPa (from top to bottom, respectively). Spectra are corrected for protein concentration. (B) Spectra are those of untreated ovalbumin (topmost line, 0.5 mg/mL) and of the soluble fraction of ovalbumin treated for 5 min at 800 MPa at concentrations of 0.5, 1.0, and 2.0 mg/mL (bottom to top, respectively) in 50 mM phosphate, pH 6.8. Spectra are corrected for protein concentration.

Table 2. Variation in Accessible Free −SH Groups in Treated Ovalbumin[a]

treatment pressure, MPa	accessible −SH groups, mol/mol of protein
untreated	0.0
450	0.5
600	2.3
800	2.3

[a] Accessible −SH groups were determined on 2 mg/mL ovalbumin treated at the given pressure for 10 min in the presence of 0.2 mM DTNB. No protein precipitation occurred. Reacted SH groups were measured spectrophotometrically on the treated solutions.

In Table 2 the number of accessible SH residues, detectable by adding DTNB to the protein prior of the pressure treatment, is correlated with the treatment intensity. No SH groups were detectable by DTNB in untreated ovalbumin, and only 0.5 mol of −SH/mol of protein became accessible to the reagent during treatment for 10 min at 450 MPa, by which no aggregates were formed even in the absence of protective agents. On the other hand, 2.3 mol of −SH/mol of protein
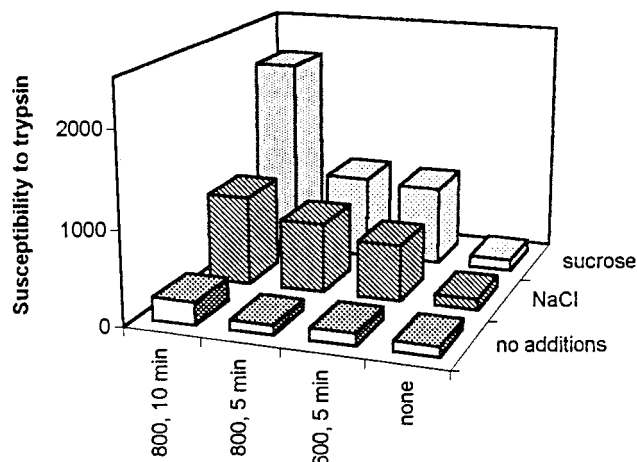
became accessible to the −SH reagent during treatment at 600−800 MPa.

Altogether, these data suggest that pressure-induced formation of insoluble aggregates involved a disulfide exchange mechanism, in which the free protein thiols made available during transient denaturation played a prominent role. This confirms what was observed for the thermal treatment of ovalbumin (Yoshinori, 1996; Tani et al., 1997) and other food proteins (Shimada et al., 1989; Iametti et al., 1996), for which a similar disulfide exchange mechanism was reported and for which a protective effect of DTNB against pressure-induced polymerization was noticed as well (Iametti et al., 1997).

Susceptibility of Treated Ovalbumin to Proteolysis. Pressure-induced conformational modifications of ovalbumin that could have practical relevance from a technological and nutritional point of view were examined by determining the susceptibility of the treated protein to trypsin action. Tryptic digestion represents a powerful method for examining the conformation of ovalbumin (Takahashi and Hirose, 1992). Native ovalbumin is completely resistant to digestion by trypsin, contrarily to heat-denatured or acid-treated ovalbumin (Ottensen and Wallevik, 1968). High-pressure treatment of ovalbumin solutions in the absence of protectants did not modify the susceptibility of the residual soluble protein to trypsin, if not for a <10% increase in susceptibility (10%) at the highest pressure/ time combinations. Pressure-insolubilized ovalbumin was not digested by trypsin.

Much different results were obtained when the ovalbumin was pressure-treated in the presence of protectants. Treatment in the presence of NaCl or sucrose resulted in a pressure-dependent increase of the susceptibility of ovalbumin to trypsin. In particular, the highest increase in proteolysis was observed for ovalbumin treated at 800 MPa for 10 min in the presence of 10% sucrose (Figure 6). Once again, the two protectants displayed different effects, suggesting that they are addressing different stabilizing forces in the protein structure.

The effects on tryptic susceptibility observed in the presence of protectants were independent of the protein concentration in the range from 0.5 to 2 mg/mL.
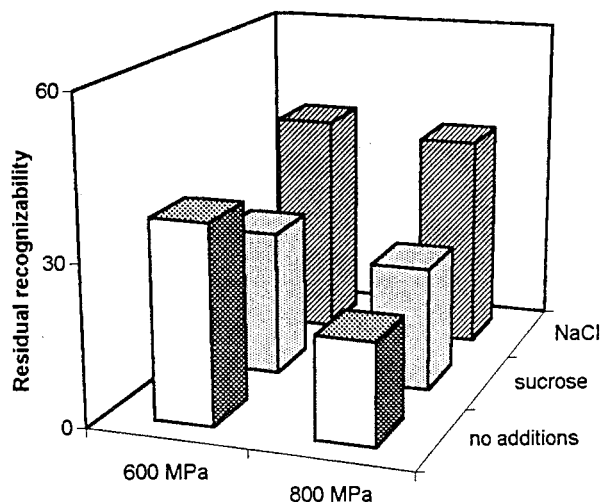
Figure 7. Recognizability of pressure-treated ovalbumin by anti-ovalbumin antibodies. Ovalbumin (2 mg/mL) was dissolved in 50 mM phosphate buffer, pH 6.8, and was treated at 600 or 800 MPa for 5 min in the absence or presence of 10% (w/w) NaCl or 10% (w/w) sucrose. Results are given as percent response with respect to the untreated protein.

Modification of Epitopes in Pressure-Treated Ovalbumin. Treatments aimed at reducing the human immune response to the administration of egg proteins in food are of great potential relevance in human nutrition, given the high number of individuals who are sensitive to these classes of products. Thermal treatment of ovalbumin present in milk-, cream-, or egg-based foods resulted in marked modifications of the recognition of the protein by suitable antibodies, likely as a consequence of severe structural modifications of the protein (Turin et al., 1994).

In the present case, immunochemical test carried out by using a direct, noncompetitive ELISA format and antibodies raised against the native protein showed that the structural modifications detected in pressure-treated ovalbumin resulted also in modifications of the epitopic regions of the protein.

As shown in Figure 7, increasing the treatment pressure resulted in an increased loss of recognizability even in conditions where no changes in solubility were observed, such as in the presence of protectants. As observed in trypsin susceptibility studies, the effect of sucrose was different from that of NaCl. Ovalbumin treated in the presence of sucrose presented a lower recognizability than the protein treated in the presence of NaCl. Indeed, as discussed before, most of the observed modifications were less extensive in the presence of NaCl than in the presence of sucrose. However, the samples treated at 600−800 MPa in the presence of NaCl showed a reactivity toward antibodies that was 40% lower than that of the untreated protein.

In all direct, noncompetitive ELISA tests discussed above the coating step was performed by using treated ovalbumin. The results obtained in these experimental conditions could have been ascribed to a different absorption of the treated protein to the styrene wells in comparison with that of the native protein. The use of native ovalbumin as a coating antigen, as done in the direct competitive ELISA format, prevented this possible artifact. Therefore, to be sure that the determined modifications in antiboddy recognition could be ascribed to real structural modifications, a competitive ELISA test was performed. The data so obtained (not shown)

were closely comparable with those obtained with the direct, noncompetitive ELISA format. Thus, pressure treatment really did modify epitopic regions of ovalbumin.

As shown in this study for most other structural changes, also the modification of epitopic regions was dependent on the protein concentration. For ovalbumin treated in the presence of sucrose, increasing protein concentration led to a decrease in the specific content of antibody recognition sites for unit mass protein. For example, in ovalbumin treated at 800 MPa for 10 min, the residual antibody recognition was 20% that of the untreated protein at 2 mg/mL, being half this figure when a 10 mg/mL solution of ovalbumin was treated. No effect of concentration was evident for the samples treated in the presence of NaCl. These results again confirm the different effects of the two protectants on structural ovalbumin modifications.

CONCLUSIONS

In the absence of protective agents, protein insolubilization was the main event in pressure treatment of ovalbumin. Residual solubility and the extent of denaturation of the protein left in solution were consistent with the latter being an intermediate in the formation of insoluble aggregates. The observation that structural changes increased and protein solubility decreased when solutions of increasing concentrations were treated suggests that aggregation of modified proteins was a driving force in assessing the reaction equilibrium.

When protectants were present, protein insolubilization did not occur even when the protein structure was sensibly modified. Neither protectant interfered with the exposure of hydrophobic regions (as seen through intrinsic fluorescence), but they acted differently on the preservation of secondary structure elements. NaCl was by far more efficient than sucrose in preventing loss of secondary structure. These observations suggest that selected regions of the tertiary structure were relevant (along with the exposure of reactive −SH groups, because no aggregates were formed when they were blocked) for aggregation of the denatured protein. Thus, also in the case of ovalbumin, stabilization of noncovalent aggregates through disulfide exchange reactions may represent the final step in the formation of insoluble aggregates upon process-induced denaturation.

Trypsin susceptibility studies also indicated that the soluble protein had a different conformation after treatment in the presence or in the absence of protectants. Putative access sites buried in the native structure of ovalbumin or in process-induced aggregates were not accessible to enzymatic action, which was by far more relevant in samples having lost their compactness but remaining in soluble form, as occurred in the presence of protectants. The increase in sensitivity to trypsin was highest for treatment at 800 MPa in the presence of sucrose, which also resulted in the highest modification of secondary structure elements among the proteins remaining in soluble form. These same samples show the lowest content in epitopic sites per unit mass of protein, suggesting that there are structural elements related to antigenic sites.

However, pressure treatment resulted in a significant decrease in the number of epitopic sites per unit mass of soluble protein in all of the samples considered here, confirming the potential of high-pressure treatment for obtaining reduced-allergenicity foods.

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Besides their possible relevance to the understanding of the mechanism of pressure-induced modifications in food proteins, the observations reported here indicate the possibility of obtaining egg-derived products with interesting features in a practically feasible process using a mixture of common food ingredients. To verify the possible practical application of this work, we plan to extend our experiments to whole egg whites.

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