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Changes in Sulfhydryl Content of Egg White Proteins Due to Heat and Pressure Treatment

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The sulfhydryl (SH) content of egg white proteins (10% v/v or 9.64 mg of protein/mL) after heat (50−85 °C) and combined heat- and high-pressure treatments (100−700 MPa, 10−60 °C) was determined using 5′,5-dithiobis (2-nitrobenzoic acid) (DTNB), both for the soluble fraction and the total protein fraction. Only irreversible changes were taken into account. Both physical treatments were performed at two pH levels: pH 7.6, corresponding to the pH of fresh egg white, and pH 8.8, corresponding to that of aged egg white. Both heat and combined heat- and high-pressure treatment resulted in an exposure of buried SH groups. These exposed SH groups were involved in the formation of disulfide bond stabilized protein aggregates, as shown by gel electrophoresis. Under severe processing conditions (above 70 °C at atmospheric pressure or above 500−600 MPa, depending on the temperature applied), a decrease in total SH content could be observed, probably due to the formation of disulfide bonds by oxidation, especially at alkaline pH when the thiolate anion was more reactive. The high degree of exposure of sulfhydryl groups, and subsequent oxidation and sulfhydryl−disulfide bond exchange reactions resulting in soluble aggregates, can explain why pressure-induced egg white gels are softer and more elastic than heat-induced ones. When pressure treatment was performed at low temperatures (e.g., 10 °C), a lower pressure was required to induce similar changes in the sulfhydryl content, as compared to higher temperatures (e.g., 25 °C), indicating an antagonistic effect between pressure and temperature in the domain studied (10−60 °C, 100−700 MPa). Treatment conditions resulting in extensive protein insolubilization were accompanied by a transfer of free sulfhydryl groups from the soluble to the insoluble protein fraction. These SH groups were mainly accessible to DTNB.

KEYWORDS: Egg white; sulfhydryl content; solubility; heat treatment; high-pressure treatment

INTRODUCTION

Disulfide (SS) bonds and sulfhydryl (SH) groups play an important role in the heat-induced gelation of proteins. While hydrophobic and electrostatic interactions initiate gel network formation after heat-induced partial denaturation of the proteins, the role of disulfide bonds in protein gelation may be related to their ability to increase the number of molecules involved in the aggregate. Inaccessible thiol groups become exposed by protein unfolding during heating (*1*). This allows the formation of new covalent inter- and intramolecular disulfide cross-links via thiol−disulfide exchange and oxidation reactions. In this way, disulfide bonds contribute to the elasticity of the formed gels since the storage modulus, related to the elasticity of a solid, is proportional to the cross-link density in the gel network (*2*).

For this reason, several studies have been conducted on the effect of heat treatment on the sulfhydryl and disulfide content of proteins from different sources (*1*, *3*−*6*). In native β-lactoglobulin, the free thiol group is buried in the hydrophobic interior of the protein and, normally, does not participate in a disulfide linkage. The reactivity of this free thiol group can be markedly increased by protein unfolding, induced by heat treatment. Then, the free SH121 group promotes SH−SS interchange reactions with the Cys66−Cys160 bond of the same or of another β-lactoglobulin monomer (*7*). Such newly formed SS bonds play an important role in the heat-induced aggregation and gelation of β-lactoglobulin. The observation by Shimada and Cheftel (*3*) that the free SH121 group in the native β-lactoglobulin, unlike the newly formed SH groups, reacts slowly with DTNB in urea when sodium dodecyl sulfate (SDS) is present allows the monitoring SH−SS interchange reactions occurring during heat treatment by measuring the reaction rate of DTNB with β-lactoglobulin SH groups in the presence or absence of SDS. At alkaline pH, these interactions involving β-lactoglobulin's exposed SH groups are mainly responsible for whey protein isolate gelation, as only a limited decrease of total SH content can be observed (*3*). In soy protein isolate, newly formed additional SS bonds and/or SH−SS interchange reactions

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contribute markedly to the strengthening of its gels at alkaline pH or at high temperatures (115−120 °C). At very high temperatures (130 °C), partial breakdown of SS bonds may contribute to gel softening (*4*).

Ovalbumin, the major protein in egg white, has been comprehensively studied as a model for the impact of processing on the physicochemical and functional properties of egg white. It is the only egg white protein to contain four free sulfhydryl groups, which are buried in the protein core. In addition to these sulfhydryl groups, the protein contains one disulfide bond (*8*). An increase in the exposed sulfhydryl groups can be observed after heat treatment, with higher levels of SH groups being obtained at longer treatment times and temperatures. At the same time, heat treatment results in a decrease of total SH groups (*1*, *5*). Hayakawa and Nakai (*6*) showed that the strength of ovalbumin gels was increased by the decrease in total sulfhydryl groups during thermal treatment.

High hydrostatic pressure is a relatively recent nonthermal technology for food processing, in particular for pasteurization purposes at room temperature. High-pressure processing avoids the formation of off-flavors and the deterioration of food components and nutrients, due to its limited effect on the rates of these deterioration reactions, that are often coupled with a positive reaction volume, and pressure generally enhances only reactions coupled with a negative reaction volume (*9*−*13*). However, pressure treatment can cause protein denaturation, depending strongly on the protein structure, pressure range, temperature, pH, and solvent properties (*14*). Pressure-induced unfolding can enhance the reactivity of buried free SH groups. Indeed, SH−SS interchange reactions due to high-pressure treatment of β-lactoglobulin have been observed (*15*). Iametti et al. (*16*) observed an increased accessibility of sulfhydryl groups to DTNB during high-pressure treatment of ovalbumin. It was suggested that adding DTNB before pressure treatment prevented the formation of insoluble aggregates, due to a sulfhydryl−disulfide exchange mechanism between DTNB and the free SH groups. No information is, however, available on the sulfhydryl content of ovalbumin after pressure treatment. Furthermore, studies on the effect of pressure on food proteins are generally conducted only at room temperature, while it has been shown that the temperature during pressure treatment is a determining factor of the degree of protein denaturation (*17*).

Chicken egg white is extensively used in the food industry, mainly because of its excellent functional properties such as foaming and gelling. For application in food products, pasteurization of egg whites is often required to ensure microbial safety. The choice of preservation technique (conventional heating or high-pressure treatment), however, will affect to a greater or lesser extent the physicochemical characteristics of the egg white and therefore also its functional properties. In the present study, the changes in sulfhydryl content were selected to investigate the effect of physical treatments (heat and high pressure) on egg white proteins, as sulfhydryl groups and disulfide bonds play an important role in gelation, one of the major functional properties of egg white. The aim of this paper was to study the effect of high-pressure treatment in a broad pressure−temperature range (100−700 MPa and 10−60 °C) on the sulfhydryl content and solubility of egg white proteins and to compare it to the effect of heat treatment. The contribution of the sulfhydryl groups to the insolubilization of egg white proteins was investigated, and the involvement of sulfhydryl groups and disulfide bonds in the formation of soluble aggregates was studied in more detail by SDS−gel electrophoresis. Furthermore, because the pH increases during storage of eggs (*18*), the effects of physical treatment were studied at two levels of pH, corresponding to that of fresh (pH 7.6) and aged egg white (pH 8.8). The information obtained can help elucidate the mechanism of aggregation of egg white proteins under different processing conditions (temperature, pressure, and pH) and explain the differences observed in gels produced by heat or high-pressure treatment (*19*).

MATERIALS AND METHODS

Materials. Eggs were obtained from a local supermarket. The egg white was separated from the egg yolk, and the chalaza was removed. The albumen was gently mixed and stored at −40 °C, without any conversion to *S*-ovalbumin, as demonstrated by differential scanning calorimetry measurements (data not shown). 5′,5-Dithiobis (2-nitrobenzoic acid) (DTNB) was from Aldrich. Reagent grade chemicals were used to prepare the following: Tris-glycine buffer (0.1 M Tris(hydroxymethyl)-aminomethane (Tris), 0.1 M glycine, and 4 mM ethylenediamine-tetraacetic acid disodium salt, pH 8.0), 5% sodium dodecyl sulfate in Tris-glycine buffer (denoted SDS-Tris-glycine), Ellman's reagent (4 mg/mL DTNB in Tris-glycine buffer), and two Tris-HCl buffers (200 mM Tris HCl, pH 7.6 and 8.8). Egg white solutions (10% v/v or 9.64 mg of protein/mL) were prepared in either of the Tris-HCl buffers.

Heat Treatment. The protein solutions (1 mL) were heated in 1.5 mL centrifuge tubes with a cap (Eppendorf, Hamburg, Germany) in a water bath at a certain, constant temperature (in the range of 50−85 °C) for 20 min. The required temperature was reached within 1 min of heating. After heating, the sample was immediately cooled in ice water to stop further denaturation and stored at 4 °C until analysis. Depending on the pH and temperature applied, clear solutions or turbid suspensions of protein aggregates were obtained.

High-Pressure Treatment. Isothermal−isobaric experiments were performed in laboratory scale eight-vessel high-pressure equipment (HPIU-10.000, serial no. 95/1994, Resato, Roden, The Netherlands). The protein solutions were filled in flexible tubes (0.7 mL, Elkay, Belgium) and were enclosed in the pressure vessels already equilibrated at the desired temperature. The pressure was built up slowly (100 MPa/min) to minimize adiabatic heating. After attaining the desired pressure, the individual vessels were isolated, and finally the central circuit was decompressed. The vessels were decompressed after 20 min of pressure treatment. In these experiments, a pressure range of 100−700 MPa and a temperature range of 10−60 °C were used. After pressure release, the samples were immediately cooled in ice water to stop any further denaturation and analyzed after 24 h of storage at 4 °C. Depending on the pH and pressure and temperature applied, clear solutions or turbid suspensions of protein aggregates were obtained.

Determination of Solubility. The soluble fraction was defined as the supernatant after centrifugation of a 10-fold diluted sample (in water) (i.e., 1% v/v) at 19 900*g* and 4 °C during 15 min. The protein content of the supernatant was determined twice using the Sigma Procedure no. TRPO-562. This method of protein quantification is based on the reduction of Cu^{2+} by protein in an alkali environment. Bicinchonininic acid (BCA) forms a colored complex with the resulting Cu^{+}. The absorbance of this complex was measured at 562 nm. The protein content was determined by comparison with a standard curve using bovine serum albumin. A 100% solubility was assigned to samples containing the same amount of protein as the untreated sample.

Determination of Sulfhydryl Groups. The concentration of sulfhydryl (SH) groups of egg white solutions was determined using Ellman's reagent (5′,5-dithiobis (2-nitrobenzoic acid)), which is based on the reaction at neutral and alkaline pH between protein sulfhydryl groups and DTNB, resulting in the formation of the thionitrophenylated protein and a yellow thionitrophenylate anion (*20*). The content of total, exposed, and buried SH groups of the total (1:10 dilution of the treated sample in H_2O) or soluble protein fraction (supernatant obtained as described previously) was measured in duplicate using both the following procedures, modifications of the methods described earlier (*21*). Procedure A (determination of exposed SH groups): to 500 μL of the total or soluble protein fraction, 500 μL of Tris-glycine buffer

was added. After a 15 min reaction with 10 μL of Ellman's reagent at ambient temperature, the reaction mixture was either centrifuged during 15 min at 19 900*g* and 4 °C (total protein fraction) or kept at 4 °C for 15 min (soluble protein fraction). Finally, the absorbance of the supernatant (total protein fraction) or the reaction mixture (soluble protein fraction) was measured at 412 nm against a reagent blank. Procedure B (determination of total SH groups): to 500 μL of the total or soluble protein fraction, 500 μL of SDS-Tris-glycine buffer and 10 μL of Ellman's reagent was added. For 15 min, the reaction mixture was kept at 40 °C in a water bath to allow the protein to unfold and all sulfhydryl groups to be accessible to DTNB. Finally, the SH determination was continued as described in procedure A.

The problem of the turbid solutions obtained after heating in the determination of the SH content of the total protein fraction was addressed by centrifuging the protein−DTNB reaction mixture after the required reaction time. A clear, yellow supernatant was obtained over a white precipitate, indicating no noticeable absorption of DTNB to the pellet (*1*). A molar extinction coefficient of 13 600 M^{-1} cm^{-1} for the thionitrophenylate anion at 412 nm was used to calculate the amount of exposed or surface (procedure A) and total (procedure B) sulfhydryl groups (*22*). The amount of buried sulfhydryl groups (inaccessible to DTNB) was calculated by subtracting the amount of exposed SH groups from the total amount of the SH groups. To avoid discussion concerning the exact value for the molar extinction coefficient (*23*), the sulfhydryl contents are expressed as the percentage of total sulfhydryl groups of the untreated, total protein fraction (*21*). Determinations were performed twice, and the average value was calculated. The standard deviation on the measurements was always less than 5% of the average value.

SDS Slab Polyacrylamide Gel Electrophoresis. A Phastsystem (Amersham Biosciences, Uppsala, Sweden) was used for SDS−PAGE of the soluble protein fraction. For protein separation, Phastgel Homogeneous 20% and Phastgel SDS buffer strips were used (Amersham Biosciences, Uppsala, Sweden). The soluble fractions were transferred to a buffer containing 2.5% SDS, with or without 5% 2-mercaptoethanol. Gel staining was performed with silver nitrate, according to Heukeshoven and Dernick (*24*).

RESULTS AND DISCUSSION

Irreversibility of the Changes in Sulfhydryl Content. Although Mine (*25*) observed a slow, progressive polymerization of soluble egg white proteins during storage at room temperature after heat treatment (2% w/v, pH 9.5), in our study, no further changes in solubility and SH content were observed after 24 h at 4 °C. This was also the case for pressure-treated egg white solutions (data not shown). Therefore, determination of these properties was performed 24 h after heat treatment and storage at 4 °C. In this way, only the irreversible changes in the sulfhydryl content of egg white proteins were taken into account.

Changes in Sulfhydryl Content of the Total Protein Fraction Due to Heat Treatment. The SH content of the egg white solutions (10% v/v or 9.64 mg of protein/mL) was measured after heat treatment in the temperature range of 50−85 °C. In the untreated egg white solution, a total amount of SH groups of 58.5 ± 3.4 μM SH/g of protein was obtained, which is in agreement with the 50.7 μM SH/g of dry weight observed by Beveridge and co-workers (*22*). Before heat treatment, these SH groups were mainly buried in the protein core and therefore inaccessible for DTNB when no denaturant (SDS) was applied, as shown by the low exposed SH content for the untreated sample (**Figure 1**). As can be observed from **Figure 1**, above 70 °C, heat-induced unfolding of the egg white proteins resulted in the exposure of buried sulfhydryl groups (calculated from the difference between total and exposed sulfhydryl groups), which was more pronounced when heat treatment was performed at a higher temperature. This was demonstrated by an increase in the amount of SH groups that

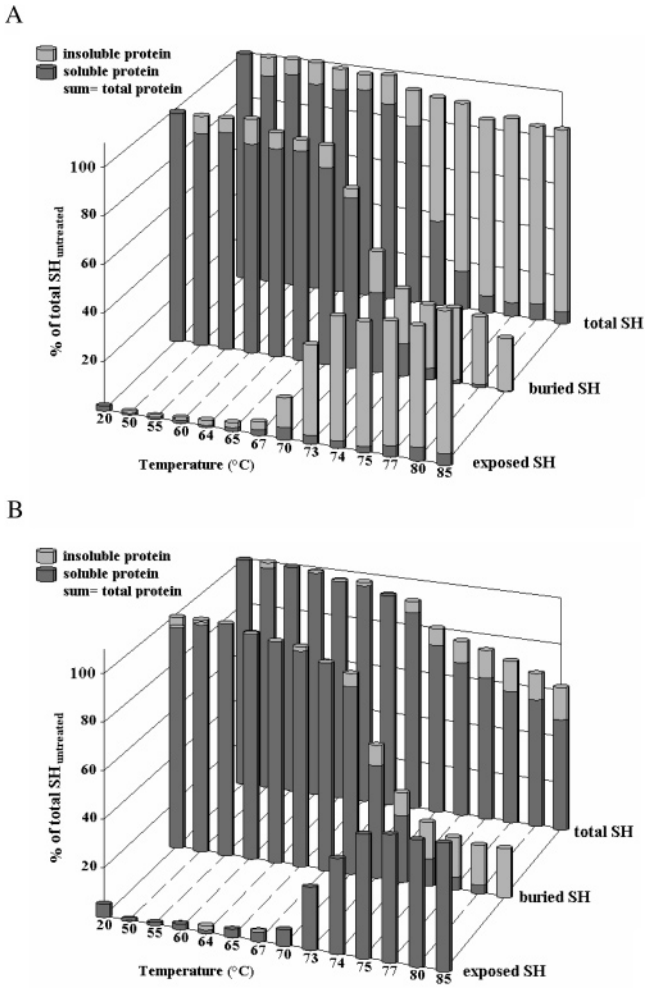

Figure 1. Changes in exposed, buried, and total SH content of egg white solutions (9.64 mg of protein/mL) after 20 min of heat treatment at pH 7.6 (**A**) and pH 8.8 (**B**). The light gray part of the bar represents the contribution of the insoluble protein fraction to the SH content, while the dark gray part represents that of the soluble protein fraction.

readily react with DTNB. These exposed sulfhydryl groups are rather reactive at neutral or alkaline pH and therefore can be expected to be involved in further interactions that can lead to protein aggregation. One possible mechanism for such further reaction is the sulfhydryl−disulfide exchange reaction, in which the exposed SH groups react with the molecule's own disulfide bond or that of another ovalbumin molecule, or even that of another SS-containing egg white protein. This can result in the formation of a protein network and, depending on the protein, salt concentration and pH in a gel (*26*). In β-lactoglobulin, this reaction mechanism can easily be demonstrated as the reaction rate of the native SH group with DTNB is low when SDS is present, in contrast to the reaction rate of DTNB with newly formed SH groups in other positions. Accelerated reaction with DTNB therefore reveals that sulfhydryl−disulfide exchange reactions have occurred during heating (*3*). In ovalbumin, however, such differences in DTNB reaction rates for the various SH populations have not been observed. When the sulfhydryl−disulfide exchange reaction is the sole reaction involving exposed sulfhydryl groups, no change in the total SH content should be expected as the free SH group merely shifts from one position to another. However, after heat treatment of egg white protein solutions, a gradual but limited decrease of total SH content could be observed (**Figure 1**). This indicates that some of the exposed SH groups were oxidized to form new

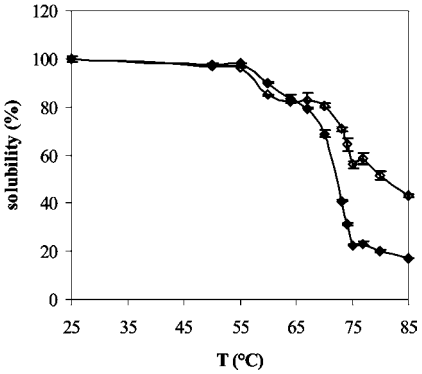

Figure 2. Changes in solubility after 20 min of heat treatment of egg white solutions (9.64 mg of protein/mL) at pH 7.6 (◆) and pH 8.8 (◇). (Mean value and error bars represent standard deviation of duplicate measurement).

disulfide bonds. It has been demonstrated that in oxygen containing egg white solutions, heat treatment induces a rapid decrease in total SH content, while no clear decrease in these groups can be observed when heat treatment is performed on nitrogen flushed solutions (*1*). As in our study, the egg white solutions were heated with a (air) headspace of 0.5 mL, sulfhydryl oxidation to disulfide bonds could be expected. Beveridge et al. (*22*) have proposed a method to determine the amount of disulfide bonds in food proteins. This method consists of the reduction of these bonds and the subsequent determination of the total cystein content using the Ellman reagent. This reagent, however, is also sensitive for reducing agents; therefore, the latter has to be adequately removed to allow determination of the content of SH groups. This approach has been shown appropriate for the determination of disulfide content in egg white powder, but anomalous results have been obtained for heat-treated egg white solutions (*1*). Therefore, in our study, no direct measurement of the disulfide content of the egg white solutions was performed. The heat-induced changes in all three types of SH groups (total, exposed, and buried) were most pronounced in the temperature range of 70−85 °C. These observations are in correspondence with those obtained by Mine and co-workers (*27*), although in a slightly lower temperature range because in their study, the heating was performed on a much shorter time scale than in ours. Under the temperature, pH, and protein concentration conditions used, no gel formation occurred; only turbid suspensions of protein aggregates were obtained after heat treatment, probably due to reactions involving the exposed SH groups. A clear difference in the changes in total SH content could be observed for the two studied pH values. At the higher pH, the decrease in total SH groups was stronger, while the increase in exposed SH groups was similar to that at pH 7.6. The higher reactivity of the thiolate anion at alkaline pH can explain this difference.

Solubility Losses Due to Heat Treatment. The solubility of the proteins in the turbid suspensions obtained after heat treatment was investigated. As is known from literature, the heat treatment of egg white can result in a major loss of solubility, depending on the pH and salt content (*26*). In **Figure 2**, the heat-induced reduction of the protein solubility of the egg white solutions is shown. At pH 7.6, aggregates formed due to heat treatment were less soluble than at a higher pH. This was demonstrated by SDS−polyacrylamide gel electrophoresis without 2-mercaptoethanol. When the soluble fraction (obtained as described previously) of heat-treated egg white solutions was applied on the gel for both pH levels, at pH 7.6, little protein was observed after silver staining, as the protein content of the soluble fraction at this pH was rather low (**Figure 2**). For pH 8.8, a clear and intense stain was observed in the stacking gel, demonstrating the presence of soluble aggregates too large to migrate through the gel. After heating at 70 °C, a small band corresponding to ovalbumin was observed, for both pH values (although more intense for pH 8.8). However, for the soluble fraction of egg white solutions treated at 85 °C, this band had completely disappeared (data not shown). From **Figure 2**, it can be observed that insolubilization of egg white proteins occurs in two steps. On the basis of the SDS−PAGE patterns, it can be assumed that the second step, from temperatures above 67−70 °C, involves the insolubilization of ovalbumin molecules, as above 70 °C the band corresponding to ovalbumin disappeared from the SDS−PAGE pattern (data not shown). A possible explanation for the lower protein solubility at pH 7.6 as compared to 8.8 is that at pH levels closer to the isoelectric point (pI = 4.5−5 for most egg white proteins), electrostatic repulsive forces between proteins are lower, resulting in stronger protein interaction, larger aggregates, and therefore lower solubility.

Although the decrease of total SH groups due to SH oxidation was more pronounced at pH 8.8 (**Figure 1**), this was not associated with a substantial loss of solubility. This might indicate that the disulfide bonds formed at pH 8.8 are predominantly intramolecular or that the aggregates formed are smaller and therefore more soluble. The latter was demonstrated by SDS−PAGE. When gel electrophoresis was performed in the absence of 2-mercaptoethanol, the soluble aggregates obtained by heating at 85 °C at pH 8.8 were retained in the stacking area, while in the presence of 2-mercaptoethanol, the aggregates were dissociated, and a faint peak of ovalbumin reappeared indicating the presence of intermolecular disulfide bonds (data not shown).

A significant negative correlation exists between SH content and gel strength of ovalbumin (depending on protein concentration), whereas no good correlation was observed between the former and the ovalbumin coagulability. The latter is probably more affected by the heat-induced increase in hydrophobicity (*6*). In our study, a positive correlation (correlation coefficient = 0.9738 and 0.9766, at pH 7.6 and pH 8.8, respectively) between residual protein solubility and total SH content of the total protein fraction was found.

Changes in Sulfhydryl Content of the Soluble Protein Fraction Due to Heat Treatment. To determine the contribution of the soluble protein fraction to the overall sulfhydryl content, dilutions (1:10) of the heat-treated egg white solutions were centrifuged (15 min at 19 900*g* and 4 °C). The supernatant consisted of the soluble protein fraction, of which the amount of total, exposed, and buried SH groups was determined. As shown in **Figure 1**, heat treatment resulted in a temperature-dependent decrease of the total SH content of the soluble fraction, which was more pronounced than the decrease observed for the total protein fraction. At pH 7.6, the loss of SH groups in the soluble protein fraction was striking, as after a treatment of 20 min at 85 °C, only 5.4% of the initial amount of SH groups could be detected in the soluble protein fraction (corresponding to 17% of the initial protein content). In the total protein fraction, however, 84% of the SH groups was still present after this treatment. This may indicate that almost no ovalbumin (the only egg white protein containing free sulfhydryl groups) was longer present in the soluble protein fraction and that all SH groups of any ovalbumin molecules still present in this fraction were engaged in disulfide bonds, either by sulfhydryl oxidation or by sulfhydryl−disulfide exchange reaction. SDS−PAGE pat-

terns showed that at pH 7.6, little ovalbumin was retained in the soluble fraction. For egg white solutions treated for 20 min at 85 °C, no band corresponding to ovalbumin could be observed, both in the absence and in the presence of 2-mercaptoethanol. At lower temperatures (70 °C), however, ovalbumin was still present in the soluble fraction, both as a single molecule and as involved in disulfide bond stabilized soluble aggregates (data not shown). Therefore, it can be assumed that after 20 min treatment at 85 °C, the insoluble protein fraction (which represents 83% of the total protein) contains the major part of the ovalbumin fraction (which amounts to 54% of the total protein fraction). This corroborates that the second phase in the solubility decrease observed is due to the insolubilization of ovalbumin and indicates that the sulfhydryl groups of ovalbumin play a major role in the insolubilization of egg white proteins.

At the higher pH, the insolubilization was less pronounced (**Figure 2**), which was reflected in the considerable amount of free SH groups remaining in the supernatant. Indeed, when SDS−PAGE was performed in the presence of 2-mercaptoethanol, the ovalbumin band was visible for the soluble protein fraction (corresponding to 56.9% of the initial protein content) of egg white solutions treated at 85 °C (data not shown). This indicates that at pH 8.8, soluble aggregates are formed by SH−SS exchange reactions and/or sulfhydryl oxidation. At both pH values, the fraction of the SH groups that was inaccessible to DTNB could be accounted for by the insoluble protein fraction (**Figure 1**). At pH 7.6, this is not surprising, as almost no SH groups remain in the soluble fraction. At pH 8.8, this indicates that all ovalbumin molecules present in the soluble protein fraction are denatured in such a way that all priorly buried SH groups have become exposed. These SH groups might be engaged in sulfhydryl−disulfide exchange reactions, but the aggregates formed remained soluble, as demonstrated by SDS−PAGE (data not shown). The SH groups in the insoluble fraction are all inaccessible to DTNB and either correspond to the original hidden groups or have become inaccessible to DTNB by aggregation, as no exposed SH groups could be detected in this fraction (**Figure 1**).

From these data, it can be concluded that heat treatment of egg white proteins results in the exposure of buried sulfhydryl groups, leading to aggregation both by SH−SS exchange reactions and sulfhydryl oxidation. Depending on the pH, these aggregates are more or less soluble. Other factors, such as electrostatic repulsion and hydrophobic interactions, can account for the differences observed in the solubility of egg white proteins treated at different pH levels.

Changes in Sulfhydryl Content of the Total Protein Fraction Due to High-Pressure Treatment. The effect of high-pressure treatment on the SH content of egg white solutions was studied at four different temperatures (10, 25, 40, and 60 °C), as earlier studies indicated that pressure-induced denaturation of egg white proteins was strongly temperature-dependent (*17*, *21*, *28*). When DTNB is added before the pressure treatment of ovalbumin solutions, it blocks the free SH groups that become exposed during the pressure treatment, thereby preventing the formation of insoluble aggregates due to a sulfhydryl−disulfide exchange mechanism between DTNB and free SH groups (*16*). Similar observations could be made for β-lactoglobulin. Exposure of its buried SH group during high-pressure treatment (up to 400 MPa) could be visualized by the increase of yellow color due to the formation of TNB when DTNB was added to β-lactoglobulin solutions before pressurizing. The increased activity of this exposed SH group is believed to induce the

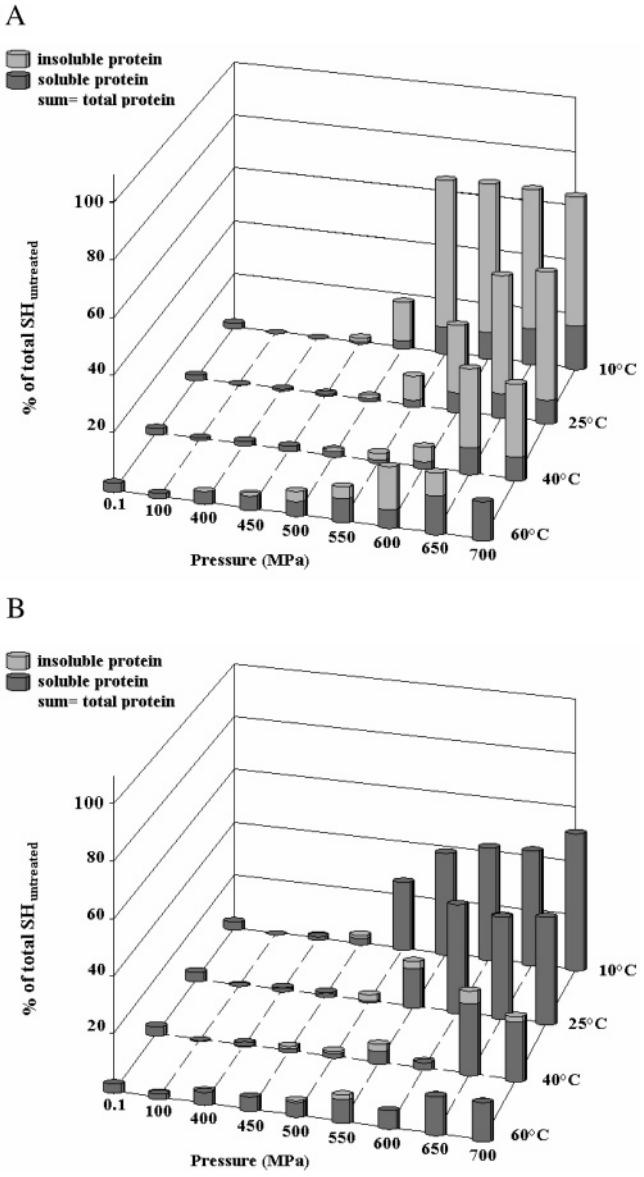

Figure 3. Changes in exposed sulfhydryl content of egg white solutions (9.64 mg of protein/mL) after 20 min high-pressure treatment at pH 7.6 (**A**) and pH 8.8 (**B**). The light gray bar represents the contribution of the insoluble protein fraction to the SH content, while the dark gray bar represents that of the soluble protein fraction.

protein's pressure-induced irreversible denaturation and to be involved in protein aggregation (*15*, *29*). However, under the conditions used in our study, pressure-treated samples showed increased turbidity, especially at pH 7.6 (data not shown). Thus, the simultaneous increase of absorbance due to the formation of the 2-nitro-5-thiobenzoate anion as well as due to the aggregation of egg white proteins would lead to conflicting results concerning the calculation of the amount of sulfhydryl groups exposed during pressurization. Therefore, in our study, DTNB was added after pressure treatment, and the contribution of turbidity to the absorbance was eliminated by centrifugation of the DTNB-reacted samples, without affecting the quantification of the SH content (*1*). By doing so, the exposed as well as the total SH remaining after pressure treatment can be determined.

High-pressure treatment resulted in a pressure-dependent exposure of buried sulfhydryl groups (**Figure 3**) and a decrease of total SH content (**Figure 4**), as mentioned in an earlier study (*21*). After treatment at 700 MPa, almost all residual SH groups

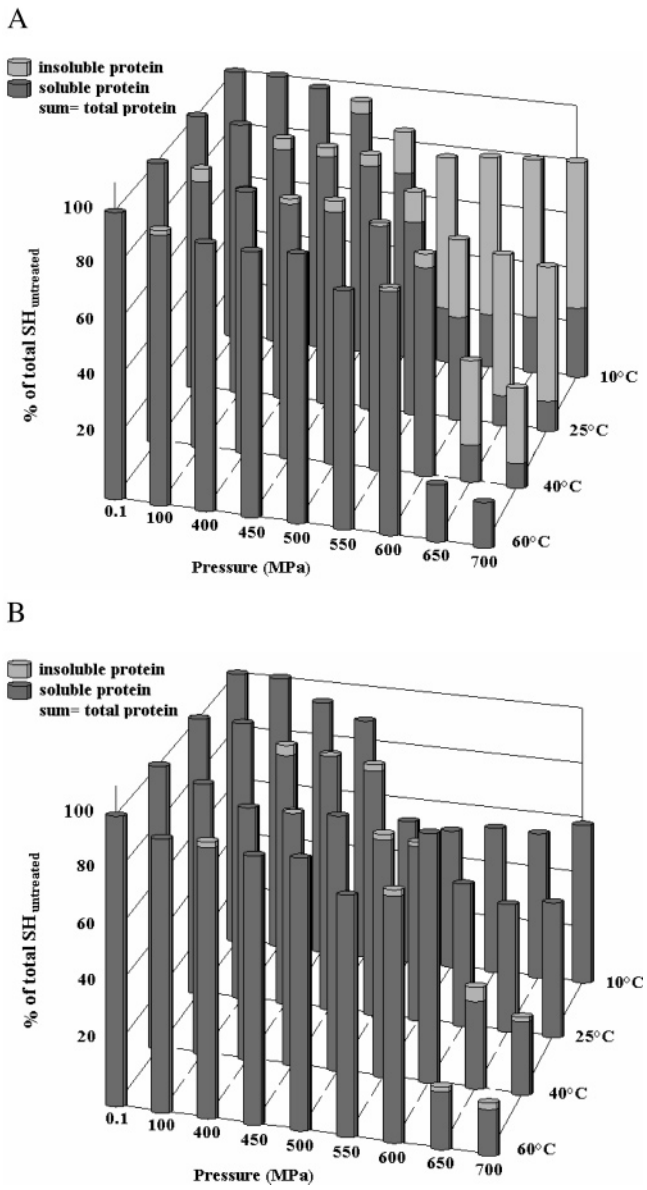

Figure 4. Changes in total sulfhydryl content of egg white solutions (9.64 mg of protein/mL) after 20 min high-pressure treatment at pH 7.6 (**A**) and pH 8.8 (**B**). The light gray bar represents the contribution of the insoluble protein fraction to the SH content, while the dark gray bar represents that of the soluble protein fraction.

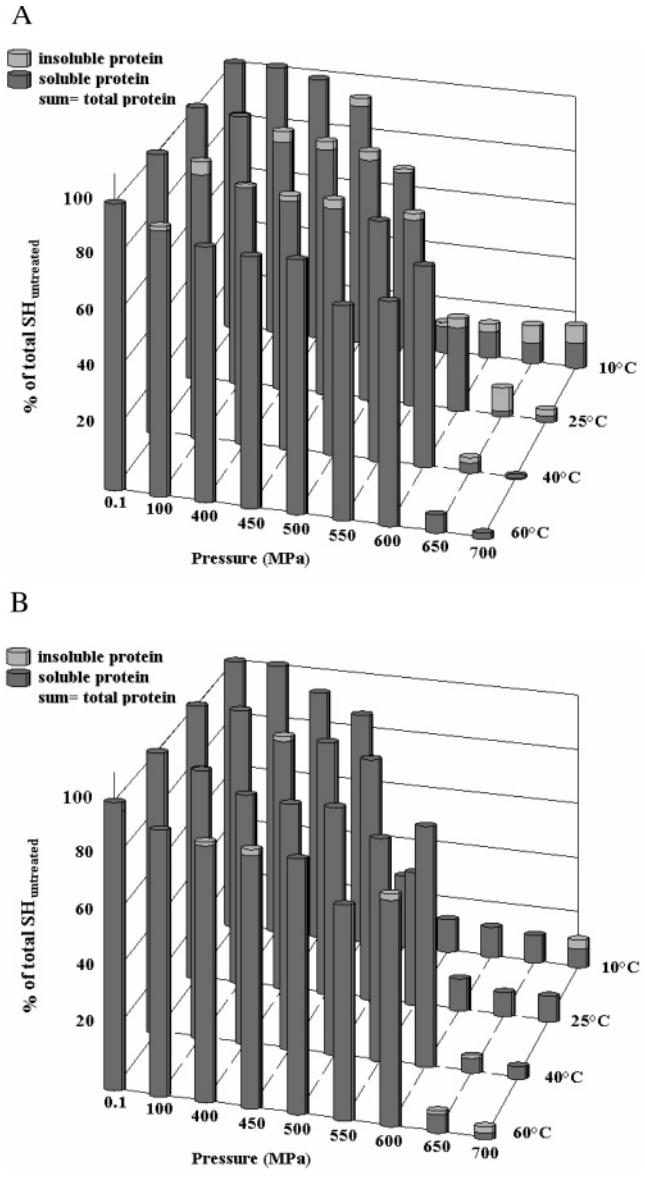

Figure 5. Changes in buried sulfhydryl content of egg white solutions (9.64 mg of protein/mL) after 20 min high-pressure treatment at pH 7.6 (**A**) and pH 8.8 (**B**). The light gray bar represents the contribution of the insoluble protein fraction to the SH content, while the dark gray bar represents that of the soluble protein fraction.

were exposed, as almost no buried SH groups could be observed (**Figure 5**). Although aggregation was observed for some pressure−temperature combinations applied, a considerable amount of exposed SH groups was retained (**Figure 4**) that, however, could have undergone sulfhydryl−disulfide exchange reactions.

As expected, a clear effect of temperature on the pressure-induced changes in SH content could be observed (**Figures 3**−**5**). At lower temperatures, the pressure needed to induce changes in the SH content was lower than at elevated temperatures. For instance, at 10 °C, from 500 MPa upward a clear increase in exposed SH groups could be observed, while at 40 °C, this only occurred above 600 MPa (**Figure 3**). Furthermore, a higher amount of accessible SH groups was obtained after pressure treatment at low temperatures. This is an indication for an antagonistic effect between pressure and temperature in the pressure−temperature domain studied. Suzuki (*17*) observed a similar antagonistic effect for the denaturation of ovalbumin. At lower temperatures, denaturation was faster than at higher temperatures (up to 40 °C). Besides a temperature dependence, an effect of pH on the pressure-induced changes in SH groups also was observed. At pH 8.8, the effect of pressure on the sulfhydryl content was more striking than at pH 7.6. This could be expected, as at a higher pH the thiolate anion is more reactive, resulting in a higher probability of sulfhydryl−disulfide interchange reactions. This exchange reaction can, however, not explain the decrease of total SH content (**Figure 4**), as in the sulfhydryl−disulfide interchange reactions the free SH group merely shifts from one position to another, and no net decrease of SH content should be expected. When oxygen is present, and above 300 MPa, the sulfhydryl groups can be oxidized under pressure (*13*), explaining the loss of SH groups at elevated pressures at both levels of pH. High-pressure treatment (in the pressure- temperature domain studied) was more effective in lowering the SH content of the egg white solutions than heat treatment at atmospheric pressure; thus, sulfhydryl groups were more sensitive to oxidation at elevated pressure.

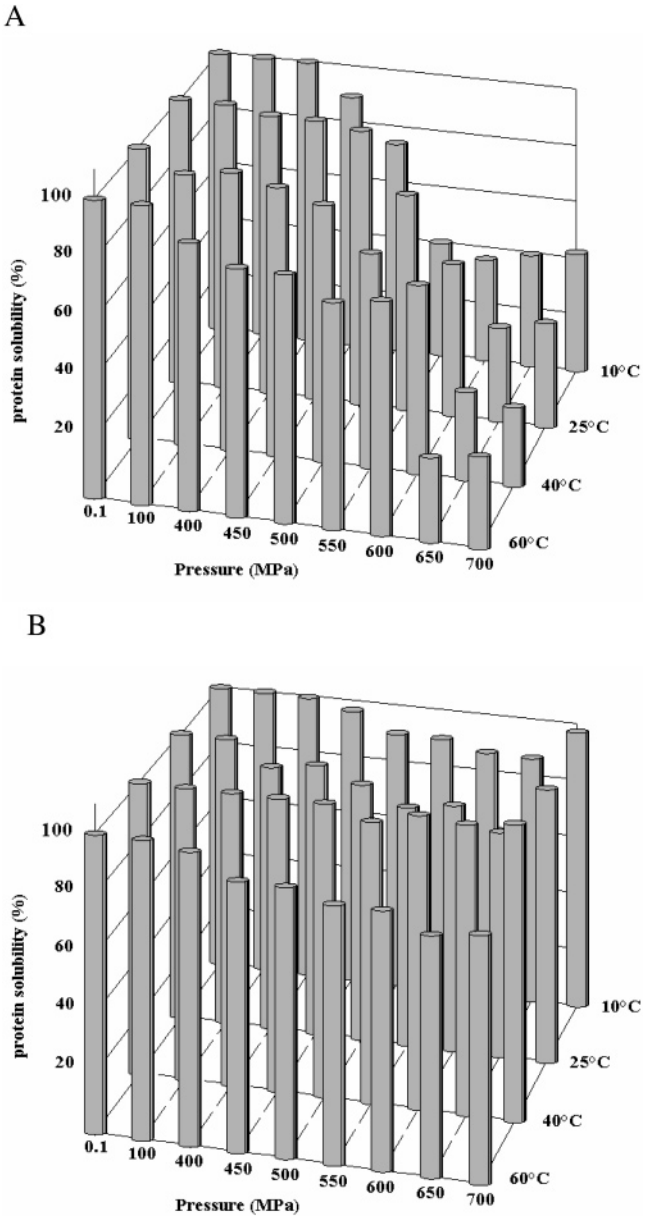

Figure 6. Changes in protein solubility of egg white solutions (9.64 mg of protein/mL) after 20 min high-pressure treatment at pH 7.6 (**A**) and pH 8.8 (**B**).

Solubility Losses Due to High-Pressure Treatment. High-pressure treatment resulted in a loss of protein solubility, as shown in **Figure 6**. However, this decrease was more pronounced at pH 7.6 as compared to pH 8.8, as was the case after heat treatment at atmospheric pressure. At the latter pH, almost no reduction in protein solubility was observed, except when pressure treatment was performed at a denaturing temperature (60 °C). Under the conditions studied, no gels were formed. In the pressure−temperature domain studied, the effect of pressure on protein solubility was less adverse than that of heat treatment at atmospheric pressure. This difference may be attributed to the stabilization of aggregates by hydrophobic interaction due to heat treatment, as this type of interaction is strengthened with increasing temperature but weakened under high pressure (*11*).

Changes in Sulfhydryl Content of Soluble Protein Fractions Due to High-Pressure Treatment. As the loss of protein solubility at pH 7.6 was quite considerable, the contribution of both the soluble and the insoluble protein fraction to the SH content was investigated (**Figures 3**−**5**). As observed for the total protein fraction, the effect of pressure on the SH groups in the soluble fraction was strongly dependent on the temperature during pressure treatment (antagonistic effect), yet the impact of the pH was even more striking. At pH 8.8, essentially all free sulfhydryl groups remained in the soluble protein fraction (**Figure 4**), as could be expected based on the high residual protein solubility (80% after 20 min at 700 MPa and 60 °C). This indicates that most of the ovalbumin molecules remained soluble, even though they might be involved in aggregation through sulfhydryl−disulfide exchange reactions or even disulfide bond formation. This was indeed demonstrated by SDS−PAGE patterns of the soluble fraction of egg white solutions pressure treated (700 MPa) at pH 8.8. In the absence of 2-mercaptoethanol, only a faint (treated at 10 and 25 °C) or no (treated at 40 and 60 °C) band for ovalbumin was observed, while a large amount of proteins was unable to migrate through the gel, due to the size of their aggregates. In the presence of 2-mercaptoethanol, these aggregates were separated, and the band of ovalbumin was intensified (at all four temperature levels) (data not shown). This indicates that at pH 8.8, pressure induces the formation of soluble ovalbumin aggregates through SS bonds. For a large extent, these bonds are formed by oxidation of SH groups, as demonstrated by the decrease of the total SH content (**Figure 4**). At atmospheric pressure and elevated temperature (above 70 °C), some insolubilization did occur at this pH, coinciding with a shift of some of the SH groups to the insoluble protein fraction, however, not as pronounced as at pH 7.6. At this lower pH, pressure treatments resulting in an extensive loss of protein solubility (**Figure 6**) induced a considerable transfer of SH-containing entities from the soluble to the insoluble fraction (**Figure 4**). Nevertheless, some ovalbumin molecules engaged in the SS bond stabilized aggregates were present in the soluble fraction, demonstrated by SDS−PAGE patterns (data not shown), however, not as numerous as at pH 8.8. After severe pressure treatment (>650 MPa), the amount of buried sulfhydryl groups in the soluble protein fraction was rather low (**Figure 5**), and even in the insoluble fraction, most of the free SH groups was exposed **(Figure 3**).

Thus, pressure treatment of egg white solutions is accompanied by a pronounced exposure of SH groups that are involved in oxidation and SH−SS interchange reactions, resulting in protein aggregation. Especially at high pH values, these aggregates remain soluble. Temperature during pressure treatment strongly affects the pressure needed to induce changes in the sulfhydryl content and solubility of egg white solutions, and an antagonistic effect was observed.

CONCLUSIONS

Both heat and pressure treatment resulted in changes in the sulfhydryl content of egg white proteins. Physical protein denaturation was demonstrated by an exposure of the ovalbumin sulfhydryl groups that were previously buried in the protein core. These exposed SH groups of ovalbumin were involved in an SH−SS exchange reaction and SH oxidation (especially at high pH and elevated pressure), resulting in the formation of aggregates, as shown by SDS−PAGE. These aggregates were more soluble at higher pH. The lower protein solubility despite the higher total SH content after heat treatment in comparison with high-pressure treatment might be attributed to the increased hydrophobic interactions at elevated temperature in contrast to the weakening effect of pressure on this type of bond.

Pressure-induced changes in the free sulfhydryl groups are strongly temperature dependent. At lower temperatures, a lower

pressure is required to obtain an exposure of SH groups. Higher levels of exposed SH groups could be obtained with high pressure treatment (>550 MPa) as compared to heat treatment, when the former was performed at low temperatures (10 °C), indicating an antagonistic effect of temperature and pressure in the temperature−pressure domain studied.

For both physical treatments, a transfer of sulfhydryl groups from the soluble fraction to the insoluble fraction was observed at pH levels where extensive protein insolubilization was observed. This indicates that even after protein aggregation, some free SH groups are retained that were not involved in oxidation reactions, although they could have been part of a sulfhydryl−disulfide exchange reaction.

The high degree of exposure of sulfhydryl groups, and subsequent oxidation and sulfhydryl−disulfide bond exchange reactions resulting in soluble aggregates, can explain why pressure-induced egg white gels are softer and more elastic than heat-induced ones.

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