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Kinetic Study on the Changes in the Susceptibility of Egg White Proteins to Enzymatic Hydrolysis Induced by Heat and High Hydrostatic Pressure Pretreatment

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A kinetic study was conducted on the effect of heat pretreatment in the temperature range of 50−85 °C at atmospheric pressure and of high hydrostatic pressure pretreatment (100−700 MPa) at four temperatures (10, 25, 40, and 60 °C) on the susceptibility of egg white solutions (10% v/v, pH 7.6) to subsequent enzymatic hydrolysis by a mixture of trypsin and α-chymotrypsin at 37 °C and pH 8.0. Both heat pretreatment at atmospheric pressure and high-pressure pretreatment resulted in an increase in degree of hydrolysis (DH) after 10 min of enzymatic reaction (DH10) of egg white solutions, as measured using the pH-stat method, which could be described by a fractional conversion model (based on an apparent first-order reaction kinetic model). The temperature dependence of the corresponding rate constants could be described by the Arrhenius equation. At elevated pressure, a negative apparent activation energy was obtained, implying an antagonistic effect of pressure and temperature. The pressure dependence of the rate constants could be described by the Eyring equation, and negative activation volumes were observed, which demonstrates the positive effect of pressure on the susceptibility of egg white solutions to subsequent enzymatic hydrolysis.

KEYWORDS: Kinetic; egg white; pH-stat; hydrolysis; heat treatment; high hydrostatic pressure; antagonistic effect

INTRODUCTION

High hydrostatic pressure is more and more being used as an alternative for thermal processing, as this nonthermal processing technique avoids the formation of off-flavors and the deterioration of food components and nutrients (*1*). However, pressure treatment can induce protein denaturation. Pressure denaturation of proteins is a complex phenomenon and depends on the protein structure, pressure range, temperature, pH, and solvent properties (*2*). Besides the inactivation of microorganisms (*3*) and the improvement or alteration of functional properties such as gelation, emulsifying, and foaming characteristics of foods (*4*), high hydrostatic pressure can be applied in enzymatic hydrolysis of food proteins. Several studies report on the enhanced enzymatic hydrolysis of proteins from various sources, due to pressure treatment either during or prior to digestion (*5*−*7*, *11*−*13*). The rate of β-lactoglobulin hydrolysis by thermolysin at elevated pressure is significantly increased by an increase of pressure up to 200 MPa (*5*). Tryptic hydrolysis of β-lactoglobulin is enhanced under high pressure, with an optimum at 300 MPa. This increased hydrolysis is due to accelerated breakdown of large intermediate hydrolysis products into final tryptic peptides. The effect of prepressurization on the subsequent hydrolysis of β-lactoglobulin by trypsin is probably limited to the facilitation of the initial proteolytic attack of the protein (*6*). High-pressure treatments furthermore enhance the hydrolysis of soybean whey proteins by pepsin, trypsin, and α-chymotrypsin (*7*).

Chicken egg white is extensively used in the food industry, mainly because of its excellent functional properties such as foaming and gelling. Ovalbumin, the major protein in chicken egg white, in its native form has a low susceptibility to digestion by trypsin and α-chymotrypsin. Heat denatured ovalbumin however, shows an increased susceptibility to these proteases (*8*−*11*). Also, pressure treatment has been shown to enhance the susceptibility to enzymatic hydrolysis of egg white proteins (*12*−*14*). Hayashi et al. (*12*) report an extensively improved digestibility by subtilisin of homogenized pressure-induced egg white gels as compared to raw egg white. The pressure-induced egg white gels are digested even more effectively than boiled egg white. Iametti et al. (*13*, *14*) observed an increased affinity of trypsin for high-pressure treated ovalbumin solutions as well as egg white. When NaCl or sucrose was added before treatment, pressure induced an even more pronounced increase of sensitivity to proteolysis.

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The aforementioned studies have been focused on a qualitative description of the effect of high hydrostatic pressure on the susceptibility of egg white to tryptic hydrolysis. However, to develop and control industrial heat or high pressure processes to obtain a microbially safe egg white with optimal nutritional quality, a quantitative approach is required. Therefore, in the present work, a kinetic study was conducted on the effect of high pressure pretreatment on the susceptibility of egg white proteins to subsequent enzymatic hydrolysis at atmospheric pressure, and a comparison with the effect of heat pretreatment was made. As temperature during pressure treatment strongly affects the pressure-induced protein denaturation, the effect of temperature during pressure pretreatment on the susceptibility to enzymatic hydrolysis of egg white solutions was studied on a kinetic basis. The kinetic data obtained will allow the prediction of changes in the susceptibility of egg white proteins due to heat and pressure pretreatment.

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 until use, without any conversion of *N*-ovalbumin to *S*-ovalbumin, as demonstrated by differential scanning calorimetry measurement (data not shown). The pH of the egg white solutions (10% v/v or 10.8 mg of protein/mL in 150 mM NaCl) was adjusted to pH 7.6 using 0.1 N HCl. α-Chymotrypsin and trypsin (from porcine pancreas, type IX-S) were obtained from Sigma (St. Louis, MO). Other chemicals were of analytical grade.

Isothermal Treatment. Isothermal denaturation was investigated in the temperature range of 50−85 °C in a temperature-controlled water bath (Haake). Isothermal treatment of the egg white solutions (1 mL) was performed in 1.5 mL flexible centrifuge tubes (Eppendorf). A thermal lag of 1 min was considered, and the properties of the sample after 1 min of heating were considered as the initial value of the susceptibility to enzymatic hydrolysis. After heating, samples were immediately transferred to an ice bath to stop further denaturation. Samples were analyzed after 24 h storage at 4 °C. In this way, the irreversible changes in the egg white properties under study were measured. Under the conditions applied (10% v/v in 150 mM NaCl), no gel formation was observed, even at the highest treatment temperatures and after prolonged heating. However, an opaque suspension of protein aggregates was observed after intensive heat pretreatment.

High-Pressure Treatment. Isothermal−isobaric experiments were performed in laboratory scale multivessel high-pressure equipment (HPIU-10.000, serial no. 95/1994, Resato, Roden, The Netherlands). The pressure medium was a glycol−oil mixture (TR15, Resato). The protein solutions were filled in tubes with a flexible stopper (3 mL, Nunc, Denmark) and were enclosed in the pressure vessels already equilibrated at the desired temperature. Introduction of the samples in the pressure equipment took exactly 3 min, after which pressure was built up slowly (100 MPa/min) to minimize adiabatic heating. After attaining the desired pressure, all individual vessels were isolated, and the central circuit was decompressed. An equilibration period of 2 min was taken into account to ensure isothermal−isobaric conditions. The sample taken at 2 min after the pressure was reached was considered as the sample at zero treatment time. The vessels were decompressed after preset holding times (which are referred to as the actual treatment time). In these experiments, a pressure range of 100−700 MPa and temperature range of 10−60 °C was used. Exactly 1 min after pressure release, the samples were cooled in ice water and analyzed after 24 h of storage at 4 °C. Under the conditions applied (10% v/v in 150 mM NaCl), no gel formation was observed, even at the highest treatment pressure or temperature and after prolonged pressurizing. However, an opaque suspension of protein aggregates was observed after intensive pressure pretreatment. No conversion of *N*-ovalbumin to *S*-ovalbumin due to pressure treatment was observed, as demonstrated by differential scanning calorimetry measurements (data not shown).

Biochemical Analysis. Activities of α-chymotrypsin and trypsin were verified by applying, respectively, the BTEE (*N*-α-benzoyl-L-tyrosine-ethylester) method and BAEE (*N*-α-benzoyl-L-arginine-ethylester) method according to Sigma.

Determination of Protein Solubility. 1:10 dilutions of the treated samples were centrifuged during 15 min at 19 900*g* and 4 °C. Protein content of the supernatant was determined using Sigma Procedure TRPO-562 and compared to that of the untreated sample. Under the pretreatment conditions applied, no gel was formed. However, after centrifugation of the opaque protein suspension obtained, a precipitate below a clear supernatant was observed for intensive treatments.

Enzymatic Hydrolysis of Egg White Proteins. The enzymatic hydrolysis of egg white proteins was determined by a pH-stat technique, described in a previous study (*8*), based on the proportionality of the base consumption during enzymatic protein hydrolysis to keep the pH constant and the amount of peptide bonds cleaved (*15*, *16*). The degree of hydrolysis (DH) was calculated based on eq 1

$$\mathrm{DH}\ (\%) = \frac{1}{h_{\mathrm{tot}}}\frac{1}{\alpha}\frac{N_{\mathrm{b}}B}{MP}100 \qquad (1)$$

where h_{tot} is the total amount of peptide bonds in the protein (calculated from the amino acid composition, B is the base consumption (mL), N_{b} is the base normality (meq/mL), MP is the mass of protein (g), and α is the degree of dissociation. An average proportionality constant between peptide bonds cleaved and base consumed (α^{-1}) of 1.300 was used (*8*).

The susceptibility to enzymatic hydrolysis was determined on aqueous dilutions of the egg white suspensions, resulting from the heat or high-pressure pretreatment. In this way, the total susceptibility (of aggregated as well as nonaggregated proteins) was measured. Aqueous dilutions (10 mL, 0.625 mg of protein/mL, pH 8.0) of the egg white suspensions were hydrolyzed at 37 °C by adding 1 mL of an enzyme solution, with a concentration in α-chymotrypsin of 18.6 BTEE-U/mL and in trypsin of 2310 BAEE-U/mL. The pH was kept at 8.0 by adding 0.01 N NaOH, using a pH-stat (Metrohm, Basel, Switzerland) with automatic dosage of the base. The DH after 10 min of hydrolysis (further referred to as DH10) was used as a parameter to measure the effect of heat and high-pressure pretreatment on the susceptibility of egg white proteins to enzymatic hydrolysis.

Kinetic Data Analysis. The fractional conversion model (a modified first-order kinetic model) was used to express the effect of heat and pressure pretreatment on the susceptibility of egg white proteins to enzymatic hydrolysis (*8*). In this model, the change of DH10 as a function of treatment time is described by eq 2

$$\mathrm{DH10}_t = \mathrm{DH10}_\infty - (\mathrm{DH10}_\infty - \mathrm{DH10}_0)\exp(-kt) \qquad (2)$$

where $\mathrm{DH10}_\infty$ is the equilibrium value for DH10 at infinite treatment time, and $\mathrm{DH10}_0$ is the DH10 of the sample at 0 min of isothermal−isobaric conditions. All parameters were estimated by nonlinear regression analysis (*17*).

The temperature dependence of the rate constant, k (min^{-1}), at constant pressure is described by the Arrhenius equation

$$k = k_{\mathrm{refT}}\exp\left(-\frac{E_{\mathrm{a}}}{R}\left(\frac{1}{T}-\frac{1}{T_{\mathrm{ref}}}\right)\right) \qquad (3)$$

in which k_{refT} is the rate constant (min^{-1}) at a reference temperature T_{ref}, R is the universal gas constant (8.31415 J/mol K), and E_{a} is the activation energy (J/mol). k_{refT} and E_{a} were obtained by nonlinear regression analysis (*17*).

The pressure dependence of the rate constant, k (min^{-1}), at constant temperature is described by the Eyring equation

$$k = k_{\mathrm{refP}}\exp\left(-\frac{V_{\mathrm{a}}}{RT}(P - P_{\mathrm{ref}})\right) \qquad (4)$$

in which k_{refP} (min^{-1}) is the rate constant at a reference pressure P_{ref} (MPa), R is the universal gas constant (8.31415 MPa cm^3/mol K), and

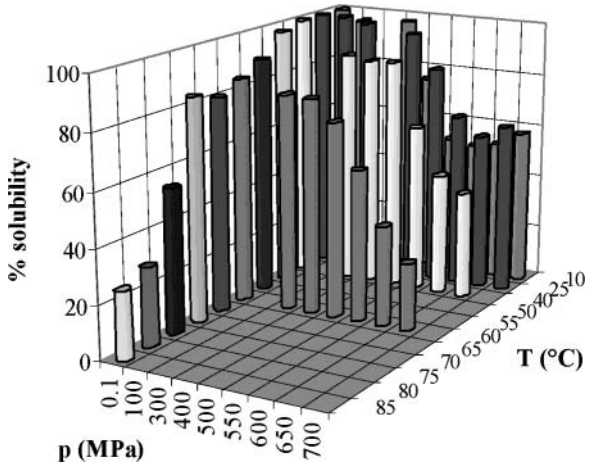

Figure 1. Effect of heat or pressure pretreatment (20 min) on the solubility of egg white solution (10% v/v, pH 7.6, 0.15 M NaCl).

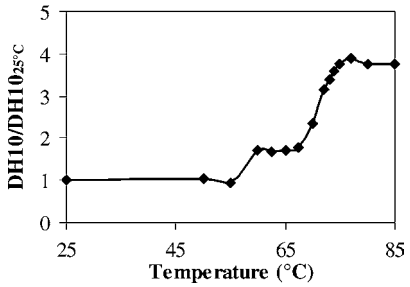

Figure 2. Effect of heat pretreatment (20 min) on the susceptibility of egg white solutions (10% v/v egg white, pH 7.6, 0.15 M NaCl). The DH after 10 min of enzymatic hydrolysis of diluted samples (0.625 mg of protein/mL) by trypsin and α-chymotrypsin at 37 °C and pH 8.0 was measured.

V_a is the activation volume (cm^3/mol). k_{refP} and V_a were obtained by nonlinear regression analysis (*15*).

RESULTS AND DISCUSSION

Effect of Heat Pretreatment on the Susceptibility of Egg White Solutions to Enzymatic Hydrolysis. Under the conditions applied (10% egg white v/v 0.15 M NaCl), no gel formation was observed due to heat pretreatment, even after prolonged heating at high temperatures. However, a turbid suspension of protein aggregates was observed. In **Figure 1**, the loss of solubility, defined as the residual protein content of the supernatant after centrifugation (15 min 19 900*g*), due to heat pretreatment of 20 min is shown. Despite the low residual solubility after treatment at 80 or 85 °C, all aggregates remained in suspension, making it feasible to determine the digestibility of the heated samples.

When the preheated, turbid suspension was subsequently hydrolyzed by a mixture of trypsin and α-chymotrypsin, an increase in susceptibility to enzymatic hydrolysis was observed (**Figure 2**). Two clear temperature ranges could be distinguished. The strongest increase could be observed between 67.5 and 77 °C. The heat denaturation of ovalbumin can be responsible for this strong increase in susceptibility to enzymatic hydrolysis. Indeed, in an earlier study it was observed that *S*-ovalbumin showed a higher susceptibility when treated for 15 min above 70 °C (*8*). Furthermore, ovalbumin composes 54% of the egg white proteins. Therefore, changes in the susceptibility of this protein will strongly affect the overall susceptibility to enzymatic hydrolysis. The absence of *S*-ovalbumin in the egg white in the present study (all ovalbumin was present as *N*-ovalbumin) can explain the somewhat lower temperature range in which these changes occur. In the second, lower temperature range, a rise in susceptibility of the egg white solution can be observed between 55 and 60 °C. This is probably due to the denaturation of the more heat sensitive egg white proteins. The two phases of changes in the sensitivity to proteolysis were not observed by Mine et al. (*10*). However, these authors studied the effect of nonisothermal pretreatments on egg white proteins (2%) at pH 9.5, and only α-chymotrypsin was used to measure the susceptibility to enzymatic hydrolysis. Nevertheless, also in that study a marked increase in sensitivity to enzymatic hydrolysis above 55 °C was observed. The further increase at temperatures above 80 °C might be explained by the higher protein solubility at pH 9.5 as compared to pH 7.6, which was used in our study, and the difference in time scale of the heat pretreatment.

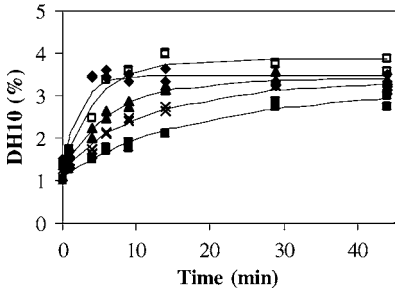

Figure 3. Time-dependent changes in the susceptibility of egg white solutions due to heat pretreatment at 72 (■), 73 (×), 74 (▲), 75 (□), and 77 °C (◆) (10% v/v egg white, pH 7.6, 0.15 M NaCl). The DH after 10 min of enzymatic hydrolysis of diluted samples (0.625 mg of protein/mL) by trypsin and α-chymotrypsin at 37 °C and pH 8.0 was measured.

The lower solubility can also explain the somewhat less pronounced increased susceptibility of the egg white solution treated at temperatures above 80 °C as compared to the previous study (*8*), as the protein concentration used during heat pretreatment was higher in the present study. Extensive aggregation might lower the accessibility of the unfolded proteins to the hydrolytic enzymes. However, even at decreasing protein solubility, an increase in susceptibility of the egg white solution due to heat pretreatment could be observed. In the study by Hayashi et al. (*12*), it was also observed that (homogenized) boiled egg white gels showed an increased digestibility by subtilisin, despite the formation of insoluble aggregates.

Kinetic Study on the Changes in the Susceptibility of Egg White Solutions to Enzymatic Hydrolysis Due to Heat Pretreatment. In the temperature range of 72−77 °C, the typical T-range for ovalbumin denaturation (*9*), the time-dependent effect of heat pretreatment on the DH10 of egg white solutions could be described by a first-order fractional conversion model (**Figure 3**). Longer treatment time resulted in a higher subsequent susceptibility to enzymatic hydrolysis, until an equilibrium value was reached (*8*). The temperature dependence of the rate constants of the fractional conversion model, shown in **Table 1**, could be accurately described by the Arrhenius equation (r^2 = 0.9943). The resulting activation energy was 365.4 ± 6.8 kJ/mol. This value is higher as compared to that of the previous study (*8*), implying a higher heat sensitivity of DH10 after heat pretreatment at higher protein concentration.

At temperatures above 77 °C, a first, strong increase in susceptibility was observed, followed by a decrease, probably due to the formation of less soluble aggregates, inaccessible to hydrolytic enzymes. Indeed, above 77 °C, protein solubility drops below 30% after less than 10 min of heating. Only heat pretreatments resulting in a loss of solubility of over 75%

Table 1. Rate Constants Estimated by Fractional Conversion Model for Effect of Heat Pretreatment[a] on Susceptibility of Egg White Proteins to Enzymatic Hydrolysis at Atmospheric Pressure

T (°C)	k (min^{-1})
72	0.0567 ± 0.0084
73	0.0886 ± 0.0081
74	0.1897 ± 0.0173
75	0.2187 ± 0.0311
77	0.4553 ± 0.1214

[a] 10% v/v egg white, pH 7.6, 0.15 M NaCl.

showed a decreasing susceptibility to enzymatic hydrolysis when the pretreatment was prolonged.

Effect of Pressure Pretreatment on the Susceptibility of Egg White Solutions to Enzymatic Hydrolysis. As pressure-induced protein denaturation strongly depends on the temperature at which pressurization occurs, the effect of pressure pretreatment was studied at four temperatures: 10, 25, 40, and 60 °C, the latter being the maximum temperature attainable in the pressure system being used. As shown in **Figure 4**, pressure pretreatment (20 min) resulted in a pressure-dependent increase in DH10, which was accompanied by a loss of protein solubility (**Figure 1**). The strongest changes in susceptibility of egg white solutions to enzymatic hydrolysis were observed above 500 MPa (20 min pretreatment). From differential scanning calorimetry data, it can be observed that above this pressure ovotransferrin and lysozyme are completely unfolded, and only ovalbumin is still partially folded. At 25 °C, after 20 min pretreatment at 700 MPa, the endothermic peak for ovalbumin denaturation could no longer be observed (data not shown). This would indicate that, as for heat pretreatment, the strongest increase in DH10 corresponds to the denaturation of ovalbumin.

This pressure-dependent increase in susceptibility to enzymatic hydrolysis is in accordance with the results of Iametti et al. (*14*), who observed a 2.5- and 4.5-fold increase of susceptibility to trypsin hydrolysis of egg white in the presence of 1.7 M NaCl after 5 min treatment at 25 °C and at 400 and 600 MPa, respectively. However, under the conditions of their study, no protein precipitation or gel formation was observed. In our study, a much lower salt concentration (0.15 M) was used, combined with a lower protein concentration, but nevertheless, a pressure-induced increase of the susceptibility to enzymatic hydrolysis of egg white solutions could be observed, however, only above 400 MPa. Differences both in protein and in salt concentration used, and therefore in residual protein solubility, might account for this dissimilarity between results. Pressurization (30 min at 25 °C) of egg white above 600 MPa results in gels with a 100% digestibility by subtilisin (*12*).

Iametti et al. (*13*) also studied the effect of pressure on ovalbumin solutions (2 mg/mL). In the presence of 1.7 M NaCl, pressure strongly increased the sensitivity to proteolysis. However, in the absence of the salt, high-pressure treatment of ovalbumin had little effect on the susceptibility of the residual soluble protein to trypsin. Pressure-insolubilized ovalbumin was not digested by trypsin.

As shown in **Figure 4**, the temperature during pressure pretreatment strongly affected the susceptibility of the egg white solutions to enzymatic hydrolysis. At temperatures below 60 °C, the lower the temperature applied during pressurization, the lower the pressure needed to increase the DH10 in subsequent enzymatic hydrolysis. As a result, at a certain elevated pressure, the susceptibility after pressure treatment was lower when the egg white solution was treated at a higher temperature. This is

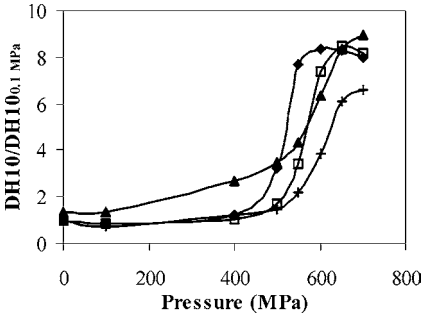

Figure 4. Effect of pressure pretreatment (20 min) on the susceptibility of egg white solutions, treated at 10 (◆), 25 (□), 40 (+), and 60 °C (▲) (10% v/v egg white, pH 7.6, 0.15 M NaCl). The DH after 10 min of enzymatic hydrolysis of diluted samples (0.625 mg of protein/mL) by trypsin and α-chymotrypsin at 37 °C and pH 8.0 was measured.

in contrast to heat pretreatment of egg white solutions at atmospheric pressure, where a higher temperature always resulted in a stronger increase in susceptibility to enzymatic hydrolysis (**Figure 2**).

This apparent antagonistic effect on the susceptibility of egg white proteins to enzymatic hydrolysis between pressure and temperature can be explained by the elliptic phase diagram of protein denaturation in the pressure−temperature diagram. A second principle governing pressure denaturation, next to Le Chatelier's principle, is that of microscopic ordering, which states that, at constant temperature, an increase in pressure increases the ordering of molecules, resulting in a decrease in entropy. However, a temperature rise leads to disordering. Therefore, the effects of pressure and temperature on proteins are antagonistic (*18*). At high temperature, pressure stabilizes the protein, while at low temperature, pressure denaturation occurs at lower pressures when lowering the temperature (*19*). This is exactly what is observed for the pressure-induced changes in the susceptibility of egg white proteins to enzymatic hydrolysis. Because of instrumental limitations, temperatures higher than 60 °C could not be studied; therefore, no information is available on the effect of pressure at these higher temperatures.

Because of the setup of the high-pressure experiment, at 60 °C the pressure pretreatment was preceded by a 3 min and followed by a 1 min heat treatment at atmospheric pressure within the high-pressure equipment. The resulting heat denaturation might explain the somewhat different course of the susceptibility with pressure as compared to the nondenaturing temperatures (**Figure 4**). Nevertheless, the strongest changes in DH10 occur at higher pressures as compared to pressure treatment at 10 °C.

In the temperature−pressure domain studied, pretreatment at elevated pressure resulted in a higher residual protein solubility as compared to heat pretreatment at atmospheric pressure (**Figure 1**). This can explain the higher susceptibility of egg white solutions to enzymatic hydrolysis that can be obtained by pretreating samples at high pressures. At elevated pressure, the same loss of protein solubility corresponded to a larger increase in DH10, as compared to pretreatment at high temperature at atmospheric pressure. This can confirm the different mechanism of protein denaturation at elevated pressure.

Kinetic Study on the Changes in the Susceptibility of Egg White Solutions to Enzymatic Hydrolysis Due to Pressure Pretreatment. The time-dependent effect of pressure pretreatment on the susceptibility of egg white solutions to enzymatic hydrolysis was studied for different combinations of pressure and temperature. As shown in **Figure 5**, the increase in DH10 due to pressure treatment at 25 °C followed a first-order

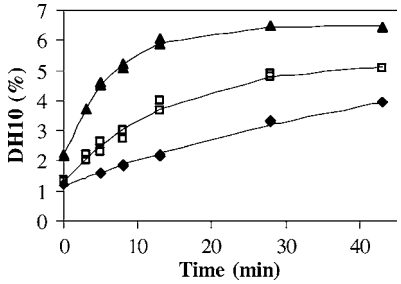

Figure 5. Time-dependent changes in the susceptibility of egg white solutions due to pressure pretreatment at 25 °C and 550 (◆), 575 (□), and 600 MPa (▲) (10% v/v egg white, pH 7.6, 0.15 M NaCl). The DH after 10 min of enzymatic hydrolysis of diluted samples (0.625 mg of protein/mL) by trypsin and α-chymotrypsin at 37 °C and pH 8.0 was measured.

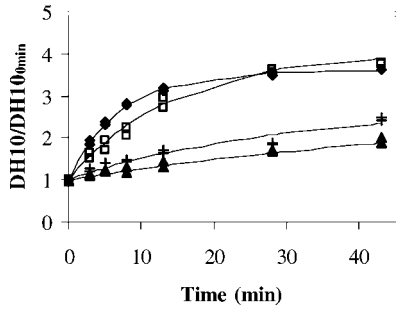

Figure 6. Time-dependent changes in the susceptibility of egg white solutions due to pressure pretreatment at 575 MPa and 10 (◆), 25 (□), 40 (+), and 60 °C (▲) (10% v/v egg white, pH 7.6, 0.15 M NaCl). The DH after 10 min of enzymatic hydrolysis of diluted samples (0.625 mg of protein/mL) by trypsin and α-chymotrypsin at 37 °C and pH 8.0 was measured.

Table 2. Rate Constants Estimated by Fractional Conversion Model for Effect of Pressure Pretreatment on Susceptibility of Egg White Proteins to Enzymatic Hydrolysis

P (MPa)	*k* (min^{-1})			
	10 °C	25 °C	40 °C	60 °C
550	0.1074 ± 0.0059	0.0168 ± 0.0029	nd	nd
575	0.1441 ± 0.0048	0.0693 ± 0.0069	0.0362 ± 0.0062	0.0177 ± 0.0087
600	0.1751 ± 0.0117	0.1541 ± 0.0051	nd	nd

[a] 10% v/v egg white, pH 7.6, 0.15 M NaCl.

fractional conversion model, as was the case for heat pretreatment. Longer pretreatment time resulted in a higher susceptibility, until an equilibrium value was reached. This equilibrium value was reached faster at higher pressure. In **Figure 6**, the time-dependent effect of pressure pretreatment on the susceptibility of egg white solutions to enzymatic hydrolysis is shown at 575 MPa at four different temperatures. It is clear that increasing temperature during pressure treatment resulted in a slower increase of susceptibility to the equilibrium value.

In **Table 2**, an overview is given of the temperature and pressure dependent rate constants of the fractional conversion model for the changes in the susceptibility of egg white solutions to enzymatic hydrolysis due to pressure pretreatment. The pressure dependence of the rate constants of the fractional conversion model at 10 ($r^2 = 0.9865$) and 25 °C ($r^2 = 0.9747$) could be accurately described by the Eyring equation. The resulting activation volumes were -22.2 ± 2.7 and -90.2 ± 14.5 cm^3/mol for 10 and 25 °C, respectively. Following Le Chatelier's principle, pressure favors reactions resulting in a volume decrease. The negative activation volumes observed for the changes in susceptibility of egg white solutions to enzymatic hydrolysis induced by pressure pretreatment confirm the positive effect of pressure on this property. The difference in activation volumes for the two temperatures under study would imply that, although the rate constants were higher at the lower temperature, these were less pressure sensitive as compared to the rate constants at 25 °C.

At elevated pressure, lowering the treatment temperature resulted in an increase of the rate constant for the changes in the susceptibility of egg white solutions to enzymatic hydrolysis. This is in contrast to heat treatment at atmospheric pressure, where a decrease of temperature resulted in a slower change in the property studied. As a result, a negative apparent activation energy (-33.8 ± 0.4 kJ/mol) was observed for the temperature dependence of the rate constants at 575 MPa, which could accurately be described by the Arrhenius model ($r^2 = 0.9994$). Suzuki (*22*) also observed a negative apparent activation energy for the pressure-induced insolubilization of ovalbumin solutions. Below 40 °C, an activation energy of −104.6 kJ/mol at 490 MPa was observed. The higher pressure and the different property under study might explain the higher activation energy in our work.

Because of instrumental limitations, temperatures higher than 60 °C could not be studied; therefore, no information is available on the effect of pressure at these higher temperatures. It can be expected that once the temperature of highest pressure stability is reached, pressure-induced denaturation will occur at a faster rate with increased temperature, as can be expected from the general elliptic phase diagram for protein denaturation. Further experiments are required to predict the effect of temperature on pressure-induced changes in the susceptibility of egg white solutions to enzymatic hydrolysis at higher temperature levels. However, at higher temperatures, gelation might start to occur; therefore, it would be of interest to study the effect of high temperature during pressure pretreatment on the susceptibility of egg white solutions to enzymatic hydrolysis under conditions were little insolubilization can be expected (*14*).

In conclusion, both heat and pressure pretreatment induced an increase in the susceptibility of egg white proteins to subsequent enzymatic hydrolysis by trypsin and α-chymotrypsin at 37 °C. The time-dependent changes in this property induced by pretreatment could be described by a first-order fractional conversion model. At atmospheric pressure, the increase in susceptibility to enzymatic hydrolysis was faster with increasing temperature, while at elevated pressure, the opposite was observed. This indicates the antagonistic effect between temperature and pressure in the temperature−pressure domain studied. In this region, residual protein solubility of pressure pretreatment was higher at prolonged treatment time as compared to heat pretreatment at atmospheric pressure. Furthermore, a higher DH10 could be obtained after pretreatment at elevated pressure, when considering a sample with the same protein solubility. From a processing point of view, during pressure pretreatment, it is interesting to work at lower temperatures, as lower pressures are needed, and the changes in the susceptibility of egg white proteins to subsequent enzymatic hydrolysis due to pretreatment are faster.

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