JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2003,** *51,* 3819−3823 **3819**

Heat-Induced Changes in the Susceptibility of Egg White Proteins to Enzymatic Hydrolysis: a Kinetic Study

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A kinetic study was conducted on the effect of heating in the temperature range of 50−92 °C, on the susceptibility of ovalbumin and albumen solutions to enzymatic hydrolysis by a mixture of trypsin and α-chymotrypsin at 37 °C and pH 8.0. Heat treatment resulted in an increase in degree of hydrolysis after 10 min of enzymatic reaction of both ovalbumin and albumen, as measured using the pH-stat method. The time-dependent change in the susceptibility to enzymatic hydrolysis after heat treatment was described by a fractional conversion model (based on an apparent first-order reaction kinetic model). Different end levels of degree of hydrolysis were obtained after heating for a long time at different temperatures, which suggests that the final degree of unfolding of the protein is temperature dependent.

KEYWORDS: Kinetic; ovalbumin; egg white; pH-stat; hydrolysis; heat denaturation

INTRODUCTION

Digestibility is an important factor in the determination of the nutritive quality of a protein, because it affects the bioavailability of its amino acids. Several biological and chemical assays exist to evaluate the in vivo digestibility of proteins. However, these methods are often too expensive and time-consuming for application in the food industry (*1*). Therefore, faster and less costly in vitro methods have been developed. Hsu et al. (*2*) described a multi-enzyme method, in which the drop of pH after 10 min of hydrolysis was correlated with the in vivo apparent protein digestibility in rats. This method was modified by Pedersen and Eggum (*3*), who estimated the protein digestibility based on the amount of base used during enzymatic protein hydrolysis to maintain constant pH, thus ensuring constant enzymatic activity.

In the food industry, enzymatic hydrolysis is often applied to obtain food proteins with better functional properties without loss of their nutritive quality. However, the reaction has to be monitored continuously, because extended hydrolysis of proteins leads to bitter peptides. Adler-Nissen (*4*) proposed a pH-stat method to calculate the degree of hydrolysis (DH) in which pH is kept constant by adding base during hydrolysis.

Technological processes, such as heating, can affect the digestibility of proteins. Guo and co-workers (*5*) reported a decrease in the nutritional properties of sodium caseinate due to thermal processsing. Enzymatic hydrolysis by trypsin of native β-lactoglobulin results in small peptides, not detectable by electrophoretic analysis, whereas the heat-treated protein is cut into larger peptides (*6*).

However, ovalbumin, the major protein in chicken egg white, in its native form, is resistant to digestion by trypsin, whereas heat denatured ovalbumin shows an increased susceptibility to this protease (*7*). 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 white is required to ensure microbial safety (*8*). This will not only affect the functional properties of egg white proteins but also an alteration of the digestibility can be expected. Hitherto, most of previous studies (*7*, *9*) have been focused on a qualitative description of the effect of thermal processing on the nutritional properties of egg white proteins. However, for industrial application, a quantitative approach is required to develop and control thermal processes in order to obtain microbially safe egg white with optimal nutritional quality.

Thus, the purpose of this research was to study the effect of thermal processing on the in vitro digestibility of egg white proteins by trypsin and α-chymotrypsin on a kinetic basis. Susceptibility to enzymatic hydrolysis was measured using a combination of the methods proposed by Pedersen and Eggum (*3*) and Adler-Nissen (*10*).

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 −25 °C, without any conversion to S-ovalbumin, as demonstrated by differential scanning

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10.1021/jf026019y CCC: $25.00
Published on Web 05/20/2003

calorimetry measurement (data not shown). Ovalbumin (grade V, lot 31K7025), α-chymotrypsin, and trypsin (from porcine pancreas, type IX-S) were obtained from Sigma (St.-Louis, MO). Other chemicals were of analytical grade.

Biochemical Analysis. Protein content was determined using Sigma Procedure No. TRPO-562. Activities of α-chymotrypsin and trypsin were verified by applying the BTEE (N-α-benzoyl-L-tyrosine-ethylester) -method and BAEE (N-α-benzoyl-L-arginine-ethylester) -method, respectively, according to Sigma.

Enzymatic Hydrolysis of Egg White Proteins. In this study, enzymatic hydrolysis of egg white proteins was determined by a pH-stat technique to follow continuously the DH during enzymatic reactions with α-chymotrypsin and trypsin under alkaline conditions, as described by Adler-Nissen (*10*). The pH was kept constant during enzymatic protein hydrolysis by adding base (NaOH), and the base consumption was proportional to the amount of peptide bonds cleaved. The DH was calculated based on **Equation 1**

$$\mathrm{DH}\ (\%) = \frac{h}{h_{\mathrm{tot}}} 100 \tag{1}$$

where h_{tot} is the total amount of peptide bonds in the protein (calculated from the amino acid composition) and h is the amount of peptide bonds cleaved during the reaction (both expressed in meq/g). The latter is proportional to the amount of base consumed and can be calculated using **Equation 2**

$$h = \frac{1}{\alpha} \frac{N_b B}{\mathrm{MP}} \tag{2}$$

where 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. The average degree of dissociation (α) of the free α-amino groups is pH dependent and can be estimated by **Equation 3**

$$\alpha = \frac{10^{\mathrm{pH}-\mathrm{p}K}}{1 + 10^{\mathrm{pH}-\mathrm{p}K}} \tag{3}$$

in which pK is the average pK of the α-amino groups liberated during the hydrolysis.

Aqueous solutions (10 mL) of ovalbumin (6.25 mg protein/ml) were hydrolyzed at 37 °C by adding 1 mL of an enzyme solution, with a concentration in α-chymotrypsin of 186 BTEE-U/ml and in trypsin of 23100 BAEE-U/ml as described in a study by Pedersen and Eggum (*3*), in which a correlation between in vivo and in vitro protein digestibility using a three-enzyme system (trypsin, α-chymotrypsin, peptidase) was found. However, because chain length has a considerable effect on the average pK, the use of peptidase (an exopeptidase) was discarded to reduce the variation in pK (and thereby in α) during the hydrolysis experiment (*10*). Because of precipitation of egg white protein during heat treatment at 6.25 mg protein/ml, the concentrations of solutions used were 10 times lower for the measurement of susceptibility to enzymatic hydrolysis of albumen (i.e., 0.625 mg protein/ml, 18.6 BTEE-U/ml α-chymotrypsin, 2310 BAEE-U/ml trypsin), without any effect on the DH measured (data not shown).

pH was kept constant by adding 0.1 N NaOH (0.01 N for hydrolysis of egg white solutions), using a pH-stat (Metrohm, Basel, Switzerland) with automatic dosage of the base. For the calibration of the pH-stat method, pH was 7.5, 8.0, or 8.5, while the pH was kept at 8.0 during the actual enzymatic protein hydrolysis experiments.

Pedersen and Eggum (*3*) observed a correlation between the base consumption after 10 min of hydrolysis and in vivo digestibility. It should be noted that this correlation was rather low for animal proteins compared to the digestibility of plant proteins. Therefore, in the present study, the DH after 10 min of hydrolysis (DH10) was used as a parameter to measure the effect of heat treatment on the susceptibility of egg white proteins to enzymatic hydrolysis,without, however, making any correlation with in vivo digestibility.

For pH-stat calibration, the proportionality constant (α^{-1}) between peptide bonds cleaved and base consumed was calculated by using the average pK as stated in **Equation 3**. The average pK was obtained by determining the increase in concentration of free α-amino groups (OPA-method) of samples taken during the pH-stat-controlled hydrolysis after various predefined hydrolysis time periods at different pH values (7.5, 8.0 and 8.5). The above-mentioned OPA-method is based on the reaction of *o*-phthaldialdehyde (OPA) and *β*-mercaptoethanol with primary amines to produce 1-alkylthio-2-alkylisoindoles with a maximum absorbance at 340 nm (*11*). The OPA-reagent was prepared daily as described by Church et al. (*12*), by mixing 50 mL of 100 mM sodium tetraborate, 5 mL of 20% SDS (w/v), 80 mg of OPA dissolved in 2 mL methanol, and 200 μL of *β*-mercaptoethanol, then diluting this with water to a final volume of 100 mL.

To measure the amount of amino groups liberated through enzymatic hydrolysis, samples of 150 μL were immediately added to 3 mL of the OPA-reagent. The solution was briefly mixed by inversion, and after 2.5 min of incubation at room temperature, the absorption was measured at 340 nm. A standard curve was obtained by replacing the samples with increasing concentrations of L-leucin (0−1.5 mM) and used to calculate the L-leucin equivalents (h_{Leu}). After correlating hydrolysis equivalents ($h_{\mathrm{pH-stat}}$) and h_{Leu}, straight lines of slope b were obtained. The pK values were calculated using **Equation 4,** in which $\mathrm{pH}_2 > \mathrm{pH}_1$, and b_1 and b_2 are the slopes of the straight line at the respective pH value (*10*).

$$\mathrm{p}K = \mathrm{pH}_2 + \log(b_1 - b_2) - \log(b_2 10^{\mathrm{pH}_2 - \mathrm{pH}_1} - b_1) \tag{4}$$

Heat Treatment of Egg White Proteins. Prior to the enzymatic hydrolysis, tubes (1 cm diameter) containing 11 mL of protein solution (pH 8.0) were heated in a water bath at various constant temperatures in the range of 50−92 °C and for certain preset times (ranging from 0 to 33 min). Sample temperature was reached within 0.1 °C of water bath temperature within 2 min under these conditions. After withdrawal from the heating bath, the tubes were immediately placed in ice water to stop thermal protein denaturation.

Statistical Analysis. Results of the pH-stat calibration were analyzed by linear regression analysis. The fractional conversion model (a modified first-order kinetic model) was used to express the effect of heat treatment on susceptibility of egg white proteins to enzymatic hydrolysis. In this model, the change of DH10 as a function of treatment time is described by **Equation 5**

$$\mathrm{DH10}_t = \mathrm{DH10}_\infty - (\mathrm{DH10}_\infty - \mathrm{DH10}_0) \exp(-kt) \tag{5}$$

where $\mathrm{DH10}_\infty$ is the equilibrium value for DH10 at infinite treatment time and $\mathrm{DH10}_0$ is the DH10 of the untreated sample. All parameters were estimated by nonlinear regression analysis. The temperature dependence of the rate constant, k (min^{-1}), is described by the Arrhenius equation

$$k = A \exp\left(-\frac{E_{\mathrm{a}}}{RT}\right) \tag{6}$$

in which A is the preexponential factor (min^{-1}), R is the universal gas constant (8.31415 J/mol · K), and E_{a} is the activation energy (J / mol). A and E_{a} were obtained by linear regression analysis of the natural logarithm of the rate constant versus the reciprocal absolute temperature (*13*).

RESULTS AND DISCUSSION

pH-Stat Calibration. A typical enzymatic protein hydrolysis curve is shown in **Figure 1**. The decrease in rate of curvature can be explained by product inhibition by oligopeptides formed at higher levels of DH (*10*, *14*).

A linear relationship between base consumption and concentration of free amino groups for each pH-value was found, both for ovalbumin and egg white solutions. On the basis of the results of the pH-stat calibration (**Table 1**, **Figures 2** and **3**), the average pK for amino groups at 37 °C was calculated. For ovalbumin solutions, a value of 7.36 was obtained, while the amino groups of egg white showed a mean pK of 7.48. These

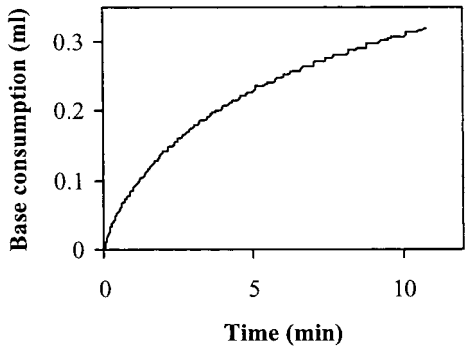

Figure 1. Hydrolysis curve of ovalbumin solution (heated for 23 min at 84 °C) during hydrolysis by trypsin and α-chymotrypsin at 37 °C and pH 8.0.

Table 1. Equation of Calibration Curves and Calculations of pK Values for Amino Groups in Egg White Proteins Hydrolysates at 37 °C

				slope		
substrate	pH	equation	corr coef r^2	b_1	b_2	p*K*
ovalbumin	7.5	$h_{Leu} = 1.1002 \cdot h_{pH-stat} + 0.6472$	0.9806	1.1002	0.6790	7.37
	8.0	$h_{Leu} = 0.6790 \cdot h_{pH-stat} + 0.5638$	0.9918	0.6790	0.7682	7.40
	8.5	$h_{Leu} = 0.7682 \cdot h_{pH-stat} + 0.5427$	0.9869	1.1002	0.7682	7.31
					mean	**7.36**
egg white	7.5	$h_{Leu} = 0.8034 \cdot h_{pH-stat} + 1{,}0093$	0.9512	0.8034	0.5369	7.47
	8.0	$h_{Leu} = 0.5369 \cdot h_{pH-stat} + 0{,}9943$	0.9803	0.5369	0.4514	7.48
	8.5	$h_{Leu} = 0.4514 \cdot h_{pH-stat} + 1{,}0138$	0.9826	0.8034	0.4514	7.48
					mean	**7.48**

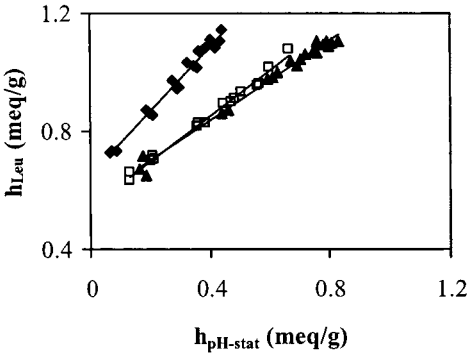

Figure 2. Calibration of pH-stat for trypsin and α-chymotrypsin hydrolysates of ovalbumin at 37 °C and pH 7.5 (◆), 8.0 (□), and 8.5 (▲).

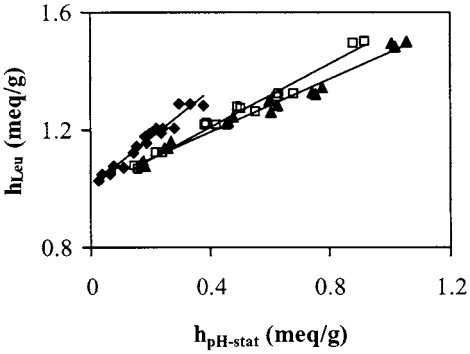

Figure 3. Calibration of pH-stat for trypsin and α-chymotrypsin hydrolysates of albumen at 37 °C and pH 7.5 (◆), 8.0 (□), and 8.5 (▲).

values are comparable to those of milk proteins (7.15) found by Dzwolak and Ziajka (*15*), who used trinitrobenzenesulfonic acid (TNBS) instead of OPA to measure the amount of liberated amino groups.

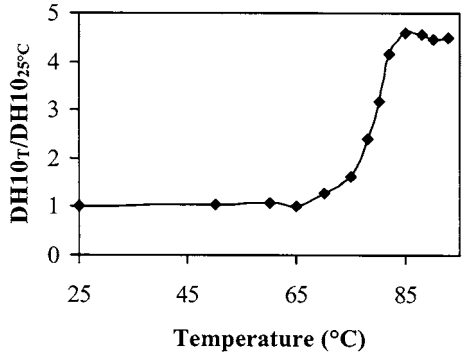

Figure 4. Thermal denaturation curve of ovalbumin solution after 15 min of heat treatment at different temperatures, obtained by monitoring the DH after 10 min of enzymatic hydrolysis by trypsin and α-chymotrypsin at 37 °C and pH 8.0.

The p*K* value of amino groups is dependent on temperature. Adler-Nissen (*10*) reported a decrease of the p*K* value of 0.23 pH-units when increasing the temperature by 10 °C. This change in p*K* is due to the large enthalpy of ionization of the amino group. However, because the pH-stat experiment was always conducted at 37 °C, the effect of temperature on p*K* was not taken into account.

The influence of the chain length of polypeptides on the p*K* value is considerable. While the p*K* is 7.5−7.8 on average for polypeptides at 25 °C, it is half a pH unit higher for di- and tripeptides (*10*). Thus, at low DH, when polypeptides are predominantly present, the p*K* value would differ from that at high DH, as oligopeptides are formed due to hydrolysis. However, when endo-peptidases such as trypsin and α-chymotrypsin are used and hydrolysis is stopped at moderate values of DH (i.e., below 20%), the amount of di- and tripeptides is relatively low. Therefore, the method of Pedersen and Eggum (*3*) for the estimation of in vivo digestibility was applied without peptidase (an exo-peptidase).

By use of **Equation 3**, the degree of dissociation (α), and subsequently the proportionality constant (α^{-1}), at pH 8.0 between peptide bonds cleaved and base consumed were calculated. Values for the latter were 1.236 and 1.300 for ovalbumin and egg white, respectively. Thus, for the same amount of peptide bonds cleaved, a larger amount of base has to be added to keep pH constant during enzymatic hydrolysis of ovalbumin compared to egg white solutions.

Influence of Heat Treatment on the Susceptibility of Ovalbumin and Albumen to Enzymatic Hydrolysis. When ovalbumin was heat-treated during 15 min, a distinct increase in susceptibility to enzymatic hydrolysis was observed at temperatures above 75 °C. At 85 °C, a 4.5-fold increase in DH10 could be observed (**Figure 4**). For albumen solutions, however, after treatment for 15 min at 75 °C, the susceptibility to enzymatic hydrolysis was 4.8 times higher than the initial value of DH10 (data not shown). Heat denaturation of ovalbumin and albumen solutions was accompanied by an increase of susceptibility to enzymatic hydrolysis, and therefore, the latter could be used as a measure for the former.

The susceptibility of egg white proteins to enzymatic hydrolysis is of practical interest, not only from a nutritional point of view but also as a measure for technological features. Egg white proteins are extensively used as emulsifiers and foaming agents because of their excellent surface properties. One of the most important structural factors of functional properties seems to be surface hydrophobicity. This is not surprising, because the adsorption at the interface between oil or air and water

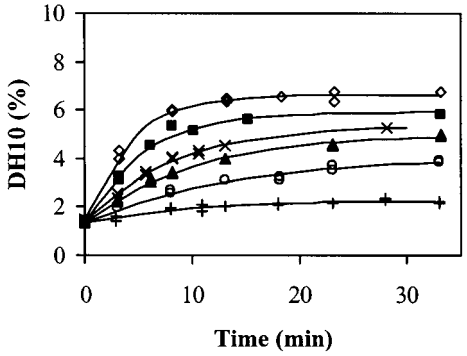

Figure 5. Thermal denaturation curve of ovalbumin solution heat treated at 75 °C (+), 78 °C (○), 79.5 °C (▲), 80.5 °C (×), 82.5 °C (■), and 84 °C (◇), obtained by monitoring the DH after 10 min of enzymatic hydrolysis by trypsin and α-chymotrypsin at 37 °C and pH 8.0.

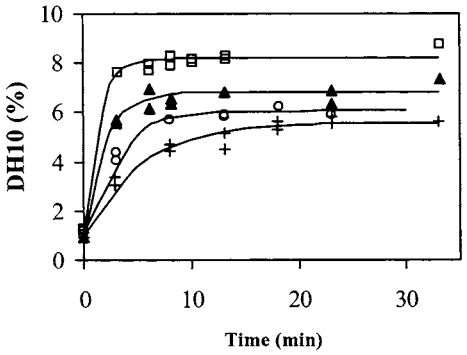

Figure 6. Thermal denaturation curve of albumen solution heat treated at 75 °C (+), 78 °C (○), 79.5 °C (▲), and 82 °C (□), obtained by monitoring the DH after 10 min of enzymatic hydrolysis by trypsin and α-chymotrypsin at 37 °C and pH 8.0.

requires both hydrophilic and hydrophobic sites. Proteins undergo surface denaturation at the oil−water and air−water interface. Thereby, the surface hydrophobicity increases, resulting in good emulsifying and foaming characteristics (*16*, *17*).

Kinetics of Thermal Denaturation of Ovalbumin and Albumen as Monitored by DH10. The time-dependent denaturation, as measured by monitoring the change in susceptibility of ovalbumin to enzymatic hydrolysis by trypsin and α-chymotrypsin, is shown in **Figure 5** for various heat treatments. The increase in DH10 due to heat treatment followed a first-order fractional conversion kinetic model. At temperatures below 78 °C, the influence of temperature on the susceptibility to enzymatic hydrolysis of ovalbumin in solution was negligible. The highest DH10 attained was 6.65%, which was much lower than 20%, so p*K* could be assumed constant during hydrolysis (*10*).

The same kinetic model as in the case of ovalbumin could be applied to describe the change in susceptibility to enzymatic hydrolysis after heat treatment of albumen proteins, as shown in **Figure 6**. It could be observed that heat treatment exerts a larger influence on the enzymatic hydrolysis of the egg white as a whole than on that of the purified ovalbumin, because higher levels of DH10 are reached at lower temperatures. This is to be expected, because ovalbumin composes only 54% of the albumen protein. Many other egg white constituents are more sensitive to heat treatment than ovalbumin, which explains the sharp rise in DH10 after heat treatment at lower temperatures (*19*). In addition, the ovalbumin in egg white was in the N-form, while the commercial ovalbumin was in the S-form, as demonstrated by DSC (data not shown). S-ovalbumin denatures

Table 2. Rate Constants Estimated by Fractional Conversion Model for Thermal Denaturation of Egg White Proteins as Measured by DH10

	ovalbumin	albumen
T (°C)	k (min^{-1})	k (min^{-1})
75.0		0.1775
78.0	0.0616	0.2391
79.5	0.1033	0.4400
80.5	0.1591	
82.0		0.7336
82.5	0.2655	
84.0	0.2175	
E_a (kJ/mol)	298 ± 19	210 ± 14

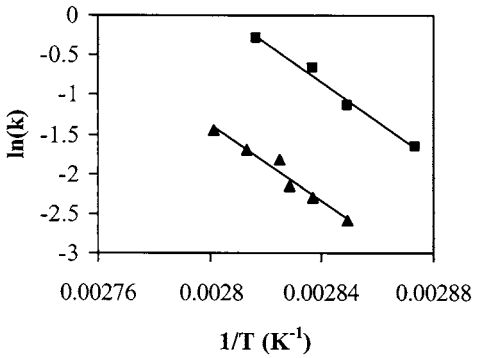

Figure 7. Temperature dependence of rate constants of the fractional conversion model for the susceptibility to enzymatic hydrolysis of ovalbumin (▲) and albumen (■), based on Arrhenius model.

at higher temperatures (92.5 °C) compared to N-ovalbumin (84 °C), but the denaturation enthalpy is the same (*20*). The DH10 of untreated egg white proteins was slightly lower than that of ovalbumin in solution (1.1% compared to 1.4%). This can be explained by the presence of ovomucoid and ovoinhibitor, which are inhibitors of trypsin, in the albumen. However, the effect of these inhibitors on the enzymatic hydrolysis of treated egg white solutions is expected to be rather negligible, because their heat stability is not so high at the pH used (*21*).

The rate constants at different temperatures of the fractional conversion model for heat denaturation of egg white proteins as measured by DH10 are given in **Table 2**. It can be clearly seen that the rate constants for the increase in susceptibility of albumen proteins to enzymatic hydrolysis by heat treatment are higher than those for ovalbumin solutions. This is due to the lower thermal stability of some of the other egg white proteins compared to ovalbumin. As can be seen in **Figure 7**, the temperature dependence of these rate constants could be described by the Arrhenius model, resulting in activation energies of 210 ± 14 kJ/mol ($r^2 = 0.967$) and 298 ± 19 kJ/mol ($r^2 = 0.988$) for albumen and ovalbumin, respectively. The lower activation energy for enzymatic hydrolysis of albumen indicates a lower temperature sensitivity of susceptibility to enzymatic hydrolysis in the temperature range studied, compared to ovalbumin solutions.

An interesting feature of the enzymatic hydrolysis of albumen and ovalbumin is the temperature dependence of the equilibrium value of DH10 ($DH10_\infty$), as shown in **Figure 8**. A linear increase in the final level of enzymatic hydrolysis with temperature was observed. This indicates that the final degree of unfolding of protein, measured by susceptibility to proteolysis, is not the same at all temperatures. At lower temperatures (for instance below 78 °C for ovalbumin), a deflection of linearity

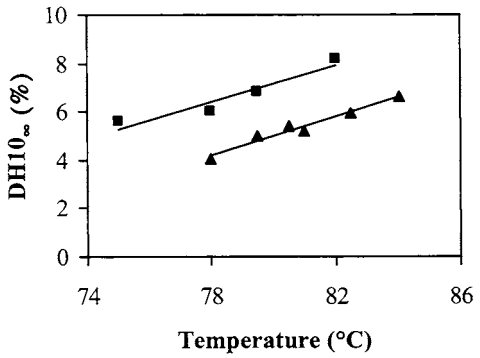

Figure 8. Temperature dependence of the equilibrium value of $DH10_\infty$ for the enzymatic hydrolysis of heat treated ovalbumin (▲) and albumen (■), by trypsin and α-chymotrypsin at 37 °C and pH 8.0.

can be expected toward a constant value, because the degree of hydrolysis of untreated protein ($DH10_0$) is 1.4 ± 0.2% for ovalbumin and 1.1 ± 0.1% for egg white solutions. Different final levels of foaming and emulsion activity can be expected after heat treatment at different temperatures, because a correlation between susceptibility to proteolysis and the functional properties was found, as reported by Kato et al. (*18*).

In conclusion, the heat-induced change in susceptibility of egg white proteins to enzymatic hydrolysis could be described by a first-order fractional conversion model. Different final levels of DH were observed after heat treatment at different temperatures, so different levels of foaming and emulsion activity can be expected. However, care should be taken to translate these results into predictions of in vivo digestibility of egg white proteins after heat treatment.

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Received for review October 8, 2002. Revised manuscript received April 10, 2003. Accepted April 24, 2003. This research has been supported by KU Leuven and the Fund for Scientific Research Flanders (FWO).

JF026019Y