MOLECULAR STUDIES OF PRESSURE & TEMPERATURE INDUCED STRUCTURAL CHANGES IN PROTEIN

THE MOLTEN GLOBULE AND EFFECT ON FUNCTIONALITY

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ABSTRACT

β-lactoglobulin in Tris-HCl buffer pH 7 50 mM was treated with combined pressure and temperature treatments using a central composite experimental design. Pressures studied were up to 294 MPa, temperatures up to 62°C and processing times up to 30 minutes. The molecular structure, at a secondary and tertiary level, was analysed post-processing using far and near-UV circular dichroism. It was found that, although the pressures applied were moderate, the far-UV circular dichroism spectra showed important changes corresponding to an increase in α-helical content. The tertiary structure was almost completely lost for the highest processing intensities. This suggests a transition to the molten globule state. The percentage change in ellipticities at 293 nm (tertiary structure) were found to be linearly connected to the three independent variables hence possibly allowing process optimisation for that response. Pressure was found to be the most important parameter to bring about the molecular changes at the two molecular levels investigated.

KEY WORDS

PROTEIN, HIGH PRESSURE, MOLTEN GLOBULE, STRUCTURE, FUNCTIONALITY

INTRODUCTION

High pressure processed foods are now commercially available with a wide range of products currently produced worldwide. For example, the Spanish company Espuña produces pressure treated ham, Ultifruit, in France produces fresh orange juice and high-pressure guacamole, manufactured by the company Avomex, has

had some success in the United States for a number of years. High pressure treatment appears to meet the increasing demands of consumers for foods that are natural, have superior organoleptic properties and longer shelf lives than those produced by thermal processing alone due to the detrimental effects heat has on flavour, texture and colour[1] Research into high pressure processing as an alternative food processing technique commenced more than 10 years ago in Japan under the influence of Hayashi and was initially used in food systems as a preservation technique. Over the last decade, however, considerable attention has been given to the impact of pressure on modifications of food and food constituents[2]. In foodstuffs, proteins are responsible for the functionality and their capabilities are unique to their structure. For example some proteins form foams whilst others have excellent emulsifying properties. Changes to protein structure during processing can alter their functionality and therefore the potential exists to apply an established technology to produce novel foodstuffs with minimal effect on flavour, colour and nutritional value[3]. β-lactoglobulin is an important functional protein as it is the major component of many diary emulsions. High pressure treatment has been reported to induce partial denaturation of β-lactoglobulin with the changes in structure resulting in protein aggregation and it was generally reported that pressure had a negative effect on the functional properties of the protein as the emulsifying capacity and foaming ability were reduced[4,5]. Various workers have investigated structural modifications and even on pressurisation at 900MPa, only minor changes were reported[6]. Combinations of pressure with low or moderate temperatures have also been investigated. Kolawski et al reported that keeping sub-zero temperatures under pressure treatment (300 MPa) protected β-lactoglobulin against unfolding and aggregation[7]. Tedford et al reported modifications in the structure of β-lactoglobulin on processing at lower pressures (100 MPa) in combination with moderate temperatures (up to 75°C)[8,9]. Galazka and Ledward reported a reduction in the functionality of β-lactoglobulin when subjected to high pressure due to the formation of aggregates[10].

Processing results in protein denaturation. The view in which it occurs through a reversible two-state process (native↔unfolded) is now thought to be an over simplification for most proteins. Several intermediate states have been shown to exist with their properties depending on the experimental conditions employed and they have been considered as being in the molten globule state[11]. The molten globule state designates a partially folded conformation that can be distinguished from the native and the fully denatured forms which has an increased enthalpy and entropy due to a higher level of disorder upon transition from the native structure to the new state[12]. The molten globule state is highly hydrated with greater side chain flexibility. A protein in the molten globule state usually retains a significant degree of secondary structure and possesses relatively few stable tertiary interactions. A molten globule has been reported for β-lactoglobulin processed using pressure with the denatured state having a higher surface hydrophobicity which could potentially result in improved functionality[13]. On formation of this reported molten globule for β-lactoglobulin, the % α-helix of the secondary structure increases due to the native β-sheet converting to non-native α-helix, the tertiary structure disappears and the surface hydrophobicity increases[13].

Currently, the commercial applications of high pressure processed foods have taken place at starting temperatures at or close to ambient (although temperature rise due to adiabatic expansion has been taken into

account). Reported set up costs are in the range of £500,000-£2,000,000 with the majority of the cost due to the required capital investment of the high pressure vessel and ancillary pipework The effects of smaller ranges of pressure in combination with temperature are therefore of particular interest[14]. Little work has been carried out on the interactive effect of these two parameters on food systems and this could potentially enable costs to be kept to a minimum whilst ensuring the desired product quality.

The main objective of this study was to investigate the effects that combined pressure and temperature processing had on the structure of β-lactoglobulin, using a 2^3 experimental design to assess the interactive effects, and to identify the formation of the molten globule state. The changes on protein structure were measured for pre and post processing using circular dichroism spectroscopy. The results were analysed for changes on structure and for identification of the molten globule state. The work reports on the existence of the 'molten globule' when processing using pressure in combination with temperature.

MATERIALS AND METHODS

Protein sample

The protein used in this study was β-lactoglobulin derived from bovine milk (chromatographically purified and lyophilized, approx. 90%) and was purchased from Sigma Chemical Co. (St. Louis, MO). The protein was prepared by dissolving in 50mM Tris-HCl buffer at pH7 to provide protein concentrations of approximately 0.5mg/ml and 2.0mg/ml for far and near-UV spectroscopy respectively. The concentrations were measured spectrophotometrically before and after processing using an absorbance coefficient of 0.96 l g^{-1} cm^{-1} [13]

Pressure-temperature treatments

The prepared samples were placed in 2ml sterile plastic microcapsules and inserted into a water filled high-pressure vessel. The temperature was regulated by immersion of the vessel in a constant temperature water bath. The vessel was pre-heated to eliminate thermal lag. The temperature increase induced by the adiabatic compression was evaluated at different starting temperatures for various pressures and taken into account. The vessel and its contents were then pressurised and held under pressure for the required length of time before the pressure was released instantaneously.

Experimental design

The samples were processed using a 2^3 central composite experimental design. The levels of the different independent variables for each of the conditions are given in table 1. The axial distance from the centre of the design for the star points was chosen as 1.215[15]. The design used allows carrying out a minimum number of experiments to assess the effects of the three independent variables. A total of 15 experiments were carried out and these were done in duplicate. After processing, the samples were stored at -4°C prior to analysis. Preliminary analysis showed that a single freezing and thawing cycle did not affect the native structure of the protein.

Circular dichroism analysis

Circular dichroism spectroscopy was used to assess the overall characteristics of protein secondary and tertiary structure for β-lactoglobulin using a Jasco J600 Spectropolarimeter (Jasco UK Ltd., Essex, UK.). The secondary structure was determined using the far-UV region (190-260nm) and the tertiary structure was determined using the near-UV region (260-320nm). The data were analysed following correction for cell pathlength, protein concentration and mean residue weight[16]. Spectra were recorded at 20°C using quartz cells with pathlenghts of 0.02cm and 0.5cm with data collection at 0.2nm intervals using a scan rate of 10 $nm.min^{-1}$ for the far and near-UV region respectively. Spectra were carried out in duplicate for both the far and near-UV analysis

Data analysis

For the near-UV circular dichroism results, it was possible to fit a polynomial equation to analyse the parameters effects. In order to do this, tertiary structures measurements were obtained from the % change in molar ellipticity at 293nm when compared with the value for the native protein. The mean values for the % change in ellipticity at 293nm were fitted to the following second-order polynomial

$$y = b_0 + \sum b_i x_i + \sum b_{ii} x_{ii}^{\ 2} + \sum b_{ij} x_i x_j \qquad (1)$$

where y is the estimated response (percentage change ellipticity at 293), x_i factor level for the independent factor and b_0, b_i, b_{ij} and b_{ij} are the equation parameter estimates corresponding respectively to constant, linear, quadratic and interactive terms[15]. The F-test was used to assess the significance of the equation parameters with a 95% minimum confidence level.

RESULTS

Near-UV Circular Dichroism

The near-UV circular dichroism spectra of the native protein and for the different processing conditions are shown on figures 1 and 2 (duplicate not shown). The processing conditions had a very important effect on the near-UV circular dichroism spectra. The spectra were compared with that of the native protein that shows the typical tryptophan and tyrosine peaks at 293 and 284 nm respectively. The double maximum of the native β-lactoglobulin diminished in intensity progressively as the intensity of the combined treatment increased. For treatments 6 (280 MPa, 40°C, 30 min.) and 9 (294 MPa, 50 °C 20 min.) they can only be guessed and these features nearly completely vanished for condition 8 which is the same as treatment 6 but at higher temperature (280 MPa, 60°C, 30 min.). This loss of near-UV circular dichroism spectrum displayed gives evidence that the combined process resulted in molecular structures with less and less rigid tertiary structure in the vicinity of the aromatic amino acids as the intensity of the treatment increased. Hence it can be thought that the well-defined tertiary structure was lost for the highest intensity treatments because of the destruction of the interactions responsible for maintaining the rigid native protein tertiary structure. This is a typical trait of the conversion into a molten globule state of globular proteins[13].

The tryptophan environment was used to investigate quantitatively the effect of the different parameters on the tertiary structure. The molar ellipticities at 293 nm and its percent change when compared to the native form are presented in table 2. Equation 2 gives the polynomial function that best fits the data.

$$y = 49.48 + 18.65x_1 + 6.72x_2 + 6.98x_3 - 5.56x_1^2 - 1.09x_2^2 - 0.24x_3^2 - 4.07x_1x_2 + x_1x_3 + 4.61x_2x_3 \quad (2)$$

The adjusted R^2 was calculated as 0.88 and table 2 shows the close agreement of the predicted and average observed responses. The significance was assessed by the F-test. Table 3 shows that the fitting was significant with 99% confidence as the F-value was higher than the table F-value with 9 and 20 degrees of freedom which is 3.46. In order to assess the significance of each coefficient independently, F-values were calculated using the individual ratio of the mean square on the residual mean square for each coefficient (table 4). Comparing these values to the F table values for 1 and 20 degrees of freedom, which are 4.35 and 8.1 with 95% and 99% confidence levels respectively, all the linear coefficients were found significant with 99% confidence. The quadratic and interaction terms were all found significant with 95% confidence level except from the pressure/time interactive term and the temperature and time quadratic terms that were not found to be significant. Hence, the best representation of the results is when Equation 2 is used ignoring the x_2^2, x_3^2 and x_{13} terms.

The three-dimensional graphical representation allowed a better visualisation of the different independent variable effects on the molar ellipticity. Figures 3, 4 and 5 show the response when one of the variables was kept constant at its mid point, i.e. pressure at 215 MPa, temperature at 50°C and with time kept at 20 minutes respectively. Figure 3 show that while temperature has not an important effect on the molar ellipticity at 293 nm with low processing time, it does with the higher times studied. The time of processing had already a significant effect at low temperature but this effect is even more important as the temperature increased. The three-dimensional graphs emphasised that pressure, when compared to time (figure 4) or temperature (figure 5), was the parameter that had the most significant effect on the near-UV circular dichroism spectra of β-lactoglobulin and hence on the tertiary structure of the globular protein. In figure 4 it can be seen that the importance of the processing time is conserved as the processing pressure is increased while in figure 5 the temperature effect present at low pressures vanished at the higher pressures studied. The importance of pressure can also be observed quantitatively using the coefficients on the equation describing the response surface for significant terms as the linear coefficient for pressure (b_1) has the largest magnitude and the most important coefficient.

Far-UV Circular Dichroism

The spectra for each of the processing conditions are shown on figures 6 and 7. The circular dichroism spectrum was typical for a protein composed of anti-parallel β-structure[17] and showed a minimum at 216 nm. As can be observed from figures 6 and 7, the changes on the far-UV circular dichroism of β-

lactoglobulin occur from the lowest intensity of treatments investigated and are gradual up to the highest intensity. These changes are drastic and the spectra are affected both in shape and amplitude. The amplitude of the spectra was greatly increased and the spectral minimum shifted from 216 nm for the native protein to 204.8 nm for the treatment that had the most effect on the far-UV spectra, i.e. number 8 (280 MPa, 60°C and 30 min.). This is actually due to a change of the shape of the spectra because of a switch in the relative importance of the small trough at 210 nm and the more important one positioned at 216 nm in the native structure spectrum. As the intensity of the process increases, the importance of the trough positioned at 210 nm in the native protein spectra increases and at some point it becomes more significant than the one at 216 nm resulting in spectra with modified shapes. Pressure seems to be an important parameter to induce these spectral changes as they are more apparent for conditions in which pressure is higher than 280 MPa (6, 7, 5, 9 and 8). This difformation is also occurring for treatments that were processed at 215 MPa (12, 13, 14 and 15) but is non-existant or very slight for combined treatments that include lower pressures. These spectral changes indicate that there has been an increase in the proportion of α-helix in the protein when it was processed. Proteins converting to the molten globule state usually show little change in secondary structure and hence conserved/native-like far-UV spectra. However, β-lactoglobulin is an exception and shows a strong change in secondary structure upon conversion to the molten globule state as evidenced previously for pressure at 50°C[13] and by the present results for a range of pressure/temperature combined processes.

DISCUSSION

The tertiary and secondary molecular structures of β-lactoglobulin were greatly modified when the protein was processed using combinations of pressure and temperature as shown by the near and far-UV circular dichroism spectra obtained post-processing. In the near-UV, the changes in the spectra show that the combined treatment had a very destructive effect on the tertiary structure. Indeed, the aromatic amino acid environment has nearly totally lost its rigid tertiary structure for the highest processing conditions. The combined treatment also had drastic effects on the far-UV circular dichroism spectra. However, rather than being destructive, this corresponded to the creation of α-helix. This agrees with the observed formation of a molten globule in which a segment of native structure shifts from one secondary structure (β-sheet) to another (α-helix)[13,17]. The secondary structure, at a given point in a protein, depends not only on the local sequence of the amino acids but also on the interactions between different parts of the molecule. Without these interactions, many parts of the β-lactoglobulin protein that are β–sheet in the native form would assume a preferred α-helix secondary structure. This is what is observed in refolding experiments where a state similar to the molten globule, the burst-phase intermediate, is detected very shortly (18 ms) after the refolding process has started[17]. This intermediate has a secondary structure that is composed of non-native α-helices that are transformed into native β-strands at a later stage of the refolding process. In the molten globule created by the application of the combined process, the interactions between distant parts of the protein may have been reduced in intensity and some β-strands reverted to a preferred α-helical structure. Hence, the combined

process induced a reversion of the structure to non-native α–helices that could be the same as those present in the early phase of the β-lactoglobulin refolding. This may be a result of weakening of interactions between distant regions of the molecule as pressure disrupts non-covalent bonds[18] and induces water infiltration in the protein structure[19].

The results at the two molecular structural levels both indicate that the combined process resulted in a characteristic molten globule state. This state is an intermediate in the unfolding pathway in which the globular protein is trapped. This state has a defined secondary structure (in the present case different from the native one) with little tertiary structure remaining. Circular dichroism spectroscopy allowed the identification of structures differing from the native protein and also differing when compared to each other.

The polynomial fitting of the near and circular dichroism spectra indicated that the effect of pressure was most significant. Although no quantitative analysis of the far-UV circular dichroism was possible in the same way, qualitative analysis of the far-UV spectra seem to indicate that pressure is as well the most important parameter for inducing the changes in the secondary structure. Temperature seemed to have a lesser effect than pressure (specialy at high pressure) but its importance must not be neglected. Indeed, when pressure alone is applied and the changes on the secondary[4] and tertiary structure[6] are monitored, little irreversible modifications are observed even at pressures as high as 900 MPa. The present results show important effects at much lower pressures than the ones used in these studies because of the combination with moderate temperature. Previous β-lactoglobulin circular dichroism studies with a combined process at higher temperatures (75°C) and lower pressures (105 MPa)[9], found that temperature was the main element affecting the molecular structure of the globular protein. In the present study pressure became the most important element. This demonstrates that it is possible to design a combined process using pressure with moderate temperatures that results in significant effects on the molecular structure of β-lactoglobulin.

The 15 processing conditions created molten globules with distinct molecular structures that corresponded to the level of processing. These different structures could have different functional properties and could be potentially very useful in the food industry. A single protein could therefore give rise to several structures with different properties[20]. The main element that could prevent the molten globules created from displaying increased functionality is aggregation. It has been demonstrated that pressure treatment resulted in the reduction of some functional properties of β-lactoglobulin such as emulsifying capacity and foamability[4]. This was though to be due to aggregation occurring during processing or upon depressurisation. This can occur mainly through covalent (disulphide bond creation) or non covalent bonds (e.g. hydrophobic interactions). Pressure [6, 21] as well as temperature[22] induce the unfolding of β-lactoglobulin making available the free thiol group for reaction and bringing hydrophobic groups to the protein surface resulting in increased aggregation. The advantage of using moderate pressures (<300 MPa) and temperatures (<62°C) at low protein concentration is that less aggregation may occur. The difficulty would be to create molten globules with higher functionality (e.g. with higher surface hydrophobicity) without the detrimental occurrence of aggregation. The fact that the parameters can be linked to the degree of unfolding linearly (at least with the near-UV circular dichroism results presented) could allow process optimisation to obtain the “best” molten

globule for a given application. Another element in favor of the use of moderate temperature is that above 65°C the secondary structure of β-lactoglobulin has lost all its α-helical elements[22]. These temperatures would not result in the creation of molten globules that have the definition used in this paper (i.e. with secondary structure and little tertiary structure) but probably to structures closer to the completely denatured state.

CONCLUSION

The combined pressure temperature processes, to which β-lactoglobulin was subjected, resulted in important effects on the molecular structure at the secondary and tertiary levels. The structural elements obtained had the characteristics molecular structure of molten globules. Different molten globules having distinct molecular characteristics were obtained through combined process at moderate pressures and temperatures. This illustrates the validity and potential of combine pressure/moderate temperature treatments. In addition, it was found that the creation of the molten globule structure could be linearly connected to the processing parameters. This could potentially allow for process optimisation to obtain the best molten globule structure for a given application.

Of course, in order to improve the functionality of the protein investigated through the combined process, significant work would be required to define a relationship between structure and a given functional property. The demonstration that the molten globules have different circular dichroism characteristics is a promising result as this could also be the case for elements more directly related to functionality such as surface hydrophobicity. Future work should concentrate on demonstrating the relationship between the molten globule and functionality. In addition, further study should characterise the amount of aggregation occurring during processing as even if no visual aggregation was detected for the processed samples in this study, soluble aggregates may have occurred.

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