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Effects of high hidrostatic pressure on the viscosity of β-lactoglobulin solution

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Summary

In this research new experimental data for the pressure dependence of the viscosity of β-lactoglobulin solution are presented. The experimental investigation is based on in-situ viscometric measurement technique which provides an observation of the high-pressure-induced changes of β-lactoglobulin solution during the treatment. This method refers to a rolling ball viscometer that is adapted for the use at high pressures and has a variable inclination angle. The estimation of the viscosity has been done in order to detect reversible and irreversible conformational changes of β-lactoglobulin. For investigation protein solutions concentration 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 g/g were used. The sample solutions are exposed to pressure of 0.1-600 MPa. The results showed that there is no significant effect of 100 MPa pressure on the viscosity of β-lactoglobulin solutions. With increasing pressure, between 100 and 300 MPa, the viscosity of β-lactoglobulin solutions increase. Pressure above 300 MPa causes further increase of the viscosity due to nonreversible and more extensive effects on protein, e.g. unfolding of monomeric proteins and aggregation. The structural changes of the β-lactoglobulin under high pressure affect the hydration of the β-lactoglobulin molecules. At pressure between 100 and 300 MPa there is a significant increase in relative hydration due to structural changes and increase in number of water molecules which are associated with protein molecules. Higher pressure cause protein aggregation due to formation of intermolecular disulfide bonds and irreversible denaturation and aggregation occurs. Because of that, there is no changes in protein hydration, moreover, the hydration of β-lactoglobulin molecules have a small decreases at pressure between 400 and 600 MPa.

Key words: β-lactoglobulin, high pressure, viscosity, in-situ technique, functional properties

Introduction

High-pressure processing as a relatively new technology in food industry has attracted considerable attention in food research last years. High-pressure treatment represents an interesting alternative to traditional heat processing, as the functional properties of macromolecules may be changed more selectively by hydrostatic pressure without the negative side effects of heat-induced changes of flavour and colour. Moreover, high pressure processing affect the stability of the structure of food constituents including proteins, vitamins, lipids, saccharides and pigments in such a way that can improve their intrinsic functional properties to meet needs in terms of colour, flavour and texture retention (Johnston et al., 1992; Kessler, 2002; Tedford et al., 1999; Trujillo et al., 2002).

Many researches have showed that the high-hydrostatic pressure is an effective method to produce microbial safe, minimally processed products with improved functional properties, and to develop

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new products of high sensory and nutritional quality (Ahmed and Ramaswamy, 2003; Hoover, 1993; Hosseini-nia et al., 2002). This is one of the most promising methods for the food treatment because of its potential to achieve new or improved functional properties of biopolymers through changes in their structure and improved control over enzyme reactions (Cheftel, 1992; Farr, 1990).

Whey proteins play an important role in controlling the texture of many food products and have grown in popularity as nutritional and functional food ingredients. They possess unique functional properties such as gelation, water binding, emulsification and foaming ability (Damodaran, 1994; Herceg et al., 2004; Jovanović et al., 2005). Different food applications, however, require different functional contributions and these co-called functional properties are assumed to relate the physical-chemical properties of proteins to desired food product characteristic. Modification of macromolecular structure and functional properties of whey proteins by high-pressure processing is now well recognized.

Among the whey proteins, β-lactoglobulin appears to be most sensitive to high pressure. β-lactoglobulin is the major whey protein in the milk of ruminants and many other mammals. Its concentration ranges from 2 to 4 g/L, which corresponds to 50 % of the total amount of whey proteins. This globular protein has been extensively studied. Many studies are focused on its biochemical and physicochemical properties and its primary, secondary and tertiary structures are well known (Botelho et al., 2000; Boye et al., 1996). It is a highly water-soluble protein, homodimer of 18 kDa and the dimer forms with a central hydrophobic cavity that is important for binding small molecules, such as retinol (native β-lactoglobulin binds retinol at a hydrophobic cavity at the subunit interface) (Perez and Calvo, 1995). As β-lactoglobulin tends to dominate the behavior of the whey protein system, controlling the effect of high-pressure treatment on this protein is of great interest in the food industry.

Pressure-induced denaturation of β-lactoglobulin in concentrated solutions can lead to extensive aggregation and gelation (Dickinson and James, 1998). For pressure studies of protein interactions it would be of significant interest to relate the measured thermodynamic parameters to specific structure features of the proteins. The high pressure treatments cause both reversible and irreversible conformational changes by disruption of hydrogen and ionic bonds and hydrofobic interactions changing the solubility and functional properties of the protein (Capellas et al., 2001; Huppertz et al., 2004; Johnston et al., 1992). However, high-pressure induced denaturation of β-lactoglobulin is a complex phenomenon that depends on the protein structure, applied pressure, temperature, pH, ionic strength and solvent composition (Dumay et al., 1994).

Protein-water and protein-protein interactions in aqueous systems are vitally important in the applications of proteins in food system. These interactions control the more operational properties of viscosity, solubility and dispersibility. The ability of proteins to bind or entrap water is also responsible for many of their desirable functional properties. The amount of water associated with protein depends upon the amino acid composition, protein conformation, the number of exposed polar groups, surface hydrophobicity, pH, etc. Globular proteins, such as β-lactoglobulin, display varying degrees of hydration because they are most sensitive to changes in environmental conditions (Herceg et al., 2002; Fox, 1989).

A very important physical-chemical property which provides basic and supplementary information on pressure induced structural changes is the viscosity. Moreover, thermodynamic and transport properties of fluid food ingredients, such as viscosity and density, are important process and design information to the food processing. These properties are, more broadly, central in the study of any process bringing into play fluid mechanics and transport phenomena (Audonet and Padua, 2004).

For a better understanding of the processes occurring under high pressure and the effects of high pressure processing on protein structure is a need to attend the dynamic viscosity of fluid food ingredients under pressure. Many studies have been performed on the variation of the dynamic viscosity with temperature and chemical composition of the food components, but most of the studies have been carried out at normal pressure. For monitoring the different stages of unfolding and aggregation of the proteins, in-situ techniques are suitable. Therefore, the aim of this work was to provide a new experimental data for the pressure dependence of the viscosity of β-lactoglobulin solution.

Materials and methods

Materials

For the experiments β-lactoglobulin A/B from bovine milk was used, approximately 90 % chromatographically purified and lyophilised, Firm Sigma-Aldrich, Germany. The samples were prepared by dissolving powdered β-lactoglobulin in distilled water. In order to prevent presence of bubbles in the solution, gas was released from distilled water by vacuum pump and magnetic stirring at temperature of 50 °C during 30 minutes. Such prepared distilled water and appropriate amount of β-lactoglobulin were mixed and magnetic stirred for 30 minutes at 40 °C. After magnetic stirring, β-lactoglobulin solutions were kept in closed glassware at 4 °C for 12 hours. Protein concentration of β-lactoglobulin solutions was 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 g/g.

Methods

The experimental investigation is based on in-situ viscometric measurement technique. This method refers to a rolling ball viscometer that is adapted for the use at high pressure and has a variable inclination angle (Picture 1).

The β-lactoglobulin solutions are exposed stepwise to pressure of 0.1-600 MPa. The pressure is generated by compressing the solution to be measured with a manual piston pump.

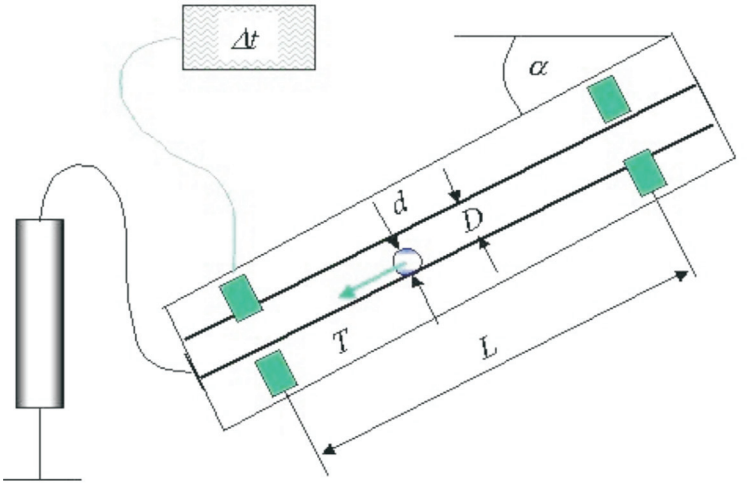
The device for measurement (rolling ball viscometer) consists of the tube (1.60 mm internal diameter), flexible capillary tube (GHT Hochdrucktechnik Hamburg, diameter 0.16 mm) and a manual piston pump. Manual piston pump produces high pressure with a maximum of 700 MPa. The temperature in tube is maintained at 20 °C by a water jacket connected to a thermostat and is connected to a pressure transducer (Wika GmbH, Klingenberg, Germany). The sphere is brought to its initial position by rotating the viscometer. The inclination angle for measurements was 30 °C. The rolling time Δt is measured by an inductive method: a small voltage peak is produced when the steel sphere passes one of two pickup-coils mounted on the outside of the high pressure tube. The rolling time of the ball at one pressure is measured until it achieves a constant value.

The viscosity of β-lactoglobulin solutions was determined according to the equation

$$\eta = K \cdot [\rho_b - \rho] \cdot \Delta t \quad \text{[Pas]} \qquad (1\text{-}1)$$

where η is the shear viscosity, K pressure and temperature dependent calibration factor, which has been determined experimentally, ρ and ρ_b the densities of liquid and the ball, and Δt the rolling time passing the distance .

The pressure dependence of the viscosity was determined relative to the viscosity at ambient pressure. Experimental measuring of the viscosity and density of β-lactoglobulin solution at ambient pres-



Picture 1. The rolling ball viscometer for in-situ viscosity measurement

sure was carried out on capillary-viscometer and oscilator (Firm Chempro, Hanau).

Density of β-lactoglobulin solution at high pressure has been determined experimentally. Experimental measuring was carried out with interferometrric Densitometry (Eder and Delgado, 2002).

Eiler's model (Foerst, 2001) was used to relate protein hydration $C_{(P,T)}$ to the relative viscosity:

$$\eta_{r(p,T)} = \left[1 - \frac{2{,}5 \cdot c_{(p,T)}}{2 \cdot \left[1 - \frac{c_{(p,T)}}{c_{\max}}\right]}\right]^2 \qquad (1\text{-}2)$$

For globular proteins such as β-lactoglobulin c_{max} (max. density of the molecules packing in solution) is 0.6 (Foerst, 2001).

Experimental data are the means of at least three independent high pressure runs and all values are presented as mean ±SD.

Calibration procedure

The calibration procedure is used in order to determine calibration parameter *K* which is introduced in eq. 1-1. At ambient pressure the calibration factor *K* is found by measuring the rolling time in fluids of known viscosity. For experiments solution with sugar concentration of 5, 10, 20, 25, 30 and 50 % were used. Calibration coefficient has been determined with inclination angles of 20, 30, 40, 50 and 60°. All measurements have been carried out at 20 °C and pressure of 0.1 MPa.

Figure 1 shows the calibration coefficient as a function of 1/dt. For each inclination angle, one equation which is used to determine the calibration coefficient of β-lactoglobulin solution is plotted.

For inclination angle 30°, the value for distilled water and β-lactoglobulin solution under pressure are also plotted. A very good agreement between these data and those obtained by calibration procedure can be observed.

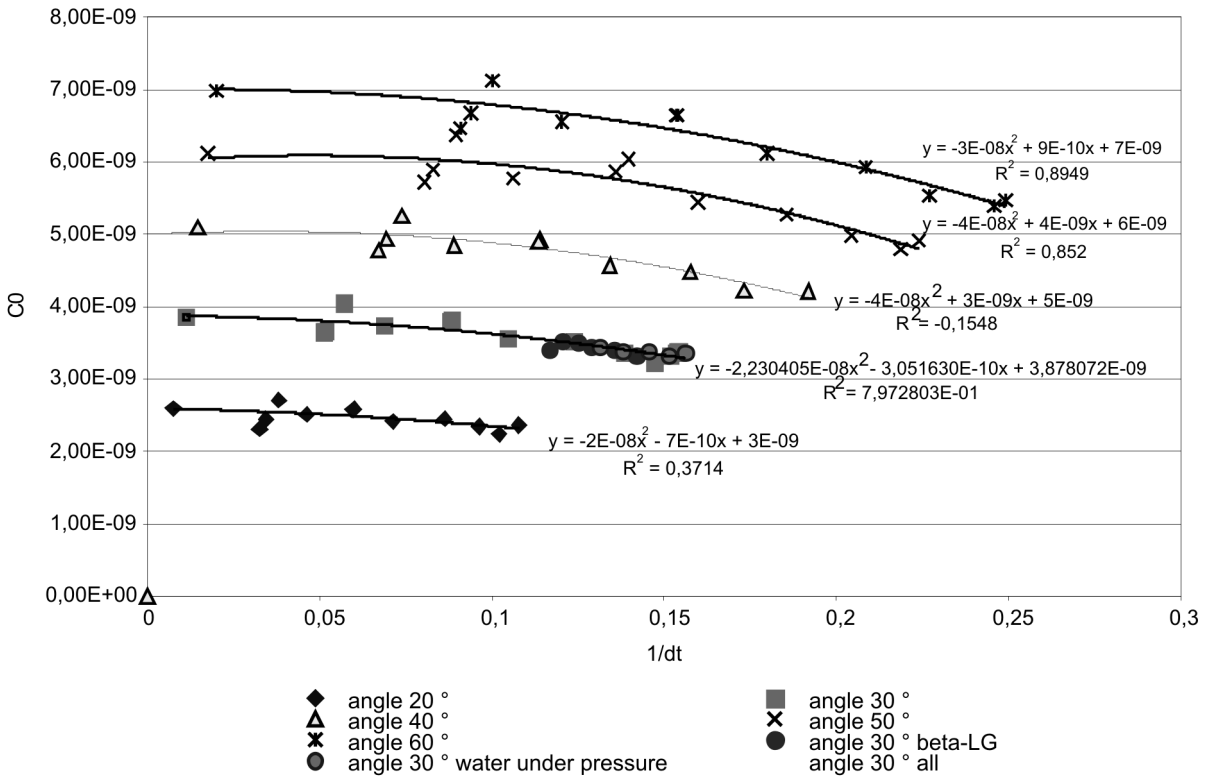


Figure 1. Calibration coefficient as a function of 1/dt

Table 1. Dynamic viscosity of the β-lactoglobulin solution under high pressure

Pressure (MPa)	Concentration of β-lactoglobulin solution (g/g)					
	0.01	0.02	0.03	0.04	0.05	0.06
	Dynamic viscosity (mPas)					
0.1	1.03±0.005*	1.07±0.006	1.14±0.005	1.22±0.003	1.27±0.003	1.33±0.003
50	1.04±0.003	1.07±0.004	1.14±0.006	1.22±0.004	1.28±0.004	1.34±0.003
100	1.05±0.003	1.08±0.008	1.15±0.011	1.25±0.005	1.31±0.006	1.37±0.003
140	1.06±0.006	1.11±0.007	1.19±0.005	1.32±0.008	1.41±0.019	1.51±0.022
180	1.09±0.004	1.15±0.007	1.27±0.006	1.44±0.011	1.67±0.024	1.82±0.050
220	1.11±0.003	1.19±0.006	1.32±0.005	1.53±0.009	1.77±0.020	2.07±0.023
260	1.15±0.003	1.23±0.006	1.38±0.007	1.63±0.013	1.85±0.021	2.33±0.047
300	1.17±0.005	1.25±0.007	1.44±0.007	1.71±0.012	2.04±0.023	2.59±0.039
400	1.25±0.003	1.36±0.007	1,55±0.008	1.86±0.010	2.30±0.012	3.05±0.064
500	1.33±0.005	1.46±0.005	1.7±0.011	2.02±0.011	2.54±0.021	3.42±0.046
600	1.42±0.005	1.57±0.007	1.87±0.013	2.22±0.012	2.80±0.016	3.81±0.018

All measurements have been carried out at 20 °C. Inclination angle is 30°

*All values are presented as mean ±SD

Results and discussion

The estimation of the dynamic viscosity has been done in order to detect reversible and irreversible conformational changes of β-lactoglobulin under high-pressure. The relative protein hydration is calculated from equation (1-2) for known values of the relative viscosity.

The effects of pressurization on dynamic viscosity of β-lactoglobulin solution are presented in Table 1. Since the high pressure affects also the dynamic viscosity of distilled water, the relative viscosity may be used for a better interpretation of pressure induced conformational changes of β-lactoglobulin solution. Experimental measuring of the dynamic viscosity of distilled water at high pressure is pre-

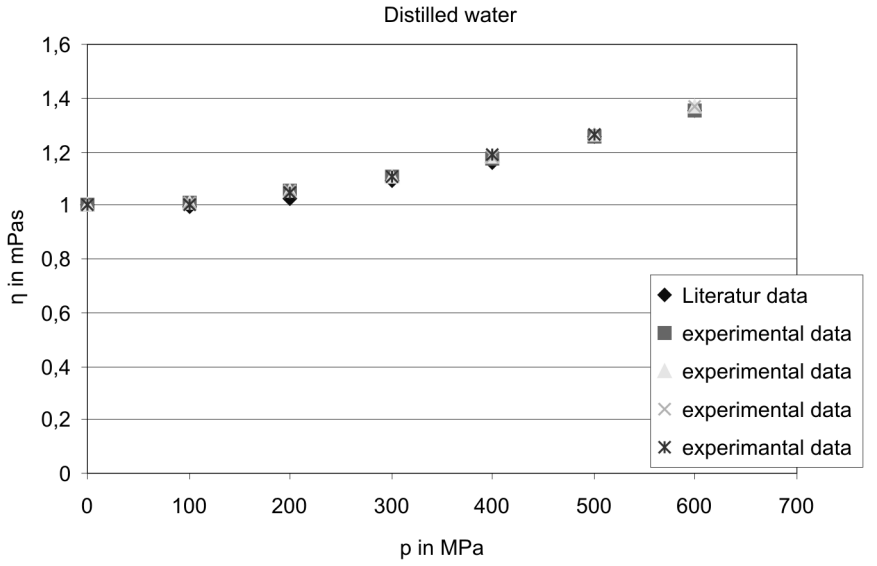


Figure 2. Dynamic viscosity of distilled water under high pressure

All measurements have been carried out at 20 °C. Inclination angle is 30°

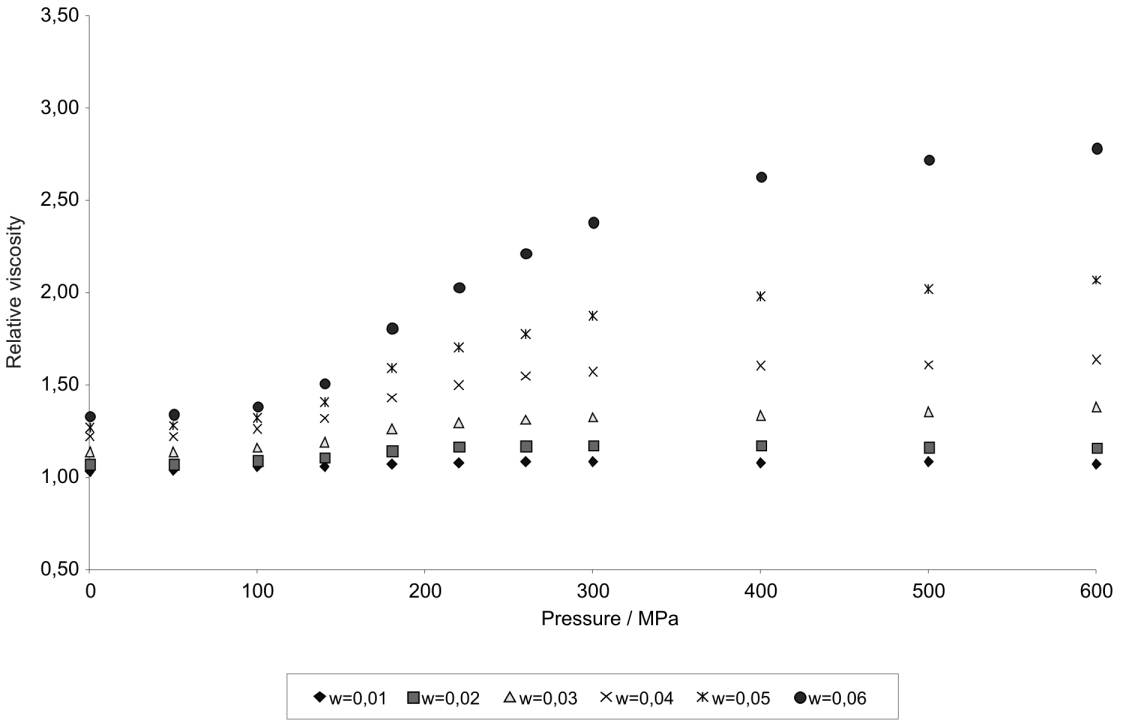


Figure 3. The relative viscosity of β-lactoglobulin solution under high-pressure

Protein concentration (g/g): w=0.01; w=0.02; w=0.03; w=0.04; w=0.05 and w=0.06. All measurements have been carried out at 20 °C. Inclination angle was 30°

sented in Figure 2. Figure 3 shows the relative viscosity of 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 g/g β-lactoglobulin solutions under high pressure.

The results showed that there is no significant effect of 100 MPa pressure on the viscosity of β-lactoglobulin solution, suggesting that no significant structural changes of β-lactoglobulin occurred. With increasing pressure, between 100 and 300 MPa, the viscosity of β-lactoglobulin solutions significantly increased probably as a result of changes in the structure of β-lactoglobulin. The β-lactoglobulin belongs to the class of β-sheet proteins; β-sheet structure predominates over other secondary structure elements in aqueous solution of β-lactoglobulin (Boye et al., 1996; Dumay et al., 1999; Knudsen et al., 2002).

The main types of molecular interactions which determine the conformation of proteins - ionic and hydrophobic interactions and hydrogen bonds are influenced by high pressure (Keim and Hinrichs, 2004; Mozhaev et al., 1996). Recent results on the effect of high-pressure on functional and structural properties of β-lactoglobulin showed that high-pressure (300-900 MPa) involves changes in the emulsification and foaming properties of β-lactoglobulin without a measurable change in the secondary structure of β-lactoglobulin (Pittia et al., 1996; Subirade et al., 1998).

Further increasing pressure (>300 MPa) cause small increase of the relative viscosity due to non-reversible and more extensive effects on protein. These include unfolding of monomeric proteins and aggregation. Aggregation effects have been observed upon treatment between 200 and 600 MPa, and formation of intermolecular disulfides at neutral pH value and the pressure up to 450 MPa has been reported (Nabhan et al., 2004; Scollard et al., 2000; Tanaka et al., 1996; Taniguchi et al., 1994). Unfolding occurs mainly as a result of disruption of hydrophobic and electrostatic interactions. Most of the –SH groups in milk are associated with the β-lactoglobulin. Pressure treatment lowers the amount of available –SH group which suggest disulphide bridge formation. Pressure denatured whey

protein could be expected to associate with itself in a similar manner to thermal denatured whey protein (Galani and Apenten, 1999; Tanaka et al.,1996; Tedford, et al., 1999).

Upon unfolding, a redistribution of bound and free water occurs concomitant with corresponding changes in the relative contributions. The main types of molecular interactions which determine the conformation of proteins (ionic and hydrophobic interactions and hydrogen bonds) are influenced by high pressure (Botelho et al., 2000; Bucheim et al., 1996). They are all liable to disruption by high pressure because of volume changes associated with their formation (Johnston et al., 2002).

However, the major part of the change in volume upon un/refolding of proteins is probably due to hydration processes. Application of high pressure to milk has recently been shown to alter the protein so as to provide increased exposure of hydrophobic regions. Several studies have shown that increases in protein hydration were caused by pressure-induced ionization, changes in amino-acid side chains and peptide bonds resulting from solvent exposure, and diffusion of water into cavities allocated in the hydrophobic core (Dufour et al., 1995; Lopez-Fandino et al., 1996; Herceg et al., 2005; Krešić et al., 2006).

The relative hydration based on hydration of untreated β-lactoglobulin molecules was used for the interpretation of the pressure-induced changes of protein hydration. The results are presented in Figure 4.

As shown in Figure 5, there is no significant effect of 100 MPa pressure on the relative hydration of β-lactoglobulin molecules suggesting that no significant structural changes of β-lactoglobulin occurred. Smaller particle have a greater solving and because of that, there is a small increasing in molecules hydration at pressure <100 MPa. At pressure between 100 and 300 MPa there is a significant increase in relative hydration at all solutions probably due to structural changes and increasing in number of water molecules which are associated with protein molecules. That is in a good agreement with the previous results which indicated that high-pressure treatment at 150-200 MPa induces a dissociation of protein dimers into monomers, a penetration of water inside the protein structure and an exposure of hydrophobic zones to the aqueous solvent. The reversible denaturation of protein molecules at 200 MPa affect the opening of the hydrophobic area which contributes to increase of protein hydration (Hinrichs and Rademacher, 2005; Krešić at al, 2008). At pressure between 300 and 400 MPa there is a small increase in relative hydration and at pressure >400 the relative hydration tend to a constant

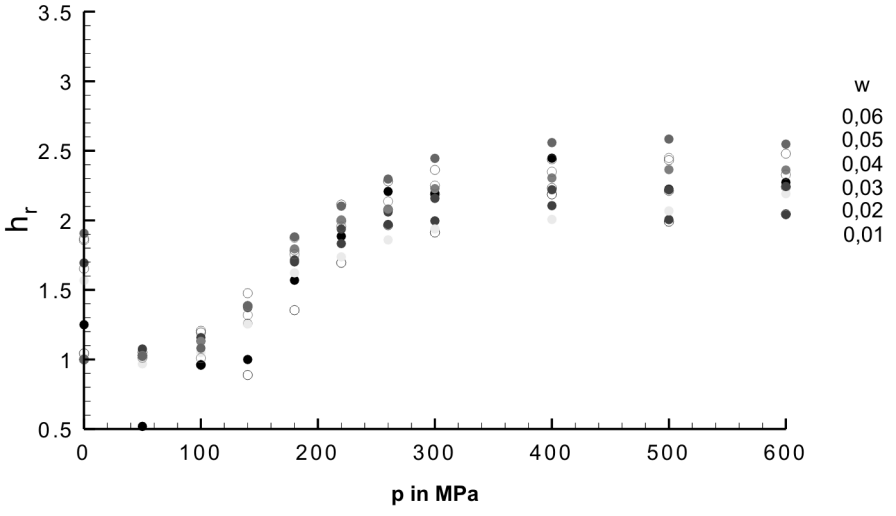


Figure 4. The relative hydration of β-lactoglobulin molecules under high pressure

Protein concentration (g/g): w=0.01, 0.02, 0.03, 0.04, 0.05 and 0.06

value. Higher pressure causes protein aggregation due to formation of intermolecular disulfide bonds. Also, irreversible denaturation and formation of β-lactoglobulin polymers occurs (Knor, 1993; Funtenberger et al., 1997). Because of that there is no changes in protein hydration, moreover the hydration of β-lactoglobulin molecules have a small decreases at pressure between 400 and 600 MPa.

The results showed that protein concentration affect the relative hydration of β-lactoglobulin molecules. One of the features that appeared most sensitive to concentration effects upon high pressure processing is the aggregation state of the protein. In solution of high protein concentration, the probability of interaction between transiently modified monomers increases, and because of that the relative hydration is smaller than in solution with low protein concentration. At low protein concentrations transiently modified monomers have a lesser chance to find an aggregation "partner" and thus there is a higher hydration of molecules. This is expected due to a higher probability of intermolecular interactions between the pressure-unfolded protein molecules with increasing concentration.

Conclusions

Modification of macromolecular structure and functional properties of proteins by high-pressure processing is now well recognized. Among the whey proteins, β-lactoglobulin appears to be most sensitive to high pressure. Pressure-induced denaturation of β-lactoglobulin in aqueous solutions can lead to aggregation and gelation. High pressure treatment under 100 MPa has no effect on the viscosity of β-lactoglobulin solution, suggesting that no structural changes of β-lactoglobulin occurred. Treatments at 100-300 MPa induce a dissociation of protein dimers into monomers, a penetration of water inside the protein structure and an exposure of hydrophobic zones to the aqueous solvent, increasing the viscosity and hydration of the β-lactoglobulin molecules. Partial unfolding induced above 300 MPa favors aggregation due to the formation of intermolecular S-S bonds through tiol/disulfide interchange reactions, followed by formation of β-lactoglobulin polymers.

The changes of the conformational structure of β-lactoglobulin would be expected to alter functional properties of the food system. Hence foaming, emulsifying, gelling and water binding capacities of the proteins may all potentially be influenced. This could lead to the development of a range of functional food ingredients prepared from milk proteins by controlled unfolding of their structure.

Utjecaj visokog hidrostatskog tlaka na viskozitet otopina β-laktoglobulina

Sažetak

U radu su prikazani novi eksperimentalni podaci o viskozitetu vodene otopine β-laktoglobulina na visokim tlakovima. Eksperimentalno ispitivanje temeljeno je na primjeni in-situ tehnike mjerenja viskoziteta otopine. Za mjerenja korišten je Rolling ball viskozimetar s različitim kutovima nagiba koji je prilagođen radu na visokim tlakovima. Određivanje viskoziteta otopine vršeno je s ciljem utvrđivanja reverzibilnih i ireverzibilnih promjena strukture molekula β-laktoglobulina. Za eksperimentalna mjerenja korištene su otopine koncentracije 0.01, 0.02, 0.03, 0.04, 0.05 i 0.06 g/g, a izložene su tlakovima od 0.1 do 600 MPa. Rezultati ispitivanja pokazuju da tlakovi do 100 MPa ne utječu bitnije na viskozitet otopine β-laktoglobulina. S povišenjem tlaka, između 100 i 300 MPa, viskozitet otopine značajno raste ukazujući na promjene strukture molekula β-laktoglobulina. Daljnji porast tlaka iznad 300 MPa dovodi do daljnjeg povećanja viskoziteta uslijed nereverzibilnih i izraženijih promjena proteina, denaturacije i agregacije. Strukturne promjene β-laktoglobulina koje su izazvane visokim tlakovima utječu i na hidrataciju molekula β-laktoglobulina. Na tlakovima između 100 i 300 MPa zapaženo je značajno povećanje relativne hidratacije, što može biti posljedica strukturnih promjena s jedne, i povećanja broja molekula vode koje su asocirane s molekulama proteina - s druge strane. Na višim tlakovima javljaju se ireverzibilna denaturacija i agregacija proteina, koja nastaje kao posljedica uspostavljanja intermolekularnih disulfidnih veza. Uslijed toga ne dolazi do povećanja hidratacije molekula β-laktoglobulina, naprotiv na tlakovima između 400 i 600 MPa hidratacija proteina blago se smanjuje.

Ključne riječi: β-lakotoglobulin, visoki tlakovi, viskozitet, in-situ tehnike, funkcionalna svojstva

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