Changes of Secondary Structure and Surface Tension of Whey Protein Isolate Dispersions upon pH and Temperature

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Abstract

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The secondary structure of proteins in unheated and heated whey protein isolate dispersions and the surface tension of the solutions were investigated at different pH. Heating protein solutions at 80°C results in an increase of unordered structure. Nevertheless, the difference between the contents of unordered structure in the unheated and heated samples increases with increasing pH of the solution. At low protein concentrations the surface tension decreased with increasing protein concentration to about 5 mg/ml. For the heated solution, a similar trend was observed in the decrease in the surface tension with increasing concentrations of protein. In both cases, the curves depicting the surface tension as a function of protein concentration could be fitted to the exponential function with a negative exponent, but with the heated solutions lower values of surface tension were observed. Studies on the surface tension of whey protein isolate solutions prove that the unfolding of whey proteins, revealed by changes in the secondary structure, causes a decrease in the surface tension.

Keywords: circular dichroism; globular protein; protein concentration

Whey protein isolate is becoming a popular food additive increasing the nutritional value and changing the functional properties (Foegeding *et al*. 2006). In the literature there is no data on the proportions of the individual secondary structures of the proteins in whey protein isolate. Circular dichroism spectrum of whey protein isolate is probably the resultant of the main whey protein spectra, namely: β-lactoglobulin, α-lactalbumin, and bovine serum albumin. The secondary structure of β-lactoglobulin is composed of nine anti-parallel structures of β-sheets and one α-helix (Creamer *et al*. 1983; Kontopidis *et al*. 2004). Indeed, CD studies of β-lactoglobulin showed characteristic minimum in the spectrum at a wave-

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length of 216 nm, indicative of secondary structure rich in β-sheet (Mercade-Prieto *et al.* 2007). The secondary structure of α-lactalbumin consists of two domains. α-Domain consists of four α-helices, while domain β contains two β-sheets (Chrysina *et al*. 2000). The secondary structure of bovine serum albumin does not include β-folded sheets and 68% of its secondary structure is composed of α-helices (Curry *et al*. 1998; Kuwata *et al.* 1999). Mercade-Prieto *et al.* (2007) have calculated that the contents of the various structures in unheated β-lactoglobulin at pH 7.5 was 32.6% of β-sheet, 16.8% of α-helix, 20% of the reversed structures, and 27% were disordered. Increasing the pH to 11 reduced the share of the organised structures and led to the corresponding increase in the unstructured ones to 60%.

Proteins tend to adsorb spontaneously from the solution at the air/solution interface. After adsorption, the natural flexibility of the protein chains favours the visibility of a previously hidden hydrophobic fragments, which can lead to interfacial denaturation of proteins (Foegeding *et al.* 2006). The surface tension of water is relatively high, i.e. about 72 mN/m at room temperature. The proteins adsorbing at the interface lower the surface tension of the solution (Nylander *et al*. 1999). The ability of proteins to reduce the surface tension was positively correlated with the ability to create foam (Wilde & Clark 1996). There is no data in the literature showing in which way the changes in the secondary structure of whey proteins in WPI influence the surface tension of the obtained dispersions.

The aim of this research was to investigate the influence of pH and heating on the secondary structure of proteins in whey protein isolate and their effects on the surface tension of the obtained dispersions.

MATERIAL AND METHODS

Whey protein isolate (WPI) LACPRODAN produced by Arla Foods Ingredients (Viby, Denmark) was used. Protein concentration (88.0%) was determined by the Kjeldahl procedure.

Circular dichroism. Whey protein isolate solutions were prepared at a concentration of 0.2 mg/ml in a solution of Britton-Robinson buffer (a mixture of 0.04M H_3BO_3, H_3PO_4, and CH_3COOH). pH of the solutions was adjusted to 3–10 with 0.2M NaOH or HCl. The samples were heated in a water bath at 80°C for 30 min and compared with the unheated samples. The spectra were recorded at 25°C using a Jasco J-815 spectropolarimetr (Jasco Analytical Instruments, Easton, USA). The spectra were measured in the wavelength range 195–260 nm and were corrected by subtracting the background from the sample spectrum and subsequently plotted as the mean molar ellipticity Θ (degree·cm^2/dmol) vs. wavelength λ (nm). The signal/noise ratio was increased by acquiring each spectrum over an average of three scans. The mean residue ellipticity [θ] (deg·cm^2/dmol) was calculated from the formula $[\theta] = (\theta_{obs}/10) \times (MRW/lc)$, where: θ_{obs} – observed ellipticity in degrees; MRW – mean residue molecular weight (molecular mass of the protein divided by the number of amino acids); l – optical pathlength in cm; c – protein concentration in g/ml. The mean residue weight of the whey protein isolate was estimated to be 114, based on the molecular masses of the three major components: β-lactoglobulin, α-lactalbumin, and bovine serum albumin. The secondary structure contents of the samples were estimated from the CD spectra using three algorithms, CONTINLL, SELCON 3, and CDSSTR, implemented in the CDPro software package (Narasimha Sreerama, Colorado State University, Fort Collins, USA) (Sreerama & Woody 2000). In the case of the CD spectra before heating, 43 soluble proteins (SP43) with known precise secondary structures were used as a reference. However, upon heating, the special reference set that additionally included denaturated proteins (SDP48) was used in CD spectra deconvolution.

Surface tension. Whey protein isolate solutions were prepared in distilled water at protein concentration of 6, 7, 8, 9, 10, and 11% at pH 6.68 (native pH value) and at a concentration of 9% of the protein at pH 2, 3, 4, 5, 6, 7, 8, 9, and 10. The study of the surface tension of the solutions was carried out at 22°C using the ring method (du Nouy'a method) employing a Kruss tensiometer K9 (KRÜSS GmbH, Hamburg, Germany). The ring was immersed in the protein solution and gradually raised above the surface. The maximum value of the force at the detachment of the ring from the surface of the solution determined the value of surface tension at the interface. Each sample was subjected to three measurements. The results were recorded using computer software Kruss (KRÜSS GmbH, Hamburg, Germany).

For the research into diluted whey protein isolate solutions, the concentrations: 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 3, 0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 mg/ml of protein in distilled water were made. The solutions were heated in a water bath at 80°C for 30 minutes. The surface tensions of these solutions were compared with those of unheated solutions at

22°C. The tests were performed by the hanging drop method using tensiometer Theta Lite (Attension/Bioline Scientific, Espoo, Finland). Theta Optical Tensiometer captures drop images and analyses the drop shape as a function of time using Young-Laplace equation implemented in OneAttension Software (Attension/Bioline Scientific, Espoo, Finland). Each sample was subjected to six measurements.

Statistical analysis. Regression analysis was performed using Excel 2007 (Microsoft, Tulsa, USA).

RESULTS AND DISCUSSION

Figure 1 shows the CD spectra in the dependence on pH, recorded without preheating at 25°C, or those of the solutions previously heated to 80°C and equilibrated at this temperature for 30 minutes. In 195–260 nm wavelength range, the changes of the protein secondary structure were revealed. Minimum at about 208 nm clearly indicates the existence of the structural motif of α-helix. However, the shape of the curve, and especially the lack of the corresponding α-helix minimum at 222 nm, replaced by barely marked inflection, suggest that in addition to α-helix there are other elements of secondary structure. After the heat treatment, the intensity of the negative band at 222 nm decreased (Figure 1), indicating a loss of the helical structure. At the same time, the intensity of the 208 nm band was slightly blue-shifted and its intensity clearly increased, which is typical of a protein that has undergone unfolding and has adopted a predominantly random coil structure. This tendency increases with the rising pH of the solution. The analysis of ellipticity values

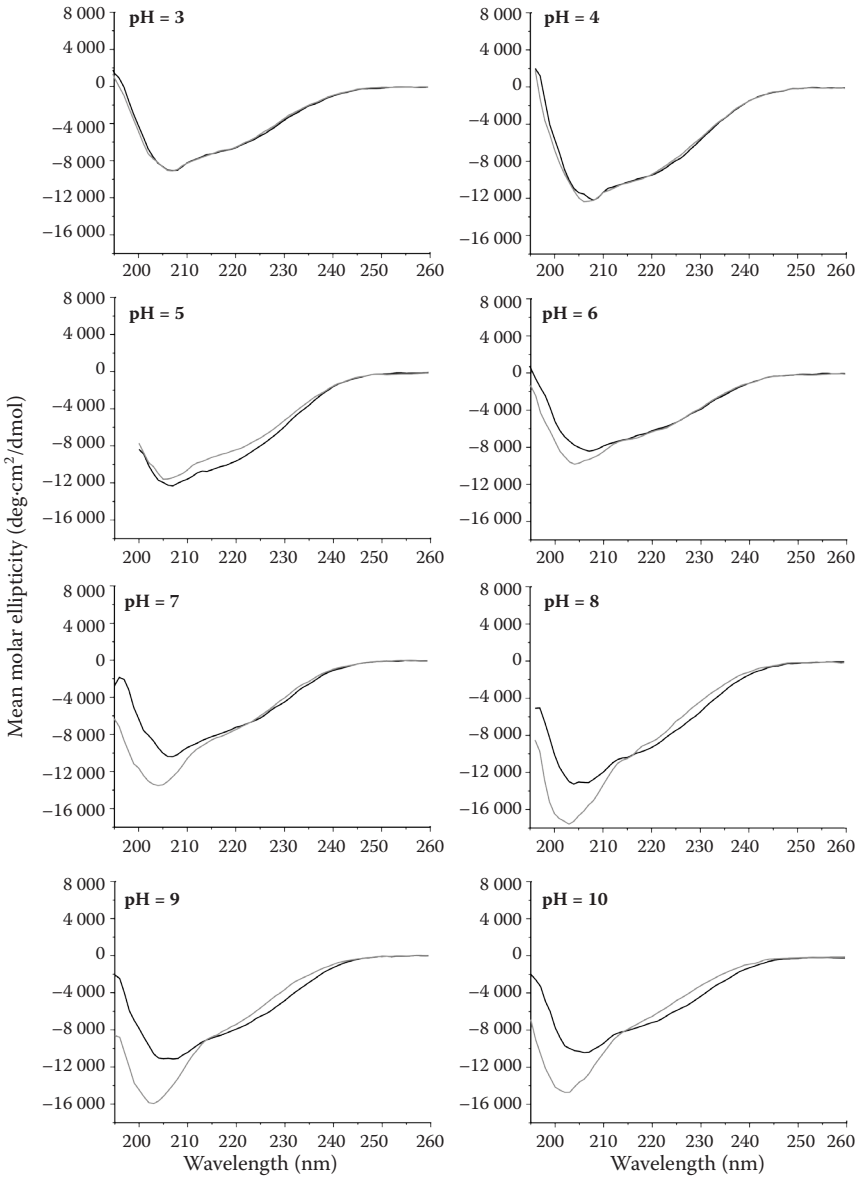

Figure 1. CD spectra of whey protein isolate dispersion obtained at different pH at a concentration of protein 0.2 mg/ml (black lines – unheated dispersions, grey lines – dispersions heated at 80°C for 30 min)

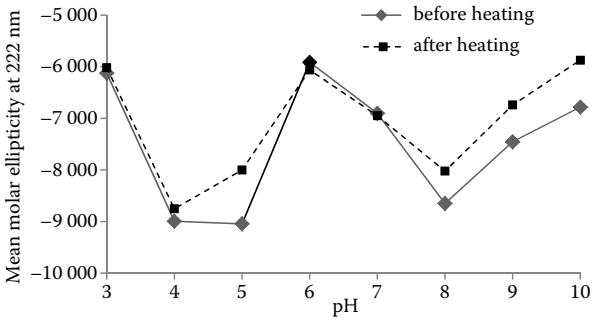

Figure 2. Change of ellipticity values of the whey protein isolate observed at 222 nm on the CD spectra before and after heating

at λ = 222 nm in the CD spectra before and after heating showed the influence of pH on the stability of helical structure (Figure 2). As obvious, marked conformational changes were observed at pH = 5 close to the isoelectric point of the mixture of whey proteins, and at basic pH (8–10). At the isoelectric point (pH = 5), the mixture of the whey proteins has a net charge of 0 and the potential for protein-protein interactions is at its highest point, which favours the aggregation processes (Xiong 1992). At basic pH, proteins carry a strongly repulsive negative charge, extensive unfolding facilitates sufficient protein-protein interactions including disulfide bond formation (Monahan *et al.* 1995).

Qi and Onwulata (2011) studied the effect of extrusion process on the structure of whey protein isolate and observed minima of CD spectra at 205 and 222 nm, which are characteristic for the structures of α-helices. At the same time, the charts contained wells in the wavelength range of 210–220 nm, proving the existence of β-sheet structures. Studies on β-lactoglobulin solutions have shown that the formation of aggregates resulted in blue-shifting the characteristic minimum (well) and lower ellipticity (Mercade-Prieto *et al.* 2007). The same phenomenon was observed for the tested whey protein isolate (Figure 1). Moro *et al.* (2011) studied the effect of β-lactoglobulin heating at 85°C on the secondary structure of the protein. They observed a blue-shift, especially between 3 and 5 min of heating. A longer heating time resulted in smaller changes in the shape of CD curves. The analysis of these curves showed that heating had caused a decline in the content of α-helices structure and β-sheet together with an increasing number of disordered structures. In order to visualise the changes in the shape of CD curves obtained for the unheated and heated whey protein isolate solutions, the results are presented in pairs for different values of pH (Figure 1). The smallest change in the curves was observed at pH 3.0. This is consistent with the studies of Wada *et al.* (2006) on β-lactoglobulin solutions heated at pH 3.0 at 80°C for one hour. They observed no change in the protein secondary structure. Studies have shown that at pH 3.0, β-lactoglobulin retains a large part of hidden hydrophobic surface after heating, and therefore denaturation under these conditions requires a higher temperature than the heating at pH 7.5 (Garcia-Hernández *et al.* 1998). The heating of β-lactoglobulin at pH 7.5 and 80°C for 1 h resulted in an increased reactivity of sulfhydryl groups of cysteine (Wada *et al.* 2006). Heating solutions of β-lactoglobulin at temperatures between 50°C and 70°C resulted in only slight changes in the ellipticity (Chen *et al.* 2005). Significant changes were observed upon heating at 80°C or above. No

Table 1. Secondary structures[a] of proteins in WPI dispersions obtained at different pH at a concentration of protein 0.2 mg/ml before and after heating at 80°C for 30 min (in %)

pH	Before heating				After heating			
	α-helices	β-sheets	β-turns	unordered structures	α-helices	β-sheets	β-turns	unordered structures
3	17.8 ± 0.6	28.9 ± 0.4	22.1 ± 0.1	31.1 ± 0.1	16.5 ± 0.2	25.2 ± 2.3	18.8 ± 1.3	39.3 ± 3.5
4	26.6 ± 2.3	20.9 ± 0.5	21.5 ± 1.3	30.9 ± 0.5	24.3 ± 0.7	16.9 ± 2.9	17.3 ± 2.2	41.1 ± 4.3
5	23.1 ± 1.2	22.9 ± 1.8	22.2 ± 0.6	31.7 ± 1.3	21.8 ± 0.2	16.9 ± 0.9	17.2 ± 0.9	44.0 ± 1.3
6	16.6 ± 0.7	28.1 ± 0.7	22.6 ± 0.3	32.8 ± 1.0	15.3 ± 0.9	18.7 ± 1.3	16.0 ± 1.0	49.9 ± 1.8
7	20.3 ± 1.2	24.4 ± 1.2	22.7 ± 0.7	32.6 ± 1.0	17.6 ± 0.5	17.8 ± 2.6	16.2 ± 1.4	48.1 ± 4.0
8	22.5 ± 1.1	21.4 ± 1.7	23.0 ± 2.3	33.0 ± 0.4	20.4 ± 2.5	18.5 ± 5.0	17.9 ± 2.5	41.8 ± 9.9
9	20.8 ± 0.6	24.2 ± 0.5	22.1 ± 0.4	32.9 ± 0.7	16.7 ± 0.7	16.5 ± 0.9	15.5 ± 1.1	51.2 ± 1.6
10	19.0 ± 0.7	25.6 ± 0.2	22.7 ± 0.4	32.6 ± 0.4	11.6 ± 1.7	18.7 ± 1.3	15.7 ± 0.2	53.5 ± 2.8

[a]percentages are averages ± standard deviations from three methods: CONTINLL, CDSSTR, and SELCON 3

such changes were observed in the case of heated α-lactalbumin solutions. The explanation for this behaviour may reside in relatively weak hydrophobic interactions that occur between the unfolded molecules of β-lactalbumin (Rattray & Jelen 1998). Foegeding *et al*. (1992) observed that the secondary structure of β-lactoglobulin heated at 72°C differed depending on whether it was dissolved in water or saline. However, the CD curves obtained for the solutions of sodium chloride and calcium chloride were identical, which indicates that the type of ions had no effect under these conditions on the secondary structure of β-lactoglobulin. Woody (1996) observed that the heating of bovine serum albumin caused a reduction in the proportion of α-helices structure. This process is associated with the progressive aggregation of the protein and the formation of β-aggregated structures (Navarra *et al*. 2009).

Table 1 present the proportions of secondary structures in proteins in solutions of whey protein isolate at different pH at 25°C, for the unheated samples and those pre-heated at 80°C for 30 min, respectively. All methods of analysing CD spectra have some limitations and assume that the spectrum of a protein can be represented by a linear combination of the spectra of its secondary structural elements, plus a noise term which includes the contribution of aromatic chromophores. CONTIN method fits the CD of unknown proteins by a linear combination of the spectra of a large data base of proteins with known conformations. The heating of whey protein isolate solutions caused a decrease in α-helices, β-sheets, and β-turns structures and an increase in unordered structures. The results may be influenced by protein aggregation, therefore, our results indicate rather the apparent content of the secondary structures. These changes are more pronounced with increased pH of a solution. This reflects the pH-dependent denaturation of proteins in the heated solution, and the formation of more flexible unfolded, predominantly unordered structure. It was observed that the heating of β-lactoglobulin solution did not result in large changes in the secondary structure, however, circular dichroism can not distinguish between the β-sheet structures of proteins and intermolecular unheated β-sheets generated during the process of aggregation induced by heating (Mercade-Prieto *et al*. 2007). Qi and Onwulata (2011) compared the secondary structure of proteins in whey protein isolate obtained by lyophilisation and spray drying. For the spray dried isolate, they observed a shift of the peaks on the CD charts, which clearly showed a partial loss of α-helix structure. Interesting phenomenon was observed in the case of heating the solutions of freeze-dried isolate in the range of 5–25°C. The secondary structure of proteins was shifted from the β-sheet structure in the α-helix structure (Qi & Onwulata 2011). β-Lactoglobulin is a protein mainly built of β-sheet structures, but it has a high tendency to move in the α-helix structure (Hamada *et al*. 1996). In the present study, the deconvolution of the CD spectra indicates that before heating, the change of pH affects mainly the equilibrium between the contents of α-helical and β-sheet structures. An increase of the proportion of β-sheet causes a decrease of the proportion of α-helical structure at the same time and vice versa. However, the same tendency is not retained upon the heating of the solutions.

Figure 3 shows the surface tension of the solutions of whey protein isolate at a concentration of 9% (w/w) protein obtained at different pH values. In the pH range 2–4 the surface tension value was

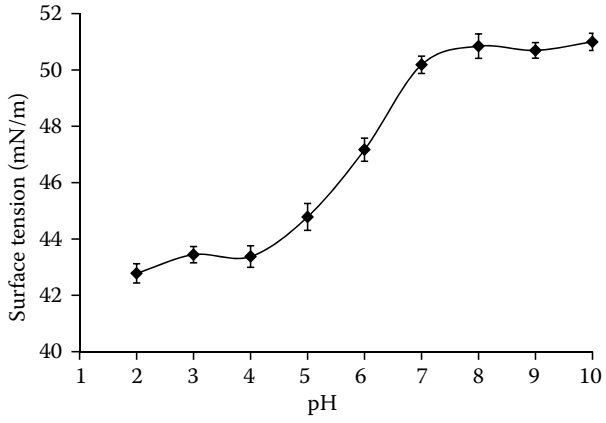

Figure 3. Influence of pH on surface tension of 9% protein WPI dispersions

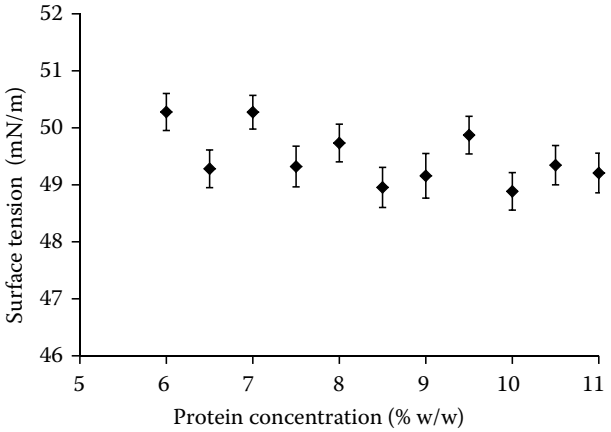

Figure 4. Influence of protein concentration on surface tension of native (pH 6.68) WPI dispersions

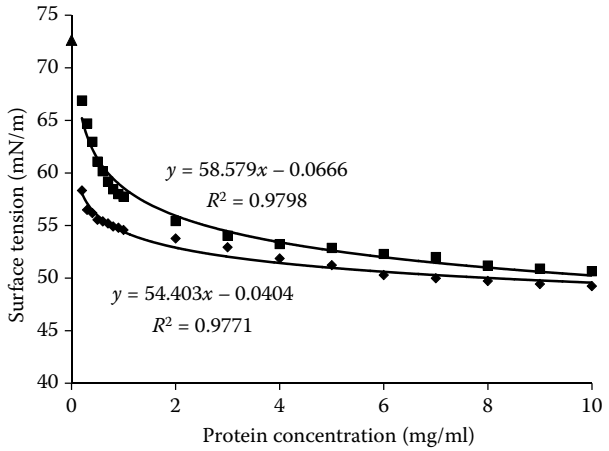

Figure 5. Influence of protein concentration on surface tension of native (pH 6.68) WPI dispersions unheated (squares) and heated at 80°C for 30 min (diamonds)

low and constant. An increase in pH from 4 to 7 resulted in an increase of the surface tension from 43 mN/m to about 51 mN/m. This value did not change with further increase in pH. The decrease of the surface tension of whey protein isolate solutions with a decrease in pH was observed by Davis *et al*. (2004). A decrease in the surface tension at pH from 10 to 5 can be explained by the decline of the total electric charge of proteins and smaller repulsion at the interface. Further lowering of pH from 5 to 2 causes a decrease in the surface tension probably due to the fact that at low pH β-lactoglobulin molecule retains a significant amount of hydrophobic groups in the interior (Garcia-Hernández *et al*. 1998). This can result in an increased adsorption of the protein at the interface solution/air and thus cause a further decrease in the surface tension. Figure 4 shows the impact of protein concentration used in practical functional applications of WPI (6–11%) on the surface tension of whey protein isolate solutions obtained at pH 6.68 (native pH). It was observed that changing the protein concentration does not affect the change in the surface tension. A similar phenomenon in relation to different protein solutions was observed by other researchers (González-Tello *et al*. 2009; López *et al*. 2010; Fernández *et al*. 2012). González-Tello *et al*. (2009) found a decrease in the surface tension with decreasing concentration of whey protein concentrate to 5%. After increasing the concentration, the surface tension increased slightly and remained constant at a concentration of 20%. In order to investigate the effect of the protein concentration on the surface tension of the WPI solutions at low protein concentrations, dispersions were made starting from 0.2 mg/ml (Figure 5). It was found that the surface tension decreased up to protein concentration about 5 mg/ml (i.e. about 0.5%). The decrease in the surface tension with increasing concentration of protein was observed by other researchers (Kratochvíl & Hrnčíř 2002; González-Tello *et al*. 2009; López *et al*. 2010; Fernández *et al*. 2012). At a certain concentration, proteins occupy the entire interface, and further increase in their concentration does not influence the surface tension any longer. Figure 5 shows also the effect of protein solution heating on the surface tension. Whey protein isolate solutions were subjected to the same heat treatment as that used in circular dichroism analysis, although a different ionic strength was applied. For the heated solution, a similar trend in the decrease of the surface tension with increasing concentrations of protein was observed. In both cases, the curves depicting the surface tension as a function of protein concentration could be fitted to the exponential function with a negative exponent (R^2 = 0.98). For the heated solutions lower values of the surface tension were observed. The proteins contained in these solutions were already partially denatured as evidenced by studies performed using the method of circular dichroism. In the heated solution of whey protein isolate in relation to the unheated solutions, a decrease in the amount of helical structure and an increase in unordered structure were observed (Table 1). Denaturation process is associated with a reduction in the number of α-helix structures and an increasing number of disordered structures (Woody 1996). Partial unfolding of the protein structure makes it more flexible, which can increase the adsorption at the interface. The increased adsorption at the interface causes a decrease in the surface tension (Cifra 2004; Fernández *et al*. 2012). Studies on the surface tension of whey protein isolate solutions prove that unfolding of whey proteins revealed by the changes in secondary structure causes a decrease in the surface tension of the proteins solutions.

CONCLUSION

Heating protein solutions at 80°C results in an increase of unordered structure. Nevertheless, the difference between the contents of unordered structure in the unheated and heated samples increases with increasing pH of the solution. An increase in pH from 4 to 7 resulted in an increase of the surface tension from 43 mN/m to about 51 mN/m. This value

did not change with further increase in pH. Changing the protein concentration from 6% to 11% (w/w) did not affect the change in the surface tension, however, at low protein concentrations the surface tension decreased up to the protein concentration of about 5 mg/ml, and the same trend was observed for the unheated and heated solutions. Heating caused a decrease in the surface tension of diluted whey protein isolate solutions. Unfolding of whey proteins by heating caused changes in the secondary structure. The obtained more flexible structure with a lower concentration of α-helix caused a decrease in the surface tension of the proteins solutions.

References

Chen W.L., Hwang M.T., Liau C.Y., Ho J.C., Hong K.C., Mao S.J.T. (2005): β-Lactoglobulin is a thermal marker in processed milk as studied by electrophoresis and circular dichroic spectra. Journal of Dairy Science, **88**: 1618–1630.

Chrysina E.D., Brew K., Acharya K.R. (2000): Crystal structure of apo- and holo-bovine α-lactalbumin at 2.2-A resolution reveal an effect of calcium on inter-lobe interactions. Journal of Biological Chemistry, **275**: 37021–37029.

Cifra P. (2004): Differences and limits in estimates of persistence length for semi-flexible macromolecules. Polymer, **45**: 5995–6002.

Creamer L.K., Parry D.A.D., Malcolm G.N. (1983): Secondary structure of bovine β-lactoglobulin. Archives of Biochemistry and Biophysics, **227**: 98–105.

Curry S., Mandelkow H., Brick P., Franks N. (1998): Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. Nature Structural Biology, **5**: 827–835.

Davis J.P., Foegeding E.A., Hansen F.K. (2004): Electrostatic effects on the yield stress of whey protein isolate foams. Colloids and Surfaces B: Biointerfaces, **34**: 13–23.

Fernández E., Artiguez M.L., de Marañon I.M., Villate M., Blanco F.J., Arboleya J.C. (2012): Effect of pulsed-light processing on the surface and foaming properties of β-lactoglobulin. Food Hydrocolloids, **27**: 154–160.

Foegeding E.A., Kuhn P.R., Hardin C.C. (1992): Specific divalent cation-induced changes during gelation of β-lactoglobulin. Journal of Agricultural and Food Chemistry, **40**: 2092–2097.

Foegeding E.A., Luck P.J., Davis J.P. (2006): Factors determining the physical properties of protein foams. Food Hydrocolloids, **20**: 284–292.

Garcia-Hernández E., Hernández-Arana A., Zubillaga R.A., Rojo-Domínguez A. (1998): Spectroscopic and thermodynamic evidence for a complex denaturation mechanism of bovine β-lactoglobulin A. Biochemistry & Molecular Biology International, **45**: 761–768.

González-Tello P., Camacho F., Guadix E.M., Luzón G., González P.A. (2009): Density, viscosity and surface tension of whey protein concentrate solutions. Journal of Food Process Engineering, **32**: 235–247.

Hamada D., Segawa S., Goto Y. (1996): Non-native α-helical intermediate in the refolding of β-lactoglobulin, a predominantly β-sheet protein. Nature Structural & Molecular Biology, **3**: 868–873.

Kontopidis G., Holt C., Sawyer L. (2004): Invited review: β-lactoglobulin: binding properties, structure, and function. Journal of Dairy Science, **87**: 785–796.

Kratochvíl A., Hrnčíř E. (2002): Correlation between the cerebrospinal fluid surface tension value and 1. Concentration of total proteins 2. Number of cell elements. General Physiology and Biophysics, **21**: 47–53.

Kuwata K., Hoshino M., Forge V., Era S., Batt C.A., Goto Y. (1999): Solution structure and dynamics of bovine β-lactoglobulin A. Protein Science, **8**: 2541–2545.

López M.L. Bengoechea C., De La Fuente J., Ruiz M., Guerrero A. (2010): Influence of the presence of monoglyceride on the interfacial properties of wheat gluten. Journal of the Science of Food and Agriculture, **90**: 1688–1694.

Mercade-Prieto R., Paterson W.R., Wilson D.I. (2007): The pH threshold in the dissolution of β-lactoglobulin gels and aggregates in alkali. Biomacromolecules, **8**: 1162–1170.

Monahan F.J., German J.B., Kinsellat J.E. (1995) Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. Journal of Agricultural and Food Chemistry, **43**: 46–52.

Moro A., Baez G.D., Busti P.A., Ballerini G.A., Delorenzi N.J. (2011): Effects of heat-treated β-lactoglobulin and its aggregates on foaming properties. Food Hydrocolloids, **25**: 1009–1015.

Navarra G., Giacomazza D., Leone M., Librizzi F., Militello V., San Biagio P.L. (2009): Thermal aggregation and ion-induced cold-gelation of bovine serum albumin. European Biophysics Journal, **38**: 437–446.

Nylander T., Hamraoui A., Paulsson M. (1999): Interfacial properties of whey proteins at air/water and oil/water interfaces studied by dynamic drop tensiometry, ellipsometry and spreading kinetics. International Journal of Food Science and Technology, **34**: 573–585.

Qi P.X., Onwulata Ch.I. (2011): Physical properties, molecular structures, and protein quality of texturized whey protein isolate: effect of extrusion temperature. Journal of Agricultural and Food Chemistry, **59**: 4668–4675.

Rattray W., Jelen P. (1998): Thermal stability of skim milk/whey protein solution blends. Food Research International, **30**: 327–334.

Sreerama N., Woody R.W. (2000): Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. Analytical Biochemistry, **287**: 252–260.

Wada R., Fujita Y., Kitabatake N. (2006): Effects of heating on neutral and acid pH on the structure of β-lactoglobulin A revealed by differential scanning calorimetry and circular dichroism spectroscopy. Biochimica et Biophysica Acta, **1760**: 841–847.

Wilde P.J., Clark D.C. (1996): Foam formation and stability. In: Hall G.M. (ed.): Methods of Testing Protein Functionality. Blackie Academic & Professional, London: 110–148.

Woody R.W. (1996): Theory of circular dichroism in proteins. In: Fasman G.D. (ed.): Circular Dichroism and the Conformational Analysis of Biomolecules. Plenum Press, New York.

Xiong Y.L. (1992) Influence of pH and ionic environment on thermal aggregation of whey proteins. Journal of Agricultural and Food Chemistry, **40**: 380–384.

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