WHEY PROTEIN DENATURATION DETERMINED BY A NOVEL APPROACH OF ANALYSIS OF DIFFERENCE-UV SPECTRA

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Abstract–A novel approach in the analysis of difference-UV spectrophotometric data was implemented for determining the degree of denaturation of bovine serum albumin and whey protein isolate. Analysis was performed by subtracting separate spectra of native and denatured protein solutions at different denaturing conditions of temperature, pH and ethanol. Data on the kinetics of protein denaturation were compared to those from differential scanning calorimetry. The results provide evidence to a new methodology of protein denaturation evaluation which is sensitive and accurate.

Keywords: *whey protein denaturation, difference-UV spectroscopy, pH, ethanol as cosolvent, DSC*

1. INTRODUCTION

In the past, numerous studies have applied difference-UV spectroscopy to study changes in the conformation of proteins induced by heat, or chemical agents [1-3]. In the case of applying this method by obtaining the spectrum of a sample of denatured protein and then subtracting it from that of the native sample, peaks appear that are attributed to the perturbation mainly of the aromatic groups tryptophan and tyrosine [4]. However, baseline drifts are observed in the difference spectrum, although this drawback has not been reported before in the literature [5]. Such drifts constitute reading the peak nominal value of a difference-UV spectrum rather erroneous. Here, a new approach in analyzing difference-UV spectra of whey proteins is described. This new method is based on the integration of the obtained difference spectra.

2. EXPERIMENTAL

Bovine serum albumin (BSA), fraction V, was purchased from Merck (Darmstadt, Germany) and whey protein isolate (WPI), 92.08% w/w protein, was a product of Davisco Foods International, Inc. (Le Sueur, MN, USA). Guanidine hydrochloride (GdHCl), 98% was purchased from Alfa Aesar (Lancashire, UK). Ethanol, 95%, was purchased from Sigma-Aldrich (St. Louis, USA). HCl and NaOH of different molarities were used to adjust the pH. Double distilled water was used in the preparation of all solutions.

The protein solutions of BSA and WPI (0.2 and 0.15%, respectively) were prepared by stirring for two hours and then kept in the refrigerator overnight for complete solubilization. The pH measurements were performed using a Bante220 portable pH-meter (Shanghai, China). For the heat treatments, glass tubes with screw caps were used in a Memmert water bath (Schwabach, Germany), equipped with a temperature controller. The UV spectroscopic analyses were performed using a PC-controlled Shimadzu UV1800 (Kyoto, Japan). Details on the implementation of the analyses can be found in previous work [5, 6]. Differential scanning calorimetry (DSC) was used to verify the difference-UV data on BSA, using denser protein solutions (10% w/w). The equipment used was a Perkin Elmer Precisely Diamond DSC (Waltham, Massachusetts, USA), with an Intracooler 2P cooling assembly. Different scan rates were used in order to apply the methods of Kissinger [7] and Borchardt & Daniels [8] for activation energy (E_a) calculations.

3. RESULTS AND DISCUSSION

Figure 1A & B shows the difference-UV curves obtained after heating at 87 and 85 ^{o}C for

different time lengths and subsequently cooling to room temperature for BSA and WPI solutions, respectively. The small differences in the peak values, or in other words the congestion of the peaks is obvious, as is also the baseline drifting for both proteinaceous systems.

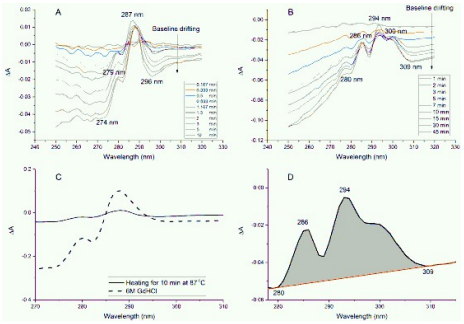

Fig.1 Difference-UV spectra: (A) BSA; (B) WPI; (C) comparison of the difference curve of BSA upon maximum denaturation upon heating and induced by GdHCl; and (D) a typical example of the proposed peak area analysis

The obtained difference-UV spectra exhibited two pronounced peaks, at 279 and 287 nm for BSA, and at 286 and 294 nm for WPI, with the differences attributed to the different content of tryptophan and tyrosine residues between the two systems. It is evident that there was a progressive increase in peak area with heating time. In Figure 1C the obtained difference curve of BSA at maximum heat denaturation is compared to the maximum unfolding of the protein molecule induced by a 6M GdHCl solution. A similar comparison was performed also for WPI (data not shown here). For the estimation of the degree of denaturation, the combined peak area of these two pronounced peaks was used, with anchor points as indicated in Figures 1A, 1B and in 1D. The obtained peak areas were then normalized against the maximum respective peak area obtained by GdHCl (2.120 and 1.621 for BSA and WPI, respectively). Figure 2A shows the results of peak area and residual protein (Figure 2B), calculated as [1-(peak area/peak area in 6M GdHCl)], with time of heating for WPI. Integrations were performed using the Origin Software.

The curves in Figure 2B are the result of non-linear (exponential) fitting, used to derive apparent rate constants (in min^{-1}). The applied fitting gave r^2 values higher than 0.99 for all the temperature conditions. As can be seen in Figure 2B, the maximum heat denaturation of WPI at 90 ^{o}C was about 40%. In the case of BSA, maximum heat denaturation was calculated to be about 17% (data not shown here).

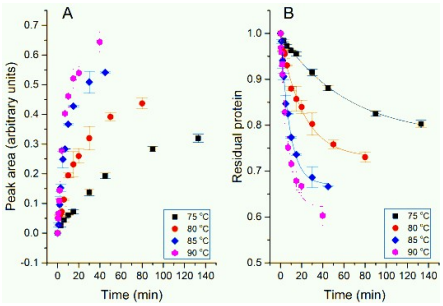

Fig. 2 (A) Evolution of peak area with heating time for WPI at different temperatures; and (B) the calculated residual protein with time at different temperatures

In an investigation of the unfolding of human serum albumin (HAS) during both chemical and thermal denaturation, using fluorescence methods, where the protein's free cystine residue was probed, it was found that GdHCl (4M) can result in complete unfolding, while heat did not allow the protein to achieve the same final conformation [9]. The percentage of 17% denaturation reported here can be said to be in agreement with the 90% reversibility of heat denaturation of 0.2% β-lactoglobulin (also a globular protein) solutions, reported in other studies [10].

Figure 3(A) is the Arrhenius plot obtained for BSA. The plot exhibits a discontinuity at around 67 ^{o}C. This effect of temperature on the reaction rate constant of BSA heat denaturation could be explained based on the assumption of two sub-processes during heat denaturation, i.e. protein unfolding at temperatures lower than this break point, and aggregation occurring at higher temperatures. Similar breaks in Arrhenius plots have been reported in the literature for other proteins [11-13]. Based on these results, two separate E_a values were calculated, namely, 79 and 246 kJ/mol, for temperatures higher and lower than the observed break point, respectively. A similar Arrhenius plot with a break point was obtained also for WPI (data not shown). Figure 3B

shows the results on kinetics obtained by DSC analysis of BSA solutions. Interestingly, DSC appears to be a method that cannot discriminate between the two different stages of unfolding and aggregation of BSA upon heating. This might be because DSC monitors the overall denaturation process and not the changes at the level of protein subgroups or domains.

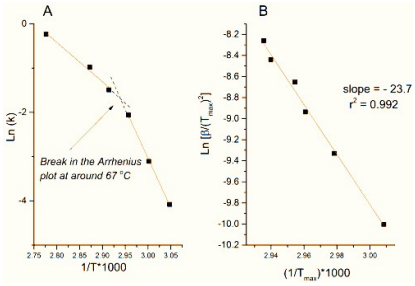

Fig. 3 Comparison of Arrhenius plots derived by using the proposed difference-UV analysis (A) and a DSC methodology (B)

PH is known to affect protein denaturation in very complex ways. In certain cases, extreme pH conditions can lead to cold denaturation. The effect of pH on the thermal denaturation of the major whey proteins has been studied in the past using DSC [14]. This study had shown that α-lactalbumin and BSA were already unfolded simply by lowering the pH before any heat treatment. Moreover, several studies have reported on the high stability of β-lactoglobulin against heat denaturation at very low pH values [15]. Figure 4 shows the effect of pH on WPI heat denaturation (85 °C) applying the herein proposed novel analysis of difference-UV spectra. Denaturation occurred even at room temperature (t=0 min) at two extreme pH conditions tested, i.e. pH 2.5 and pH 8.6. There is considerable denaturation (35%) at pH 2.5, and can be attributed to the acid denaturation of BSA and α-lactalbumin, which are present in WPI. This is in agreement with other studies that have reported substantial unfolding of these proteins by lowering the pH and prior to any heating [14]. Experiments conducted in our laboratory showed that BSA exhibits considerable initial denaturation at pH 2.5 (around 75% acid denaturation). Despite the increased initial acid denaturation, at pH 2.5, WPI exhibited the lower rate constant compared to other pH conditions tested, indicating that heating does not have a significant supplementary contribution at pH 2.5. Solvents containing alcohols are known to denature proteins [16]. A sharp transition in β-lactoglobulin in the ethanol concentration range of 20-50% has been reported [17].

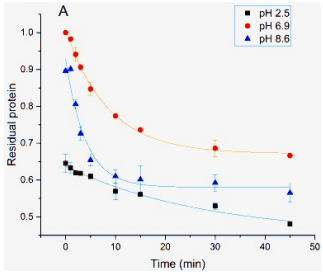

Fig. 4 Changes in residual WPI at three different pH values upon heating at 85°C

Despite the numerous studies involving individual whey proteins, the literature on the effect of alcohols on whey protein mixtures is scarce. To apply the newly developed method of analysis in the study of the effect of ethanol on WPI, the effect of time of incubation in a 1:1 water/ethanol solvent was evaluated. Figure 5A shows that there is no difference in the measured peak areas upon different incubation times.

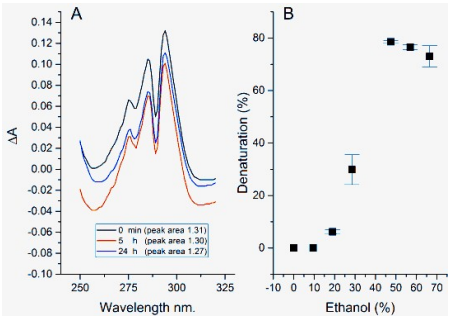

Fig. 5 Effect of time of incubation in ethanol on: (A) WPI peak area and; (B) denaturation (%) of WPI at different ethanol concentrations

Figure 5B shows the effect of ethanol at different concentrations. It was found that ethanol had a sharp effect on WPI conformation in the concentration range of 20-50%, which is in agreement with the work of other researchers mentioned above [16-18]. The mechanism behind these results may be the promotion of α-helical structures between alcohol concentrations of 20 and 50% [19].

4. CONCLUSIONS

A recently developed method of analysis of difference-UV spectra was successfully applied in the study of the denaturation of a single globular protein, BSA, as well as in a mixture of whey proteins, WPI. The sensitivity of this method allows the study of different factors involved in conformational changes of protein molecules, such as heating, pH and ethanol as cosolvent.

REFERENCES

[1] R.K.O. Apenten, S. Khokhar, D. Galani, Stability parameters for beta-lactoglobulin thermal dissociation and unfolding in phosphate buffer at pH 7.0, Food Hydrocolloids, 16 (2002) 95-103.

[2] A. Bonincontro, S. Cinelli, T. Comaschi, G. Onori, Influence of urea on thermal denaturation of lysozyme investigated by optical and dielectric spectroscopies, Physical Chemistry Chemical Physics, 6 (2004) 1039.

[3] N.K. Kella, J.E. Kinsella, Enhanced thermodynamic stability of beta-lactoglobulin at low pH - A possible mechanism, Biochemical Journal, 255 (1988) 113-118.

[4] F. Schmid, Biological Macromolecules: UV-visible Spectrophotometry, Encyclopedia of Life Sciences, (2001) 1-4.

[5] A. Nikolaidis, T. Moschakis, Studying the denaturation of bovine serum albumin by a novel approach of difference-UV analysis, Food Chemistry, 215 (2017) 235-244.

[6] A. Nikolaidis, M. Andreadis, T. Moschakis, Effect of heat, pH, ultrasonication and ethanol on the denaturation of whey protein isolate using a newly developed approach in the analysis of difference-UV spectra, Food Chemistry, 232 (2017) 425-433.

[7] H.E. Kissinger, Variation of peak temperature with heating rate in differential thermal analysis, Journal of Research of the National Bureau of Standards, 57 (1956) 217-221.

[8] H.J. Borchardt, F. Daniels, The application of differential thermal analysis to the study of reaction kinetics, Journal of the American Chemical Society, 79 (1957) 41-46.

[9] K. Flora, J.D. Brennan, G.A. Baker, M.A. Doody, F.V. Bright, Unfolding of acrylodan-labeled human serum albumin probed by steady-state and time-resolved fluorescence methods, Biophysical Journal, 75 (1998) 1084-1096.

[10] N. Poklar, G. Vesnaver, S. Lapanje, Studies by UV spectroscopy of thermal denaturation of β-lactoglobulin in urea and alkylurea solutions, Biophysical Chemistry, 47 (1993) 143-151.

[11] J.N. Dewit, G.A.M. Swinkels, A differential scanning calorimetric study of the thermal denaturation of bovine β-lactoglobulin - thermal behavior at temperatures up to 100 oC, Biochimica Et Biophysica Acta, 624 (1980) 40-50.

[12]D. Galani, R.K.O. Apenten, Heat-induced denaturation and aggregation of beta-Lactoglobulin: kinetics of formation of hydrophobic and disulphide-linked aggregates, International Journal of Food Science and Technology, 34 (1999) 467-476.

[13] A. Tolkach, U. Kulozik, Reaction kinetic pathway of reversible and irreversible thermal denaturation of β-lactoglobulin, Le Lait, 87 (2007) 301-315.

[14] J.N. Dewit, G. Klarenbeek, Effects of various heat treatments on structure and solubility of whey proteins, Journal of Dairy Science, 67 (1984) 2701-2710.

[15] E.P. Schokker, H. Singh, D.N. Pinder, L.K. Creamer, Heat-induced aggregation of beta-lactoglobulin AB at pH 2.5 as influenced by ionic strength and protein concentration, International Dairy Journal, 10 (2000) 233-240.

[16] M. Buck, Trifluoroethanol and colleagues: cosolvents come of age. Recent studies with peptides and proteins, Quarterly Reviews of Biophysics, 31 (1998) 297-355.

[17] V.N. Uversky, N.V. Narizhneva, S.O. Kirschstein, S. Winter, G. Löber, Conformational transitions provoked by organic solvents in β-lactoglobulin: can a molten globule like intermediate be induced by the decrease in dielectric constant?, Folding and Design, 2 (1997) 163-172.

[18] K. Shiraki, K. Nishikawa, Y. Goto, Trifluoroethanol-induced stabilization of the α-helical structure of β-Lactoglobulin: Implication for non-hierarchical protein folding, Journal of Molecular Biology, 245 (1995) 180-194.

[19] E. Dufour, P. Robert, D. Renard, G. Llamas, Investigation of beta-lactoglobulin gelation in water/ethanol solutions, International Dairy Journal, 8 (1998) 87-93.