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Thermochimica Acta 246 (1994) 387–403

thermochimica acta

Thermal denaturation and heat-induced gelation properties of β-lactoglobulin. Effects of some chemical parameters

Tong Xun Liu, Perla Relkin *, Bernard Launay

ENSIA, Département Science de l'Aliment, Laboratoire de Biophysique, 1, Avenue des Olympiades, 91305 Massy, France

Received 29 June 1993; accepted 20 April 1994

Abstract

The thermal denaturation of β-lactoglobulin solutions (8.5% protein concentration of which 5.8% was β-lactoglobulin) has been studied by differential scanning calorimetry. For solutions of pH between 1.5 and 8, the denaturation temperature T_d was maximum at pH 3.5, and the apparent enthalpy change ΔH_{app} seemed to be constant from pH 1.5 to 6.6, and decreased significantly at pH 8.0. With addition of *N*-ethylmaleimide (NEM) to the β-lactoglobulin solution (pH 7) in a molar ratio of up to 1:1, the peak temperature T_p increased slightly and then remained constant, for higher molar ratio, while ΔH_{app} decreased. With addition of dithiolthreithol (DTT), both T_p and ΔH_{app} decreased drastically. The kinetic parameters of heat denaturation were determined by the Borchardt and Daniels method as a function of pH. At pH 1.5 and 2.5, the denaturation process presented some degree of reversibility which increased with the scan rate of the first heating of the solution. The process could be described as a second-order reaction at $pH < pH_i$ and at pH 8.0. It seemed to be a first-order reaction at pH 6.0 and 6.6. In the case of irreversibility of the denaturation process, the half-life time was verified by an isothermic method.

The heat-induced gelation properties, the gelation time (heat treatment at 80°C) and gelation temperature (heat treatment from 40 to 90°C at 0.1°C min^{-1}) were studied with an empirical test and an Instron machine, respectively. The addition of NEM (0–20 mM) and DTT (0–32 mM) gave rise to increasing and reducing gelation time (t_g) at 80°C, respectively. The onset gelation temperature, determined from the first increase of the apparent Young's modulus, seemed to be lower than that of the denaturation temperature determined by extrapolation to 0.1°C min^{-1} of peak temperatures obtained at various scan rates. It was

* Corresponding author.

SSDI 0040-6031(94)01878-K

higher than that of the onset denaturation temperature, determined from the increasing edge of the heat flow measured at 5°C min^{-1} (7.5°C for pH 8 solution). The maximum apparent Young's modulus E_{app} observed with increasing heat treatment displayed a higher value at pH 6.6.

The experimental results are compared to published data, and they are discussed in terms of electrostatic and hydrophobic interactions and of SH/S–S interchange reactions.

Keywords: Denaturation; DSC; Gelatinization; Lactoglobulin; pH

Introduction

The effect of pH on the thermal denaturation and gelation properties of whey proteins has been studied extensively [1–8]. These studies revealed that the denaturation and gelation processes were dictated by extrinsic factors such as pH. It has been reported that gelation of globular proteins is a two-stage process, where the initial denaturation or conformational change is followed by association of the denatured molecules to form an ordered gel matrix [9]. Furthermore, Hillier et al. [3] observed that at acidic pH, the thiol group of β-lactoglobulin was relatively inert while, as the pH increases to 6.8, it became reactive. More recently Shimada and Cheftel [10] and Xiong and Kinsella [11] reported results on the heat-induced covalent cross-linking of whey proteins via intermolecular oxidation of SH to S–S bonds and/or SH/S–S interchange reactions. It was well established that the heat-set of globular proteins were correlated to their net charge, but few studies have been devoted to the effect of the pH on both thermal denaturation and gelation properties [7,12–14].

The aim of this paper is to establish a relationship between heat-induced conformational change and gelation properties as a function of pH, and of addition of *N*-ethylmaleimide (NEM) or dithiolthreithol (DTT).

2. Materials and methods

2.1. Solutions

β-Lactoglobulin (β-lg) concentrates prepared by ion-exchange chromatography (IEC) from acid whey were supplied by the Compagnie Laitière Européenne, Condé/Vire, France. They contained 87.5% (dry matter basis) proteins of which about 63% were β-lg, 7% α-lactalbumin (α-la), 7.3% salts and less than 1% fat (J. Moulin, personal communication). The powdered samples were dispersed in distilled water (10% w/w) without further purification. The final pH was attained by addition of 6 M NaOH or HCl, and the protein concentration was determined by the method of Lowry et al. [15] with bovine serum albumin (BSA) as a standard.

The NEM solutions were prepared by dissolving NEM in ethanol according to Zirbel and Kinsella [16]. The solution at pH 7 was heated at 100°C for 30 min and distilled water was slowly added to bring the final concentration to 32 mM NEM in 2% ethanol. β-Lg concentrate was added to produce 8.5% protein concentration in 0–20 mM NEM solutions after stirring for 30 min and cooled to 20°C.

DTT was added to β-lg solution (8.5% proteins) in the concentration range between 0 and 32 mM. This protein solution was stirred for 30 min and then the pH was readjusted to 7 [16].

2.2. Solubility and turbidity

The turbidity of the protein solutions (4.25%, pH from 1.5 to 8) was measured with a Perkin-Elmer spectrophotometer (Lambda 3) at 600 nm using distilled water as reference. After centrifugation at 15 000 g for 15 min, the protein content of the supernatant was determined by the method of Lowry et al. [15] and the solubility was expressed in % of total protein concentration.

2.3. Determination of total SH groups as a function of heat treatment

The samples of β-lg solution were heated at 80°C for 30 min. β-Lg gels or heated solutions were solubilized in 0.086 M Tris–0.09 M glycine–4 mM ethylenediamine tetraacetic disodium salt (Na_2EDTA) at pH 8.0 (standard buffer) and containing 8 M urea and 0.5% SDS [10]. The protein solutions were adjusted to 0.1%, homogenized, and centrifuged at 3200 g for 15 min. The total SH groups were determined with DTNB by the method of Beveridge et al. [17] according to Ellman [18]. To a 4 ml aliquot of the protein supernatant was added 0.04 ml of Ellman's reagent solution (40 mg of DTNB in 10 ml of standard buffer) at zero time. The absorbance (A) was read at 412 nm against a reagent blank. The total concentration of SH groups was calculated according to the equation

$$[SH] = 73.53AD/C \quad (1)$$

where D is the dilution factor and C the protein concentration [17].

2.4. Heat denaturation properties

2.4.1. Thermodynamic parameters

The thermal behaviour of the protein solutions was determined by differential scanning calorimetry (Perkin-Elmer DSC-7). Calibration of temperature, enthalpy change and thermal resistance R of the apparatus was carried out by analysing the calorimetric trace of indium melting at 10°C min^{-1} heating rate. Stainless steel pans (75 μl volume and 0.16 J $°C^{-1}$ heat capacity) were used. The reference pan contained heat-denatured β-lg [19]. A scan rate ranging from 2.5 to 12.5°C min^{-1} was selected to study the pH effects along the thermal history

$$20°C\ (1\ min) \xrightarrow[\text{first heating}]{dT/dt} 105°C\ (0\ min) \xrightarrow[\text{cooling}]{250°C\ min^{-1}} 20°C\ (4\ min) \xrightarrow[\text{second heating}]{dT/dt} 105°C$$

The sample temperature, which was assumed to be uniform in the pan, was deduced from the programmed temperature T according to [20]

$$T_{\text{sample}} = T - (\mathrm{d}T/\mathrm{d}t)RmC_p \tag{2}$$

where $\mathrm{d}T/\mathrm{d}t$ (°C s^{-1}) is the scan rate, R (40°C W^{-1}) the thermal resistance, and mC_p (0.16 J $°C^{-1}$) the heat capacity of the sample (about 40 mg) before denaturation. The peak temperature (T_p) corresponding to maximum heat flow, and the apparent enthalpy change (ΔH_{app}) accompanying the heat-induced process were constant within ± 0.25°C and ± 15 kJ mol^{-1}, respectively.

The onset denaturation temperature T_s was determined from the increasing edge of the heat flow observed at 5°C min^{-1}. The denaturation temperature T_d was determined by extrapolating the peak temperatures T_p, obtained at various scan rates, to 0.1°C min^{-1}.

The reversibility of the denaturation process was checked with a second heating run. The ratio of the apparent enthalpy changes ($\Delta H_2/\Delta H_1$) between the second and first heating was taken as the reversibility degree of the denaturation process.

2.4.2. Kinetic parameters

Kinetic parameters were determined by the method of Borchardt and Daniels [21] using Perkin-Elmer TAS7 kinetic software and the equation

$$\ln(\mathrm{d}\alpha/\mathrm{d}t) = \ln Z - E\alpha/RT + n(\ln(1-\alpha)) \tag{3}$$

where $\mathrm{d}\alpha/\mathrm{d}t$ is the reaction rate of denaturation, α the progress of the reaction, $\ln Z$ the pre-exponential factor (min^{-1}), R the gas constant (8.31 J mol^{-1} K^{-1}), n the reaction order, and E_a the activation energy (kJ mol^{-1}). The half-life time ($t_{1/2}$) of the denaturation process at a given temperature was accordingly calculated and verified with the methodology described elsewhere [19].

2.5. Heat gelation properties

2.5.1. Isothermal test

Aliquots of 1 ml of β-lg solution were heated in covered test tubes by immersion in a water bath at 80°C. The solutions were inspected for gelation at 30 s intervals. Gelation was assumed to have occurred when the test tube could be inverted without loss of the contents, after cooling to 4°C.

2.5.2. Scanning test

The rheological properties of heat-induced gelation were studied with a compression machine (Instron 1121) interfaced with an HP 3421 computer (Fig. 1). The protein samples were placed in the double container (8 cm in height and 8.4 cm in diameter), which were connected to a programming water bath. It was covered with a fine layer of oil to avoid evaporation. The piston (29 mm diameter) was set to contact the solution so that the interfacial forces were just balanced by the buoyancy. The compression cycles (1.75%, 10 mm min^{-1} crosshead speed) started at 40°C, then the solution was heated to 90°C at 0.1°C min^{-1}. The force exerted on

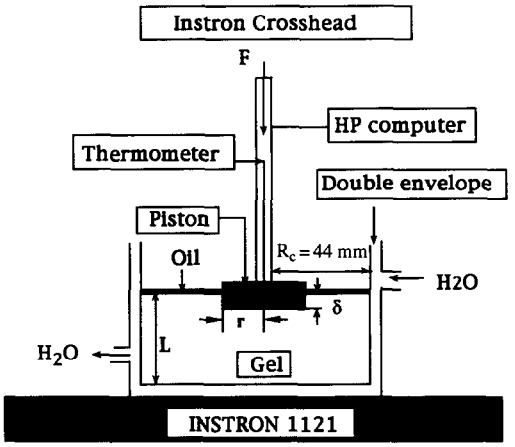

Fig. 1. Experimental arrangement of Instron 1121 used for the heat-induced gelation of β-lg solution.

the piston was converted into the Young's modulus according to Oakenfull et al. [22] and using Eq. (4) for non-semi-infinite conditions

$$E_{app} = fFL/2\pi r^2\delta \quad (4)$$

where E_{app} is the apparent Young's modulus (N m^{-2}), f the correction factor ($f = 0.47$), F the force exerted on the piston, L the solution height, r the radius of the piston (14.5 mm) and δ the amplitude of the piston displacement (0.7 mm).

The temperatures of the water bath and of the sample were controlled by a thermocouple connected to an HP 3421 A data acquisition/control unit. The temperature of the sample measured below the piston and the force exerted on the piston were recorded in 1 min steps during the heat treatment.

3. Results and discussion

3.1. Turbidity and solubility

For 4.25% protein concentration, the minimum solubility was observed at pH between 4.8 and 5.2, where the turbidity was maximum (Fig. 2). The electrostatic repulsion being minimum in this pH range, the precipitation reactions were favored. They therefore produced a decrease in solubility and an increase in turbidity, as observed visually. In the pH $\geq$ 6.0 or pH $\leq$ 3.5 ranges, because electrostatic repulsion predominates, the solutions were clear [7,23].

3.2. Denaturation parameters

Fig. 3 shows the DSC traces at 5°C min^{-1} (7.5°C min^{-1} for pH 8) obtained from solutions of various pH. The parameters of denaturation are reported in Table 1.

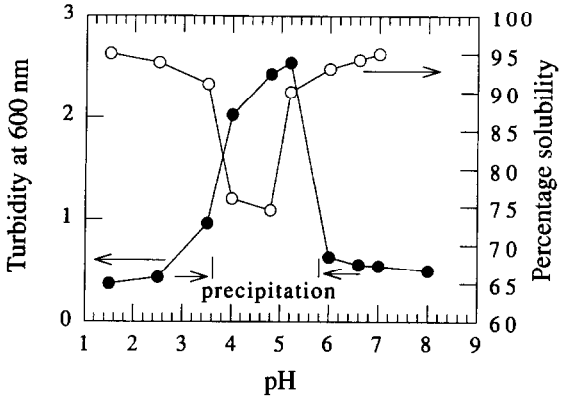

Fig. 2. Turbidity (●) and solubility (○) variation as a function of pH (4.25% protein).

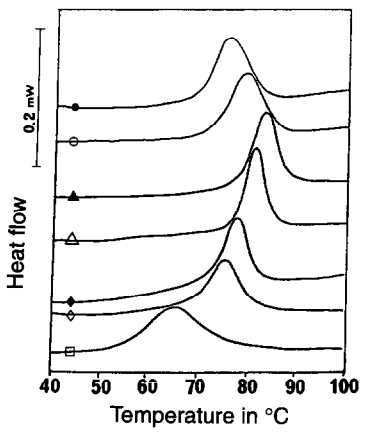

Fig. 3. Example of thermograms of β-lg samples at different pH: 40 mg, 5°C min^{-1} for pH 1.5–6.6 and 7.5°C min^{-1} at pH 8.0, heating from 40 to 105°C. Symbols: ●, pH 1.5; ○, pH 2.5; ▲, pH 3.5; △, pH 4.8; ◆, pH 6.0; ◇, pH 6.6; and □, pH 8.0.

At pH 4.8, the peak was rather narrow, as reflected by its half-width value ($\Delta T_{1/2}$). On both sides of the pH range investigated, $\Delta T_{1/2}$ was higher. The $\Delta T_{1/2}$ value is an indication of the degree of cooperativity of the denaturation process [24]; accordingly, our experimental data indicated that the cooperativity of the denaturation was dependent on pH and was maximum at about the isoelectric point where the net charge is minimum. DSC measurements on β-lg solutions obtained from ultrafiltration allowed Relkin and Launay [19] to suggest that the cooperativity at pH 3.2 seems to depend on protein concentration. Their $\Delta T_{1/2}$ value for solution (pH 3.2, 8.8% concentration) was equal to 9°C which meant less cooperativity than for β-lg concentrate obtained from IEC ($\Delta T_{1/2} = 5.7$°C for 8.2% protein concentration and pH 3.5). This difference in cooperativity could be due to higher salt content (7.3%) in the case of IEC concentrate.

Table 1
Thermodynamic and kinetic parameters of heat denaturation

Buffer	Conc./%	pH	β/ (°C min^{-1})	T_p [a]/ °C	T_d [b]/ °C	$\Delta T_{1/2}$/ °C	ΔH_{app}/ (kJ mol^{-1})	n	E_a/ (kJ mol^{-1})	Ref.
SMUF [c]	6.2	6.7	10	76	72.8	–	227	–	–	[6]
0.07 M Phosphate	10	6.75		–	70.4	–	230	–	–	[25]
Distilled water	4.4	6.0	10	82	78	–	269	–	–	[2]
Distilled water	10	3.5	5	83	–	–	238	–	–	[1]
		6.0		75	–	–	221	–	–	
		6.5		74	–	–	205	–	–	
		7.0		72	–	–	190	–	–	
		7.5		68.5	–	–	170	–	–	
		8.5		65.5	–	–	150	–	–	
0.05 M Potassium biphthalate	10	4.0	10	83	–	–	306	2.7	497.4	[5]
0.05 M Potassium phosphate	10	6.0		85.5	–	–	356	2.3	530.9	
		7.0		84	–	–	362	1.9	585.2	
		8.0		81.5	–	–	357	2.4	526.7	
SMUF	5.0	6.0	10	78	75.1	–	184	–	–	[27]
		7.0	10	76	66.0	–	285	–	–	
Distilled water	3.5	3.2	5	89.7	88.1	10.6	307	2	392	[19]
	8.8	3.2		88.9	86.5	9.0	269	2	465	
	10.8	3.2		86.9	85.4	7.5	285	2	497	
	12.1	3.2		86.9	–	7.3	283	2	497	
	24	3.2		84.55	83.4	7.2	238	2	547	
HCl–glycine	1.1	3.5	1	88.55	–	–	214	–	–	[26]
	1.1	2.3	1	81.3	–	–	205	–	–	
Distilled water	3.5	3.5		–	87.6	–	255	–	–	[20]
		2.3		–	78.2	–	185	–	–	
		1.7		–	74.1	–	165	–	–	
Distilled water	8.2	1.5	5	74.6	73.6	7.8	266	2	492	This
	8.2	2.5		77.5	76.9	8.1	272	2	457	work
	8.2	3.5		82.1	81.7	5.7	286	2	615	
	8.1	4.8		80.5	79.1	5.0	252	2	774	
	8.4	6.0		77.2	76.1	6.0	260	1	358	
	8.6	6.6		74.7	73.5	7.3	262	1	262	
	8.5	8.0		65.3	64.6	12.2	187	2	272	

[a] T_p, peak temperature at mentioned scan rate. [b] T_d, temperature extrapolated to 0°C min^{-1}. [c] SMUF, simulated milk ultrafiltrate.

The heat denaturation temperature (estimated by extrapolation to 0.1°C min^{-1} heating rate of the peak temperatures observed at various scan rates ranging from 2.5 to 12.5°C min^{-1}) was maximum for pH 3.5. The enthalpy change seemed to be constant for $1.5 < pH < 6.6$, and drastically decreased at pH 8.0. Our results confirmed those of Hegg [2] who observed a maximum thermal stability between pH 3 and 5. The denaturation temperature at pH 6.6 was somewhat higher than the values reported by De Wit and Swinkels [25] and Rüegg et al. [6], while at pH 3.5 and 6.0, we observed T_d values somewhat lower than those found by Schwarz [26] and Paulsson et al. [27]. De Wit and Klarenbeek [1] also compared endotherms of β-lg denaturation in the pH range 3.5–8.5. As pH increased from 3.5 to 8.5, the peak temperature decreased, indicating a decrease in thermal stability (Table 1). Our data agreed with their observation for both T_p and ΔH_{app}. In contrast, Park and Lund [5] found that the thermal stability was maximum at $pH \approx 6.0$, with ΔH_{app} independent of pH.

The denaturation process seemed to be partly reversible for a scan rate as low as 5°C min^{-1} for pH 1.5 and 2.5. For scan rates between 5 and 12.5°C min^{-1}, the degree of reversibility increased linearly with the scan rate (Fig. 4). For pH above 2.5, the trace of the second heating run did not show an endothermic peak. Relkin et al. [20] observed for β-lg purchased from Sigma the same behaviour of reversibility and tentatively explained the effect of the scan rate by taking into account that the slower the scanning rate, the longer the time for intermolecular SH/S–S interchange reactions to complete. The denaturation process would therefore become irreversible because of formation of covalent linkages. The other hypothesis proposed by these authors was formation of soluble aggregates during the first scan; the peak observed during the second heating run would accordingly correspond to their melting. The measurement of free SH content as a function of pH (Fig. 5) indicating a slight decrease in free thiol groups with heating time at 80°C (acidic

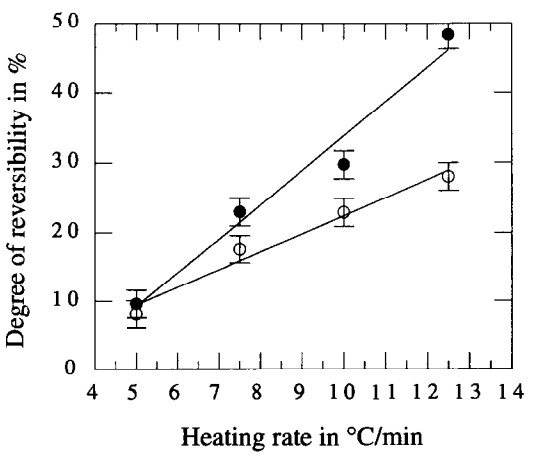

Fig. 4. Degree of reversibility versus heating rate at pH 1.5 (●) and 2.5 (○).

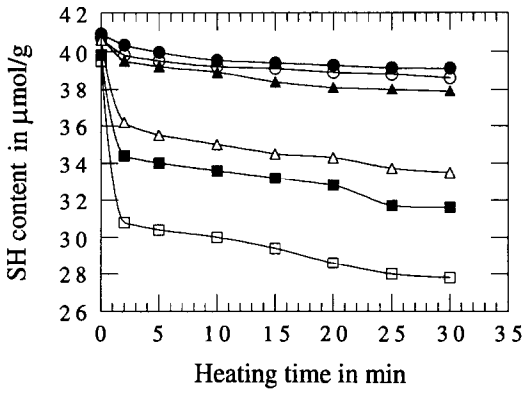

Fig. 5. The free thiol group content versus heating time at 80°C for different pH: ●, 2.5; ○, 3.5; ▲, 4.8; △, 6.0; ■, 6.6; and □, 8.0.

pH) supported the first hypothesis. In favour of the second hypothesis, Harwalkar's [28,29] results should be mentioned: this author ovserved formation of irreversible precipitation by heating (90°C, 20–30 min) purified β-lg solution (1%) at pH 4.5 and 6.5, and soluble gel at pH 2.5; after readjusting the system at pH 4.5, at least two molecular species coexisted in solution. These results were compatible with ours: reversibility was observed at pH 1.5 and 2.5, whereas no reversibility occurred at pH 3.5 in solutions of β-lg prepared with IEC which showed an opposite behaviour with respect to solutions of either ultrafiltration-fractionated β-lg or Sigma β-lg (three times crystallized).

3.2.1. Effect of NEM

As discussed above, the SH groups played an important role in the denaturation of β-lg at basic pH. To check the effect of a blocking intermolecular disulphide bond, we added NEM (1–20 mM) to 8.5% protein solution (about 2.5 mM β-lg) at pH 7. For NEM concentration up to 2.5 mM, the peak temperature increased by about 0.5°C and remained constant for higher concentrations (Table 2). This agreed with the hypothesis that 2.5 mM NEM was sufficient to react with all the free SH groups of β-lg. In contrast, the apparent enthalpy change decreased with

Table 2
Thermodynamic parameters of heat denaturation process (8.5%, pH 7.0, 10°C min^{-1}) in the presence of NEM (0–20 mM) or DTT (0–32 mM)

NEM/ mM	T_p/ °C	ΔH_{app}/ (kJ mol^{-1})	DTT/ mM	T_p/ °C	ΔH_{app}/ (kJ mol^{-1})
0	71.8	237	0	71.8	237
2.5	72.3	210	4	71.3	189
5.0	72.4	210	8	69.8	159
10	72.3	200	16	58.5	58.0
20	72.2	189	32	53.2	67.0

increasing NEM concentration. This experimental result was due to the presence of the ethanol used for the preparation of NEM solution (data not shown).

3.2.2. Effect of addition of DDT

To determine the effects of reduction of disulphide bonds on T_p and ΔH_{app}, DTT (0–32 mM) was added to the protein solution. We observed that T_p and ΔH_{app} drastically decreased (Table 2).

3.3. Kinetic parameters

The apparent reaction order was equal to 2 for $1.5 < pH < 4.8$ and at pH 8.0, while it was equal to about 1 at pH 6 and 6.6. The activation energy was maximum at pH 4.8 (774 kJ mol^{-1}) and it decreased on both sides of the isoelectric point, pH_i. In contrast, Park and Lund [5] described denaturation of β-lg between pH 6.0 and 9.0 either as a second- or third-order process at $6.0 < pH < 9.0$ or at $pH < 5.0$, respectively; the relevant activation energy seemed practically constant within the range $4 < pH < 8$ (Table 1). Harwalkar [29] studied the denaturation process by specific optical rotation and loss of solubility. The thermal denaturation of β-lg (1%, pH 2.5) obeyed either a pseudo-first-order or two consecutive first-order reaction kinetics. Relkin and Launay [19] showed that the thermal behaviour of β-lg (3.5%–24% concentration and pH 3.2) was the result of both a denaturation process (endothermic) and interchain interactions (exothermic). A second-order reaction mechanism was proposed for the protein denaturation at pH 3.2, whatever the protein concentration investigated. In the present study, we observed that the denaturation of β-lg could be described as a second-order reaction at $pH < pH_i$ and as a first-order reaction at $pH \geq 6$. More recently, just when this work was finished, Gotham et al. [30] reported that at pH 6.6 intramolecular disulphide intermediates were responsible for irreversibility in thermal behaviour of β-lg solutions (6%–13% concentration). From the effect of heating rate, these authors also determined kinetic parameters using Kissinger's relationship between the denaturation temperature, protein concentration and heating rate, ranging from 0.3 to 20°C min^{-1}. From their results, ΔH_{app} appeared independent of scan rate only in the range 3–20°C min^{-1}. In the same range of temperature at pH 6.6, our experimental result ($E_a = 297$ kJ mol^{-1} and $n = 1$) agreed with theirs ($E_a = 260$ kJ mol^{-1} and $n = 1$). For $pH > 5$ the thermal behaviour corresponding to the endothermic peak was irreversible; the kinetic parameters were verified via the half-life time of the reaction at a constant temperature using the methodology described elsewhere [19]. Table 3 shows that the progress of the reaction was about 50% after a heat treatment of the sample lasting as long as the half-life time calculated for the temperature considered. At pH 1.5 and 2.5, the reversibility of the observed behaviour corresponded to too low a degree of reaction when calculated as above.

3.4. Gelation properties

Figs. 6a and 6b show the evolution of Young's modulus at different pH as a function of the heating time or of temperature from 40 to 90°C at 0.1°C min^{-1}

Table 3
Half-life of the reaction of heat denaturation and verification of the kinetic parameters (8.5%, 5°C min^{-1} for pH 1.5–6.6 and 7.5°C min^{-1} for pH 8.0)

pH	1.5	2.5	3.5	4.8	6.0	6.6	8.0
ΔH_1 in kJ mol^{-1}	266	272	286	252	260	262	187
$T_{isotherm}$ in °C	70	75	80	75	70	70	60
$t_{1/2}$ in min^{-1}	3.7	1.6	0.82	11.6	4.6	2.7	2.6
ΔH_2 in kJ mol^{-1}	97.1	108.8	146.7	128.5	125.1	141.0	101.0
Percentage reaction[a]	36.5	40.0	51.3	51	48.1	53.8	54.0

[a] Percentage reaction = $100(\Delta H_1 - \Delta H_2)$. $\Delta H_1/\Delta H_1$ is calculated from the first scan rate and ΔH_2 after isothermal treatment for $t_{1/2}$ [19].

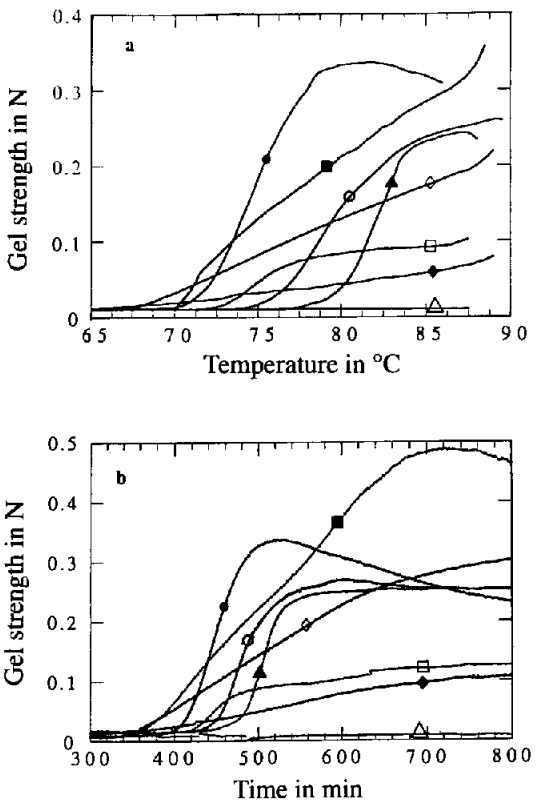

Fig. 6. Evolution of the gel strength as a function of (a) temperature and (b) heating time from 40 to 90°C at 0.1°C min^{-1}; pH symbols: ●, 1.5; ○, 2.5; ▲, 3.5; △, 4.8; □, 6.0; ■, 6.6; ◇, 7.0; and ◆, 8.0.

heating rate, respectively. The temperature at which the apparent Young's modulus began to deviate from the baseline was defined as the onset gelation temperature [31].

According to Ferry [9], the gelation mechanism of globular proteins could be modelled according to the scheme

$$x(P_n) \overset{\text{heating}}{\longleftrightarrow} x(P_d) \overset{\text{heating}}{\longrightarrow} (P_d)_x$$

where P_n is the native protein, and P_d the denatured protein.

At pH 4.8, β-lg water solution did not develop any gel structure (but a coagulum was observed at about 50°C), while the solution of the protein in citrate buffer induced formation of a strongly inhomogeneous coagulum about 80°C [13]. Using a dynamic rheological method (shear strain amplitude, 15%), Paulsson et al. [14] observed moderate gel texture even at the isoelectric point of β-lg solution (5% in SMUF) and Gault et al. [32] determined the Young's modulus (12 kPa) for 10% WPC in deionized water solution using a compression machine (deformation, 2%). The difference between our results and those obtained by the other authors was probably caused either by the different buffers used or by protein composition differences.

The onset gelation temperatures were largely dependent on pH (Fig. 7). They seemed to be lower than for denaturation temperatures T_d and somewhat higher than for the onset denaturation temperatures T_s. The minimum DSC heating rate in this work was 2.5°C min^{-1}, while for the gelation, a rate of 0.1°C min^{-1} was selected. Because heat denaturation of globular proteins cannot be reliably determined at very low heating rate with our DSC equipment (small volume of pans), we extrapolated peak temperatures observed at various scan rates (from 2.5 to 12.5°C min^{-1}) to 0.1°C min^{-1} to assess T_d. If this considered T_d is referred to as the starting point of heat denaturation it becomes impossible to state whether or not the onset of denaturation precedes gelation, except in the case of solutions at pH $\geq$ 7.0 where $T_g - T_s \geq 6$°C.

Paulsson and Dejmek [33] found that the onset temperature of gelation was somewhat higher than the peak temperature of denaturation and slightly dependent on pH (3°C between pH 4.5 and 7.5); the same authors [27] reported a large variation in the peak temperature of denaturation (17°C between pH 4 and 8).

Fig. 8 shows that Young's modulus was also dependent on pH. The transparence changes observed were correlated with the modification in the equilibrium between

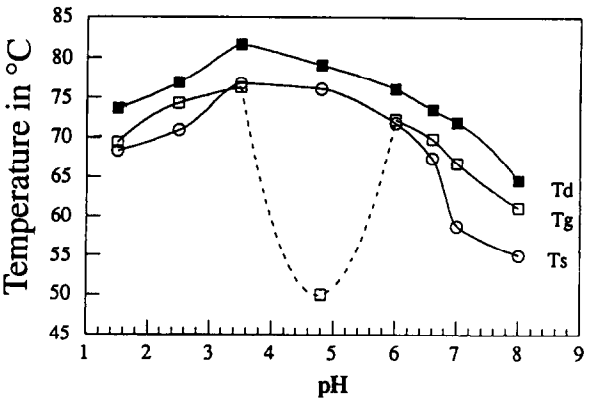

Fig. 7. Variation curves of gelation (T_g), denaturation (T_d) and onset denaturation temperatures (T_s) versus pH.

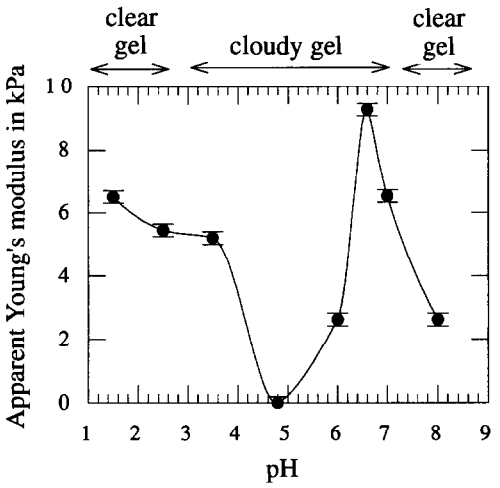

Fig. 8. Maximum apparent Young's modulus as a function of pH.

repulsive and attractive forces for $pH < pH_i$ and $pH > pH_i$. At pH between 3.5 and 7.0 the gels were opaque, and at extreme pH they were clear. Under acidic conditions ($pH < pH_i$), once the Young's modulus started to deviate from the baseline, the rising rate was high until completion of the gelation process. E_{max} measured at the beginning of the plateau seemed to be not significantly dependent on pH ($E_{max} = 6.5$, 5.5 and 5.2 kPa for pH 1.5, 2.5 and 3.5, respectively). In contrast, at basic pH, the rising rate of the Young's modulus was moderate and E_{max} was more dependent on pH: $E_{max} = 9.3$ and 6.7 kPa at pH 6.6 and 7, respectively. At pH 6.0 and 8, E_{max} was lower (2.6 kPa). Near the isoelectric point ($pI = 5.2$), the protein solutions underwent coagulation rather than gel formation; when heated in distilled water, due to excessive repulsion at pH 8, the denatured protein molecules were apparently prevented from associating to form a strong network. Mulvihill and Kinsella [4] also observed an increase in viscosity of β-lg solutions (10%) when heated at pH 8: however self-supporting gel was not formed unless salts, such as NaCl or $CaCl_2$, were added. This result also agrees with our previous observations on β-lg concentrate (7.3% salts) dispersed in phosphate (pH 6) and glycine (pH 8) buffers, the highest plateau of the Young's modulus being observed for pH 8 [13].

Generally, the forces involved in the structure of native protein in solution are also involved in network formation during gelation [34]. The maximum apparent Young's modulus at pH about 6.6 may reflect the fact that the optimal balance between attractive and repulsive forces is reached at this pH, and a more ordered gel network is formed, which in turn enhances the gel tightness. At acidic pH, the lower reactivity of the free thiol groups (Fig. 5) in this range of pH cannot contribute to the formation of the network and the forces involved are probably hydrogen, ionic and/or hydrophobic interactions. In contrast, at the higher pH, the free SH groups become highly reactive, and SH/S–S interchange probably causes

the formation of covalent cross-links, which tighten the gel network. Although we could not exclude the involvement of hydrogen bonds and of electrostatic or intermolecular hydrophobic interactions, the data reported in Fig. 5 confirmed the hypothesis that the SH groups were involved in the gelation process for pH > 6. Before the heat treatment, the SH content slightly decreased with increasing pH, possibly because of the partial oxidation of the SH during the preparation of the sample at basic pH. In the case of heat treatment at 80°C for pH 2.5–4.8, the SH content slightly decreased during the first 2 min, then remaining constant up to 60 min. This indicated lower reactivity of the SH groups in acidic conditions. However, at pH > 6.0, the decrease in SH groups was enhanced by heat treatment. The formation of the intermolecular bonds resulting from the SH/S–S interchange reaction supported by the higher reactivity of SH may also be responsible for the decrease in T_d and ΔH_{app} (Fig. 5) and for the irreversibility of the heat denaturation at basic pH. These results confirmed the hypothesis that the thermal denaturation and gelation in acidic conditions would imply fewer reactive free thiol groups than at alkaline pH.

From the kinetic parameters shown in Table 1, we calculated the denaturation degree at 90°C using the isothermal methodology [19]. We observed that 100% of β-lg was denatured after 10 min at 90°C at any pH investigated. The ratio between the value of Young's modulus measured at 90°C $E_{(90°C)}$ and that measured at the plateau E_{max} was calculated. The probability of gel formation at 90°C evaluated from the ratio $E_{(90°C)}/E_{max}$ seemed to be equal to unity in cases where pH ≤ 6, while it was about 50% at pH > 6. From the loss rate of the SH groups at 80°C as a function of pH (Fig. 5), and from the kinetics of the gelation process reported in Fig. 6, we could argue that the heat-set gels were more affected by SH oxidation reactions at basic pH than at acid pH: at acid pH, free SH groups decreased after 30 min heat treatment at 80°C by less than 10%; at basic pH, the decrease was about 30% after a few minutes of heat treatment and the free SH oxidation reactions seemed to continue after 30 min. The kinetics of the denaturation process as evaluated from the half-life time of the reaction at 70°C was faster at pH 6.6 ($t_{1/2}$ = 2.7 min) than at pH 6.0 ($t_{1/2}$ = 4.6 min). The gel strength (Fig. 6a) attained a flat trend at about 80°C at pH 6.0 and the gelation process seemed still in progress after several hours at 90°C (Fig. 6b). At pH 1.5 and 2.5, the thermograms observed on reheating and the isothermal heat treatment at 70 and 75°C, respectively, indicated some degree of reversibility of the denaturation process (Table 3); the gel strength reached the plateau at about 78 and 85°C for pH 1.5 and 2.5, respectively. From these observations we could conclude that the decrease in the free SH concentration with the progress of the heat treatment was responsible for the reduced thermoreversibility of the protein solution at pH > 6. The kinetics of the denaturation process was slower at acid pH than at basic pH, whereas the kinetics of the gelation process seemed to follow an opposite trend.

The gelling time of β-lg solutions (pH 7) in the presence of NEM increased as the SH groups were progressively blocked (Fig. 9). No difference in the opacity of the gels formed in the presence of NEM was observed. Similar results have been observed for the gelling time of whey protein in the presence of NEM [3,11].

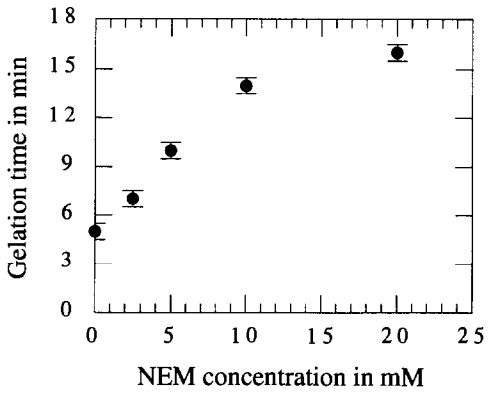

Fig. 9. Gelation time at 80°C in the presence of NEM (pH 7.0, 8.5%).

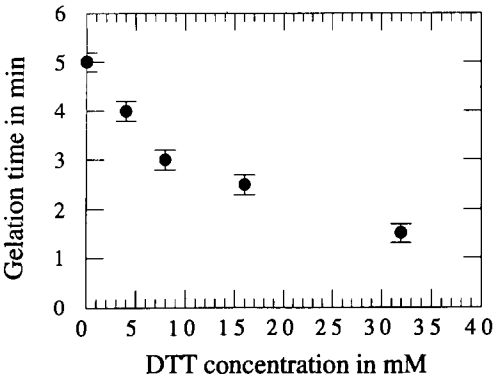

Fig. 10. Gelation time at 80°C in the presence of DTT (pH 7.0, 8.5%).

Because of binding to NEM, fewer SH groups became available for the SH/S–S interchange, and therefore, fewer cross links were formed. As a result, the gelling time was delayed. These results agreed with published data which indicate that thiol groups are involved in the gelation of β-lg at pH 7.

The gelling time seemed to follow the opposite trend with DTT added to protein solutions at pH 7 (Fig. 10). As DTT reduced disulphide bonds, more free thiol groups were available for more SH/S–S interchange reactions, implying an increase in the number of linkages between the other proteins (sample containing $\approx 7\%$ α-lactalbumin) in the gel matrix [35]. Thus, this phenomenon could enhance the protein denaturation at lower temperature and therefore results in a lower gelling time.

4. Conclusions

From the above results, we may conclude that the thermal denaturation of β-lg is pH-dependent. The low reactivity of SH groups and the partial reversibility of

heat denaturation at acidic pH show that β-lg is more thermally resistant than at basic pH.

The reactivity of the SH groups upon heat treatment is enhanced at pH 6.6, where possible SH/S–S interchange reactions favour the irreversibility of the denaturation process and gel network formation (maximum strength at 90°C).

The onset gelation temperature seems to be higher than the onset denaturation temperature, whatever the pH range, except at pH 3.5 and 6 where they are of the same order. At acidic pH, the gel formation was completed at a temperature corresponding to 100% denatured proteins (Fig. 6a), while at basic pH, the network formation continued although 100% of the denaturation of β-lg was reached.

These results provide further information about the protein structure development upon thermal gelation, in relation to the denaturation process.

Acknowledgments

Financial support from Le Ministère Français de l'Agriculture et de la Pêche is gratefully acknowledged (DGER "Formation par la Recherche", under grant, R 91/15).

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