Estimation of Lysine Damage in Heated Whey Proteins by Furosine Determinations in Conjunction with the Digestion Cell Technique

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Lysine damage in whey proteins heated in the presence of lactose was estimated by the digestion cell technique in conjunction with furosine determinations. The digestion cell technique consists of an in vitro digestion with pepsin and pancreatin under specific conditions of pH and temperature with continuous dialysis of the digestion products. Combinations of temperature (75, 100, 121 °C), time (50, 500, 5000 s), and a_w (0.3, 0.5, 0.7, 0.97) were tested with respect to lysine damage. Heating whey at 121 °C for 5000 s destroyed over 50% of lysine (advanced Maillard reaction) at a_w 0.3, 0.5, or 0.7. At a_w 0.97, much less damage occurred. Furosine is generated from the hydrolysis of the Amadori compound upon acid hydrolysis and can be measured on the aminograms. In the advanced Maillard reaction, no furosine is produced, leading to an overestimation of chemically available lysine. Furosine determination in digestion products allows the estimation of inactivated lysine and, hence, permits quantification of enzymatically available lysine (digestible lysine) at any stage of the Maillard reaction. After whey was heated at 75, 100, and 121 °C for 5000 s in dry form, 75, 40, and 45% lysine, respectively, were chemically available, whereas 62, 25, and 20% lysine, respectively, were enzymatically available. At a_w 0.97, lysine was 93% available chemically and only 76% available enzymatically. Such results cannot be obtained by sole use of any one chemical method of available lysine determination.

Thermal processing or even storage can have deleterious effects on the nutritional quality of proteins, mainly as a result of Maillard reaction (Hurrell and Carpenter, 1981). The nutritional changes induced by this series of reactions include decreased protein digestibility, reduced availability of lysine (Lys) as well as of several other essential amino acids, and possible formation of growth inhibitors or toxic compounds (Satterlee and Chang, 1982; Gumbmann et al., 1983).

Among the numerous methods proposed to estimate the extent of protein nutritional damage, the in vitro digestion technique developed by Gauthier et al. (1982) and Savoie and Gauthier (1986) offers promising advantages. After the stepwise proteolysis of a food sample with pepsin and pancreatin with simultaneous dialysis, the digestion products are analyzed for their amino acid content, thus giving information on the digestibility of all amino acids. This technique was successfully used to study the impact of alkaline treatments on the enzymatic release of amino acids (Vachon et al., 1983) and the effect of phytic acid on the digestibility of rapeseed protein and amino acids (Serraino et al., 1985) as well as the effect of different heat treatments on delactosed whey proteins (Desrosiers et al., 1987a,b).

In in vitro dialysates, Lys is present both in the free form and in oligopeptides having a molecular weight smaller than 1000 (Gauthier et al., 1986). Subsequent acid hydrolysis performed for the determination of amino acid content of this material can regenerate, when present, some of the Lys inactivated in the Amadori compound (Finot et al., 1981). Classical chemical methods to measure reactive Lys in processed foods, such as the 1-fluoro-2,4-dinitrobenzene method, are not applicable to the determination of reactive Lys in the free form or in small peptides, since the chemical reagent used is likely to react with both α- and ϵ-amino groups of free Lys (Carpenter and Booth, 1973). The complexation of α-NH_2 group of Lys with copper was not found to be fully efficient (Desrosiers et al., 1988). In contrast, the furosine (FUR) method (Finot and Mauron, 1972) should allow the quantitative estimation of Lys in the free form or in small peptides, as well as the proportion of Lys inactivated in the Amadori compound. That method was based on the observation that in heated milk proteins, ϵ-deoxylactulosyllysine (lactulosyl-Lys) is the only biologically unavailable molecule formed between Lys and lactose (Bujard and Finot, 1978). According to these authors, lactulosyl-Lys does not regenerate Lys upon enzymatic hydrolysis. However, upon acid hydrolysis in 6 N HCl, free N^{ϵ}-deoxyketosyllysine derivatives of glucose and lactose produced 20.3% FUR and 10.4 pyridosine and regenerated 49.5% of the Lys originally present in the compound (Finot and Mauron, 1972). Furosine elutes after arginine on aminograms and can easily be measured. An equation has been proposed, which compensates for the level of FUR formation and for Lys regeneration from the Amadori compounds bound to proteins (Finot, 1973; Bujard and Finot, 1978). This method allows the quantitative determination of reactive Lys (Lys residue with its ϵ-NH_2 group free) and of inactivated Lys either as its Amadori compound (early Maillard reaction) or as other Lys derivatives resulting from the advanced Maillard reaction, which do not regenerate Lys upon acid hydrolysis (destroyed Lys). The FUR method has been used as a measure of heat intensity in ultra-heat-treated (UHT) milks (Erbersdobler et al., 1987).

The combination of the FUR method (Bujard and Finot, 1978) with the digestion cell technique (Savoie and Gauthier, 1986) should allow the estimation of the impact of heat treatments on the different forms of Lys. Therefore, the purpose of this work was to estimate the relative proportion of reactive Lys, Lys inactivated as lactulosyl-Lys, and destroyed Lys in whey proteins subjected to various heat treatments in the presence of lactose, some of which being representative of those used in industry.

MATERIALS AND METHODS

Sample Preparation. Whey protein concentrate was obtained from a dairy plant (Laiterie Etchemin, PQ), where it was prepared by ultrafiltration of cheddar cheese whey on Romicon's modular dairy system (PM_{50}) at 40 °C. That material was further diafiltered at Université Laval pilot plant (Ste-Foy, PQ) in order to remove most of the lactose according to the procedure described by Desrosiers et al. (1987b). After the fifth dilution, the lactose content reached 2.4 (±0.1%) on a dry weight basis (dwb) as determined by the phenol reaction (Gerhardt, 1981) and the

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proportion of protein in the sample was 81.1% (N × 6.38) dwb. The diafiltered retentate was freeze-dried (FTS Systems Inc., Stone Ridge, NY). A 500-g portion of freeze-dried diafiltered whey was added to an aqueous lactose solution (100 g of lactose/30 L of water) in order to make a 1:1 Lys to lactose molar ratio. The mixture was thoroughly stirred, freeze-dried, and kept in the freezer until utilization. This lactose concentration (18.7% dwb) represents about half that normally present in whole milk (37.7% dwb).

Portions of 125 g of freeze-dried whey samples were adjusted to the desired water activity (a_w) values (a_w 0.3, 0.5, 0.7, 0.97) as follows: Various aqueous sulfuric acid solutions of suitable concentration were put into the bottom of a desiccator and whey samples exposed to the vapor in order to provide an atmosphere of known relative humidity (Stokes and Robinson, 1949; Labuza, 1984). Water activity values were measured at 1-h intervals on a precalibrated hygrometer (Rotronic, ag Hygroskop DT, Kaymont Instrument Corp., Zurich), using 35, 50, 65, and 95% relative humidity calibration standards (Rotronic, Zurich), until two consecutive readings varied by less than 0.01 a_w unit (approximately 8 h). Moisture content of samples was determined by weight gain after equilibrium was reached. Whey samples were rapidly put into thermal death time (TDT) cans (6-cm i.d. × 1-cm depth) provided by Continental Can Co. of Canada Ltd. (Montreal, Qc) by portions of 3 g (a_w 0.3, 0.5, 0.7) or 10 mL (a_w 0.97), sealed, and labeled. Heat treatments were performed as described by Desrosiers et al. (1987b) at the desired temperatures (75, 100, 121 °C) and times (50, 500, 5000 s). Since the heat treatments were performed within the next 16 h after the equilibrium was reached, it is unlikely that variations in a_w values caused by un-steady-state conditions would significantly affect the digestibility values. Moreover, the a_w values of control (unheated) samples varied by 0.04 a_w unit only after 1 year of storage in the cans at −18 °C.

Digestion Cell Technique. Pepsin (EC 3.4.23.1, hog stomach mucosa, 1:60000), pancreatin (hog pancreas 5×), and thimerosal were purchased from ICN Pharmaceuticals, Life Science Group (Cleveland, OH), and sodium phosphate monobasic (ACS) from Fisher Scientific Co. (Montreal, Qc). Pepsin activity was 3152 units/mg of protein (Anson, 1938). (p-Tolylsulfonyl)-L-arginine methyl ester (TAME) and N-acetyl-L-tyrosine ethyl ester (ATEE) were used as substrates for the measurement of trypsin and chymotrypsin activities in pancreatin. Trypsin and chymotrypsin activities of pancreatin were 5.65 TAME units/mg of pancreatin (Hummel, 1959) and 4.57 ATEE units/mg of pancreatin (Schwert and Takenaka, 1955), respectively. Tubular dialysis membranes (Spectra/Por 6, 1000 molecular weight cutoff) were provided by Spectrum Medical Industries Inc. (Los Angeles CA). Upon receipt, those membranes were cut to the appropriate length and stored at 4 °C in a sodium benzoate solution for further use.

Forty milligrams of whey nitrogen (N × 6.38 = 255.2 mg of whey protein) were suspended in 16 mL of 0.1 N HCl (50 ppm thimerosal) in a flat-bottom glass tube, and the mixture was stirred magnetically for 5 min at 37 °C. The pH was adjusted to 1.9, the solution made up to 19 mL with deionized water, and 1 mL pepsin solution (1 mg/mL of 0.1 N HCl) added. After 30 min, the digestion was stopped with 0.5 mL of 1 N NaOH. The pH was adjusted to 7.5 and the volume made to 22 mL with deionized water. The protein mixture was poured into the dialysis membrane fitted on the inner compartment of the digestion cell, and the tube was rinsed with 2 mL of deionized water (Savoie and Gauthier, 1986; Gauthier et al., 1986). Digestion started with the addition of 1 mL of pancreatic enzymes (10 mg of pancreatin/mL) prepared in phosphate buffer (0.01 M, pH 7.5). Dialysates were collected over 6 h by the continuous circulation of the phosphate buffer (1.6 mL/min) by a peristaltic pump.

Total Lysine and Furosine Determination. Nitrogen content of the freeze-dried whey samples was measured by the Kjeldahl method, using a Kjel-Foss autoanalyzer (Model 16210; Foss Co., Denmark). Nitrogen contents of samples collected during digestion (dialysates) were determined by a micro-Kjeldahl technique (Technicon Auto Analyzer II continuous-flow analytical system; Technicon, Tarrytown, NY), using Method No. 329-74 W/B.

To each protein sample (4 mg of protein, N × 6.38) was added 2.0 mL of 6 N HCl in hydrolysis tubes (19-mm i.d. × 100-mm height; Pierce, Rockford, IL). An 80-μL sample of norleucine solution internal standard (10 μmol/mL) was added. For enzymatic dialysates, a volume containing 0.2 mg of N was evaporated (flash evaporation) to dryness at 40 °C. Samples were recuperated in 2.0 mL of 6 N HCl in hydrolysis tubes with 40 μL of norleucine.

The tubes were saturated with oxygen-free nitrogen, sealed, frozen in an acetone–liquid nitrogen bath (−80 °C), and allowed to thaw slowly under vacuum. This step was repeated twice, and hydrolysis was carried out in an oven (Hycel Thermal Block; Hycel Inc., Houston, TX) at 110 °C for 24 h. Acid hydrolysates were evaporated, suspended in 2.0 mL of NaS buffer (sample dilution buffer; Beckman Instruments, Palo Alto, CA), and filtered through membrane filter (0.22-μm filter type GS; Millipore Corp., Bedford, MA). Lysine and FUR were determined by ion-exchange chromatography (high-performance amino acid analyzer, System 6300; Beckman Instruments, Palo Alto, CA) according to Finot and Mauron (1972). Due to the difficulty of synthesizing FUR in an adequate state of purity, a standard is not available on the market. Furosine content was calculated from the surface area of FUR on chromatograms, taking Lys as a reference.

Chemical and Enzymatic Determinations of Available Lysine. In heated milk proteins, Lys can be present in three different forms: reactive Lys and Schiff's base, which are biologically available, and lactulosyl-Lys and destroyed Lys, which are biologically unavailable (Finot et al., 1981). Lysine is partially regenerated from lactulosyl-Lys upon acid hydrolysis, and a known proportion of FUR is produced (Finot and Mauron, 1972). The FUR method, by using the levels of Lys (total Lys) and of FUR after acid hydrolysis, allows the estimation of the level of Lys blocked as lactulosyl-Lys (early Maillard reaction) or in other forms that do not regenerate Lys. The latter forms, resulting from the advanced Maillard reaction, represent destroyed Lys, and are estimated by difference.

From the levels of Lys and FUR generated in hydrolyzed milk proteins, Finot et al. (1981) have derived three equations. In protein:

$$\text{Lys as lactulosyl-Lys} = 3.1 \times \text{FUR} \quad (1a)$$

This equation has been derived from the correlation between the level of unavailable Lys and the FUR content in heated milks, and it was shown that lactulosyl-Lys generated 32% FUR (1/3.1 FUR × 100) (Bujard and Finot, 1978). However, the proportion was found to be 20.3% FUR in the case of free deoxyketosyl-Lys (Finot and Mauron, 1972) and therefore in dialysates

$$\text{Lys as free deoxyketosyl-Lys} = 4.9 \times \text{FUR} \quad (1b)$$

and in protein

$$\text{reactive Lys} = \text{total Lys} - 1.24 \times \text{FUR} \quad (2a)$$

In heated milk, 40% of the Lys is regenerated from lactulosyl-Lys, assuming that all unavailable Lys is in the form of a deoxyketosyl derivative. Free deoxyketosyl-Lys (form most likely to be found in enzymatic dialysates) regenerates 49.5% of Lys (Finot and Mauron, 1972). Therefore, in dialysates

$$\text{reactive Lys} = \text{total Lys} - 2.4 \times \text{FUR} \quad (2b)$$

Finally, destroyed Lys, which results from advanced Maillard reaction, is obtained by difference and is calculated as follows:

$$\text{destroyed Lys} = \text{init Lys} - (\text{reactive Lys} + \text{Lys as lactulosyl-Lys}) \quad (3)$$

Initial Lys is the amount of Lys present in unheated whey proteins. No destroyed Lys can be found in dialysates.

From those equations, chemically available lysine values (ALV_{chem}) can be calculated in lactose-added heated whey proteins according to

$$ALV_{chem}\ (\%) = \frac{\text{total Lys} - 1.24 \times \text{FUR}}{\text{total Lys (unheated protein)}} \times 100 \quad (4)$$

Enzymatically available lysine values (ALV_{enz}) are calculated as

$$ALV_{enz}\ (\%) = \frac{\text{total Lys (dialysate)} - 2.4 \times \text{FUR}}{\text{total Lys (dialysate of unheated protein)}} \times 100 \quad (5)$$

$$ALV_{enz}\ \text{loss}\ (\%) = 100 - ALV_{enz}\ (\%) \quad (6)$$

Table I. Destroyed and Chemically Available Lysine in Heated Whey Proteins at Various a_w Levels

a_w	treatment: temp (°C), time (s)	TLV,[a] mg %	FUR,[b] mg %	inact Lys as lactulosyl-Lys, mg %	destroyed Lys,[d] mg %	ALV_{chem},[e] %
	control (unheated)	9.75 (±0.23)[f,a]	0.00 (±0.00)[a]	0.00 (±0.00)[a]	0.00 (±0.00)[a]	100.0 (±0.00)[a]
0.3	75, 5000	8.59 (±0.24)[b]	1.02 (±0.02)[b]	3.16 (±0.06)[b]	0.00 (±0.52)[a]	75.3 (±0.9)[b]
	100, 500	8.13 (±0.83)[b]	1.00 (±0.04)[b]	3.10 (±0.12)[b]	0.00 (±1.14)[a]	70.7 (±7.2)[b]
	100, 5000	5.47 (±0.08)[d]	1.16 (±0.14)[b]	3.60 (±0.43)[b]	2.12 (±0.59)[b]	41.3 (±1.3)[c]
	121, 500	6.28 (±0.47)[c]	1.75 (±0.43)[c]	5.43 (±1.33)[c]	0.21 (±1.56)[a]	42.2 (±8.2)[c]
	121, 5000	4.37 (±0.33)[e]	0.07 (±0.05)[a]	0.22 (±0.15)[a]	5.25 (±0.61)[c]	43.9 (±2.9)[c]
0.5	75, 5000	8.49 (±0.41)[g]	0.69 (±0.13)[g]	2.14 (±0.40)[g]	0.00 (±0.90)[a]	78.3 (±3.7)[g]
	100, 500	8.26 (±0.22)[g]	0.79 (±0.09)[g]	2.45 (±0.28)[g]	0.02 (±0.74)[a]	74.7 (±1.4)[g]
	100, 5000	5.38 (±0.11)[i]	1.46 (±0.12)[h]	4.53 (±0.37)[b]	1.65 (±0.46)[g]	36.6 (±1.5)[i]
	121, 500	6.37 (±0.53)[h]	2.00 (±0.11)[i]	6.20 (±0.34)[i]	0.00 (±0.87)[a]	39.9 (±5.6)[hi]
	121, 5000	4.50 (±0.33)[j]	0.04 (±0.00)[a]	0.12 (±0.00)[a]	5.18 (±0.33)[b]	45.6 (±2.3)[h]
0.7	75, 50	9.88 (±0.48)[a]	0.02 (±0.00)[a]	0.06 (±0.00)[a]	0.00 (±0.48)[a]	101.0 (±2.5)[a]
	75, 500	9.50 (±0.83)[a]	0.23 (±0.00)[m]	0.71 (±0.00)[m]	0.00 (±0.83)[a]	94.5 (±6.3)[a]
	75, 5000	8.25 (±0.58)[m]	1.01 (±0.14)[n]	3.13 (±0.43)[n]	0.00 (±0.72)[a]	71.8 (±4.2)[m]
	100, 50	9.59 (±0.61)[a]	0.31 (±0.12)[m]	0.96 (±0.37)[m]	0.00 (±0.73)[a]	94.4 (±5.2)[a]
	100, 500	8.24 (±2.62)[amn]	0.97 (±0.26)[n]	3.01 (±0.80)[n]	0.00 (±2.88)[a]	72.2 (±27.8)[am]
	100, 5000	5.63 (±0.08)[n]	1.22 (±0.02)[n]	3.78 (±0.06)[n]	1.85 (±0.10)[m]	42.2 (±0.00)[n]
	121, 50	8.65 (±0.19)[m]	0.94 (±0.22)[n]	2.91 (±0.68)[n]	0.00 (±0.41)[a]	76.8 (±2.4)[m]
	121, 500	6.07 (±0.57)[n]	1.59 (±0.38)[n]	4.93 (±1.18)[n]	0.72 (±0.95)[a]	42.0 (±1.0)[n]
	121, 5000	4.37 (±0.83)[n]	0.02 (±0.02)[a]	0.06 (±0.06)[a]	5.34 (±0.85)[n]	44.6 (±8.4)[n]
0.97	75, 5000	9.78 (±0.09)[a]	0.01 (±0.00)[a]	0.03 (±0.00)[a]	0.00 (±0.09)[a]	100.3 (±1.4)[a]
	100, 500	10.34 (±0.43)[a]	0.00 (±0.00)[a]	0.00 (±0.00)[a]	0.00 (±0.43)[a]	106.0 (±1.9)[a]
	100, 5000	9.60 (±0.18)[a]	0.11 (±0.00)[x]	0.34 (±0.00)[x]	0.05 (±0.18)[a]	97.1 (±0.1)[x]
	121, 500	9.64 (±0.58)[a]	0.08 (±0.02)[x]	0.25 (±0.06)[x]	0.00 (±0.60)[a]	97.8 (±3.8)[ax]
	121, 5000	9.17 (±0.44)[a]	0.12 (±0.08)[x]	0.37 (±0.24)[x]	0.36 (±0.52)[a]	92.5 (±3.1)[x]

[a] TLV = total lysine value, measured from the surface of lysine on the aminograms of whey proteins. [b] FUR = furosine, measured from the surface of furosine on the aminograms of whey proteins taking lysine as a reference. [c] Inactivated Lys as lactuloysl-Lys calculated from eq 1a (Materials and Methods). [d] Destroyed lysine calculated from eq 1a, 2a, and 3 (Materials and Methods). [e] ALV_{chem} = chemically available lysine value, calculated from eq 4 (Materials and Methods). [f] Each value is the average of two determinations (±SD). Means bearing a common leter are not different ($p < 0.05$) according to the LSD test.

Statistical Analyses. The effect of heat treatments on nitrogen digestibility and total Lys of whey proteins was analyzed by a randomized incomplete block design, having a 3 × 4 factorial arrangement of temperature and time and three replicates. Statistical comparisons between unheated and heated samples, with respect to total Lys, nitrogen digestibility, and available Lys losses were subjected to the general linear model (GLM) procedure of the Statistical Analysis System (SAS, 1982). Least significant difference (LSD) values were calculated in order to locate differences among means (SAS, 1982).

RESULTS

Maillard reaction occurs faster as temperature and duration of heating are increased. Table I shows a decrease in total Lys values (TLV) upon increasing temperature when the heating time was 5000 s and also upon increasing time at 100 or 121 °C at a_w 0.3, 0.5, and 0.7. Although TLV are necessary to perform subsequent calculations, FUR and destroyed Lys values have more analytical and biological interest than TLV. It can be seen from Table I that FUR was generated upon heating whey. The amount of FUR produced and of Lys inactivated as lactulosyl-Lys increased with the severity of heat treatments up to a certain limit that seemed to be reached when whey was heated at 121 °C for 500 s at a_w 0.3, 0.5, and 0.7. Excessive heat treatment at those a_w values produced almost no more FUR and very small amounts of Lys inactivated as lactulosyl-Lys. At a_w 0.97, only a small amount of FUR was generated and very little Lys was inactivated.

Some destruction of Lys occurred after severe heating (100 °C, 5000 s) at a_w 0.3, 0.5, and 0.7 and after excessive heating (121 °C, 5000 s) of whey at all a_w values. It is noteworthy that severe heating of whey at 121 °C for 500 s did not destroy a large proportion of Lys, in spite of FUR and TLV approaching values obtained upon heating whey at 100 °C for 5000 s.

Chemically available Lys values (ALV_{chem}) were calculated in all samples. It can be seen (Table I) that even an excessive heat treatment at a_w 0.97 only slightly decreased ALV_{chem}, whereas the damaging effect of heating whey on ALV_{chem} was much more pronounced at lower a_w values, even under milder heating conditions (100 °C, 500 s). Increasing the temperature from 100 to 121 °C did not significantly decrease ALV_{chem} for the longest heat treatment.

In dialysates (Table II), total Lys liberated by in vitro digestion also decreased with the severity of heat treatment. Water seems to have a protective effect on Lys as can be seen from higher TLV at a_w 0.97 than at lower a_w values at any given heat treatment. The amount of FUR found in dialysates increased according to heating intensity except under excessive heating conditions at a_w 0.3, 0.5, and 0.7, where no FUR was produced. Very low amounts of FUR appeared in dialysates when whey was heated in aqueous form (a_w 0.97).

Total lysine values and FUR were used to evaluate the proportion of Lys liberated by proteolytic enzymes, i.e. enzymatically available lysine values (ALV_{enz}) or digestible Lys. An important discrepancy exists between ALV_{chem} (Table I) and ALV_{enz} (Table II). Those results show that ALV_{chem} may overestimate the amount of available Lys in heated whey.

The fate of Lys upon heating whey at various temperatures for 5000 s under different a_w values is illustrated in Figure 1A–D. At 75 °C, the proportion of destroyed Lys in the protein was undetectable at all a_w values but increased drastically ($p < 0.05$) as the temperature increased to 100 and 121 °C at a_w 0.3, 0.5, and 0.7. At 121 °C, the proportion of destroyed Lys was less than 5% at a_w 0.97 but reached 54% at lower water activities.

At a_w 0.3, 0.5, and 0.7, ALV_{chem} steeply decreased as the temperature was raised to 100 °C and slightly increased at 121 °C. Enzymatically available lysine values were even lower than ALV_{chem}. At a_w 0.3 and 0.5, an increase in heating temperature from 100 to 121 °C did not significantly decrease ALV_{enz}, but at a_w 0.7, ALV_{enz} underwent

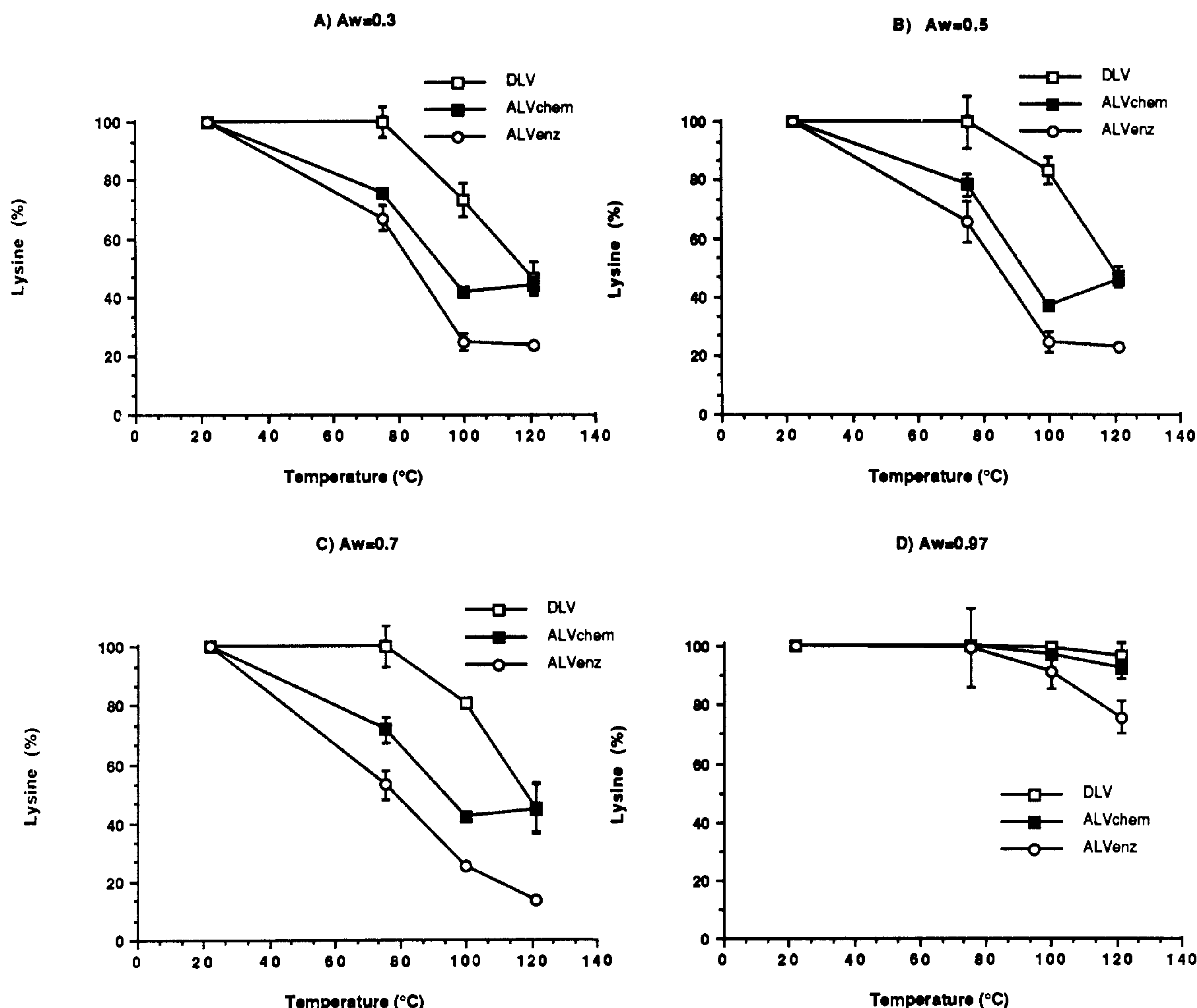

Figure 1. Distribution among destroyed, chemically available, and enzymatically available lysine values as a function of heating temperature and various water activities (heating period 5000 s). DLV = destroyed lysine values. Area above line represents the proportion of destroyed lysine. ALV_{chem} = chemically available lysine values. Area under line represents the proportion of chemically available lysine. Area between ALV_{chem} and DLV represents the proportion of lysine as lactulosyllysine. ALV_{enz} = enzymatically available lysine values. Area under line represents the proportion of enzymatically available lysine.

an additional 12% drop. At a_w 0.97, heating at 121 °C for 5000 s caused only 25% ALV_{enz} losses as compared to 76–86% at lower water activities.

Figure 2 depicts ALV_{enz} losses as affected by moderate, severe, and excessive heat treatments at various a_w values. For all those heat treatments, heating at a_w 0.97 caused significantly less ($p < 0.05$) ALV_{enz} losses than the same heat treatments at lower a_w values. Available Lys losses were either negligible or smaller than 10% for moderate and severe heat treatments at a_w 0.97 but steeply increased to 24% in whey heated at 121 °C for 5000 s. The greater loss in ALV_{enz} occurred after excessive heating of whey at a_w 0.7. At lower a_w values (0.3, 0.5, 0.7), ALV_{enz} losses increased with the severity of heating but seemed to level off at around 75% loss.

DISCUSSION

It is generally recognized that the rate and intensity of the Maillard reaction increase as a function of temperature and heating time (Maillard, 1916; Ellis, 1959; Adrian, 1974). As the heating period was prolonged for whey heated at 100 or 121 °C, more Lys was inactivated as lactulosyl-Lys and destroyed Lys (Table I). Frangne and Adrian (1972) have observed that available Lys losses leveled off after glutenine or ovalbumin was heated at 120 °C for 10 h. Our results showed that, at that temperature, ALV_{chem} tended to level off after a much shorter heating period (5000 s) (Figure 1), whereas longer heat treatment would probably have been necessary to obtain complete destruction or a stabilization of Lys destruction. According to Lea and Hannan (1950), in casein–glucose model systems, the formation of brown pigments occurs at temperatures above 80 °C, particularly between 100 and 120 °C. Our results indicate that at a_w 0.3, 0.5, and 0.7, the amount of total Lys started to significantly decrease in whey heated at 100 °C for 500 s as compared to unheated whey, or even at 75 °C, provided the duration of heating was long enough (5000 s) (Table I).

Saltmarch and Labuza (1982) have obtained Q_{10} values ranging between 1.5 and 4.7 for available Lys losses in whey powders stored at 25, 30, and 45 °C at a_w 0.33, 0.44, or 0.65. In our study, Q_{10} for ALV_{enz} losses at a_w 0.3 and 0.5 (calculated from Table II) ranged between 6 and 16. At higher a_w values, Q_{10} decreased for equivalent heat treatments. The effect of temperature on the Maillard reaction depends on the food system, the temperature range at which

Table II. Enzymatically Available Lysine in Dialysates of Heated Whey Proteins at Various a_w Levels

a_w	treatment: temp (°C), time (s)	TLV,[a] mg %	FUR,[b] mg %	ALV_{enz},[c] %
	control (unheated)	7.30 (±0.27)[da]	0.00 (±0.00)[a]	100.0 (±0.0)[a]
0.3	75, 5000	5.39 (±0.47)[b]	0.21 (±0.03)[b]	66.9 (±4.4)[b]
	100, 500	4.25 (±0.86)[b]	0.26 (±0.04)[b]	49.7 (±1.8)[c]
	100, 5000	2.39 (±0.25)[cd]	0.25 (±0.04)[b]	24.5 (±3.0)[de]
	121, 500	2.60 (±0.31)[c]	0.26 (±0.02)[b]	27.1 (±3.5)[d]
	121, 5000	1.74 (±0.39)[d]	0.01 (±0.01)[a]	23.5 (±0.5)[e]
0.5	75, 5000	5.26 (±0.67)[g]	0.20 (±0.02)[g]	65.5 (±7.0)[g]
	100, 500	4.70 (±1.00)[g]	0.21 (±0.06)[g]	57.5 (±12.3)[g]
	100, 5000	2.37 (±0.32)[h]	0.24 (±0.02)[g]	24.6 (±3.3)[h]
	121, 500	2.65 (±0.07)[h]	0.26 (±0.01)[g]	27.8 (±0.1)[h]
	121, 5000	1.68 (±0.80)[h]	0.01 (±0.01)[a]	22.7 (±1.0)[h]
0.7	75, 50	7.33 (±0.78)[a]	0.00 (±0.00)[a]	100.4 (±6.9)[a]
	75, 500	7.25 (±0.73)[a]	0.06 (±0.05)[m]	97.3 (±7.0)[a]
	75, 5000	4.09 (±0.49)[m]	0.09 (±0.03)[m]	53.1 (±5.1)[n]
	100, 50	5.74 (±0.68)[a]	0.06 (±0.02)[m]	76.7 (±6.7)[m]
	100, 500	4.35 (±0.60)[m]	0.25 (±0.06)[n]	51.4 (±7.1)[n]
	100, 5000	2.40 (±0.15)[n]	0.23 (±0.01)[n]	25.3 (±0.2)[o]
	121, 50	4.55 (±0.34)[m]	0.23 (±0.02)[n]	54.8 (±0.3)[n]
	121, 500	2.36 (±0.87)[n]	0.25 (±0.10)[n]	24.1 (±1.0)[o]
	121, 5000	0.99 (±0.16)[o]	0.00 (±0.00)[a]	13.6 (±0.2)[p]
0.97	75, 5000	7.25 (±0.99)[as]	0.00 (±0.00)[a]	99.3 (±13.5)[a]
	100, 500	7.42 (±0.75)[a]	0.01 (±0.01)[a]	101.3 (±6.6)[a]
	100, 5000	6.69 (±0.71)[as]	0.00 (±0.00)[a]	91.6 (±6.3)[s]
	121, 500	7.14 (±2.34)[as]	0.04 (±0.02)[s]	96.5 (±28.7)[a]
	121, 5000	5.65 (±0.59)[s]	0.05 (±0.04)[s]	75.8 (±5.8)[t]

[a] TLV = total lysine value, measured from the surface of lysine on the aminograms of dialysates. [b] FUR = furosine measured from the surface of furosine on the aminograms of dialysates, taking lysine as a reference. [c] ALV_{enz} = enzymatically available lysine value, calculated from eq 5 (Materials and Methods). [d] Each value is the average of three determinations (±SD). Means bearing a common letter are not different ($p < 0.05$) according to the LSD test.

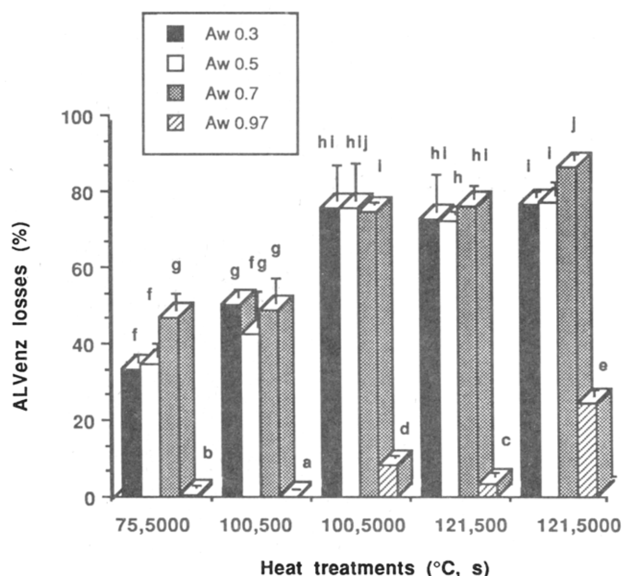

Figure 2. Results are expressed as enzymatically available lysine losses (%). Each bar is the average of three determinations (±SD). Means bearing a common letter are not different according to the LSD test (95% confidence level).

the food was processed or stored, and the method by which protein quality was estimated (Saltmarch and Labuza, 1982). The much higher temperature range used in this study may explain the higher Q_{10} values obtained.

Water activity also had an impact on available Lys. Available Lys values for any given heat treatment were lower at a_w 0.3, 0.5, or 0.7 than at a_w 0.97 (Tables I and II). Saltmarch and Labuza (1982) observed that Maillard reaction in whey powders was optimal at a_w 0.44 as a result of lactose recrystallization. Once crystallization is initiated, lactose molecules become tightly packed and the amount of water that can be held within the crystals decreases. That water could be given up to other food constituents such as proteins and cause a higher rate of Lys loss (Saltmarch and Labuza, 1980). The relationship between a_w and reactive Lys loss has not always been found when monitored at elevated temperatures because of a rapid shift in a_w caused by the high processing temperatures under the conditions used (Wolf et al., 1977; Kaanane and Labuza, 1985). Also, temperature and lactose interactions probably masked the effects of a_w (Wolf et al., 1977). This may explain why no significant difference was observed in ALV_{chem} at a_w 0.3, 0.5, and 0.7 for most heat treatments. According to Lorient (1978) and Labuza and Saltmarch (1981), as a_w is increased over 0.8, Maillard reaction rate decreases. The high water content at those a_w's probably prevents the temperature from increasing above the boiling point of water; also, the diluting effect of water on the reactants (Lys and lactose) does not favor Maillard reaction (Saltmarch and Labuza, 1982). That would explain the higher ALV_{chem} obtained in whey heated at a_w 0.97 as compared to similar heat treatments at a_w 0.3, 0.5, and 0.7. The presence of FUR on chromatograms of heated whey indicated that an early Maillard reaction had taken place. Figure 1 illustrates that, upon heating, some Lys was inactivated in a form such as lactulosyl-Lys (area between ALV_{chem} and DLV), while another proportion of Lys was destroyed (advanced Maillard reaction). Table I shows that early and advanced Maillard reactions often overlap, since heat treatments can both produce lactulosyl-Lys (as measured by FUR) and destroy some Lys.

According to Finot and Magnenat (1981), in an unknown product, the estimation of Lys blockage in the advanced Maillard reaction is difficult, since when the Amadori compound starts to degrade, some Lys is destroyed, and it becomes impossible to determine the initial Lys content. Assuming that the Lys content of an intact protein represents the initial Lys value and that all inactivated Lys as lactulosyl-Lys produces FUR upon acid hydrolysis, it is possible, by the digestion cell technique, to measure Lys damage due to Maillard reaction and also to estimate losses in Lys availability caused by structural changes in the protein (Desrosiers et al., 1987b). Therefore, for mild heat treatments, one would expect that ALV_{enz} would be equal to ALV_{chem}, if, at that stage, available Lys losses were caused only by the formation of lactulosyl-Lys, as indicated by the presence of FUR on aminograms. The smaller values obtained for ALV_{enz} showed that structural changes were already involved. Those structural modifications were more pronounced after moderate or excessive heat treatments and were accompanied by some Lys destruction. The smaller values found for ALV_{chem} than for ALV_{enz} could be explained by Lys inactivation in cross-links or by protein aggregation. Those cross-links would be cleaved by acid hydrolysis, but not by digestive enzymes. The greatest difference arose for whey heated at 121 °C for 5000 s at low a_w values. Those observations, therefore, suggest that, at this temperature, the Amadori compound was destroyed, causing Lys destruction, and also, the aggregation and polymerization reactions further reduced Lys digestibility. From Figure 1D, it is clear that, at a_w 0.97, Maillard reaction is not the main cause of Lys damage, since the proportion of inactivated Lys is very small. The heat-induced conformational changes may have promoted cross-linking, which would not destroy Lys but would significantly reduce its release by digestive enzymes. In

fact, even when very little residual lactose was present, a significant reduction in Lys digestibility was observed in whey heated in liquid form at 121 °C for 5000 s as compared to the unheated sample in the same physical state (Desrosiers et al., 1987a).

Furosine determinations of heated whey proteins without the measurement of Lys liberation by proteolytic enzymes would not have taken into consideration heat-induced conformational changes of the protein and, consequently, would have been limited to the early Maillard reaction. Acid hydrolysis of whey proteins, performed in order to measure total Lys, does not give a true estimate of the nutritional quality of a protein since it can cleave peptide linkages that would normally be resistant to mammalian digestive enzymes and would partially regenerate Lys from the Amadori compound (Hurrell and Carpenter, 1981). The digestion cell technique, therefore, allows the measurement of the extent of enzymatic liberation of nitrogen and amino acids of a protein, and the correction for the diffusion of lactulosyl-Lys in dialysates can be made by FUR evaluation.

The decrease in digestible Lys obtained upon heating, especially at lower a_w values (Figure 2), may indicate a reduced accessibility of digestive enzymes to the peptide bonds of the protein as a result of heat-induced modification of protein conformation or steric hindrance created by cross-linking (Darcy, 1984; Kinsella, 1984; Kilara and Sharkasi, 1986). Lysine has a strong preference for the outside of the protein as a result of its polar NH_2 group located at the end of a large nonpolar side chain. Not only the polar part but the entire side chain of Lys is usually oriented toward the outside of protein (Wertz and Scheraga, 1978). Therefore, Lys is usually liberated easily by digestive enzymes. As heating was itensified, Lys losses increased (Figure 1). Mauron (1981) pointed out at least three mechanisms responsible for such a decrease in Lys availability: the involvement of Lys side chain in the Maillard reaction, the formation of cross-links between peptide chains, and the decreased overall digestibility of the protein. The new bond formed between Lys and lactose would be resistant to enzymatic hydrolysis due to the narrow specificity of trypsin for the peptide bonds linking the carboxyl group of Lys to the amino group of any other amino acid (Hansen and Millington, 1979). Also, the low molecular weight fraction of a heated glucose–Lys mixture was found to cause some inhibition, not only of trypsin but of other pancreatic enzymes such as carboxypeptidases A and B (Oste et al., 1986).

Results of this study suggest that damage to the protein, caused by heat treatments in the presence of lactose, involved not only Lys destruction but also Lys inactivation in the form of lactulosyl-Lys. Lysine inactivated in the Amadori compound (lactulosyl-Lys) is not bioavailable and can be found in appreciable amounts in heated whey. The combined use of FUR determinations with the digestion cell technique enables detection of nutritional damage before anything is visible to the naked eye, i.e. before the appearance of brown pigments. The mechanism responsible for the decrease in enzymatically available Lys would also affect the enzymatic release of other amino acids as will be shown in a subsequent paper.

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Reduced Stability and Accelerated Autoxidation of Tuna Myoglobin in Association with Freezing and Thawing

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The mechanisms involved in accelerated discoloration of frozen tuna meat after thawing were investigated. Bluefin tuna myoglobin (Mb) was dissolved in 50 mM phosphate buffer (pH 5.5–7.4) with or without 0.2 M NaCl and quickly frozen in dry ice–acetone, followed by a 2-h storage at −80 °C and subsequent thawing at 20 °C. The "frozen/thawed" Mb solution thus prepared, along with unfrozen (intact) Mb, was examined for the free energy for unfolding, helical content, and autoxidation rate. In 50 mM phosphate buffers, the free energy for unfolding (ΔG_D) was generally lower in frozen/thawed Mb than in unfrozen Mb, with the highest value (7.3 kcal/mol) at pH 6.5. Similar tendency was observed in 50 mM phosphate buffers containing 0.2 M NaCl, although $\Delta G_D^{H_2O}$ values were generally lower. Helical contents of frozen/thawed Mb were practically the same as those of unfrozen Mb, regardless of pH. Frozen/thawed Mb showed a higher autoxidation rate than unfrozen Mb. It was concluded that, during freezing and thawing, Mb suffered some conformational changes in the nonhelical region, resulting in a higher susceptibility to both unfolding and autoxidation.

It is empirically known that tuna meat, once frozen and thawed, discolors more quickly than unfrozen meat during subsequent storage, by an unexplained reason. Myoglobin (Mb), so far reported, shows a pH-dependent autoxidation, the rate increasing with decrease of pH (George and Stratmann, 1952; Matsuura et al., 1962; Brown and Mebine, 1969). As reported previously, however, we found a unique pH dependency of autoxidation in bluefin tuna Mb, associated with freezing and thawing: The formation of metmyoglobin (metMb) was minimum in the pH range 6.0–6.5, depending upon buffer species (Chow et al., 1985, 1987). On the other hand, freezing and thawing accelerated insolubilization of the Mb, with a minimum ratio in the same pH range. These findings suggested that some pH-dependent conformational changes of Mb might have occurred during freezing and thawing.

Free energy required to denature a protein completely is one of the best parameters to evaluate the degree of denaturation or "stability" against chemical denaturant (Ahmed and Bigelow, 1982). Guanidine hydrochloride (Gdn·HCl) and urea induce proteins into a randomly coiled denaturation state (Tanford, 1970). Gdn·HCl is a more potent denaturant, unfolding proteins at several times lower concentrations than does urea (Greene and Pace, 1974). In this connection, Fosmire and Brown (1976) found that yellowfin tuna Mb was much more susceptible to urea denaturation than sperm whale Mb. Balestrieri et al.

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