Degradation of Tryptophan in Heated β-Lactoglobulin−Lactose Mixtures Is Associated with Intense Maillard Reaction

Véronique Moreaux and Inès Birlouez-Aragon*

Laboratoire de Chimie Analytique, Institut National Agronomique, 16 rue Claude Bernard, 75005 Paris, France

The Maillard reaction that occurs during food processing is believed to induce the formation of radical species at high temperatures. This study was carried out to determine whether radical-sensitive Trp is affected during the glycation of β-lactoglobulin (βLG) by lactose in the presence of iron and vitamin C at 115 °C for 6 min. The early glycation, measured as furosine, increased nonlinearly with time, but the formation of fluorescent advanced glycated products (AGEs) was almost exponential, explaining most of the blockage of lysine residues (up to 70%). At the same time, 46−55% of Trp residues were lost, and the Trp concentration was negatively correlated with the fluorescent AGEs. The Trp fluorescence quantum yield decreased considerably due to changes in the protein conformation upon heating with lactose. Trp degradation and AGEs formation were significantly greater in proteic fraction soluble at pH 4.6 than in insoluble fraction. The radicals released during the advanced Maillard step could be responsible for Trp oxidation.

Keywords: *β-Lactoglobulin; heat denaturation; tryptophan; Maillard reaction*

INTRODUCTION

Protein glycation occurs frequently during food processing and storage and depends on the concentration and structure of the protein, the sugar content, and the water activity (O'Brien, 1995). Recent studies on mixtures of proteins or amino acids and sugars have shown that radical species are formed during glycation (Hayashi and Namiki, 1981; Gillery et al., 1988; Kawakishi et al., 1990; Hunt and Wolff, 1991; Cheng et al., 1991; Yim et al., 1995). These radical species could interact with the Trp residues of the protein, but little information is available on the oxidative damage on Trp during the advanced Maillard reaction. Trp has been shown to be very stable in milk powder (Nielsen et al., 1985), but no study has been carried out on liquid milk. However, the free radicals formed during lipid peroxidation or metal-mediated vitamin C oxidation are known to catalyze the oxidation of Trp in proteins in solution (Hidalgo and Kinsella, 1989; Reyftmann et al., 1990; Giessauf et al., 1995; Miura et al., 1992; Uchida and Kawakishi, 1988, 1989). We now have evidence that Trp residues are lost from the acetate-soluble proteins of milks supplemented with lactose and iron (Birlouez-Aragon et al., 1996a). This study was carried out to verify whether the radicals that could be formed during the lactose-induced glycation of β-lactoglobulin (βLG) solutions, the major whey protein, favored Trp degradation. This protein is particularly sensitive to heat denaturation, and its high lysine content predisposes it to glycation. The high Trp content of βLG plus the probable exposure of these hydrophobic residues to the environment during the heat-induced structural modification (Mills, 1976) should favor Trp degradation. The possible degradation of Trp is particularly important because it is an essential amino acid, and some of its degradation products could be toxic (Sugimura, 1985).

* Author to whom correspondence should be addressed (telephone 01-44-08-16-49; fax 01-44-08-16-53; e-mail birlouez@.com).

MATERIALS AND METHODS

Reagents. β-Lactoglobulin (A + B) from bovine milk (three times crystallized and lyophilized) and ascorbic acid were purchased from Sigma (St. Quentin Fallavier, France). $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was from Prolabo (Paris, France). The Coomassie reagent was a Pierce product purchased from Interchim (Paris, France). All other reagents were of analytical grade.

Heat Processing of β-LG Solutions. Duplicate or triplicate samples of βLG (5 g/L) were incubated in three independent repetitions of the same experiment. The effect of the incubation time was analyzed at only 2, 4, and 6 min on one experiment; the other experiments studied only the modifications of βLG after 2 and 6 min of heating. The buffer was 100 mM phosphate buffer (pH 7.4) containing 116 mM NaCl, 4.9 mM KCl, and 1.2 mM $MgSO_4$. The experiment was carried out using an experimental design. The three variable factors were set at the low/high levels of 40/80 g/L for lactose, 0/14 mg/L for iron, and 0/100 mg/L for vitamin C. Two milliters of the solution was placed in glass screw-capped tubes, heated in an oil bath at 155 °C for 2−6 min, and rapidly cooled to room temperature in ice-cold water. The temperature profile of the solution was measured with a thermometer inserted in a tube capped with a perforated rubber stopper.

The heat denatured proteins were precipitated by adding 9 volumes of 0.1 M acetate buffer (pH 4.6). The solution was centrifuged at 9000*g* for 10 min at room temperature. Aliquots of the supernatant containing the acetate-soluble proteins (ASP) were used for fluorescence analysis and protein measurement.

Analytical Procedure. Early glycation was assessed by furosine, the acid hydrolysate of ε-lactulosyllysine. Samples (200 μL ≃ 1 mg of βLG) were deproteinized with 0.1 volume of 50% trichloroacetic acid. The pellet was hydrolyzed with 2 mL of 7.8 N HCl at 110 °C for 18 h under nitrogen. Furosine was quantified by HPLC on a C_{18} Nucleosil 250 × 4.6 mm column with detection at 280 nm as described elsewhere (Birlouez et al., 1996b) and adapted from Schleicher and Wieland (1981).

The fluorescence of tryptophan and advanced glycated end products (AGEs) was measured on total proteins (TP) and acetate-soluble proteins (ASP) using a Fluoromax-Spex spectrofluorometer (Jobin-Yvon, France). Trp fluorescence was measured at 340 nm with excitation at 280 nm. Two AGEs fluorophores were found at excitation of 350 and 375 nm with

S0021-8561(96)00500-6 CCC: $14.00

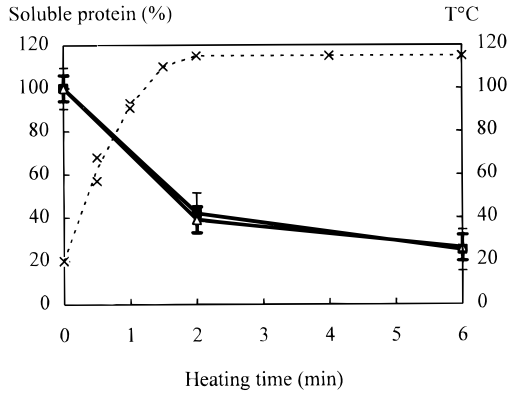

Figure 1. Percentage of ASP of βLG (5 g/L) during heating with 40 (■) and 80 g/L (△) lactose and temperature profile in the tube (dotted line) during heating in an oil bath at 155 °C.

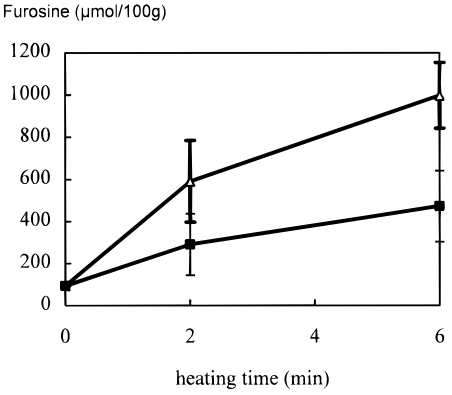

Figure 2. Furosine content in βLG as a function of time of heating in the presence of 40 (■) and 80 g/L (△) lactose. Points represent the mean of three samples with standard deviation.

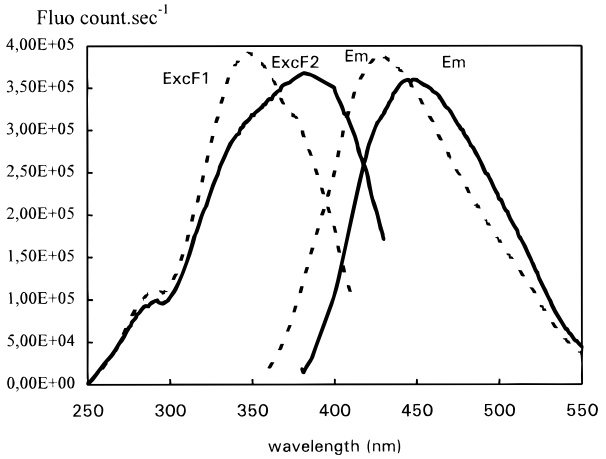

Figure 3. Fluorescence spectra of the two fluorophores of βLG heated with lactose: first AGEs fluorescence with excitation/emission at 350/425 nm (dotted line) and the second fluorophore 375/460 nm (solid line).

emission at 425 and 450−460 nm, respectively. AGEs fluorescence was quantified by the emission fluorescence at 425 nm with excitation at 350 nm in counts per second after division by the protein concentration of the solution.

The concentration of ASP was measured according to the Coomassie protein assay, adapted from the Bradford method (Bradford, 1976). This assay was not influenced by the concentration of lactose after the 100-fold dilution.

The percentage of available lysine in the protein was evaluated with a fluorescamine-based assay (Yaylayan et al., 1992). The free NH_2 groups of the protein dissolved in 0.2 M borate buffer (pH 8.5) immediately reacted with the fluorescent probe, fluorescamine (2 M in acetone). The fluorescence of the resulting complex was immediately measured at 475 nm with excitation at 390 nm.

Peptide Trp concentration was assessed by HPLC after akali hydrolysis with a method derived from that of Landry and Delhaye (1992). Proteins (600−900 μL) were precipitated with 0.1 volume of 50% trichloroacetic acid. The pellet (3−4.5 mg) was hydrolyzed with barium hydroxide octahydrate (2.7 N) for 16 h in an oven at 110 °C in a sealed glass tube flushed with nitrogen in the presence of thiodiglycol. The solution was partially neutralized with HCl/AcOOH ($10^{-2}/10^{-1}$ N), and 20 μL was injected onto a Lichrospher (Merck, Nogent-sur-Marne, France) 100 RP 18 column (250 × 4.6 mm, 5 μm). The eluant was 13.5 mM ammonium acetate/methanol (85/15) brought to pH 4.5 with formic acid, and the flow was 0.7 mL min^{-1} up to 6 min and 1 mL min^{-1} up to 30 min. Trp was monitored at 280 nm. Trp concentration was measured in total protein and in acetate-soluble and insoluble proteins. 5-Methyltryptophan was used as an external standard and tyrosine, which is stable during heating, as an internal standard.

The regression lines were calculated with Excell5 (Microsoft) and nonparametric tests were used to compare the experiments with high and low lactose with Unistat software.

RESULTS

The experimental design revealed no significant effect of either iron or vitamin C. The only influencing factor was lactose. Therefore, samples with the same lactose concentrations were analyzed together, without taking into account the other parameters.

Heat Denaturation of β-LG. Figure 1 shows the mean temperature profile during the 6 min heating in the oil bath at 155 °C and the parallel decrease in the proteins remaining soluble at pH 4.6. The temperature rose to 110 °C during the first 2 min of incubation, after which it remained stable at 115 °C. Protein denaturation essentially occurred during the first 2 min, except for one of the three experiments, in which it was maximal at 6 min. The final percentage of ASP for the three experiments was 26.0 ± 8.3% (n = 23) and was not significantly affected by the lactose concentration (26.6% and 25.4% for high and low levels of lactose, respectively). When βLG was heated alone, the percentage of ASP remained much higher, 69% (± 9) (n = 3) at 2 min and 52.0% (±0.64) (n = 2) at 6 min.

Glycation of β-LG by Lactose upon Heating. *Early Glycation.* The furosine concentration was measured during one of the three repetitions of the experiment. It seemed to increase nonlinearly with time with a reduction of the rate of early glycation after 2 min. It was twice as high for 80 g/L lactose compared to 40 g/L lactose (Figure 2).

Advanced Glycation. Browning of the solution upon heating was associated with the appearance of two fluorophores; the major one (F1), with excitation at 350 nm and emission at 425 nm, could be produced by an advanced Maillard reaction, and named AGEs fluorescence (Figure 3). The second fluorescence (F2) had maximum excitation/emission around 375−385/450−460 nm and evolved similarly to the first fluorescence (F2 = 0.91 ±0.02 × F1, r = 0.93; n = 20). Figure 4 shows the development of AGEs fluorescence (425 nm) in total and acetate-soluble proteins as a function of time and lactose concentration in the only experiment in which the three incubation times where analyzed. The relative fluorescence of AGEs from βLG increased in an exponential way with time in ASP but almost linearly in total proteins. Furthermore, at 6 min, the intensity of AGEs fluorescence was 2.5 times more intense in the ASP than in the total proteins. The fluorescence of AGEs from βLG was not significantly

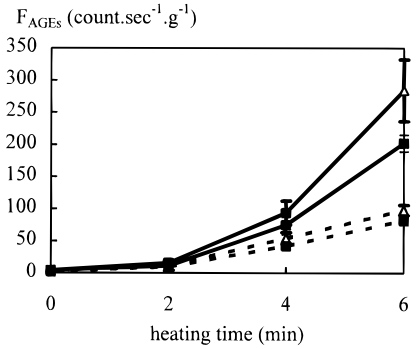

Figure 4. AGEs fluorescence (350/425 nm) of βLG in total protein (dotted line) and in ASP (solid line) in the presence of 40 (■) or 80 g/L (△) lactose in the lone experiment studied as a function of time. Points represent a mean of three repetitions with the standard deviation.

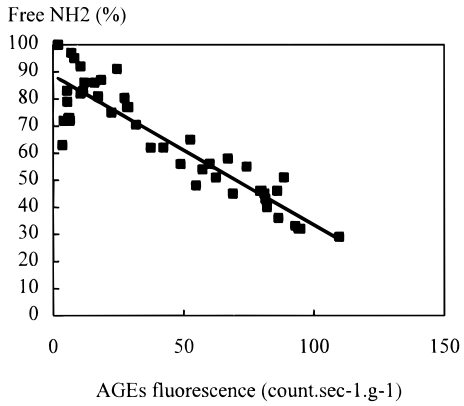

Figure 5. Percentage of remaining free amino groups of βLG as a function of AGEs fluorescence in all experiments.

influenced by either vitamin C or iron, but the intensity was significantly higher in the samples with the highest lactose concentration: after 6 min of heating and considering the three independent repetitions of the experiment, the AGEs fluorescence was 37 ± 19% (n = 15) higher with 80 g/L lactose than with 40 g/L lactose in total proteins and 45 ± 19% (n = 15) in ASP. The percentage of βLG free NH_2 decreased linearly as a function of the AGEs fluorescence (Figure 5) (r = 0.916, n = 44).

Trp Degradation upon Heating and Glycation. Trp residues were quantified by HPLC. Heating βLG without lactose, vitamin C, and iron caused denaturation and change in fluorescence quantum yield, but the Trp concentration was unchanged, remaining at the initial value of 14.8 mmol/100 g. On the other hand, all samples containing lactose lost Trp residues. At 6 min, the loss of Trp residues in total proteins was 1.2 times greater for high than for low levels of lactose (Figure 6) (56.4 ± 2.3% and 45.5 ± 6.4%, respectively) and decreased almost linearly with time, but was not influenced by iron or vitamin C. There was a parallel 86% decrease in Trp fluorescence.

The fluorescence quantum yield (Q_f) of Trp residues was calculated by dividing the Trp fluorescence by the concentration of Trp residues evaluated by HPLC. We arbitrarily set the Q_f of the native protein at 100 to calculate the changes in Q_f during heating. The Q_f values of βLG heated in the presence of various lactose concentrations are shown in Table 1. Without lactose, the Q_f of βLG increased during the 6 min heating, while

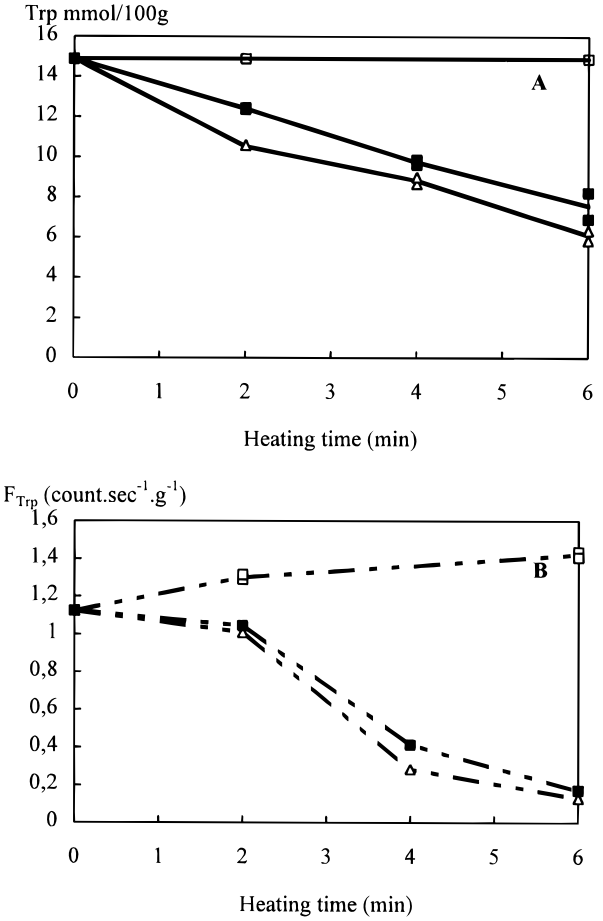

Figure 6. (A) Trp residues of βLG as a function of heating time in the presence of 0 (□), 40 (■), and 80 g/L (△) lactose; (B) Trp fluorescence of βLG as a function of heating time in the presence of 0 (□), 40 (■), and 80 g/L (△) lactose. This figure takes into account the result of the only experiment studied versus time.

Table 1. Q_f of Trp of βLG in Total Protein (PT) and Acetate-Soluble Protein (ASP) Heated with Various Lactose Concentrations

	heating time (min)	lactose concn (g/L) 0 (n)	40 (n)	80 (n)
PT	0	100	100	100
	2	118 ± 1 (3)	155 ± 20 (5)	153 (1)
	6	130 ± 1 (2)	54 ± 12 (5)	42 ± 7 (5)
ASP	0	100	100	100
	2	135 ± 7 (3)		
	6	147 ± 3 (2)	77 ± 17 (3)	70 ± 2 (3)

in the presence of lactose, it first increased during the first 2 min but considerably decreased at 6 min of heating. In ASP, the Q_f of Trp also increased at 2 min and decreased at 6 min but in a less extensive way than the Q_f of Trp in total protein.

At 6 min of heating, the total Trp residues in ASP was 52.9 ± 5.5% (n = 4) and 44.9 ± 8.94% (n = 3) lower in the presence of 80 and 40 g/L lactose, respectively, than in the native protein and was 30.4 ± 8.9% (n = 3) and 28.8 ± 1.5% (n=3) lower in insoluble proteins with 80 and 40 g/L lactose, respectively.

Figure7A shows the linear decrease (r = −0.905, n = 22, p = 0.01) in Trp residues as a function of AGEs fluorescence in total proteins. The Trp fluorescence decreased in an exponential way (Figure 7B) as a function of the AGEs fluorescence (F_{AGEs}) similarly in ASP and total proteins (Figure 7B). The decrease in

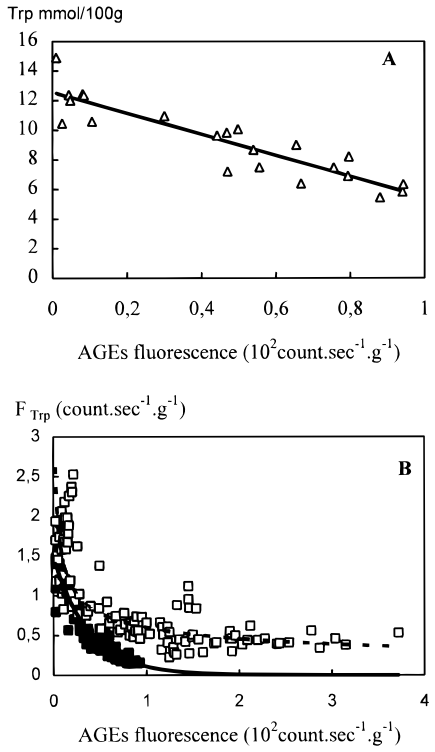

Figure 7. (A) Trp residues of βLG as a function of AGEs fluorescence (350/425 nm); (B) Trp fluorescence of βLGactoglobulin as a function of AGEs fluorescence upon heating with lactose: total protein (■); ASP (□). This figure represents all results obtained in the different repetitions of experiment.

FTrp could be fitted to the following equations:

in total proteins:

$$F_{\mathrm{Trp}} = 1.4875 \times \exp(-2.624F_{\mathrm{AGEs}}) \qquad (r = 0.96,\ n = 118)$$

in soluble proteins:

$$F_{\mathrm{Trp}} = 1.2842 \times \exp(-0.5392F_{\mathrm{AGEs}}) \qquad (r = 0.75,\ n = 118)$$

DISCUSSION

Globular proteins such as βLG are denatured by heating, and their physicochemical properties change (Mills, 1976; Jelen and Rattray, 1995). Heating first dissociates dimer proteins into monomers (Laligant et al., 1991), having Trp quantum yield (Q_f) at 20 °C 1.73 times greater than that of the dimer (Mills, 1976). This type of dissociation may be responsible for the increase in the Q_f of Trp that occurs when βLG is incubated at 115 °C for up to 6 min in the absence of lactose, whereas the total concentration of Trp is unaffected. However, unpublished preliminary analyses by exclusion chromatography and mass spectrometry suggest that βLG is present in our solution as the monomer and, upon heating, first forms a dimer and then high molecular weight aggregates (>100 000). Therefore, protein−protein associations (De Wit and Klarenbeek, 1981), mainly via disulfide bonds (Sawyer, 1968), better explain the fluorescence changes that occur under our conditions. As a consequence, Trp fluorescence cannot be used to quantify Trp and direct HPLC measurements are needed to monitor the concentration of Trp during heating with lactose. The total Trp concentration drops considerably in the presence of lactose, linearly as a function of AGEs formation, but is not greatly affected by iron or ascorbate. At the same time, the presence of lactose causes a 50% increase in the Trp Q_f of βLG at 2 min and twice as much protein denaturation as without lactose. The denaturation rates are similar at 40 and 80 g/L lactose. The first increase in the Trp Q_f is followed by a large drop over the next 4 min. This could be due to aggregation of unfolded βLG monomers into oligomers through Maillard cross-links after 2 min, as indicated by the large increase in the fluorescence of AGEs. In contrast to the Trp concentration, the Trp fluorescence per gram of protein decreases exponentially with the fluorescence of AGEs, thus overestimating the loss of Trp. This is explained by the simultaneous decrease in the Q_f of Trp (fluorescence intensity per residue) and Trp concentration. In ASP, the decrease in the Q_f of Trp is smaller than in total proteins, explaining the less rapid decrease of Trp fluorescence per gram of protein as a function of AGEs fluorescence.

The loss of available lysine is linearly correlated with the fluorescence of AGEs, indicating that the advanced step of the Maillard reaction could be responsible for blocking the free NH_2 of lysine in βLG (70% at 6 min). The blockage of lysine by ϵ-lactulosyllysine, the early product of the Maillard reaction, was calculated from the furosine concentration to be 3−5%, taking into account that the recovery of ϵ-lactulosyllysine as furosine is only 32% (Bujard and Finot, 1978) and that βLG contains 81.7 mmol/100 g of lysine (Gordon et al., 1961). The extreme heating could have given rise to the extensive browning of the βLG solution and the rapid conversion of ϵ-lactulosyllysine into the advanced Maillard products, as indicated by the shapes of the furosine and AGEs time curves (more logarithmic-like for furosine and exponential for AGEs fluorescence). Similar curves were obtained at 37 °C with a very high concentration of glucose (1250 mM instead of 111−220 mM; Birlouez et al., 1996b). Doubling the lactose concentration doubles the increase in Amadori product but, surprisingly, is associated with only a 1.3−1.4-fold increase in the fluorescence of AGEs and loss of available lysine.

The loss of Trp upon heating with lactose could be due either to a direct glycation of the Trp indole (Nyhammar and Pernemalm, 1985; Saito et al., 1986) or to a radical oxidation during the advanced Maillard step (Hayashi and Namiki, 1981; Gillery et al., 1988; Kawakishi et al., 1990; Hunt and Wolff, 1991; Cheng et al., 1991; Yim et al., 1995). The low reactivity of the Trp indole group and the strong competition with the much more reactive ϵ-amino group of lysine suggest that direct glycation of Trp is unlikely. On the other hand, oxygen radicals derived from ascorbate−copper or lipids have been shown to decrease Trp fluorescence of protein in vitro (Reyftman et al., 1992; Uchida et al., 1988, 1989; Miura et al., 1992) and, in particular, in βLG incubated with lipid peroxide radicals (Hidalgo and Kinsella, 1989). Furthermore, the fluorescence of the βLG−lactose mixture at 460 nm produced by excitation at 375 nm is similar to that of free Trp incubated with iron and vitamin C, in which hydroxyl radicals are involved (Moreaux et al., 1996) and suggests that the Trp in βLG is oxidized. The supernatant obtained after the protein precipitation contains a product with the same retention time and spectral characteristics as hydroxy-3-anthranilic acid (data not shown). This could be split out from the protein (Pirie and Dilley, 1974) and indicates that Trp is oxidized during advanced glycation.

The early glycation of lysine produces radical species prior to the formation of the advanced glycated end products at high temperature in the absence of metals (Hayashi et al., 1981). Others have observed reactive intermediates (Yim et al., 1995) and oxygen radicals (Hunt and Wolff, 1991) during the transformation of the Amadori product into the advanced compounds in the presence of metal ions at low temperatures. In turn, the formation of fluorescent advanced Maillard products is considered to involve radicals, such as the transition metals (Kato et al., 1981; Ahmed et al., 1993), reactive intermediates of glycation products (Hayashi et al., 1981; Yim et al., 1995), or oxygen radicals (Le Guen et al., 1992).

In turn, the free radicals produced during glycation could be responsible for Trp degradation, as indicated by the linear relationship between the loss of Trp and the fluorescence of advanced Maillard products in βLG solutions. In the acetate-soluble βLG, a greater glycation is also associated with a greater degradation of Trp as compared to the insoluble aggregates. That could result from the favored exposure of the lysine and tryptophan residues of acetate soluble βLG to the environmental lactose. The severe heating conditions used in the present study probably led to oxidative reactions which, under milder conditions, would require the presence of catalysts such as metals ions. The susceptibility of Trp to the advanced Maillard reaction has already been evidenced with N′-acetylated Trp. It does not react with glucose but is destroyed in the presence of glucose−glycine reaction products (Finot et al., 1982). Le Guen et al. (1992) have also shown that *N*-formylkynurenine, an oxidative degradation product of Trp, is formed in glycated albumin.

These results suggest that intensive Maillard reactions that occur in many processed liquid foods, and particularly in lactose- and iron-enriched liquid infant formulas, could favor Trp degradation, as suggested by the loss of Trp residues from the ASP of such milks (Birlouez-Aragon et al., 1996a).

LITERATURE CITED

Ahmed, N.; Liggins, J.; Furth, A. M. Metal-catalysed oxidation and post-amadori reactions of serum albumin and other model proteins. *Biochem. Soc. Trans.* **1993**, *21*, 93S.

Birlouez-Aragon, I.; Moreaux, V.; Nicolas, M.; Ducauze, C. Evidence for acivation of the Maillard reaction and decreased tryptophan concentration in acetate-soluble proteins of iron and lactose supplemented milks. *Food Addit. Contam.* **1996a**, in press.

Birlouez-Aragon, I.; Tessier, F.; Mompeyssin, V.; Baciuska, J. Lack of effect of copper on advanced Maillard reaction and glucose autoxidation at physiological concentrations of albumin. *Redox Rep.* **1996b**, *2*, 127−132.

Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Bujard, E.; Finot, P. A. Mesure de la disponibilité et du blocage de la lysine dans les laits industriels (Quantification of lysine availability and blocking in industrial milk). *Ann. Nutr. Alim.* **1978**, *32*, 291−305.

Cheng, R. Z.; Tsunehiro, J.; Uchida, K.; Kawakishi, S. Oxidative damage of glycated protein in the presence of transition metal ion. *Agric. Biol. Chem.* **1991**, *55*, 1993−1998.

De Wit, J. N.; Klarenbeek, G. A differential scanning calorimetric study of the thermal behaviour of bovine b-lactoglobulin at temperatures up to 160 °C. *J. Dairy Res.* **1981**, *48*, 293−302.

Finot, P. A.; Magnenat, E.; Guignard, G.; Hurrell, R. F. The behaviuor of tryptophan during "early" and "advanced" Maillard reactions. *Int. J. Vitam. Nutr. Res.* **1982**, *52*, 226.

Giessauf, A.; Steiner, E.; Esterbauer, H. Early destruction of tryptophan residues of apolipoprotein B is a vitamin E-independent process during copper-mediated oxidation of LDL. *Biochim. Biophys. Acta* **1995**, *1256*, 221−232.

Gillery, P.; Monboisse, J. C.; Maquart, F. X.; Borel, J. P. Glycation of proteins as a source of superoxide. *Diabete Metab.* **1988**, *14*, 25−30.

Gordon, W. C.; Basch, J.-J.; Kalan, E. B. Amino acid composition of β- lactoglobulin A, B, AB. *J. Biol. Chem.* **1961**, *236*, 2908−2912.

Hayashi, T.; Namiki, M. On the mechanism of free radical formation during browning reaction of sugars with amino compounds. *Agric. Biol. Chem.* **1981**, *45*, 933−939.

Hidalgo, F. J.; Kinsella, J. E. Changes induced in β-lactoglobulin B following interactions with linoleic acid 13-hydroperoxide. *J. Agric. Food Chem.* **1989**, *37*, 860−866.

Hunt, J. V.; Wolff, S. P. Oxidative glycation anf free radical production: a causal mechanism of diabetic complications. *Free. Radical Res. Commun.* **1991**, *12*, 115−123.

Jelen, P.; Rattray, W. Thermal denaturation of whey proteins. In *Heat-Induced Changes in Milk*, 2nd ed.; Fox, P. F., Ed.; Internatonal Dairy Federation: Brussels, 1995; pp 66−85.

Kato, Y.; Watanabe, K.; Sato, Y. Effect of some metals on the Maillard reaction of ovalbumin. *J. Agric. Food Chem.* **1981**, *29*, 540−543.

Kawakishi, S.; Okawa, Y.; Uchida, K. Oxidative damage of protein induced by the Amadori compound-copper ion system. *J. Agric. Food Chem.* **1990**, *38*, 13-17.

Laligant, A.; Dumay, E.; Casas, C.; Cuq, J. L.; Cheftel, J. C. Surface hydrophobicity and aggregation of β-lactoglobulin heated near neutral pH. *J. Agric. Food Chem.* **1991**, *39*, 2147−2155.

Landry, J.; Delhaye, S. Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. *J. Agric. Food Chem.* **1992**, *40*, 776−779.

Le Guen, C. A.; Jones, A. F.; Barnett, A. H.; Lunec, J. Role of reactive oxygen species in the generation of fluorescence by glycation. *Ann. Clin. Biochem.* **1992**, *29*, 184−189.

Mills, O. E. Effect of temperature on tryptophan fluorescence of β-lactoglobulin B. *Biochim. Biophys. Acta* **1976**, *434*, 324−332.

Miura, T.; Muraoka, S.; Ogiso, T. Oxidative damage to bovine serum albumin induced by hydroxyl radical generating systems of xanthine oxidase + EDTA-Fe^{3+} and ascorbate-EDTA-Fe^{3+}. *Chem. Biol. Interact.* **1992**, *85*, 243−254.

Moreaux, V.; Birlouez-Aragon, I.; Ducauze, C. Copper chelation by tryptophan inhibits the copper-ascorbate oxidation of tryptophan. *Redox Rep.* **1996**, *2*, 191−197.

Nielsen, H.; De Weck, D.; Finot, P. A.; Liardon, R.; Hurrell, R. F. Stability of tryptophan during food processing and storage. 1. Comparative losses of tryptophan, lysine, and methionine in different model systems. *Br. J. Nutr.* **1985**, *53*, 281−292.

Nyhammar, T.; Pernemalm, P. A. Reaction of *N*-acetyl-DL-tryptophan amide with D-xylose or D-glucose in acidic solution. *Food Chem.* **1985**, *17*, 289−296.

O'Brien, J. Heat-induced changes in lactose: isomerization, degradation, Maillard browning. In *Heat-Induced Changes in Milk*; Fox, P. F., Ed.; International Dairy Federation: Brussels, 1995; pp 134−169.

Pirie, A.; Dilley, K. J. Photo-oxidation of *N*′-formylkynurenine and tryptophan peptides by sunlight or simulated sunlight. *Photochem. Photobiol.* **1974**, *19*, 115−118.

Reyftmann, J. P.; Santus, R.; Mazière, J. C.; Morlière, P.; Salmon, S.; Candide, C.; Mazière, C.; Haigle, J. Sensitivity of trytpophan and related compounds to oxidation induced by lipid autoperoxidation. Application to human serum low- and high-density pipoproteins. *Biochim. Biophys. Acta* **1990**, *1042*, 159−167.

Saito, G.; Okitani, A.; Hayase, F.; Kato, H. Characterization of tryptophan derivatives from the reaction of *N*-α−acetyltryptophan with carbonyl compounds. *Agric. Biol. Chem.* **1986**, *50*, 2315−2323.

Sawyer, W. H. Heat denaturation of bovine β-lactoglobulins and relevance of disulfide aggregation. *J. Dairy Sci.* **1968**, *51*, 323−329.

Schleicher, E.; Wieland, O. H. Specific quantification of protein(lysine) bound glucose in human serum albumin and other glycosylated proteins. *J. Clin. Chem.* **1981**, *19*, 81−87.

Sugimura, T. Carcinogenicity of mutagenic heterocyclic amines formed during the cooking process. *Mutat. Res.* **1985**, *150*, 33−41.

Uchida, K.; Kawakishi, S. Selective oxidation of tryptophan and histidine residues in proteins through the copper-catalyzed autooxidation of L-ascorbic acid. *Agric. Biol. Chem.* **1988**, *52*, 1529−1535.

Uchida, K.; Enomoto, N.; Itakura, K.; Kawakishi, S. The hydroxyl radical generated by an tron(II)/EDTA/ascorbate system preferentially attacks tryptophan residues of protein. *Agric. Biol. Chem.* **1989**, *53*, 3285−3292.

Yaylayan, V. A.; Huyghues-Despointes, A.; Polydorides, A. A fluorescamine-based assay for the degree of glycation in bovine serum albumin. *Food Res. Int.* **1992**, *25*, 269−275.

Yim, H. S.; Kang, S. O.; Hah, Y. C.; Chock, P. B.; Yim, M. B. Free radicals generated during the glycation reaction of amino acids by methylglyoxal. A model study of protein-cross linked free radicals. *J. Biol. Chem.* **1995**, *270*, 28228-28233.

Received for review July 9, 1996. Revised manuscript received January 23, 1997. Accepted February 20, 1997.[⊗]

JF9605005

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1997.