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Intermediary and/or advanced Maillard products exhibit prooxidant activity on Trp: In vitro study on α-lactalbumin

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Abstract

Complex glycoxidation reactions take place during heat treatment of proteic foods leading to protein oxidative damage and nutritional loss. This study investigates the oxidative effect of Maillard products on Trp in α-lactalbumin incubated at 90 °C, and compares it with that of radical generating model systems. Trp oxidation rates were measured, on the one hand during the Maillard reaction—by incubation in the presence of lactose or preformed intermediary and advanced Maillard products—and on the other hand, in the presence of H_2O_2/FeII and ascorbate/FeIII mixtures. After 2 h at 90 °C, similar Trp degradations were observed in the different systems, but exclusively in the undenatured proteins soluble at pH 4.6. Initial degradation rates were, however, much higher in the radical-generating systems, and in the presence of pre-formed Maillard products, than in the samples where the Maillard reaction developed. These results suggest that Maillard products are responsible for Trp degradation, probably as a consequence of production of oxygen radical species during the intermediary and advanced Maillard reaction. © 2001 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Tryptophan; Maillard reaction; Radical-mediated oxidation; α-Lactalbumin

1. Introduction

The Maillard reaction (MR) is known to occur frequently during food processing and storage and this complex glycation reaction affects the nutritional value of food proteins (O'Brien, 1997). Lysine residues are preferential targets because of the high reactivity of the ϵ-NH_2 groups in the presence of reducing sugars. This reaction is activated by oxygen radical species and, since some steps of the reaction, such as degradation of intermediary dicarbonyl compounds, are self-producing radical species in the presence of transition metal ions (Cheng, Tsonehiro, Uchida, & Kawakishi, 1991; Gillery, Monboisse, Maquart, & Borel, 1988; Wolff, Bascal, & Hunt, 1988), propagation of the reaction occurs under such conditions. Furthermore, transition metals, such as iron and copper, are chelated by Maillard products (Saxena, Saxena, Wu, Obrenovich, Weiss, & Monnier, 1999), inducing both activation of the MR (Kato, Watanabe, & Sato, 1981; Tessier & Birlouez-Aragon, 1997) and site-specific oxidation (Stadtman & Oliver, 1991). That explains why intense MR in milk products has been associated with protein oxidative damage, namely on tryptophan (Trp) (Finot, Magnenat, Guignard, & Hurrell, 1982; Moreaux & Birlouez-Aragon, 1997). This interaction between glycation and oxidation is commonly named glycoxidation. As a consequence, in addition to lysine damage, specifically due to the MR, Trp degradation and decrease in digestibility may, in such oxidizing conditions, contribute to the loss of protein nutritional quality (Birlouez-Aragon, 1999).

Such glycoxidation conditions are favoured in infant formulas because of supplemental lactose, iron and vitamin C, present in the formula for nutritional purposes (Birlouez-Aragon, Moreaux, Nicolas, & Ducauze, 1997). The nutritional importance of such formulas demands particular attention to all reactions liable to decrease the protein nutritional value and safety.

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Amongst the numerous reactions possibly taking place via glycoxidation, the oxidative damage of Trp seems of particular importance, because this essential amino-acid is often limiting in infant protein nutrition (Fazzolari-Nesci, Domianello, Sotera, & Raiha, 1992; Domianello, Sotera, & Raiha, 1992; Heine, Radke, Wutzke, Peters, & Kundt, 1996; Sarwar, Peace, & Botting, 1989; Steinberg, O'Connell, Hatch, Picciano, & Birch, 1992). Moreover, Trp oxidative degradation products have been reported to trigger some potential toxic activity (Friedman & Cuq, 1988).

That Trp degradation may occur during the MR is well documented (Leahy & Warthesen, 1983; Moreaux & Birlouez-Aragon, 1997; Nielsen, De Weck, Finot, Liardon, & Hurrell, 1985) and the role of iron metal ions is strongly suggested by inactivation following chelation of iron by lactoferrin (Bihel & Birlouez-Aragon, 1998). However, which step of the MR is implicated and whether Trp is degraded by oxidative damage is still unclear.

This study was carried out to investigate the association between Trp degradation and different steps of the MR in vitro, and to compare the resulting Trp oxidation rates with those derived from hydroxyl radical-generating systems. For that purpose the particularly Trp-rich milk-derived α-lactalbumin (AL) was incubated at 90 °C in the presence of lactose (models L), or preformed early and advanced Maillard products (MP; models M), or in the presence of the radical-generating systems H_2O_2/iron II or ascorbate/iron III (models R). The latter allow generation of hydroxyl radicals by the Fenton reaction (R1) or the Udenfriend reaction (R2; Udenfriend, Clark, Axelrod, & Brodie, 1954), where the first step is ascorbate oxidation, giving rise to production of hydrogen peroxide and reduction of iron III, allowing the Fenton reaction to take place. In the model L, the MR develops during incubation time (3 h). Two lactose levels (L1 with 20 $g.l^{-1}$ and L2 with 40 $g.l^{-1}$) were used to produce two proportional levels of MPs. The models M differ from the previous studies by the fact that preformed MP were added at the beginning of the incubation time. In the M1 model, the complete mixture of early, intermediary and advanced MP were present whereas, in the M2 model, the mixture was further dialyzed in order to obtain only the advanced Maillard products (AMP). Trp concentration was evaluated by means of HPLC analysis or by direct fluorescence at 290/340 nm. The advanced Maillard reaction was followed by means of either fluorescence at 350/440 nm (in the total solution) or by the FAST method (Fluorescence of Advanced Maillard products and Soluble Tryptophan; in the pH 4.6-soluble protein fraction containing only the undenatured proteins). This last method has been proposed for rapidly evaluating the MR developed in milk during sterilization (Birlouez-Aragon, Nicolas, Metais, Machond, Grenier, & Calvo, 1998). The FAST index is defined as the ratio between AMP fluorescence to Trp fluorescence in the pH 4.6-soluble fraction of the solution ($F_{AMP}/F_{Trp} \times 100$) and is well correlated to furosine or lactulose in UHT milk samples (Birlouez-Aragon, Sabat, & Gouti, 2001).

2. Materials and methods

2.1. Materials

The protein, composed of 85% AL and 15% β-1actoglobulin, was provided by Armor Protein, France.

Pre-formed MP were prepared as follows: a proteose-peptone fraction (PP) was prepared from pasteurized commercial milk incubated for 5 h at 80 °C. The milk was brought to pH 4.6 with HCl for precipitation of caseins and denatured whey proteins. The absence of characteristic whey proteins (β-lactoglobulin and α-lactalbumin) in the supernatant of centrifugation was checked by exclusion chromatography (as indicated in analytical methods; levels lower than 0.1 $g.l^{-1}$). Further incubation of the PP fraction for 16 h at 110 °C allowed the development of a brown colour and fluorescence associated with MR. The incubated PP solution contained lactose (45 $g.l^{-1}$), early and intermediary MP and brown fluorescent AMP (Model M1). Dialysis of this solution (pore size around 3500 daltons) allowed isolation of the protein-linked MP, excluding lactose and intermediary free dicarbonyl compounds (Model M2). Exclusion chromatography showed the exclusive presence of high molecular aggregates ($>60\,000$ daltons) in the M2 fraction. The concentration of MP in those solutions could not be measured because of the complex mixture of Maillard compounds formed under those conditions. However, the fluorescence at excitation 350 nm and emission 440 nm (AMP fluorescence) is an accurate way to evaluate the extent of MR (Birlouez-Aragon et al., 2001). AMP fluorescence was 1.6 times greater in the non-dialysed fraction than in the dialyzed fraction, because of a dilution during dialysis.

2.2. Incubation assays

The different solutions all contained 6 $g.l^{-1}$ AL in 100 mM phosphate buffer (final pH = 7.5) and were incubated at 90 °C in Pyrex tubes immersed in an oil bath for 3 h. This heat treatment allowed AMP fluorescence (350/440 nm) to develop at levels similar to those observed in some UHT infant formulas. Aliquots were taken for analysis at different times of incubation.

For the radical generating models, 1 mM iron II ammonium-sulfate prepared from a 10 mM stock solution in 250 mM sulfuric acid, and either 1 mM hydrogen peroxide (R1 model) or 1 mM ascorbic acid (R2 model) were added to the protein solution just before

incubation. The final pH was checked to be the same in all samples, despite addition of sulfuric acid.

For the Maillard reaction models, either 20 $g.l^{-1}$ lactose (L1 model) or 40 $g.L^{-1}$ lactose (L2 model), or preformed Maillard products, prepared as described earlier, were added at the beginning of the incubation time for models M1 and M2. The fluorescence levels (excitation 350 nm and emission 440 nm) were adjusted by dilution in buffer to those accumulated in the lactose models: the non-dialyzed fraction, M1, had a similar fluorescence to the lactose 40 $g.l^{-1}$–AL mixture at the end of the incubation time [0.796×10^7 counts of photons emitted per second (cps)], whereas the dialyzed fraction M2 had a fluorescence intensity similar to that of the lactose 20 $g.l^{-1}$–AL mixture after 3 h at 90 °C, i.e. 0.476×10^7 cps.

2.3. Analytical procedures

2.3.1. Protein quantification

Soluble proteins were isolated by precipitating the denatured proteins with 0.2 M acetate buffer, pH = 4.60 (1:9 v:v). Protein concentrations in the total samples and in the acetate supernatant were quantified by the Lowry colorimetric method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.3.2. Protein aggregation and denaturation

Exclusion chromatography was performed on a Waters Protein Pak 200 W glass column, 8×300 mm (MW between 60 000 and 5000 daltons), eluted with a 80 mM acetate buffer (pH = 6.2) at a flow rate of 0.8 ml min^{-1}. Proteins were detected by absorbance at $\lambda = 250$ nm.

2.3.3. Evaluation of advanced Maillard reaction

AMP accumulation was evaluated by specific fluorescence. Fluorescence measurements were made on the complete solution, or on the pH 4.6-soluble protein fraction, after convenient dilution, on a Fluoromax-Spex spectrofluorometer (Jobin-Yvon, France). Two major AMP fluorophores were seen, one with excitation at 350 nm and emission at 435–440 nm, and the other one with excitation at 330 nm and emission at 420 nm. The relative analytical error of fluorescence measurements is 2.5%

2.3.4. Analysis of proteic Trp

Trp quantification was assessed by HPLC after alkali hydrolysis, on an isocratic HPLC system (Waters 486, Saint Quentin en Yvelines, France) equipped with a C18 Spherisorb reverse-phase column (250×4.6 mm, 5 μm) and a fluorescence detector (FL 3000, Thermo-Separation-Products, Les Ulis, France), according to the procedure of Landry and Delhaye, 1992.

Samples (1.5 ml) and 5-methyl tryptophan (MeTrp) as extemal standard (0.1 ml, 1.25 mM) were hydrolyzed with barium hydroxide octahydrate (840 mg) for 16 h in an autoclaver at 110 °C in sealed glass tubes. The solution was neutralized with HCl, diluted to 25 ml, and filtered through on Nylon 0.45 μm filters for HPLC analysis. The eluant was composed of ammonium acetate/methanol (65/35; v/v), to pH 4.5 with formic acid, and the flow rate was set at 0.8 ml min^{-1}. Trp and MeTrp were detected at $\lambda_{ex}/\lambda_{em}$ = 290/350 nm and tyrosine at 270/310 nm. The relative analytical error was 1.8%, and the experimental error was 7.2%. Trp fluorescence was measured on the total and soluble protein fractions at excitation 290 nm and emission 340 mn with a relative analytical error of 2.5%.

2.4. Statistical analysis

Kinetic data were calculated on Prism software from modelization of the curves, plotting the concentrations or fluorescence as a function of incubation time as lines or exponentials. Comparison of means used non-parametrical tests.

3. Results

3.1. Protein denaturation

Protein denaturation was almost maximal after the first hour of incubation, as shown by the denaturation curves, plotting the protein concentration of the pH 4.6-soluble fraction as a function of the incubation time (Fig. 1). Exclusion chromatograms indicate a proportional decrease of native AL and formation of two aggregate fractions, with mean molecular weights (MW) of 55 000 and higher than 100 000. In alI models, the lower MW aggregates appeared sooner (at 30 min) than the higher MW aggregates, only present after the first hour of incubation.

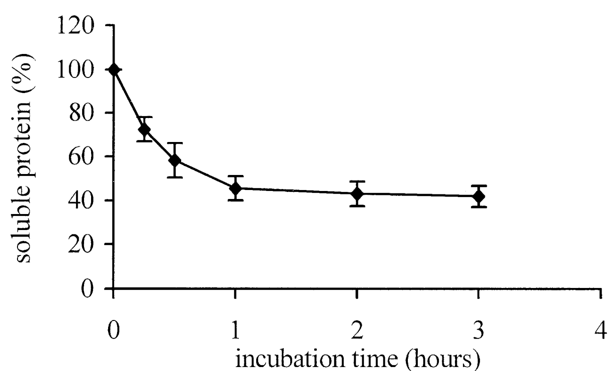

Fig. 1. Protein denaturation during incubation of AL solutions at 90 °C. Note: mean values and standard deviations are calculated from the six different AL systems.

3.2. Evolution of AMP fluorescence

Two main fluorophores were seen similarly in the proteose-peptone fraction previously incubated at 110 °C and in the AL-lactose solutions incubated at 90 °C. The major one (F1) had maximal excitation and emission wavelengths at 350/435–440 nm and the other one (F2) at 330/420 nm. These fluorophores are considered to be associated with AMPs.

The two fluorophores increased exponentially as a function of incubation time in AL–lactose mixtures, and were linearly correlated ($F1 = 1.3744 \times F2 - 0.0084$, $r^2 = 0.982$, $n = 12$). The kinetics of AMP accumulation in the AL-lactose model (Fig. 2) were calculated to be :

$$F_{AMP} = 0.0659\exp(0.902\ t), \quad r^2 = 0.960(L1);$$

$$F_{AMP} = 0.1041\exp(0.604\ t), \quad r^2 = 0.980(L2),$$

depending on the lactose concentration, 40 or 80 g l^{-1} for L1 and L2, respectively (t being the incubation time in min).

When AL was incubated without lactose, no fluorescence appeared, the control and the R1 and R2 model samples had very low fluorescence levels in this wavelength range.

In the AL solutions incubated in the presence of preformed MP, AMP fluorescence remained almost stationary during incubation, i.e. at the fluorescence level reached after 3 h of incubation in the L1 and L2 systems for M1 and M2 model systems, respectively (Fig. 2).

3.3. Trp degradation during AL incubation

Peptide Trp decreased significantly in the pH 4.6-soluble protein fraction only, whatever the system, except in the control solution (AL incubated alone) where Trp concentration remained unchanged, as indicated by HPLC analysis (Fig. 3). In the total protein samples, no significant degradation of Trp could be seen. Trp degradation in the pH 4.6 AL-soluble fraction was not significantly different between the models after 3 hours of incubation, with a final mean relative Trp concentration of $72.3 \pm 4.89\%$ ($n = 8$).

The main difference between the samples was the much more rapid degradation in the systems, where the radical species were generated at the beginning of the incubation period (R1, R2; almost 90% of the total degradation reached at 30 min), than in those where the MR developed during the incubation time (L1 and L2; 90% of total degradation reached after 2 h). The M1 system was closer to the R systems, and the M2 one closer to the L system (Fig. 3).

Initial reaction rates were calculated for each model from a semi-logarithm plot of the Trp concentration for the first hour of incubation. The linear regressions obtained ($r^2 > 0.93$) suggest a first-order reaction kinetic and allowed calculation of the first-order rate constants for Trp oxidation under the different conditions.

Table 1 summarizes the kinetic equations and the first-order rate constants kTrp for each model. For the radical-generating models, R1 and R2, the very rapid Trp degradation obliged evaluation of the initial rate for the first 15 min of incubation only (two data of Trp

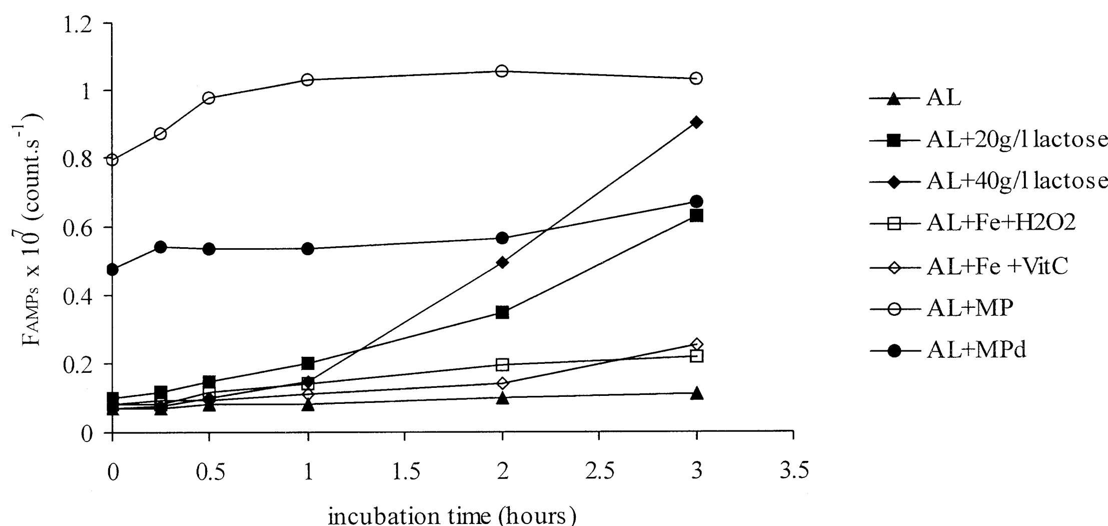

Fig. 2. Development of fluorescence ($\lambda_{ex} = 350$ nm, $\lambda_{em} = 435$–440nm) in AL samples during incubation under various conditions. Note: each sample was measured in duplicate, the mean relative error of repeatability was lower than 2.5%. MPs are pre-formed Maillard products of the proteose-peptone fraction (M1); MPd are the dialysis fractions of MP (M2).

Table 1
Kinetic equations and first-order rate constants (in 10^{-3} min^{-1}) of Trp degradation in α-lactalbumin incubated under various conditions

System	Kinetics equation ln[Trp] = f(t)	Correlation coefficent (r)	First-order rate constant k_{Trp} (10^{-3} min^{-1})
L1	y = -0.0021×−1.688	0.884	2.11±0.11
L2	y = -0.0032×−1.699	0.975	3.24±0.09
M1	y = -0.0129×−1.689	0.998	13.0±0.06
M2	y = -0.0047×−1.670	0.961	4.66±0.33
R1[a]	y = -0.0228×−1.682		22.9
R2[a]	y = -0.0184×−1.682		18.5

L: systems incubated in the presence of lactose at 20 mg.l^{-1} (L1) and 40 mg.l^{-1} (L2); M: systems incubated in the presence of pre-formed advanced maillard product (AMP), total fraction (M1) and dialyzed melanoidins (M2); R: systems incubated in the presence of H_2O_2 iron II (R1) and and ascorbate iron II (R2).

[a] As indicated in the text, the initial rate constant could only be estimated in these two cases associated with very rapid degradation using the two first points of the degradation curve.

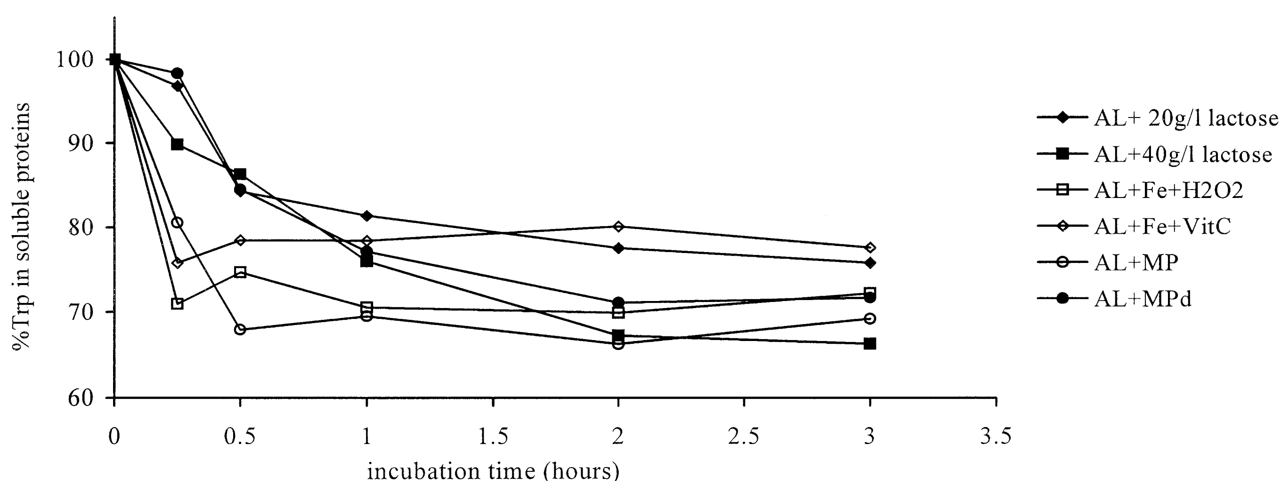

Fig. 3. Relative degradation of Trp residues in pH 4.6-soluble proteins of AL solutions incubated under various conditions. Note: Trp was quantified by HPLC and divided by the protein content of the pH 4.6 soluble fraction. The Trp content is expressed as the percentage of the concentration in the control sample, where no degradation occurred. Each sample was measured in duplicate, the mean relative error of repeatability was lower than 3.5%. MPs are pre-formed Maillard products of the proteose-peptone fraction (M1); MPd are the dialysis fractions of MP (M2).

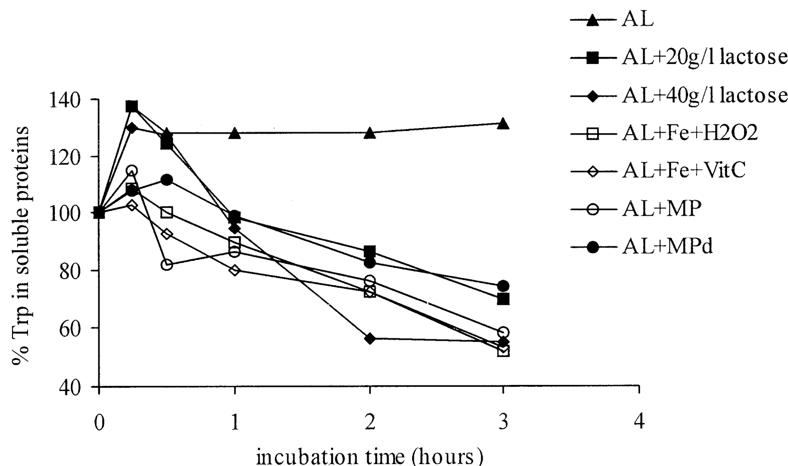

Fig. 4. Decrease in Trp fluorescence ($\lambda_{ex}/\lambda_{em}$ = 290/340 nm) in the pH 4.6-soluble protein fraction of AL incubated under various conditions. Note: Trp fluorescence was divided by the concentration of protein in the pH 4.6-soluble fraction. Each sample was measured in duplicate; the mean relative error of repeatability was lower than 2.5%. MPs are pre-formed Maillard products of the proteose-peptone fraction (M1); MPd are the dialysis fractions of MP (M2).

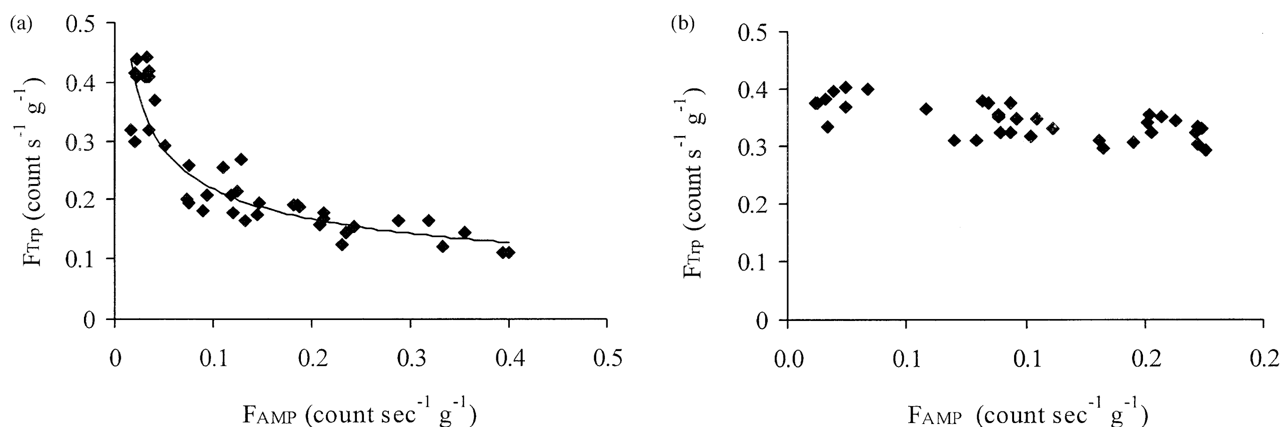

Fig. 5. Correlation between Tryptophan (F_{Trp}) and advanced Maillard product (F_{AMP}) fluorescence in the pH 4.6-soluble protein fraction (a) and in the total solution (b) of AL incubated with lactose.

concentration available and hypothesis of a first-order reaction).

The rate constants increased as a function of lactose concentration but were higher in the presence of pre-formed MP (Table 1). In the Fenton and Udenfriend model systems, the initial rate constants were much higher than in the other systems.

Similarly, Trp fluorescence in the soluble protein fraction was stable in the control sample but decreased significantly in the other model systems (Fig. 4). However, for the first 30 min of incubation, a transient increase in Trp fluorescence was observed, probably due to protein structural modifications affecting the fluorescence quantum yield. This was particularly visible in the control and the lactose model because of the slow Trp degradation (Fig. 4). In the R and M models, the rapid Trp degradation obscured this phenomenon. Consequently, Trp fluorescence was not exactly in agreement with the concentration measured by HPLC.

In pH 4.6-soluble proteins, Trp fluorescence was inversely correlated with AMP fluorescence ($r^2=0.90$, $n=36$; Fig. 5a). In the total solution, in contrast, the increase in AMP fluorescence during incubation the time was not associated with Trp fluorescence change (Fig. 5b).

4. Discussion

Previous studies on milk (Birlouez-Aragon et al., 1997) and on β-lactoglobulin (Moreaux & Birlouez-Aragon., 1997), supplemented with lactose and an iron–vitamin C mixture, suggest a possible association between intense Maillard reaction and Trp degradation. Brown advanced MPs are known to produce radical species and to chelate metals (Qian, Liu & Eaton, 1998; Saxena et al., 1999), explaining a pro-oxidant activity, which was proposed to be promoting protein fragmentation (Gaucheron, Molle, Briad, & Leonil, 1999), and oxidize sensitive amino-acids, such as Trp (Nielsen, De Weck et al., 1985; Nielsen, Finot, & Hurrell, 1985). Our purpose was to better understand which step of the MR, the intermediary or the advanced stage, is involved in Trp degradation. For that purpose, AL was chosen as a model protein, because of its high Trp content (26.7 mg g^{-1}). MPs were produced by incubation of the PP-lactose fraction of milk, because the very low Trp content of this heat-resistant protein fraction of whey, allowed the Trp concentration to remain equal in all systems after addition of MP to the protein solution.

Trp oxidation rates were compared, depending on whether the MR developed during the incubation period in the presence of lactose, or whether pre-formed MP were added at the beginning of the incubation period. Furthermore, kinetics of Trp degradation, associated with the presence of MPs, were compared with those derived from oxygen-radical species produced in the Fenton and Udenfriend systems, based on interaction between iron and either H_2O_2 or ascorbate, added at concentrations compatible with infant formulas.

Results confirm that incubation of AL in the presence of lactose induces both the formation of fluorescent AMPs and Trp degradation, but the latter occurs, only in the pH 4.6-soluble fraction of the AL solution, containing undenatured proteins. As expected, the H_2O_2 iron II and ascorbate iron III models were also associated with extensive Trp degradation in the soluble protein fraction. Again, no significant change was observed in the total AL solution containing denatured plus undenatured proteins, confirming the observations already reported in infant formulas and in a β-lactoglobulin model system (Birlouez-Aragon et al., 1997; Moreaux & Birlouez-Aragon, 1997). This could be explained by a protection of Trp residues embedded in the hydrophobic core of the high molecular weight aggregates formed during heat-induced protein denaturation, as shown by exclusion chromatography. Consequently, the radical species produced from the Fenton

or Udenfriend reaction, or from pre-formed MP, could not reach the Trp residues. Hence, the rate of formation of pH 4.6-insoluble high molecular aggregates was higher than the rate of Trp oxidation, probably impeding Trp oxidation before the protein aggregated.

Trp fluorescence was in agreement with HPLC analysis only after a 30 min period, during which Trp fluorescence increased in all systems, and especially in the control. An increase in the Trp fluorescence quantum yield explains this observation which can be interpreted by the structural modification of the heat-denatured protein. Protein denaturation is exclusively dependent on heat-treatment and not influenced by the chemical reactions taking place in the various model systems (Mills, 1976; Pico, 1997).

In whey proteins, however, Trp fluorescence appears well correlated with Trp concentration (not published) so that, in mildly heat-treated milk (pasteurization and UHT in the absence of extra iron added), Trp is not degraded and Trp fluorescence is a good indicator of the pH 4.6-soluble whey protein concentration (Birlouez-Aragon et al., 1998; Birlouez-Aragon, Sabat et al., 2001). By way of contrast, in the case of infant and growth formulas, where part of the soluble peptide Trp is degraded, because of intense MR and addition of iron, the Trp-to-whey protein ratio decreases as compared with cow's milk and indicates the extent of Trp damage upon glycoxidation (Birlouez-Aragon, Sabat, Lutz, Leclere, & Nicolas, 1999).

The hypothesis of a direct implication of brown advanced MPs is supported by the similar Trp degradation levels at the end of the incubation time in the presence of pre-formed MP (M models) and during development of the MR (L models), whereas no degradation occurred in the absence of lactose or MP. The inverse non-linear correlation observed between Trp concentration and AMP-associated fluorescence suggests a relation between Trp degradation and MR. The much higher Trp degradation rate in the presence of pre-formed MP supports the hypothesis of a direct oxidative effect of some MP, similarly to what is observed in the radical-generating model systems R1 and R2. Furthermore, the initial rate constant was proportional to the lactose concentration and to the fluorescence associated with pre-formed MP. In the lactose-containing systems, the rate constant was more related to the development of the AMP-associated fluorescence accumulated during incubation (area under the curves, Fig. 2) than to the lactose concentration. Despite a twice higher lactose concentration in the L2 system than the L1 system, total AMP fluorescence was only 1.43 times higher in L2 than in L1, and similarly, Trp degradation constant rate was 1.53 times higher in the L2 than in the L1 model system.

The radicals produced by the oxidant systems R1 and R2 (essentially HO^- and O_2^-) (Halliwell, 1978; Halliwell & Gutteridge, 1984) are expected to react immediately with the target Trp. AMP are also supposed to produce radical species and the Trp degradation observed in M1 and M2 model systems supports this hypothesis. It still remains to determine whether the oxidant species are ene-diol compounds formed during the intermediary reaction, or the brown and fluorescent products formed during the advanced reaction. The former are expected to produce H_2O_2 and oxygen radical species in the presence of trace amounts of transition metals (present in our solutions; Umetsu, Ikeda, & Nguyen, 1999; Wolff et al., 1988). Brown products and melanoidins are better known as antioxidants, because of the strong HO^-, $H_2O_2^-$ and O_2^- scavenging activities of their reductone structures (Hayase, Hirashima, Okamoto, & Kato, 1989). But a pro-oxidant activity was also suggested in the presence of traces of metal ions (Cheng et al., 1991; Umetsu et al., 1999). The presence of carbonyl and carboxyl groups in the structure of different melanoidin fractions is related to their oxidative properties (Kato, Kim, & Hayase, 1986; Umetsu et al., 1999).

In our study, MP containing low weight intermediary compounds, in addition to AMP, seemed to be more able to degrade Trp than the dialyzed fraction, containing only AMP-linked to proteins (MW > 3500 daltons). Confirmation of the molecular structures involved in this radical-mediated oxidation and elucidation of the catalyzing role of metals chelated by those structures are needed.

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