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Fortification of milk with iron-ascorbate promotes lysine glycation and tryptophan oxidation

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Abstract

Infant formulas are supplemented with iron and ascorbate for nutritional purposes, but activation of the advanced Maillard reaction by iron and production of hydroxyl radicals by the iron-ascorbate system may damage the proteins with possible nutritional loss. In this study, we evaluate the deleterious actions of iron and ascorbate, namely lysine damage and tryptophan oxidation on whey-lactose samples incubated at 60 °C. Iron-catalysed ascorbate oxidation enhanced lysine blockage mainly by ascorbylation, whereas lactosylation was little influenced. A three-fold increase in the accumulation rate of fluorescent advanced Maillard products (AMP) was observed in the presence of iron/ascorbate and AMP fluorescence was well correlated to carboxymethyllysine concentration in the samples. The tryptophan degradation rate was 2.5-fold higher in the presence of iron-ascorbate and the loss of this amino-acid was exponentially correlated with AMP fluorescence, similarly whatever iron-ascorbate was present or not. Application of milder heat treatments to infant formulas would allow to decrease the extent of these undesirable reactions.

Keywords: Iron; Ascorbate; Maillard reaction; Lysine; Tryptophan

1. Introduction

Infant and growth formulas are usually fortified with iron in order to prevent anaemia, which is one of the most frequent nutritional problems in children. As iron bioavailability is very low in cow milk (2–4%), addition of ascorbic acid is also recommended, as it increases 2–4-fold iron absorption (Gillooly et al., 1984). However, ascorbic acid is known to act as a pro-oxidant in the presence of iron. Transition metals catalyse the oxidation of ascorbic acid into dehydroascorbate, whereas hydrogen peroxide is produced. The Fenton reaction may then take place inducing both hydroxyl radical production and recycling of the metal in the oxidized form (Almaas, Rootwelt, Oyasaeter, & Saugstad, 1997; Halliwell & Gutteridge, 1984). These radicals are extremely reactive and oxidize target molecules, such as unsaturated fatty acids (Halliwell & Gutteridge, 1984) and proteins (Kocha, Yamaguchi, Ohtaki, Fukuda, & Aoyagi, 1997).

The most sensitive amino acids are histidine (Uchida & Kawakishi, 1988), tryptophan (Trp; Friedman & Cuq, 1988) particularly its pyrrole moiety (Simat & Steinhart, 1998), tyrosine and sulfur amino-acids (Davies, Delsignoret, & Lin, 1987). Tryptophan is an essential amino acid which regulates numerous physiological functions and its oxidative products trigger possible antinutritional and toxicological properties (Friedman & Cuq, 1988), so that degradation during food processing could be of nutritional and toxicological importance.

Moreover metal ions are known to activate the Maillard reaction (Kato, Watanabe, & Sato, 1981), particularly the formation of carboxymethyllysine (CML; Ahmed, Thorpe, & Baynes, 1986). By accelerating ascorbate oxidation, iron(III) also favours protein ascorbylation. Indeed, ascorbate degradation products, such as L-Threose (Ortwerth, Speaker, Prabhakaram, Lopez, Yinan Li, & Feather, 1994), are 20–100 times more reactive in terms of glycation than glucose or lactose (Lee, Mossine, & Ortwerth, 1998). Consequently, in addition to the high lactosylation level of proteins in infant formulas (Evangelisti, Calcagno, Nardi, & Zunin, 1999; Birlouez-Aragon, Moreaux, Nicolas, & Ducauze,

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1997), ascorbylation in the presence of 100–200 mg l^{-1} (near the concentration added in infant formulas) can become significant and further decrease lysine bioavailability. These complex glycation and oxidation reactions are essentially temperature dependent.

Furthermore, iron is strongly chelated on glycated proteins (Qian, Liu, & Eaton, 1998) and can generate oxygen species, efficiently reacting with the amino acid residues located near the metal binding site (Amici, Levine, Tsai, & Stadtman, 1989; Stadtman & Oliver, 1991). This oxidative process may explain the degradation of Trp in the presence of advanced Maillard products (Moreaux & Birlouez-Aragon, 1997).

This study was conducted in order to evaluate the kinetics of the early and advanced Maillard reaction, and of the consecutive Trp oxidation, using a simple whey-lactose system added or not with iron-ascorbate and incubated at a mild temperature of 60 °C. Such a low temperature is not relevant to industrial processes but allows to precisely quantify the kinetic rates. The respective contributions of the different reactions involved, and the oxidative effect of iron chelated on glycated proteins is discussed. The paper focuses on the nutritional impact of the heat treatment of infant formulas, such as lysine loss and Trp damage.

2. Material and methods

2.1. Chemicals

Lactose, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, barium hydroxide octahydrate, L-ascorbic acid and fluorescamine were from SIGMA (Saint Quentin Fallavier, France). Sodium tetraborate anhydrous and sodium acetate trihydrate were from FLUKA. Hydrochloric acid, orthophosphoric acid, methanol, ammonium acetate and ammonium-iron(II) sulfate were from PROLABO.

Lactose-free powder whey was prepared in order to obtain a 80% protein mixture enriched with β-lactoglobulin (85% β-lactoglobulin and 15% α-lactalbumin). β-lactoglobulin contains 890 μmol lysine per gram protein and α-lactalbumin contains 898 μmol lysine per gram protein (Veisseyre, 1966).

2.2. Preparation of samples

Whey samples containing 8 g l^{-1} proteins (7.2 mM lysine) and lactose 60 g l^{-1} in phosphate buffer 0.1 M, pH 7.4 were incubated in stoppered tubes Pyrex at 60 °C in an oil bath in the absence (in triplicate) and in the presence (in duplicate) of iron 10 mg l^{-1} and ascorbate 200 mg l^{-1}. Aliquots (4 ml) were taken at different times ($t = 0$, 3, 6, 9, 24, 31, 48, 55, 120 h and $t = 0$, 3, 6, 24, 31, 48, 55, 72 h, in the absence and in the presence of iron/ascorbate, respectively) and stored at −20 °C until analysis. Samples without iron/ascorbate were incubated for a longer period of time than samples with iron/ascorbate in order to compensate for the slower Maillard reaction rate in the former.

Whey samples containing 4 g l^{-1} proteins (3.6 mM lysine) were incubated with increasing lactose levels (0, 30, 60, 80, 100, 120 g l^{-1}) in stoppered tubes at 60 °C in an oil bath in the absence and in the presence of iron 20 mg l^{-1} and ascorbate 200 mg l^{-1}. Aliquots were taken at 89 h incubation and stored at −20 °C until analysis.

2.3. Fluorescamine assay

This method is described by Yaylayan, Huyghues-Despointes, and Polydorides (1992) to evaluate the ratio of blocked amino groups in glycated proteins by comparison with a control sample. Of the incubation mixtures 200 μl were added to 2.2 ml of a 0.2 M sodium tetraborate buffer, pH 8.5 in 10-ml tubes. While the tubes were vigorously shaken on a vortex type mixer, 100 μl of fluorescamine solution (15 mg/100 ml acetone) were rapidly added and fluorescence measured after 5 min on a SPEX Fluoromax™ DM 3000 F apparatus (Jobin Yvon, France) in 4-face acryl cuves 10×10×48 mm (Sarstedt, France). The excitation/emission wavelengths were set at 390/475 nm. The intrinsic fluorescence of a sample blank without fluorescamine was subtracted because of interfering fluorescent AMP in the incubated samples.

The standard deviation of analytical repeatability (two analysis) [100×(S.D./mean)] varied between 2.5 and 3.4%. The standard deviation of experimental repeatability was calculated from the results obtained from the samples incubated independently and varied between 3.2 and 8%.

2.4. Fluorescence of advanced Maillard products (AMP)

Samples (200 μl) were diluted in 2.3 ml borate buffer (0.2 M, pH 8.5) and AMP fluorescence was quantified in counts of photons emitted per second (cps; SPEX, Jobin-Yvon). The maximum excitation wavelength was 345 nm and the maximum emission wavelength was 412 nm for whey samples without iron/ascorbate and 425 nm for the samples added with iron/ascorbate, because the ascorbylation and lactosylation products exhibit slightly different emission spectra. The standard deviation of experimental repeatability (independent incubations) was 4%.

2.5. Fluorescence of soluble tryptophan

Trp fluorescence was determined in the pH = 4.6 soluble fraction of samples prepared as follows: nine

volumes of acetate buffer (0.1 M, pH = 4.6) was added to 1 volume of sample. After centrifugation at 4000 ***g*** for 10 min, Trp fluorescence in the supernatant was measured (λ_{exc} 290 nm; λ_{em} 340 nm) on a Spex fluorimeter (Jobin-Yvon). The standard deviation of experimental repeatability was 3.5%.

2.6. Furosine determination

Aliquots (330 μl) were hydrolysed for 18 h at 110 °C with 670 μl of hydrochloric acid 11.65 N (7.8 N final) in stoppered tubes and furosine was quantified on a Waters 486 HPLC system equiped with an Hypersil C18 column (250×4.6 mm) and detected at 280 nm, as described elsewhere (Birlouez et al., 1997). The standard deviation of experimental repeatability was 6%.

2.7. Protein aggregation and denaturation

The concentration of native α-lactalbumin and β-lactoglobulin and the formation of aggregates after incubation were determined by exclusion chromatography on a Waters Protein Pak 200 W Glass column (8×300 mm) coupled to absorbance (214 nm) and fluorescence (λ_{ex} = 290 nm and λ_{em} = 340 nm). The flow rate was 0.8 ml min^{-1} and the eluant was 80 mM ammonium acetate pH 6.2.

2.8. Trp quantification by HPLC

Trp quantification was assessed by HPLC after alkali hydrolysis of the protein as follows: 1.6 ml of the pH 4.6 soluble fraction of samples were hydrolysed with 0.840 g barium hydroxide octahydrate for 16 h in an oven at 110 °C in sealed glass tubes. The sample was then neutralized with 6 N HCl and diluted at 25 ml. Trp was quantified on a Waters 486 HPLC system equiped with a C18 Spherisorb reverse-phase column (250×4.6 mm, 5 μm). The eluant was 14.5 mM ammonium acetate/methanol (65/35) set at pH 4.5 with formic acid. The flow rate was 0.8 ml min^{-1} and the fluorimetric detection wavelengths were: λ_{ex} = 270 nm/λ_{em} = 310 nm for the first 5 min (tyrosine detection) and at λ_{ex} = 290 nm/λ_{em} = 350 nm for the following 15 min (tryptophan detection). The standard deviation of analytical repeatability (two analysis) was 5.7%.

2.9. CML determination by CG/MS

CML was determined in 6 samples: whey proteins (8 g l^{-1}) + lactose (60 g l^{-1}), in presence or absence of iron (10 mg l^{-1}) and ascorbic acid (200 mg l^{-1}), incubated for 0, 24 and 48 h at 60 °C.

The samples were first dialysed against distilled water during 3 days. Then, the samples containing iron were mixed (1:1) with a solution of DTPA 10 mM and dialysed again during 3 days.

Samples were concentrated (2 mg of proteins) and hydrolysed with 1 ml HCl 6 N during 24 h at 100 °C. Hydrolysate (0.5 ml) was dried and resolubilized with 100 μl of water containing 1 mM cycloleucine as internal standard. After drying under nitrogen, the samples were esterified with anhydrous *n*-methanol in the presence of HCl (anhydrous) during 40 min at 100 °C, dried under a stream of nitrogen at room temperature and then acylated by 80 μl ethylacetate (Merck) and 20 μl anhydride trifluoroacetic acid (Merck) at 100 °C during 30 min. The derivatized samples were evaporated under nitrogen and dissolved in 100 μl ethylacetate for GC/MS analysis performed on Hewlett-Packard 5890 GC using a 25 m×0.2 mm i.d.×0.33 μm film thickness (polydimethylsiloxane) Hewlett- Packard capillary column, interfaced to a Hewlett-Packard MS Engine 5989.

A standard of CML was synthetised as follows: 200 mM hydrochloride poly-L-lysine (Sigma) was incubated in a saline phosphate buffer (NaCl:130 mM, KH_2PO_4:1.5 mM, Na_2HPO_4:2.8 mM) pH 7.3, containing 400 mM of glucose, during 21 days at 37 °C.

The CML content was determined by selected ion monitoring (single ion monitoring). The *m*/*z* 392 ion was used to detect and determine CML.

2.10. Iron determination

After dialysis of the samples against water during 3 days, iron was transformed in ferrous form by reaction with ascorbic acid. In the presence of the dye, N(4-(2,4-bis(1,1-dimethylpropyl)phenoxy)butyl)-5-methoxy-6((2,3,6,7-tetrahydo-8-1H,5H-benzoquinolizine-9-yl)azo)-3-pyridine sulfamide, a complex is formed and then quantified by a colorimetric method on a Vitros 750 automate (Ortho Clinical Diagnostics) at a wavelength of 600 nm. The samples with iron-ascorbate were dialysed a second time after being mixed (1:1) with a solution of DTPA 10 mM and iron was quantified again.

2.11. Statistical analysis

Linear regressions were calculated with GraphPad Prism 3 software. Comparison of means and slopes were done with non parametrical tests (GraphPad Prism 3 software).

3. Results

Our objective was to study the effect of addition of iron(II)/ascorbate to whey samples on the early and advanced Maillard reaction and on tryptophan degradation.

Reactions were studied either as a function of incubation time (0–120 h) in samples containing a given lactose concentration (8 g l^{-1} whey proteins, 60 g l^{-1} lactose) or as a function of lactose concentration (0–120 g l^{-1}) for a single incubation time (89 h, whey proteins 4 g l^{-1}). The incubation temperature was always 60 °C.

3.1. Study of the whey-lactose system as a function of incubation time

3.1.1. Unavailable lysine

In the samples without iron/ascorbate, fluorescamine-reactive lysine was stable for the first 55 h so that no blockage could be evidenced. At $t = 120$ h, a blockage of $36.5 \pm 0.8\%$ ($n = 2$) was observed (Fig. 1).

In the presence of iron/ascorbate, lysine blockage was detected from 24 h incubation and increased linearly with incubation time, up to 55 h before slightly slowing down (Fig. 1).

3.1.2. Furosine concentrations

After a first increase between $t = 0$ and $t = 24$ h of incubation, furosine reached a steady state concentration (slope not significantly different from zero) in both samples, as shown in Fig. 2. The level of furosine at the plateau was calculated from all the points included in the horizontal portion of the curve and was significantly ($P < 0.02$, Student t test) higher in the whey samples containing iron/ascorbate: 352 ± 26 mg/100 g proteins ($n = 10$) and 310 ± 38 mg/100 g proteins ($n = 11$) in the presence and absence of iron/ascorbate, respectively.

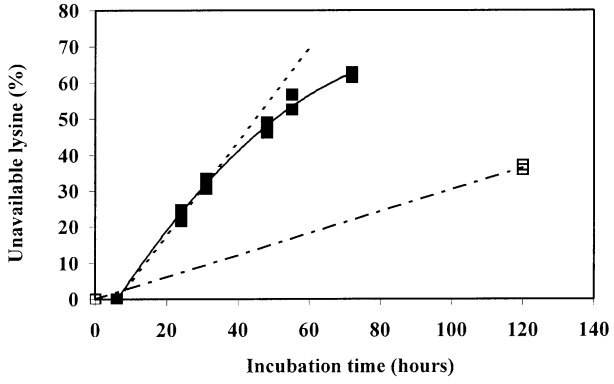

Fig. 1. Percentage of lysine blocked by the Maillard reaction as a function of incubation time in whey-lactose samples incubated in the presence ■ or absence □ of iron-ascorbate. Note: whey protein concentration was 8 g l^{-1} and lactose 60 g l^{-1}. Lysine blockage was measured as the difference (in per cent) between initial and experimental fluorescamine reactive lysine (conditions detailed in Section 2). Blocked lysine in iron-ascorbate containing samples followed a polynomial curve ($y = -0.0083x^2 + 1.6038x - 9.6933$, $R^2 = 0.9989$) but can be modelized as a linear regression during the first hours of incubation (----) ($y = 1.2845x - 7.68$). For the samples without iron-ascorbate, lysine blockage was only detected at the end of the incubation time, so that the hypothesis of a linear increase was made. (–·–·–·–) ($y = 0.3043x$).

3.1.3. AMP fluorescence

AMP fluorescence increased linearly with incubation time ($R^2 = 0.995$ and $R^2 = 0.997$ in the presence and absence of iron/ascorbate, respectively), describing a zero order reaction (Fig. 3). The rate constant of AMP fluorescence accumulation (deduced from the slopes of the linear regression) was about threefold more important in the samples with iron and ascorbate [$k = 0.194 \pm 0.005$ ($\times 10^5$) cps h^{-1}, $n = 16$] than in the samples without iron/ascorbate [$k = 0.067 \pm 0.001$ ($\times 10^5$) cps h^{-1}, $n = 26$].

3.1.4. Tryptophan degradation

Tryptophan degradation in the pH 4.6 soluble protein fraction was estimated both by direct fluorescence at 290/340 nm and by HPLC-fluorescence after alkali hydrolysis on eight representative samples. A good correlation ($R^2 = 0.985$, $n = 8$) was found between both methods; consequently only fluorescence, more rapid and simple, was measured on the other samples. As the protein concentration in the samples also decreased because of denaturation with incubation time, degradation of Trp residues was calculated by dividing Trp fluorescence (cps) in the surpernatant by the protein concentration (g l^{-1}) determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

As shown in Fig. 4A, a slight increase in Trp fluorescence per gram protein was observed during the first hours of incubation (3 and 6 h) in the samples without iron/ascorbate. But rapidly then Trp fluorescence decreased exponentially as a function of time describing a first order kinetic. The rate constant was deduced from the curve equation. It was almost 2.5-fold higher in the presence of iron/ascorbate [$k = 0.0286 \pm 0.0009$ h^{-1} ($n = 16$)] than in its absence [$k = 0.0115 \pm 0.0008$ h^{-1} ($n = 26$)].

As shown in Fig. 4B, AMP fluorescence decreased exponentially as a function of Trp fluorescence ($R^2 = 0.91$).

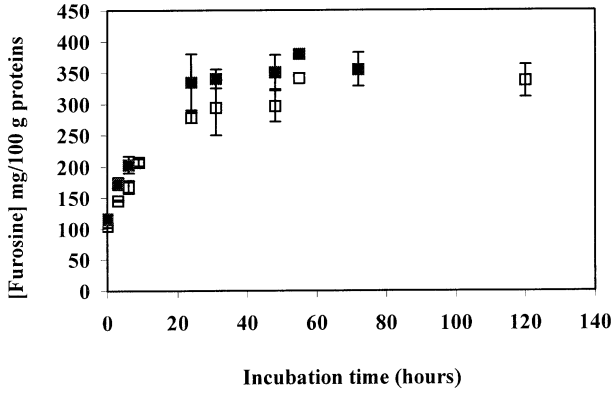

Fig. 2. Furosine concentrations as a function of incubation time in whey protein samples incubated with lactose, in the presence ■ and absence □ of iron-ascorbate. Note: conditions are specified in Section 2. Whey protein concentration was 8 g l^{-1} and lactose 60 g l^{-1}.

3.1.5. Determination of carboxymethyllysine

CML levels increased with incubation time and were 2.5–3-fold higher in iron-ascorbate containing samples (Table 1). CML was linearly correlated to AMP fluorescence independently of the presence or absence of iron/ascorbate ($R^2 = 0.99$, $n = 11$).

3.1.6. Chelation of iron

The quantity of iron chelated on the dialysed proteins was not significant in the absence of Maillard products (time zero in the absence of additional iron). In the other samples, where advanced Maillard reaction had developed, as illustrated by the formation of CML, almost 100% of the iron present in the sample was chelated (Table 1): 0.18 mg iron were chelated per gram proteins after 48 h corresponding to a final iron concentration of 1.46 mg l^{-1}, slightly higher than expected level in whey; and 1.43 mg iron were chelated per gram proteins in the iron-ascorbate sample, corresponding to a final concentration of 11.46 mg l^{-1}, equivalent to 10 mg l^{-1} added and 1.46 mg l^{-1} already present in the whey sample. Before incubation, 82% iron was already chelated on proteins, but this iron-ascorbate sample also contained a significant amount of CML.

Addition of DTPA 10 mM to the samples before dialysis allowed migration of the iron-DTPA chelates to the dialysis buffer, remaining only traces of iron in the sample (< 0.022 mg l^{-1}, below the detection limit of the method).

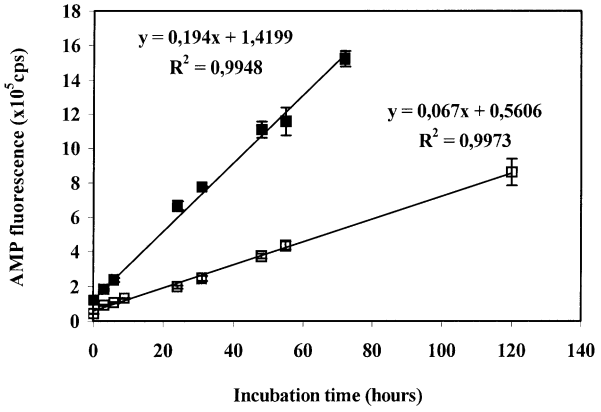

Fig. 3. Fluorescence of AMP as a function of incubation time in whey protein samples incubated with lactose, in the presence ■ and absence □ of iron-ascorbate. Note: experimental conditions are described in Section 2. Whey protein concentration was 8 g l^{-1} and lactose 60 g l^{-1}. The emission wavelength was 412 nm in the absence of iron-ascorbate and 425 nm in its presence (excitation wavelength 345 nm).

3.2. Study of the whey samples as a function of lactose concentration

The level of fluorescamine-reactive lysine decreased with lactose concentration until reaching a maximal blockage of 60% (Fig. 5A). In the absence of lactose, iron/ascorbate induced a 30% lysine blockage, and increasing lactose concentration further increased this level, despite a diminishing difference between the curve in the absence and presence of iron-ascorbate as a function of lactose concentration (Fig. 5A).

As shown in Fig. 5B, furosine concentration increased linearly with lactose. The slope of the regression line was slightly but significantly higher in the presence of iron/ascorbate than in its absence [4.21 ± 0.21 ($n = 10$) and 3.44 ± 0.14 ($n = 12$), respectively, $P = 0.006$].

AMP fluorescence increased linearly with lactose concentration with the same slope in the absence and in the presence of iron-ascorbate (0.044 ± 0.004, $n = 12$ and 0.043 ± 0.004, $n = 12$, respectively; Fig. 5C).

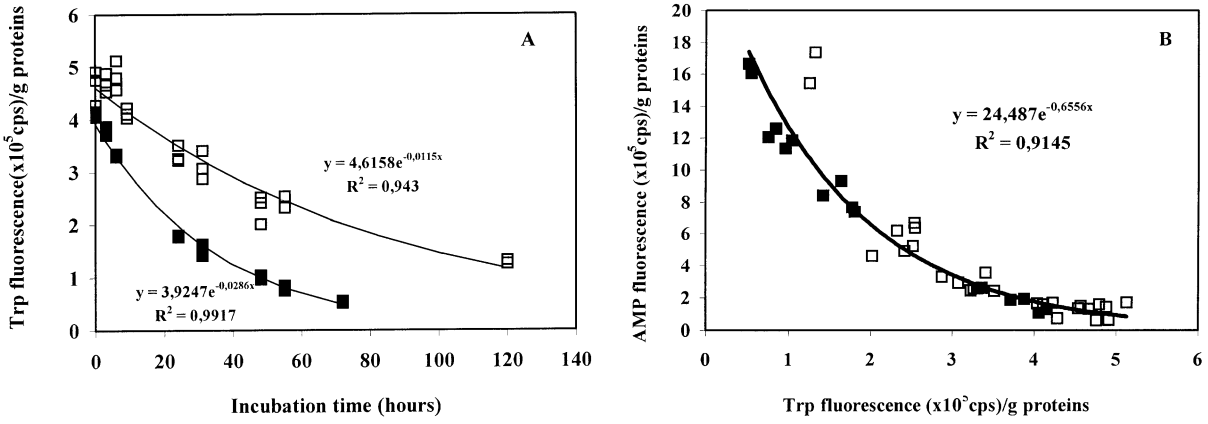

Fig. 4. (A) Degradation of peptidic tryptophan (Trp) as a function of incubation time in the pH 4.6 soluble fraction of whey samples incubated with lactose, in the presence ■ and absence □ of iron-ascorbate. Note: experimental conditions are described in Section 2. Whey protein concentration was 8 g l^{-1} and lactose 60 g l^{-1}. Trp was evaluated by fluorescence in counts of photons emitted per second (cps) per gram protein ($\lambda_{ex} = 290$ nm and $\lambda_{em} = 340$ nm). (B) AMP fluorescence as a function of Trp fluorescence in the pH 4.6 soluble fraction of whey samples incubated with lactose, in the presence ■ and absence □ of iron-ascorbate. Note: experimental and analytical conditions are described in Section 2. Whey protein concentration was 8 g l^{-1} and lactose 60 g l^{-1}.

Table 1
Concentration of carboxymethyllysine (CML) and chelated iron in the whey-lactose samples depending on the presence or absence of iron-ascorbate

Samples	Incubation time (h)	CML (pmol)/mg proteins	Chelated iron (mg/g)
Whey proteins 8 g l^{-1} + lactose 60 g l^{-1}	0	67.4±23.3	0.03±0.004
	24 h	1929.3±124.7	0.15±0.030
	48 h	2604.7±134.3	0.18±0.017
Whey proteins 8 g l^{-1} + lactose 60 g l^{-1} + iron 10 mg l^{-1} + ascorbic acid 200 mg l^{-1}	0	844.7	1.17±0.045
	24 h	4842.7±24.9	1.85±0.138
	48 h	8456.1±692.3	1.43±0.119

The mean of two independently incubated samples is given.

4. Discussion

The protein nutritional value and safety of infant formulas is extremely important because they are often the sole source of protein for children. The simultaneous addition of iron and ascorbate—for nutritional purpose—has however been shown to produce hydroxyl radicals (Almaas et al., 1997) which may damage proteins (Birlouez-Aragon, 1999; Birlouez-Aragon et al., 1997; Davies, 1987). These radical species can directly oxidize proteic amino acids, namely Trp, whose oxidation products are known to be mutagens (Friedman & Cuq, 1988). Furthermore, iron by itself has been reported to accelerate the formation of AMP from the Amadori product (Kato et al., 1981), the latter being already present at higher concentration in infant formulas as compared with cow milk because of lactose addition (Birlouez-Aragon et al., 1997; Evangelisti et al., 1999). Iron is also known to accelerate ascorbate oxidation, producing dehydroascorbate and degradation products with a strong glycating effect.

In order to precisely quantify the action of iron on these different reaction pathways and determine the possible nutritional damage on proteins in infant for-

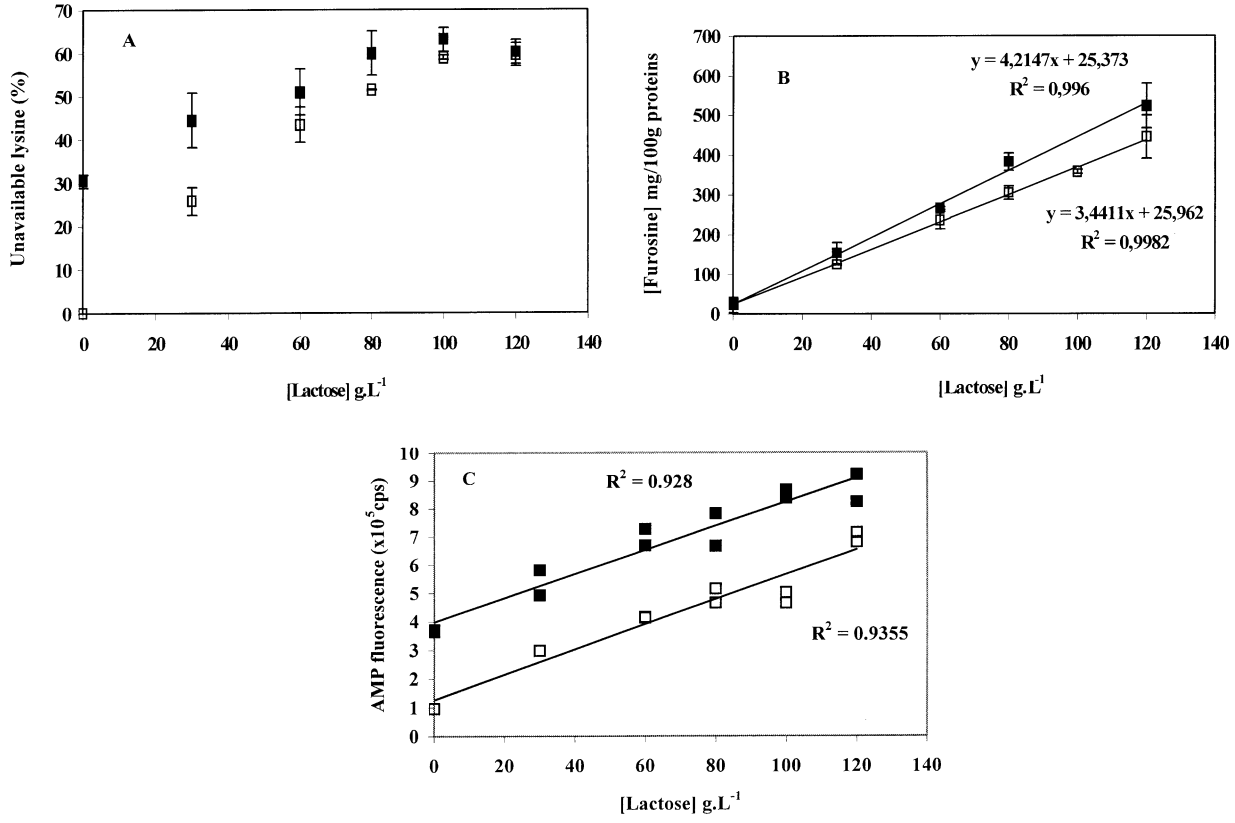

Fig. 5. Development of the Maillard reaction in whey protein samples as a function of lactose concentration, depending on the presence ■ or absence □ of iron-ascorbate. A, lysine blockage; B, furosine; and C, AMP fluorescence. Note: incubation time was 89 h at 60 °C. Whey protein concentration was 4 g l^{-1}.

mulas upon sterilization and storage, a simple whey protein model was studied where protein concentration was set at two levels (4 and 8 g l^{-1}) and lactose either constant (60 g l^{-1}) or between 0 and 120 g l^{-1}. The effect of an iron-ascorbate mixture added in concentration equivalent to what is found in infant formulas is evaluated. A low incubation temperature of 60 °C was used, in order to accurately differentiate the early step from the advanced step of the Maillard reaction.

Iron-ascorbate had a slight activating effect (+18%) on the formation of lactulosyllysine, significantly detected above a lactose concentration of 60 g l^{-1}. This activation can be explained by the catalytic effect of iron on lactose oxidation into the much more reactive dicarbonyl derivative (Wolff & Dean, 1987).

A much stronger effect of iron-ascorbate was observed on lysine blockage. In the absence of lactose, addition of iron-ascorbate to whey proteins induced a strong decrease in the chemically reactive lysine after 89 h incubation. This 30% blockage probably results from lysine glycation by the oxidative products of ascorbate which are known to react 100 times faster with lysine (Lee et al., 1998) than glucose or lactose. After 89 h at 60 °C, 200 mg l^{-1} ascorbate—almost totally oxidized—had a similar glycating effect than 40 g l^{-1} lactose. It can be calculated that 36 μM lysine is ascorbylated by 37.5 μM ascorbate in the medium, i.e. 1 mol oxidized ascorbate glycate 1 mol lysine.

In the high whey protein solution, lysine blockage rate was 4 times more rapid in the presence of lactose and iron-ascorbate than in the presence of lactose alone. However, in the low whey protein samples, increasing the lactose concentration of the solution up to 120 g l^{-1} was associated to a decreasing activation effect of iron-ascorbate on lysine blockage, as indicated by the fluorescamine assay. Maximal lysine blockage indicated by this assay was 60% of total lysine residues. This may result from a steric hindrance impeding fluorescamine to reach 40% lysine residues of the whey proteins. Whether they also are inaccessible to lactose or oxidative products of ascorbate, and consequently not involved in the Maillard reaction, cannot be deduced from this study. However, the linear increase in AMP fluorescence as a function of lactose concentration at a high incubation time (89 h), suggests that the Maillard reaction is not slowed down by limiting lysine residues, despite the low ratio between lysine and glycating substrates in this system: 3.6 mM lysine, 0–350 mM lactose and 1.13 mM ascorbate.

Some ascorbylated lysine products are fluorescent as indicated by the strong fluorescence level observed at 425 nm (excitation 345 nm) in the whey sample incubated in the presence of iron-ascorbate and without lactose. Nagaraj and Monnier (1995), identified a fluorescent condensation product formed between the epsilon-amino group of lysine and two molecules of threose, one of the major degradation product of ascorbate (Lopez & Feather, 1992). The total fluorescence of ascorbylated whey proteins is even 1.5 times more fluorescent than lactosylated whey proteins in our experimental conditions (30% ascorbylated lysine triggered the same fluorescence as 45% lactosylated lysine).

Carboxymethyllysine is a non fluorescent advanced Maillard product, which can be produced exclusively under oxidative conditions, in the presence of transition metals such as iron (Ahmed et al., 1986). It can be formed from various early Maillard products, as well from lactulosyllysine as from ascorbyllysine (Dunn et al., 1990). CML was present in all samples and was linearly correlated to the fluorescence of advanced Maillard products ($R^2 = 0.99$, $n = 11$). The formation of CML in the absence of iron, is explained by the presence of traces of metal ions in the phosphate buffer and perhaps chelated on the whey proteins (up to 1 mg l^{-1}). The 2.5–3 times higher CML concentration in the presence of iron-ascorbate can be explained either by the iron-mediated degradation of lactulosyllysine, or by the degradation of ascorbyllysine under oxidative conditions. The second hypothesis seems more probable, as before incubation (but after 1 month storage at −20 °C), only traces of CML were detected in the sample without iron-ascorbate, whereas more than 10 times higher concentrations were found in the presence of iron-ascorbate. Lactosylation cannot take place at so low temperatures, but ascorbate is known to oxidize in such conditions, giving rise to ascorbylation.

We conclude that, under oxidative conditions, iron-ascorbate has a negligible effect on the conversion of lactulosyllysine into advanced Maillard products (Kato et al., 1981), and essentially acts by promoting lysine ascorbylation. In the absence of specific ascorbylation products, only fluorinated ascorbate could help confirming this hypothesis.

Besides this glycating action, iron-catalysed ascorbate oxidation is also responsible for hydroxyl radical formation in the presence of iron, as evidenced by Almaas et al. (1997). Tryptophan is particularly sensitive to these radical species. Since we previously showed that Trp was stable in aggregated proteins (Birlouez-Aragon et al., 1997; Moreaux & Birlouez-Aragon, 1997), but was well sensitive to oxidizing species when present in soluble whey proteins, we investigated Trp degradation in the pH 4.6 soluble fraction. Despite the low incubation temperature (60 °C), 50% denaturation occurred after 60 min and analysis by exclusion chromatography allowed to evidence that, at this time, almost all the β-lactoglobulin (80–90%) had disappeared, forming high molecular weight aggregates. Trp was evaluated by means of fluorescence at 290/340 nm (excitation and emission wavelengths) in counts of photon emitted per gram protein. This rapid determination was shown to well correlate with Trp quantification by HPLC after

alkaline hydrolysis. Trp degradation followed a first order kinetic and the rate constant was 2.5 times higher in the presence of iron-ascorbate than in its absence: up to 85 and 50% Trp had disappeared after 60 h incubation. Trp degradation was exponentially related to AMP formation confirming previous results (Moreaux & Birlouez-Aragon, 1997), and the equation of the curve was the same in the absence and in the presence of iron-ascorbate. The latter condition only induced more AMP formation and proportionally more Trp degradation.

It can be hypothesized that AMP directly induce Trp degradation. Glycation of the Trp indole (Nyhammar & Pernemalm, 1985; Saito, Okitani, Hayase, & Kato, 1986) is unlikely to take place under the mild conditions used in this study. The Amadori product, depending on its chemical structure, has been reported to generate reactive oxygen species and to promote oxidative damage of the protein in the presence of ascorbate-Cu or Fe (Umetsu, Ikeda, & Nguyen, 1999). Our previous study also indicates that incubation of Trp in the presence of pre-formed dialysed Maillard products is sufficient to induce Trp degradation (not published) demonstrating that radical species are well produced by Maillard products. Whether iron-ascorbate amplify Trp destruction rate by increasing the concentration of radical-generating Maillard products, or by directly producing hydroxyl radicals via the Udenfriend reaction cannot be directly deduced from this study. However, our results strongly suggest that the higher Trp degradation in the presence of iron-ascorbate should result from the additive pro-oxidant effects of ascorbylated and lactosylated products. This pro-oxidant effect is surely not independent of the strong chelation of iron by the Maillard products (up to 100% as compared with 16% iron chelated on the control whey sample not modified by the Maillard reaction) also reported by Qian et al. (1998). Specially CML was recently demonstrated to bind divalent metal ions (Saxena, Saxena, Wu, Obrenovich, Weiss, & Monnier, 1999) and to catalyse radical-mediated oxidation (Requena & Stadtman, 1999), namely of ascorbate (Saxena et al., 1999). The results of this study also support the hypothesis of a site-specific metal-catalyzed oxidation of Trp consecutive to the chelation of iron on glycated lysine similarly to what was proposed by Amici et al. (1989).

These findings corroborate the previous results obtained on infant formulas from various dairy or pharmaceutical companies (Birlouez-Aragon, 1999; Birlouez-Aragon et al., 1997; Birlouez-Aragon, Sabat, Lutz, Leclère, & Nicolas, 1999) and point out the need of improving their nutritional quality. The two amino acids, lysine and Trp altered during the heat treatment of milk proteins in the presence of iron/vitamin C are also found of decreased bioavailability in in vivo studies (Sarwar & Botting, 1999; Sarwar, Peace, & Botting, 1989). As the nutrient composition is under regulation, improvement of infant formulas quality implies optimization of the sterilization technology for liquid milk, and of the storage conditions for the powders.

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