Monitoring of Heat-Induced Proteolysis in Milk and Milk-Resembling Systems

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Peptide mapping from the soluble fractions of skimmed milk and a milk-resembling system, obtained either by treatment with trichloroacetic acid (TCA, 40 and 120 g/L) or by adjusting the pH to 4.6, were analyzed by RP-HPLC with UV (205, 214, and 280 nm) and fluorescent detection (excitation at 280 and 270 nm and emission at 340 and 310 nm). Two peptides (p21 and p25), both soluble in 120 g/L TCA, were chosen for following the extent of the heat-induced proteolysis in milk and milk-resembling systems. Both peptides were absent in skimmed raw milk, but were detected in low amounts in Na-caseinate/lactose solution. The rate of formation of p21 and p25 peptides could be fitted to zero-order kinetics. The p25 peptide was formed more rapidly in skimmed milk than in the Na-caseinate/lactose system. As for the p21 peptide, other factors apart from temperature and heating time should be considered for understanding its thermal behavior. Possibly, p21 and p25 peptides could be applied for assessing previous industrial processing.

Keywords: *Milk; model system; heat-induced proteolysis; HPLC; fluorescence*

INTRODUCTION

Heating of milk causes many chemical and physical effects on the milk constituents, milk proteins being among the most sensitive to heat (Singh, 1995). Casein is less sensitive than the whey proteins to heating, due to the small secondary and tertiary structures of the former. However, it is well-known that casein in milk and model systems undergoes changes at high temperatures such as dephosphorylation, proteolysis, aggregation, glycation, interaction with whey proteins (i.e. κ-casein and β-lactoglobulin) and covalent modifications (Alais et al., 1966; Fox, 1982; Guo et al., 1989). It has been reported that concentrated skim milk heated above 110 °C (Fox et al. 1967) or UHT skim milk heated above 146 °C (Morr, 1969) showed disaggregation of casein micelles with formation of nonsedimentable casein. It has been postulated that disaggregation of casein micelles occurs ordinarily in the following two cases: (i) removal of calcium or colloidal calcium phosphate and (ii) disruption of hydrogen and hydrophobic bonds (Aoki et al., 1974). This is the reason calcium chelating agents disaggregate casein micelles. During heating, all the casein fractions (α_s, β, and κ) are degraded in different ways, forming nondialyzable peptides and low molecular (dialyzable) weight peptides (Alais et al., 1966; Hindle and Wheelock, 1970; Guo et al., 1989). Fox (1981) showed that α_{s2}-casein was the casein most susceptible to thermal degradation.

In raw milk, caseins are precipitated at pH 4.6, while whey proteins remain in the supernatant. Most of the proteins in the soluble fraction are globular and heat labile. Except for the native heat-stable proteose−peptone fraction, during heating of milk at temperatures higher than 60 °C whey proteins increasingly lose their globular conformation and precipitate at pH 4.6, associated with caseins through disulfide or hydrophobic interactions (Dalgleish, 1990). The proteose−peptone fraction (25% of total whey protein) has a molecular weight between 4100 and 40 800 Da. The proteose−peptone fraction is classically defined as those proteins (peptides) which remain soluble at pH 4.6 after heat treatment of skim milk at 95−100 °C for 30 min, but which are precipitated by 80−120 g/L trichloroacetic acid (TCA) mainly the highest molecular mass peptides (including proteose−peptone component 5) (Whitney et al., 1976; Groves, 1969; Walstra and Jenness, 1984). It has been shown by Alais et al. (1967) that when isolated κ-casein is heated at 120 °C for 20 min a glycopeptide is released which is similar to that released by the action of chymosin on casein. Walstra and Jenness (1984) described that heating of milk at 120 °C/30 min promotes an 5−7% N increase in the proteose−peptone fraction (PPN), while other workers show that the proteose−peptone fraction is hardly affected by heating (Ismail et al., 1970; de Vilder and Martens, 1976).

The methods so far employed for assessing the extent of protein degradation in heat-treated milk products are, among others, the determination of soluble N at pH 4.6 and in 120 g/L TCA fractions, DEAE−cellulose chromatography, SDS−PAGE, or urea−PAGE. They have frequently been used to follow the heat-induced hydrolysis of individual protein fractions and to detect the appearance of specific casein breakdown residues (Alais et al., 1967, 1974; Guo et al., 1989; Law et al., 1994).

The most widely studied reactions related to heat-induced changes in milk proteins have been the Maillard reaction, and thiol−thiol, and thiol−disulfide interactions and aggregation, but little attention has been devoted to heat-induced proteolysis. This is partly due to a lack of sensitive analytical techniques for determining the concentration of these proteolysis products. The existence of a labile linkage in casein could be useful

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for explaining the heat stability phenomena which occur during milk processing. On the other hand, this fact could be important in industrial practice because peptides exhibit important sensory (Kato et al., 1981) and technological properties (Shimizu et al., 1989), and have also been reported as compounds with biological activity (Kato et al., 1981; Kaiser et al., 1992), e.g. inhibiting the extent of lipolysis during storage of milk (Joshi and Sharma, 1990).

In the present investigation we have studied the release, upon heat treatment of milk and milk resembling systems, of peptides which are soluble in the TCA filtrate and of peptides soluble at pH 4.6. The main aim of this work was to monitor the extent of the heat-induced proteolysis in milk and milk-resembling model systems by applying an RP-HPLC technique with fluorescent detection to describe the thermal history of the sample.

MATERIALS AND METHODS

Chemicals. All chemicals used were the purest available. β-Lactoglobulin, α-lactalbumin, and L-tryptophan were purchased from Sigma (St. Louis, MO); L-tyrosine, trifluoroacetic acid (TFA), and lactose 1-hydrate, from Merck (Darmstadt, DE); and acetonitrile HPLC grade, from Scharlau (Barcelona, ES). Sodium caseinate caseinate (spray-dried, 94% of total protein (N × 6.38 in the dry matter)) was obtained from DMV-International (Veghel, NL).

Samples and Heat Treatments. Simulated milk ultrafiltrate solutions (SMUF) were made with 30 g/L sodium caseinate and 50 g/L lactose monohydrate dissolved in a milk salt solution (Jenness and Koops, 1962) and the pH was adjusted to 6.65. Bulk milk was obtained at farm (El Pardo, Madrid, ES), then thermized (65 °C/10 s) and skimmed at 40 °C until 1 g/L total fat content and 31.7 g/L total protein content was obtained. Then, the solution was homogenized at 35 °C at 30 MPa bar for the first stage and 8 MPa bar for the second stage in a high-pressure laboratory homogenizer from APV (8.30H−Rannie, Albertslund, DK). Skimmed milk (SM system) and simulated milk solution (SMUF system) were heated in an oil bath in tightly stoppered stainless steel test tubes (120 × 7 mm^2) at controlled temperatures between 110 and 150 °C for up to 30 min.

Commercial Milk Samples. Freshly processed commercial samples of skimmed, directly heated UHT treated (Rossi-Catelli equipment, 145 °C/4 s, 29.0 g/L total protein and 3 g/L fat content) and sterilized (Stork equipment, 120 °C/14 min, 29.0 g/L total protein and 3 g/L fat content) milks were obtained from Spanish dairies in the same day of production, stored at 4 °C and analyzed within 2 days.

Sample Preparation. Sodium acetate buffer (0.1 M, pH 4.6, 4.5 mL) was added to 0.5 mL of well-mixed sample. Solutions were left for 1 h at room temperature until precipitation of proteins was observed. A fraction was centrifuged at 2000*g* for 10 min at 4 °C. The supernatant was then filtered through a 0.45 μm acetate filter (13 mm, MSI Inc., Westboro, MA) and the sample was HPLC analyzed.

HPLC Analysis. Fractions soluble at pH 4.6 or in TCA (40 or 120 g/L) were analyzed by RP-HPLC. Sample (40 μL) were injected into a PLRP-S column (15 × 0.46 cm, 8 μm particle size, 100 Å pore size, Polymer Laboratories, Church Stretton, UK) kept at 30 °C. Peptides were eluted using a binary gradient program at a flow rate of 1 mL/min. Eluent A was Milli-Q water (Millipore Corp.) and B was acetonitrile; both contained 1 mL/L of TFA. Linear gradient expressed as solvent B was 3% at 0 min, 50% at 38 min, 3% at 40 min, and 3% at 44 min. Detection was performed by UV at 205, 214, and 280 nm (0.1 AUFS and 0.2 s response time) and by fluorescent response of the tryptophanyl residues (excitation at 280 nm and emission at 340 nm) and tyrosyl residues (excitation at 270 nm and emission at 310 nm). Amounts of unknown p21 and p25 peptides, eluted at 21 and 25 min respectively, are given as relative peak area. Tryptophan concentration was calculated by the external standard procedure with standard of L-tryptophan. Standards of β-lactoglobulin and α-lactalbumin were applied for identification.

A Kontron Instruments (Milan, IT) chromatographic system was used, with a pump (MD-420), fluorescence detector (SMF-25), UV−vis detector (MD-432), and a DT-450/MT-2 v.3.90 computing integrator connected to a PC.

Statistical Analysis. Analysis of variance and Chi-square test for normality was performed by applying Statgraphic v.7.0 statistical package (Statistical Graphics Corp., Rockwille, MD). All the statistical procedures were performed at a significance level of 95%. All the analyses were performed at least by duplicate.

RESULTS AND DISCUSSION

Procedures for Protein Precipitation. The distribution of the peptides released during thermal degradation of milk proteins was studied in skimmed milk heated at 130 °C for up to 30 min and in two industrial processed skimmed milks, namely directly UHT-treated and sterilized milk. Before peptide analysis, the micellar proteins, lipids, and other interfering material had to be removed. Two classical fractionation procedures were carried out, precipitation with 40 or 120 g/L TCA and acidification until reaching the isoelectric point of caseins. Fractionation of the milk with TCA to a final concentration of 40 g/L just caused precipitation of the highest molecular mass peptides, some of them derived from the proteolysis of β-casein (Andrews and Alichandis, 1983). Furthermore, Alais et al. (1967) have described the presence of glycopeptides released by thermal degradation of κ-casein soluble in 120 g/L TCA. The RP-HPLC technique was applied to monitor the peptide distribution of the soluble fractions, also designed as peptide mapping. Frequently, chromatographic conditions for analysis of peptides involve a mixture of water and acetonitrile in the presence of a low percentage of TFA as an ion-pairing reagent. Several gradient programs were tested, the best resolution conditions being described in the Materials and Methods section. Typically, 214 nm is chosen for universal peptide detection and 254 nm for selective detection of the aromatic amino acid residues (i.e., phenyalanyl, tyrosyl, or tryptophanyl residues). Aromatic amino acids have characteristic UV absorption bands between 250 and 290 nm. Alternatively, fluorescence is a very sensitive technique for detecting tryptophanyl and tyrosyl residues. All in all, absorption of the samples at 205, 214, and 280 nm in the UV range and fluorescence detection for tryptophanyl and tyrosyl residues was used.

Figure 1 shows the different chromatographic profiles obtained from the soluble fraction of the sterilized milk sample deproteinized by adding TCA to a final concentration of 40 g/L or by adjusting the pH to 4.6. The chromatograms show the profile of the solution recorded at 214 and 280 nm and the fluorescent response of the tryptophanyl residues. The fraction soluble in 120 g/L TCA showed a chromatographic profile, irrespective of the detection method, close to that of the 40 g/L TCA fraction for peaks eluted before 30 min (data not shown). Three different groups of peaks can be distinguished in the chromatogram (Figure 1), a first group eluted before 20 min, a second group showing a characteristic pattern of peptides eluting between 20 and 30 min, and the third group for large or more hydrophobic molecules, eluted after 30 min. The main difference of the 40 g/L TCA

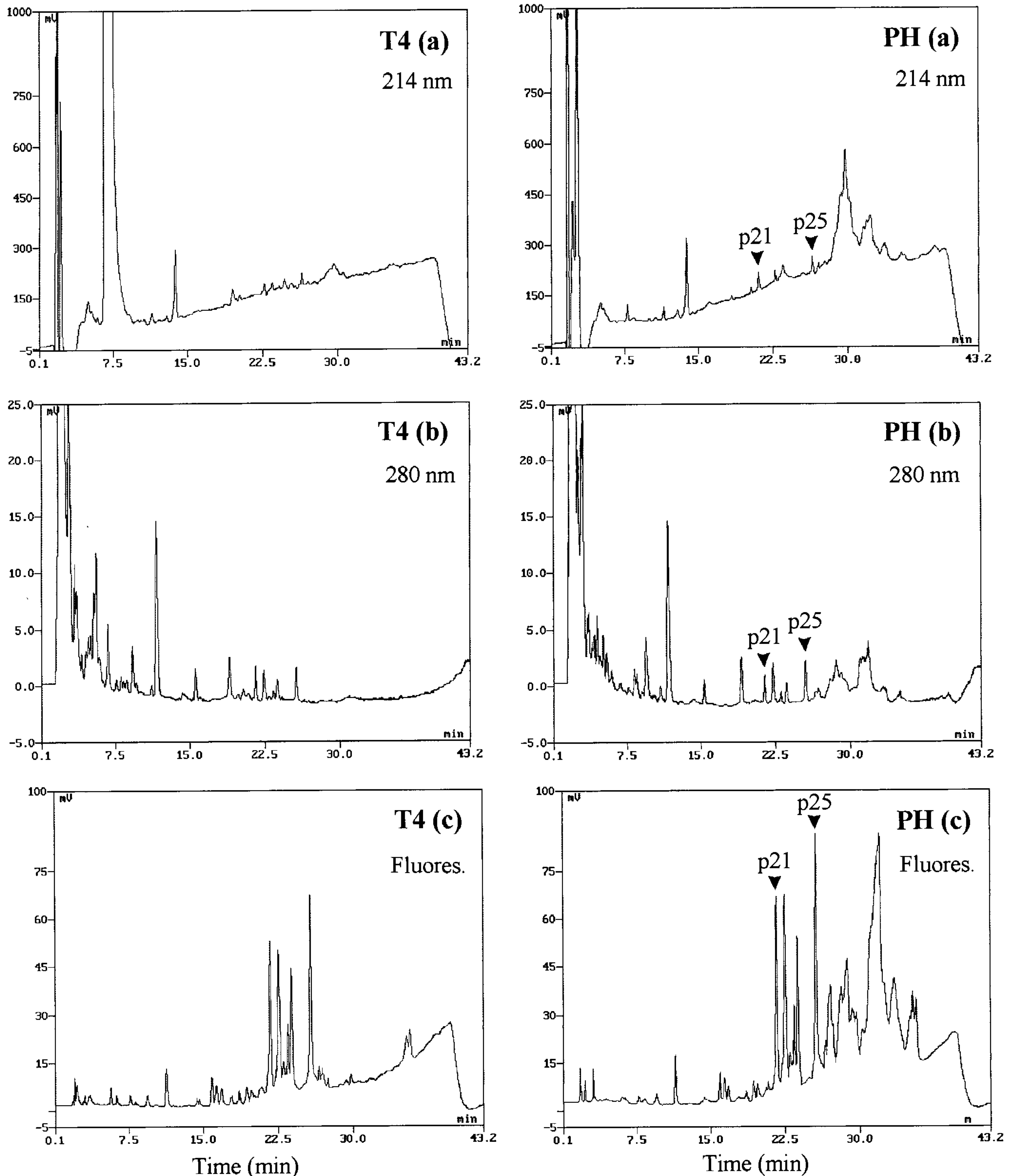

Figure 1. RP-HPLC chromatograms of the fraction soluble in 40 g/L TCA (T4) and at pH 4.6 (PH) from sterilized skimmed milk detected at 214 nm (a) and 280 nm (b) and by fluorescent response of tryptophanyl residues (c). p21 and p25 peaks are peptide components eluted at 21 and 25 min, respectively.

soluble fraction with respect to the fraction soluble at pH 4.6 appeared in the third group of peaks, corresponding to large peptides. The 40 g/L TCA soluble fraction was free of large peptides. Nevertheless, the fraction soluble at pH 4.6 was not free of large peptides, showing the highest differences in the third chromatographic region (that eluted after 30 min) as compared with the precipitation procedures with TCA. The larger differences between samples according to the heat treatment applied were observed with the fluorescence detection with excitation at 280 nm and emission at 340 nm.

Two peaks designated as p21 and p25, eluted at around 21 and 25 min (Figure 1b), showed a clear dependence with the temperature (Table 1). The total area of the second region (20−30 min) increased with

Table 1. Relative Concentrations of p21 and p25 Peptides, Calculated According to Tryptophan Response, in the Fractions Soluble in 40 g/L TCA (T4), 120 g/L TCA (T12), and at pH 4.6 (PH)[a]

	[p21 peptide]		[p25 peptide]	
sample	μmol/L	(se)	μmol/L	(se)
T4-2	0.47	(0.01)	1.31	(0.04)
T4-10	3.86	(0.07)	8.97	(0.26)
T4-20	7.95	(0.17)	15.95	(0.49)
T4-30	10.66	(0.31)	24.75	(0.74)
T4-UHT	0.77	(0.02)	1.24	(0.03)
T4-EST	4.18	(0.13)	6.59	(0.16)
T12-2	0.00	(0.00)	0.64	(0.01)
T12-10	3.82	(0.07)	3.89	(0.10)
T12-20	6.69	(0.10)	10.16	(0.24)
T12-30	8.64	(0.14)	18.27	(0.52)
T12-UHT	0.44	(0.01)	0.52	(0.02)
T12-EST	2.55	(0.05)	3.54	(0.14)
PH-2	0.07	(0.01)	0.50	(0.01)
PH-10	6.37	(0.16)	10.13	(0.18)
PH-20	10.28	(0.25)	20.88	(0.45)
PH-30	10.68	(0.29)	29.07	(0.76)
PH-UHT	0.72	(0.01)	2.07	(0.07)
PH-EST	5.28	(0.10)	6.83	(0.23)

[a] For a skimmed milk sample heated at 130 °C for 2, 10, 20, and 30 min and a directly UHT-treated and sterilized skimmed milk. Standard error in brackets.

the heat treatment of the sample which means that compounds other than p21 and p25 peptides are formed during heating of milk. These two well-resolved peaks could be observed with all the detection wavelengths used, both in UV and fluorescence as well. For comparison purposes, a final 4-fold dilution of samples was used. The relative concentrations of the p21 and p25 peptides, expressed as tryptophan concentration (micromolar), are depicted in Table 1. Both peptides were detected in all the soluble fractions analyzed but higher amounts were found in the fraction soluble at pH 4.6. Overall, these results agree with the studies of Guo et al. (1989) who reported that little soluble N was formed at 110 °C in a Na-caseinate model system, but that the levels increased progressively and almost linearly with the heating temperature, pH 4.6−soluble N increasing more sharply than the 120 g/L TCA−soluble N.

Effect of the Heat Treatment. It has been reported that the total release of peptides, as determined by the increase in N soluble in the pH 4.6 fraction and in the 120 g/L TCA filtrate of whole milk and sodium caseinate, increases with both the duration and the temperature of the heat treatment (i.e., Hindle and Wheelock, 1970; Guo et al., 1989). However, it could be useful to characterize specific peptides related to heat-induced proteolysis of milk proteins during thermal treatment of milk.

The effect of the heat treatment on p21 and p25 peptides was studied in two systems, skimmed milk (system SM) and a lactose−caseinate system dissolved in a simulated ultrafiltrate milk salt solution (system SMUF), both heated from 110 to 150 °C for up to 30 min. Figure 2a shows the chromatographic profile of the unheated samples of systems SM and SMUF. Residual peak areas of 2.04 and 1.38 were found for p21 and p25 peptides, respectively, for the unheated sample in the SMUF system, whereas no traces of p21 or p25 peptides were found in the system SM. In the fraction soluble at pH 4.6 from the SM system some of the undenatured whey proteins, α-lactalbumin (α-LAC) and β-lactoglobulin (β-LG) can be identified. Figure 2b shows the separation of a skimmed milk sample heated

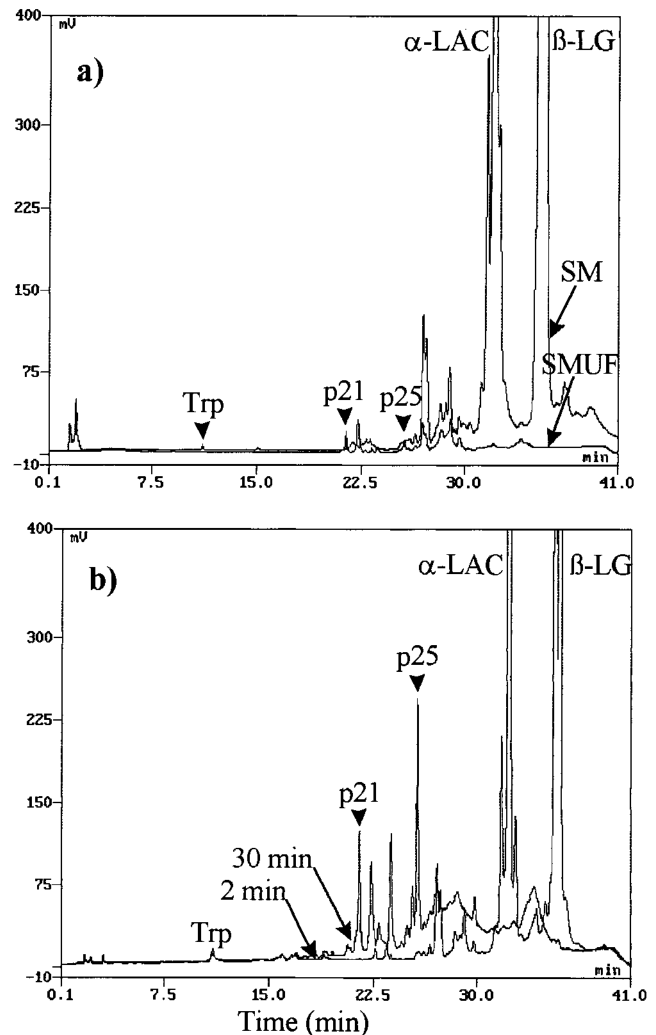

Figure 2. RP-HPLC separation of an unheated sample of systems SM and SMUF (a) and a milk sample heated at 130 °C for 2 and 30 min (b). Fluorescent detection at 280 nm excitation and 340 nm emission. β-LG (β-lactoglobulin), α-LAC (α-lactalbumin), and Trp (tryptophan).

at 130 °C for 2 and 30 min. It can be observed that p21 and p25 peptides showed the clearest relationship with the thermal conditions applied. The formation of both peptides increased with the severity of the heat treatment. Figure 3 shows the relative peak areas of p21 and p25, recorded by fluorescent response of its tryptophanyl residues, as a function of temperature and heating time.

Analysis of variance, assuming homocedasticity (similar variance between groups) and normality for the set of measurements, was performed to determine statistical differences between the two systems (results not shown). With regard to the p21 peptide, ANOVA found a statistically significant difference ($p < 0.0000$) between the two groups (SM and SMUF systems), the formation of p21 peptide in the SMUF system being higher. The p25 peptide formation also showed a significant difference ($p < 0.0000$) between the two model systems. Higher levels of p25 peptide were found in the SM system. The fit of both analyses was acceptable, with a significance level higher than 0.05 for the test of normality (Chi-square) on the residuals of the model. Additional information can be obtained from the analysis of the interactions between factors (temperature, heating time, and composition of model system). It is

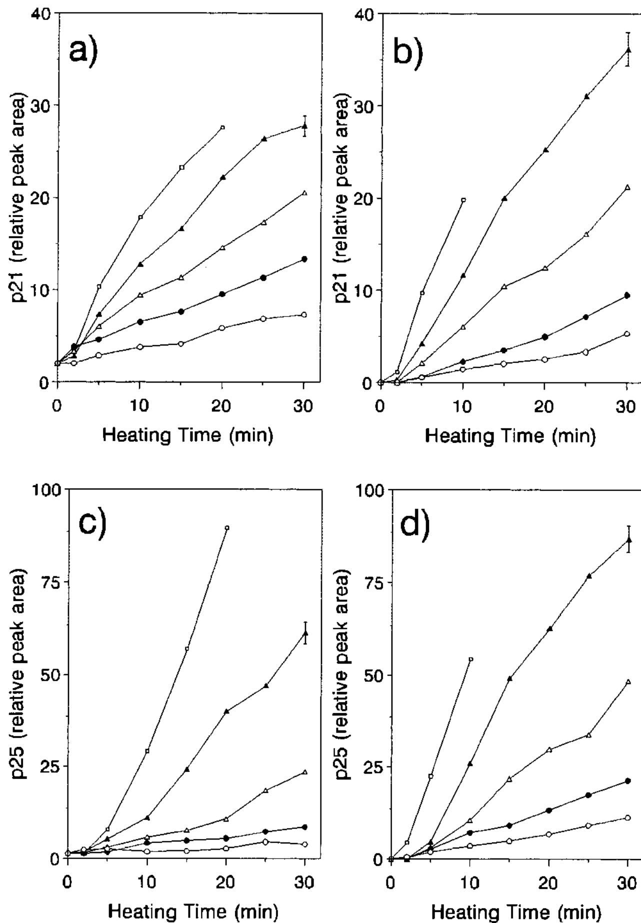

Figure 3. Relative peak area values of p21 and p25 peptides formed in the SMUF system (a and c) and in the SM system (b and d) heated at 110 °C (○), 120 °C (●), 130 °C (△), 140 °C (▲), and 150 °C (□) for up to 30 min. Error bars, included on the 140 min, represent the maximum standard deviation observed between duplicate measurement for all results.

clear that the p21 peptide is formed rapidly in the SMUF system at heating times lower than 20 min, but progressively the differences with the SM system decrease until they reach similar values at 30 min. The temperature has the same effect as heating time on the p21 peptide, the main differences between systems appearing at lower temperatures. From these results it could be reasonable to expect a higher apparent energy of activation for the formation of p21 peptide in the SM system. With regard to the p25 peptide, for all temperatures and heating times (excluding 2 min) its formation is higher in the SM system as compared with the SMUF model system.

Kato et al. (1981) detected small amounts of free amino acids (aspartic acid, threonine, serine, glutamic acid, glycines and alanine) during heating of casein with or without glucose, both in the powder state and in solution, by amino acid analysis after acidic hydrolysis of sample in 6 M HCl. In this work, the presence of tryptophan in the soluble fraction was also measured and a release of tryptophan in the most severely heat treated samples in the SMUF system was detected. Detectable concentrations of 0.38, 0.50, and 1.00 μmol/L tryptophan were just obtained in the samples heated at 140 °C/20 min, 150 °C/15 min, and 150 °C/20 min, respectively. The milk system has 0.26 μmol/L of tryptophan in the unheated Sample Liberation of tryptophan increased slowly during heat treatment, reaching the higher value of 2.54 μmol/L at 140 °C/30 min.

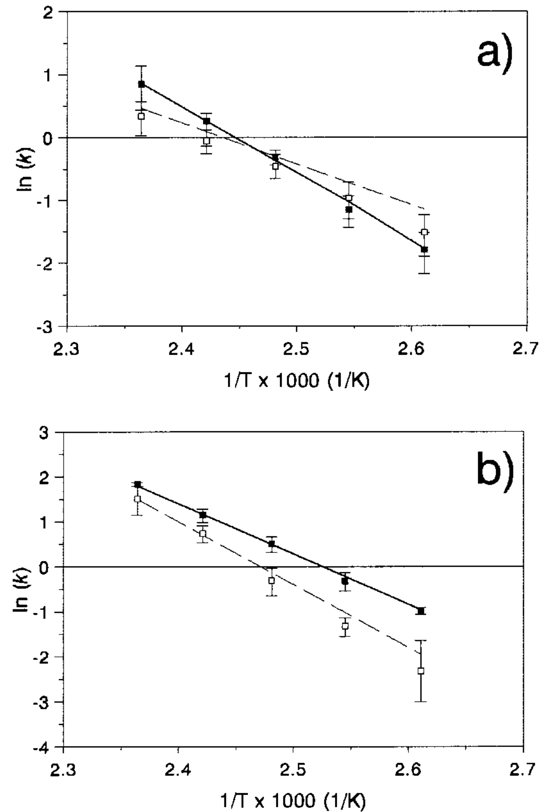

Figure 4. Arrhenius plot for the formation of p21 (a) and p25 (b) peptides in the systems SM (■ and solid line) and SMUF (□ and dotted line). Points represent experimental rate constants; error bars, standard deviations; and lines, the Arrhenius model calculated for zero-order kinetics.

Our observations point out a zero-order reaction for the heat-induced formation of p21 and p25 peptides (Figure 3). Several reactions orders between 0 and 2 were tried. Therefore, the temperature dependence for the formation of p21 and p25 peptide was calculated, assuming a zero-order model because it produced the lowest standard error of the fit (results not shown). Figure 4 a,b shows the Arrhenius plot for the formation of p21 and p25 peptides in systems SMUF and SM. Points represent experimental rate constants directly calculated from the raw data, and the lines, the Arrhenius model calculated by nonlinear regression analysis (one-step procedure). From a statistical point of view, it is better to derive the apparent energy of activation (E_a) and the preexponential factor (A_0) parameters by nonlinear regression of the reparametrized Arrhenius equation, using all measurements at once (Van Boekel, 1996). Apparent energies of activation of 54.4 $\pm$ 2.91 kJ/mol and 87.1 $\pm$ 3.23 kJ/mol were obtained from the p21 peptide in the SMUF and SM systems, respectively. E_a values for the p25 peptide were 116.0 $\pm$ 5.6 kJ/mol and 93.1 $\pm$ 3.00 kJ/mol in the SMUF and SM systems, respectively. Both p21 and p25 peptides fitted the model better in the SM system. The SMUF system showed the highest standard deviation in the reaction rates constant for both peptides at lower temperatures (see Figure 4). This effect could be due to the presence of residual amounts of p21 and p25 peptides in the unheated SMUF sample, in contrast to the SM system. It should be realized that some heat-induced changes in casein observed in model systems cannot be extrapo-

lated directly to situations where casein occurs as micelles in milk.

Conclusion. Some decades ago it was shown that on prolonged heating at high temperatures, some proteolysis of casein occurs, as indicated by an increase in the level of proteose-peptone nitrogen and in nonprotein nitrogen. Kato et al. (1981) pointed out the importance of proteins as a source of amino acids and low molecular weight peptides which could improve the flavor properties in processed foods.

In this work the analysis of peptides soluble at pH 4.6 provided a useful approach for analyzing the thermal history of the sample. The peptide analysis should be carried out immediately after sample processing since enzymic proteolysis could appear during the storage period. Samples with a high level of protein degradation produced by thermal treatment gave rise to chromatograms with a characteristic pattern or mapping of peptides. Two of them, the p21 and p25 peptides (both soluble in 120 g/L TCA), had a characteristic thermal behavior. The size of p21 and p25 peptides should not be large, because both were soluble in 120 g/L TCA and they were eluted in the intermediate chromatographic region (20−30 min). The origin of the p21 and p25 peptides is difficult to elucidate and is not the purpose of this research. Additional experiments with individual milk proteins should be carried out in the future. Hindle and Wheelock (1970) reported that the glycopeptides and peptides released by chymosin action are not the same as those released by heat treatment, and can be derived or not from κ-casein.

It is reasonable to think that p21 and p25 peptides (both absent in raw milk) have a different route of formation in model systems consisting of skimmed milk and lactose−caseinate. In skimmed milk, p25 peptide is formed more efficiently at all the temperatures and heating times as compared with the SMUF system. However, during formation of the p21 peptide factors other than temperature and heating time should be considered, because significant differences in the energy of activation appeared between the SMUF and SM systems. The kinetic study gave more insight on this aspect since p25 peptide shows again a similar thermal behavior (110−150 °C) in both model systems, but the apparent energies of activation for the p21 peptide are significantly different. It can be assumed that the presence of whey proteins or the native state of casein in the SM system can be influencing the formation of p21 peptide.

In conclusion, two peptides analyzed by RP-HPLC by fluorescent detection (excitation at 280 nm and emission at 340 nm) were chosen for following the extent of the heat-induced proteolysis in milk and milk-resembling systems. This approach is promising for assessing the thermal damage in milk or milk-based products, and may provide a useful tool for understanding the changes appeared in milk proteins after thermal treatment. Caseins and caseinates are widely used as functional food proteins (milk-based beverages, cheese analogues, coffee creamers, dietary formulations, meat products, etc.) in the food industry where a thermal treatment is needed for increasing the shelf life of product. A future task could be to develop techniques to improve the extraction and concentration of those peptides by application of solid-phase extraction (SPE) before HPLC analysis, as well as peptide derivatization with a fluorescent marker (*o*-phthaldialdehyde or fluorescamine) in order to achieve their isolation and identification.

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