Disulfide Reactivity and In Vitro Protein Digestibility of Different Thermal-Treated Milk Samples and Whey Proteins

Marina Carbonaro,* Marsilio Cappelloni, and Stefania Sabbadini, Emilia Carnovale

Istituto Nazionale della Nutrizione, Via Ardeatina 546, 00178 Roma, Italy

The susceptibility of disulfides to reduction and the in vitro protein digestibility were analyzed in raw, pasteurized, ultrahigh-temperature (UHT)-treated, and sterilized milk samples and whey proteins. Both parameters increased from raw or pasteurized to UHT-treated or sterilized milk samples. Whey proteins had impaired digestibility compared with that of the corresponding whole milk samples, especially when extracted from UHT-treated and sterilized milk samples. The amino acid composition of proteins in whey indicated that the ratio of whey proteins to casein gradually decreased in the extracts from raw or pasteurized to sterilized milk samples. Disulfide reactivity of whey proteins was inversely related to the intensity of thermal treatment of milk. The results were consistent with the progressive heat-induced increase in the amount of highly stable proteins in whey from raw to sterilized milk samples. Stability of whey proteins was likely either intrinsic in the native conformation or conferred during heating by thiol−disulfide interchange reactions also involving *k*-casein. The possibility of using disulfide reactivity as a parameter in the characterization of thermal-treated milk is considered.

Keywords: *Milk; whey proteins; disulfide reactivity; protein digestibility; thermal treatment*

INTRODUCTION

Thermal treatment of milk applied in the stabilization process preceding distribution and consumption can adversely affect the nutritional quality of the resulting product (Lyster, 1979; Carnovale et al., 1982; Finley, 1985; Burton, 1988). In an attempt to minimize nutrient loss in milk and milk products, researchers (Lyster, 1979; Andrews, 1984; Buser and Erbersdobler, 1985; Resmini et al., 1985; Tripiciano et al., 1986; Pagliarini et al., 1990; Guingamp et al., 1993) are studying reliable analytical methods that can detect specific compounds and thus monitor the severity of the thermal processing. Both endogenous thermolabile components of milk and products of heat-induced reactions can be efficiently used as indicators of heat damage, provided they are susceptible to changes in concentration in the low temperature range of the most widely used stabilization techniques: pasteurization (72−85 °C for 15−30 s), ultrahigh temperature (UHT) treatment (142−145 °C for 2−5 s), and sterilization (115−120 °C for 10−30 min).

Whey proteins have recently been recognized as one of the most useful thermolabile compounds of milk as an analytical marker of heat damage (De Wit and Klarenbeek, 1984; Kinsella and Whitehead, 1989). Indeed, the extent of heat damage can be evaluated by assessing the changes in some physicochemical properties (solubility, hydrophobicity) that occur upon denaturation and that strictly depend on whey protein conformation (Lyster, 1979; Resmini et al., 1985; Tripiciano et al., 1986; Pagliarini et al., 1990). However, most of the parameters examined so far do not allow a well-resolved distinction among raw, pasteurized, UHT-treated, and sterilized milk samples. Some of the proposed methods have been therefore suggested to be used in combination to achieve definitive results (Pagliarini et al., 1990). The availability of new analytical indexes of heat damage would therefore be important. The choice parameters that are especially susceptible to the conformational changes occurring in milk protein upon heating appears to be a promising approach.

Among the stabilizing factors that are involved in maintaining protein conformation, disulfide bonds play a major role. Because the chemical reactivity of disulfide bonds depends on the position in the three-dimensional structure of the protein, reactivity is altered by changes in protein conformation (Anfinsen and Scheraga, 1975). Modifications in disulfide reactivity have previously been observed after denaturation of protein in food (Carbonaro et al., 1992).

Cysteine is quite unevenly distributed in milk proteins. Whey proteins, nutritionally the most important proteins in milk because of their high content of essential amino acids, also represent the fraction containing most of the cysteine, mainly in the form of disulfide bridges (Kinsella and Whitehead, 1989). The major disulfide-rich proteins in bovine milk whey—β-lactoglobulin, α-lactalbumin, serum albumin, and immunoglobulins—differ remarkably in structure and properties (De Wit and Klarenbeek, 1984; Kinsella and Whitehead, 1989). These proteins undergo different time- and temperature-dependent denaturation: α-lactalbumin is the most stable, followed by β-lactoglobulin, serum albumin, and immunoglobulins (De Wit and Swinkels, 1980; De Wit and Klarenbeek, 1984; Kinsella and Whitehead, 1989). Moreover, according to several studies (Haque et al., 1987; Parnell-Clunies et al., 1988; Kinsella and Whitehead, 1989; Dalgleish, 1990) on the mechanism of whey protein aggregation subsequent to denaturative modifications, intermolecular disulfide linkages formed by thiol oxidation and thiol−disulfide interchange reactions are likely involved in the formation of new structures formed upon heating. Therefore, the susceptibility of disulfides to reduction in milk proteins possibly was affected differently as a function of thermal milk treatment.

On the basis of these considerations, the susceptibility of disulfides to chemical reduction in raw, pasteurized, UHT-treated, and sterilized milk samples and in the

* Author to whom correspondence should be addressed (fax +39-6-5031592).

S0021-8561(95)00828-4 CCC: $14.00

corresponding whey protein extracts was analyzed after proteolytic digestion.

MATERIALS AND METHODS

The study was carried out on 18 samples of whole milk. Fresh raw milk (four samples) was obtained from a local dairy. Commercial samples of pasteurized milk (eight samples), UHT-treated milk (four samples), and sterilized milk (two samples), for which heat treatment conditions were known, were used. Pasteurized milk samples were produced in a plate apparatus by heating for 15 s at different temperatures: 72 °C (sample A), 75 °C (samples B, E, F, and G), 78 °C (samples C and H), and 80 °C (sample D). UHT-treated milks were processed in a direct steam injection system at 145 °C for 5 s (samples A and B) and for 3 s (samples C and D). Samples A and B of sterilized milk were indirectly preheated at 145 °C for 5 and 3 s, respectively, and then sterilized within the bottle by autoclaving at 118 °C for 12 min (sample A) and at 116 °C for 10 min (sample B).

All milk samples were freeze-dried and stored at 4 °C before use. Protein content (nitrogen (N) X 6.38) was determined by the Kjeldahl method (AOAC, 1990).

In Vitro Enzymatic Digestion. Milk samples and whey proteins (extraction report follows) were subjected to in vitro enzymatic digestion according to the multienzyme technique of Bodwell et al. (1980) that measures in vitro protein digestibility. Porcine pancreatic trypsin (type IX; 15310 unit/mg protein), bovine pancreatic chymotrypsin (type II; 48 unit/mg solid), porcine intestinal peptidase (P-7500; 115 unit/g solid), and bacterial protease (type XIV; 4.4 unit/mg solid), all from Sigma Chemical Company (St. Louis, MO) were used for the enzymatic digestion. For each sample, 10 mg of N were suspended in 10 mL of distilled water, the suspension was equilibrated at 37 °C, and the pH was adjusted to 8.0 with 1 M NaOH. One milliliter of a three-enzyme solution in water (1.58 mg of trypsin, 3.65 mg of chymotrypsin, and 0.45 mg of peptidase) was added to the sample, and digestion was allowed to proceed for 10 min at 37 °C. After addition of 1 mL (1.48 mg) of bacterial protease solution, the digestion was continued for 9 min at 55 °C. The pH was measured after a further 1 min of incubation at 37 °C and used to estimate the in vitro protein digestibility with the equation $Y = 234.84 - 22.56X$, where Y is the in vitro protein digestibility (percentage) and X is the pH of the suspension after 20 min of digestion (Bodwell et al., 1980). By addition of $HClO_4$ (0.3 M final concentration), the enzymatic digestion was stopped immediately. After 30 min, the digests were filtered and then brought to pH 7.0 by addition of solid $KHCO_3$. The precipitate was discarded, and the supernatant was assayed for recovery of N to check for the formation of peptides that are insoluble in perchloric acid after the enzymatic hydrolysis (Pieniazek et al., 1975). Recovery of N was always 100%, except for whey protein extracted from UHT-treated and sterilized milk samples (88 and 80%, respectively).

Disulfide Reactivity Assay. Chemical reactivity of disulfides (susceptibility to reduction) was determined by the method of Zahler and Cleland (1968) with the supernatant obtained after the enzymatic digestion of milk and whey proteins. Disulfide bonds were reduced by a 30-min incubation at room temperature (20 °C) with dithioerythritol (DTE, Sigma) at final concentrations of 0.43, 1.3, and 2.6 mM. The resulting thiol groups were determined by adding 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma) in the presence of DTE after this reagent was trapped with a 25-fold molar excess of arsenite. Absorption at 412 nm was continuously measured, and the thiol groups were determined from absorption extrapolated to zero time (Zahler and Cleland, 1968). The contribution to the total value of thiol groups originating from the enzymes used in the digestion was determined in appropriate blanks at the different DTE concentrations and subtracted from the corresponding values obtained for each sample. Disulfide reactivity was expressed as percentage of the total cysteine, the latter determined by amino acid analysis (described later). A control without DTE was performed to test the initial levels of SH groups in the proteolytic digest.

Whey Protein Extraction. Whey proteins were extracted from one sample of each milk class by the method of Resmini et al. (1985), after precipitation of casein at pH 4.6. Whey protein solubility (N × 6.38) was calculated after nonprotein N (N soluble in 12% trichloroacetic acid) was subtracted from the total N content of the extract. Soluble whey proteins were expressed as percentage of the total protein content of corresponding milk sample (Resmini et al., 1985). The extracts were freeze-dried and reanalyzed for protein content before use in the subsequent analyses.

Amino Acid Analysis. Amino acid composition of whey proteins was determined after hydrolysis under reduced pressure with 6 N HCl at 110 °C for 24 and 72 h. Amino acids were analyzed with a Beckman 118BL amino acid analyzer (Beckman Instruments, Fullerton, CA) after reaction with ninhydrin (Moore et al., 1958). The total cysteine and methionine contents of milk samples and whey proteins were determined as cysteic acid and methionine sulfone, respectively, after oxidation with performic acid (Schram et al., 1954). The recovery of cysteine and methionine after acid hydrolysis was controlled (MacDonald et al., 1985).

Statistical Analysis. Data were subjected to analysis of variance. The significance of the differences between means was obtained by Student's *t*-test.

RESULTS AND DISCUSSION

Whole Milk. The total protein, cysteine, and methionine content and in vitro protein digestibility of raw, pasteurized, UHT-treated, and sterilized milk samples are shown in Table 1. Total protein (2.72−3.5 g/100 mL) and total cysteine and methionine content (0.84−0.93 and 2.29−2.6 g/16 g of N, respectively) were within the ranges already reported on the basis of data obtained from several milk samples (Carnovale et al., 1982; Resmini et al., 1985; Tripiciano et al., 1986). Change in mean sulfur amino acid content of the various milk groups from intensity of heat treatment was not significant. Modifications in cysteine and methionine content from partial destruction are seldom evidenced in milk, probably because the variations involved are too small to be detected easily (Pieniazek et al., 1975; Carnovale et al., 1982). A comparison among the mean values calculated from the in vitro protein digestibilities of each milk class (Table 1) pointed out a significant increase ($p < 0.05$) from raw (80.38%) to pasteurized milk (81.09%) and from pasteurized to UHT-treated (83.15%) or sterilized milk (83.06%), possibly as a consequence of different extents of thermal denaturation. Heat treatment generally induced gross changes in protein conformation with subsequent exposure of previously inaccessible sites to proteolytic cleavage (Lyster, 1979; Finley, 1985). This change is considered to mainly account for the protein digestibility increase usually observed after heating of foods.

Disulfide reactivity in the different milk groups, as detected after in vitro mutienzymatic digestion, is depicted in Figure 1. No SH groups were determined in the samples before treatment with the reducing agent, suggesting that cysteine was either engaged in disulfide bridges or unreactive under the conditions used. After heating, the susceptibility of disulfide to reduction is expected to increase because of enhanced accessibility of disulfide in the denatured protein compared with the native conformation of the protein (Anfinsen and Scheraga, 1975). The raw milk samples (Figure 1A) presented similar profiles of disulfide reactivity: the percentage of reduced disulfides was ∼50−60% at the lowest concentration of DTE and leveled off slightly above 70% at the next concentration of the reducing agent. The incomplete reduction of disulfides in raw milk might indicate that, in the

Table 1. Total Protein, Cysteine, and Methionine Content and In Vitro Protein Digestibility of Raw, Pasteurized, UHT-Treated, and Sterilized Milk Samples[a]

sample		total protein (g/100 mL)	total cysteine[b] (g/16 g of N)	total methionine[b] (g/16 g of N)	in vitro protein digestibility (%)
raw	A	3.07 ± 0.01	0.88 ± 0.05	2.40 ± 0.01	80.35 ± 0.61
	B	3.11 ± 0.04	0.85 ± 0.04	2.46 ± 0.18	80.47 ± 0.38
	C	3.14 ± 0.03	0.85 ± 0.03	2.43 ± 0.15	80.31 ± 0.72
	D	3.13 ± 0.01	0.84 ± 0.01	2.26 ± 0.01	80.40 ± 0.81
pasteurized	A	3.36 ± 0.10	0.88 ± 0.05	2.35 ± 0.04	80.77 ± 0.89
	B	3.26 ± 0.01	0.93 ± 0.05	2.29 ± 0.09	80.75 ± 0.32
	C	3.14 ± 0.10	0.88 ± 0.06	2.35 ± 0.02	81.20 ± 0.64
	D	3.03 ± 0.01	0.84 ± 0.06	2.34 ± 0.83	81.77 ± 0.80
	E	3.50 ± 0.05	0.85 ± 0.01	2.50 ± 0.13	81.12 ± 0.83
	F	3.13 ± 0.02	0.83 ± 0.04	2.36 ± 0.08	80.76 ± 0.92
	G	3.14 ± 0.03	0.84 ± 0.01	2.42 ± 0.35	80.81 ± 0.73
	H	3.02 ± 0.04	0.83 ± 0.07	2.30 ± 0.74	81.55 ± 0.45
UHT-treated	A	2.72 ± 0.02	0.88 ± 0.03	2.38 ± 0.05	82.29 ± 0.93
	B	3.43 ± 0.20	0.87 ± 0.05	2.60 ± 0.08	83.23 ± 1.20
	C	3.19 ± 0.14	0.86 ± 0.02	2.50 ± 0.23	83.76 ± 1.43
	D	3.33 ± 0.05	0.83 ± 0.06	2.55 ± 0.10	83.35 ± 0.95
sterilized	A	3.11 ± 0.06	0.93 ± 0.05	2.30 ± 0.16	82.65 ± 1.08
	B	3.18 ± 0.01	0.85 ± 0.04	2.36 ± 0.11	83.46 ± 1.27

[a] Means and standard deviations of four replicates. [b] Corrected for 5% loss.

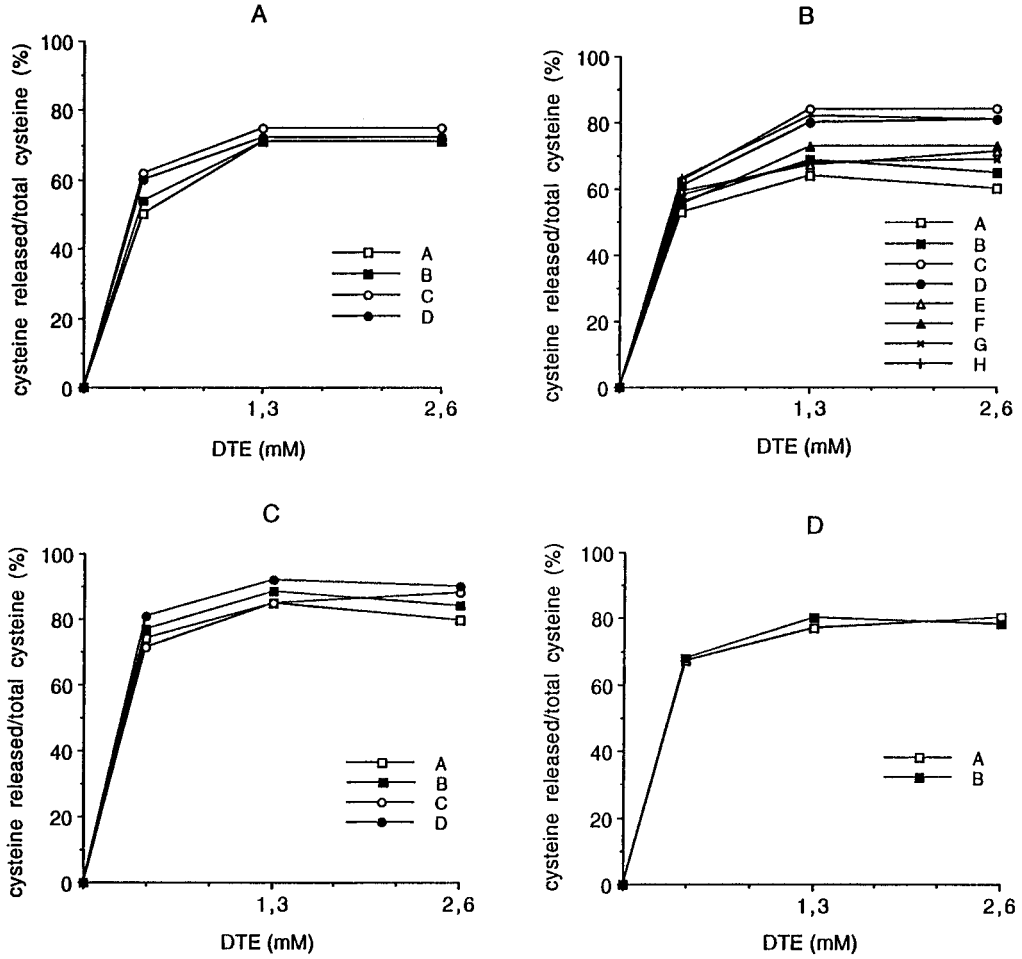

Figure 1. Disulfide reduction in proteolytic digests of raw (A), pasteurized (B), UHT-treated (C), and sterilized (D) milk samples by dithioerythritol (DTE). The values are the mean of four replicates (variability coefficient <5%).

proteolytic digests, disulfides are partially buried inside peptides or undigested proteins and thus are unreactive toward DTE. This supposition would be consistent with the reported very high stability of whey proteins in the native conformation (De Wit and Klarenbeek, 1984; Kinsella and Whitehead, 1989). With our conditions, SH groups were undetectable in the undigested samples, either before or after reduction by DTE. Among the pasteurized milk samples (Figure 1B), only three of eight (samples C, D, and H) showed a significantly ($p < 0.05$) higher maximum disulfide reactivity than in raw milk. Samples C, D, and H were also significantly different ($p < 0.01$) from the other pasteurized samples in maximum mean percentages of reduced disulfides (82% from samples C, D, and H versus 69% from samples A, B, E, F, and G; Figure 1B). The wide range covered by the percentage of reduced disulfides in the eight milk samples may reflect slight variations in

Table 2. Total Protein, Solubility, and In Vitro Protein Digestibility of Whey Protein Extracts (WP) from Samples of Raw (Sample B), Pasteurized (Sample E), UHT-Treated (Sample C), and Sterilized (Sample B) Milk[a]

sample	total protein (% of dry weight)	protein solubility (% of total protein)	in vitro protein digestibility (%)
raw WP	10.40 ± 0.61	17.39 ± 0.60	69.34 ± 0.90
pasteurized WP	9.42 ± 0.31	13.47 ± 0.85	67.29 ± 0.85
UHT-treated WP	6.20 ± 0.49	5.67 ± 1.10	62.54 ± 1.19
sterilized WP	5.51 ± 0.36	4.44 ± 0.92	60.48 ± 0.99

[a] Means and standard deviations of four replicates.

pasteurization conditions. In fact, pasteurized milk samples can be divided into two groups, by lower (samples A, B, E, F, and G) and higher (samples C, D, and H) relative disulfide reactivity, possibly depending on milder (72−75 °C, 15 s) and more severe (78−80 °C, 15 s) pasteurization conditions, respectively. Previous analyses of soluble whey proteins of several commercial pasteurized milk samples (Resmini et al., 1985; Tripiciano et al., 1986) have already pointed out a great degree of variability; some samples resembled raw milk and others resemble UHT-treated milk. However, when pasteurized milk samples that had been subjected to heat treatment in a known temperature range were compared, the higher the pasteurization temperature, the lower was the whey protein solubility (Resmini et al., 1989).

The mean percentage of disulfides reduced by DTE, in the range 0.43−2.6 mM, significantly increased from raw or pasteurized to UHT-treated ($p < 0.025$; Figure 1C) or sterilized milk ($p < 0.05$; Figure 1D). At the highest concentrations of DTE, the profiles obtained for UHT-treated and sterilized milk samples and those from pasteurized samples C, D, and H overlapped considerably (Figure 1B), further supporting the possibility that a higher temperature of processing was responsible for the increased disulfide reactivity observed for these pasteurized milk samples (Figure 1B).

According to literature concerning the effect of heat on milk protein denaturation, at up to 75 °C, extensive changes in whey protein conformation do not occur, as monitored by both decline in solubility (Dalgleish, 1990) and changes in calorimetric parameters of whey proteins (Kinsella and Whitehead, 1989). However, whey proteins are completely denatured in a short time at temperatures >90 °C (Dalgleish, 1990). Thus, the thermal behavior of whey proteins could explain the differences in disulfide reactivity observed among the differently thermal treated milk samples (Figure 1).

Whey Proteins. The total protein content, solubility, and in vitro digestibility of whey proteins obtained from the different milk classes are shown in Table 2. Total protein content and solubility percentage were significantly higher ($p < 0.05$ and $p < 0.01$, respectively) in whey proteins obtained from raw milk than in those extracted from pasteurized milk, which in turn presented values higher than those typical of whey proteins obtained from UHT-treated or sterilized milk samples. All percentages of soluble whey proteins in the different milk classes were within the ranges established to distinguish among raw, pasteurized, and UHT-treated (or sterilized) milk samples (Resmini et al., 1985; Tripiciano et al., 1986). The impaired solubility of whey proteins is likely responsible for the decrease in the values of total protein extracted from raw to UHT-treated or sterilized milk samples.

The in vitro digestibility of whey proteins (Table 2) was always lower than that of the corresponding whole milk (Table 1). The low digestibility of whey proteins extracted from raw milk can be explained by their structural properties, because most are stabilized by the presence of a number of disulfide bonds (Kinsella and Whitehead, 1989). Whey proteins have been demonstrated to be quite resistant to proteolysis under several conditions and, for β-lactoglobulin, this feature has been related to its proposed functional role as a retinol-binding protein (Papiz et al., 1986; Reddy et al., 1988). On the other hand, casein, the major protein in bovine milk, is well digested because of its poor secondary structure (Bodwell et al., 1980; De Wit and Klarenbeek, 1984; Eggum et al., 1989), which accounts for the differences in protein digestibility between whole milk (Table 1) and whey protein fraction (Table 2).

Table 3. Amino Acid Composition (g/16 g of N) of Whey Protein Extracts (WP) from Samples of Raw, Pasteurized, UHT-Treated, and Sterilized Milks[a]

amino acid	raw WP	pasteurized WP	UHT-treated WP	sterilized WP
lysine	8.47 ± 0.27	8.59 ± 0.18	7.37 ± 0.08	6.64 ± 0.20
histidine	1.73 ± 0.32	1.68 ± 0.45	1.88 ± 0.38	2.12 ± 0.03
arginine	2.70 ± 0.01	2.64 ± 0.29	2.47 ± 0.21	3.31 ± 0.35
aspartic acid	11.77 ± 0.36	10.81 ± 0.38	11.11 ± 1.29	8.30 ± 0.78
threonine	4.67 ± 0.15	4.65 ± 0.27	4.45 ± 0.29	4.61 ± 0.53
serine	4.98 ± 0.22	4.85 ± 0.33	4.94 ± 0.25	5.63 ± 0.86
glutamic acid	17.71 ± 0.42	18.15 ± 0.73	19.51 ± 1.33	22.29 ± 1.59
proline	5.07 ± 0.49	5.04 ± 0.22	6.11 ± 0.72	8.25 ± 0.79
glycine	2.25 ± 0.04	2.42 ± 0.23	2.75 ± 0.29	2.68 ± 0.18
alanine	4.34 ± 0.34	4.39 ± 0.21	3.24+0.22	3.05 ± 0.20
half cystine[b]	2.45 ± 0.42	2.77 ± 0.16	1.71 ± 0.26	0.70 ± 0.05
valine	5.27 ± 0.29	5.25+0.01	5.18 ± 0.04	5.67 ± 0.01
methionine[b]	1.94 ± 0.14	1.99 ± 0.16	1.70 ± 0.08	1.72 ± 0.12
isoleucine	4.89 ± 0.26	5.09 + 0.01	5.16 ± 0.09	4.82 ± 0.04
leucine	11.15 ± 0.33	11.38 ± 0.57	9.21 ± 0.11	7.26 ± 0.24
tyrosine	3.41 ± 0.03	3.41 ± 0.04	2.08+0.05	1.84 ± 0.06
phenylalanine	4.39 ± 0.05	4.29 ± 0.07	4.93 ± 0.06	4.44 ± 0.11
tryptophan	ND[c]	ND	ND	ND

[a] Means and standard deviations of four replicates. [b] Corrected for 5% loss. [c] ND = Not determined.

However, compared with that for whole milk (Table 1), even the in vitro digestibility of whey proteins was even impaired ($p < 0.01$) when they were extracted from UHT-treated and sterilized milk samples (Table 2). Calorimetric studies on thermal behavior of whey proteins stated that, above a critical temperature (75 °C), irreversible conformational changes occur, and the denaturation kinetics change as a result of the starting of the aggregation process (De Wit and Swinkels, 1980; Kinsella and Whitehead, 1989). Denatured whey proteins can either bind to caseins or self-aggregate;l both size and stability of these aggregates increase as a function of heating time and temperature, first through hydrophobic and electrostatic interactions and then because of the formation of disulfide bonds (Haque et al., 1987; Parnell-Clunies et al., 1988; Dalgleish, 1990). We therefore supposed that modifications in both composition and aggregation state of proteins recovered in whey from heat-treated milk samples after isoelectric precipitation of casein were responsible for the observed decline in protein digestibility.

Amino acid analysis of whey protein extracts from raw, pasteurized, UHT-treated, and sterilized milk samples (Table 3) indicated that thermal treatment induced substantial changes in the protein composition of whey. In fact, the amino acid composition reported as being typical of whey proteins (Greenberg and Dower, 1986) was obtained only when this fraction was extracted from raw or pasteurized milk samples. When whey proteins were extracted from UHT-treated and sterilized milk samples, the amino acid composition was markedly changed. Lysine, alanine, cysteine, and leucine decreased significantly ($p < 0.05$), and histidine,

glutamic acid, and proline increased concomitantly from raw or pasteurized to UHT-treated and from UHT-treated to sterilized whey protein extracts (Table 3). These changes, as already reported (Douglas et al., 1981), indicated the presence of casein in the whey protein fractions, suggesting that an interaction between whey proteins and caseins had occurred upon heating. Among whey proteins, β-lactoglobulin had the highest affinity for caseins, especially *k*-casein (Greenberg and Dower, 1986; Parnell-Clunies et al., 1988; Kinsella and Whitehead, 1989; Dalgleish, 1990). Interaction between β-lactoglobulin and *k*-casein, the extent of which depends on the availability of the monomeric reactive form of *k*-casein, is thought to be promoted by heating through the dissociation of dimeric *k*-casein (Haque et al., 1987). The decrease in the ratio of whey protein to casein in whey upon heating has been reported to be accompanied by an increase in the ratio of α-lactalbumin to β-lactoglobulin (Douglas et al., 1981; Parnell-Clunies et al., 1988). This relationship was probably a consequence of the high apparent resistance of α-lactalbumin to thermal denaturation that has been ascribed to the high degree of renaturation of the protein, which is effective in the presence of specifically bound Ca^{2+} (De Wit and Swinkels, 1980; Hiraoka et al., 1980; Kinsella and Whitehead, 1989). With the exception of α-lactalbumin, no other disulfide-rich milk proteins, such as serum albumin and immunoglobulins, have been recovered in considerable amounts in whey from thermal-treated milk samples, probably because of their high susceptibility to irreversible thermal denaturation (Douglas et al., 1981; Parnell-Clunies et al., 1988; Dalgleish, 1990). Therefore, both α-lactalbumin and aggregates of β-lactoglobulin and *k*-casein (and probably of whey proteins themselves), which had been stabilized by disulfides bonds, were probably present in increased percentage in whey protein extracts from pasteurized to sterilized milk samples. This result could explain the reduction in the in vitro digestibility of protein (Table 2), because the presence of disulfide bonds in food proteins has often been related to low digestibility (Boonvisut and Whitaker, 1976; Reddy et al., 1988).

The susceptibility of disulfides to reduction in the proteolytic products of whey proteins from raw, pasteurized, UHT-treated, and sterilized milk samples (Figure 2) was clearly related inversely to the intensity of thermal treatment because it gradually decreased from raw to sterilized milk. In whey proteins from raw milk, the percentage of reduced disulfides was 70% at the lowest concentration of DTE tested and slightly increased as the concentration of the reducing agent rose. When extracted from pasteurized milk, disulfide reactivity of whey proteins was significantly ($p < 0.05$) reduced; that is, only half of the disulfides were reduced at 0.43 mM DTE, and 70% was the maximum value achieved at the next concentration of reductant. Whey proteins from UHT-treated milk decreased further ($p < 0.05$), with a maximum value of reduced disulfides of 50%, and whey proteins from sterilized milk had the lowest ($p < 0.025$) disulfide reactivity (essentially constant value: 20%; Figure 2).

Because the accessibility of disulfide bonds inside the protein molecule directly affects their susceptibility for reduction, the more compact the conformation of proteins in whey [as for heat-stable proteins (Privalov, 1979)] and their heat-induced aggregates, the lower is the reactivity of disulfides toward the action of the reducing agent. Among the different whey protein complexes that are formed upon heating, those consisting of β-lactoglobulin disulfide linked to *k*-casein have resulted in very high stability toward both dissociation or disulfide reduction (Haque et al., 1987). Thermal treatment, therefore, likely affects protein composition of whey by increasing the proportion of proteins with the highest stability, either intrinsic or induced by specific associations promoted by heating through thiol−disulfide interchange reactions. As a consequence, both protein digestibility and disulfide reactivity are impaired. Evidence for the presence of covalent complexes between different whey proteins or between whey proteins and caseins in extracts from pasteurized milk has been provided by SDS-PAGE (Carbonaro et al., 1996). Our results are in good agreement with previous findings showing that surface hydrophobicity values and thiol content of whey proteins unexpectedly decrease with increasing heating of milk (Parnell-Clunies et al., 1988). Such changes in composition and aggregation of proteins in whey can also account for the differences in disulfide reactivity between whey proteins (Figure 2) and the corresponding whole milk samples (Figure 1).

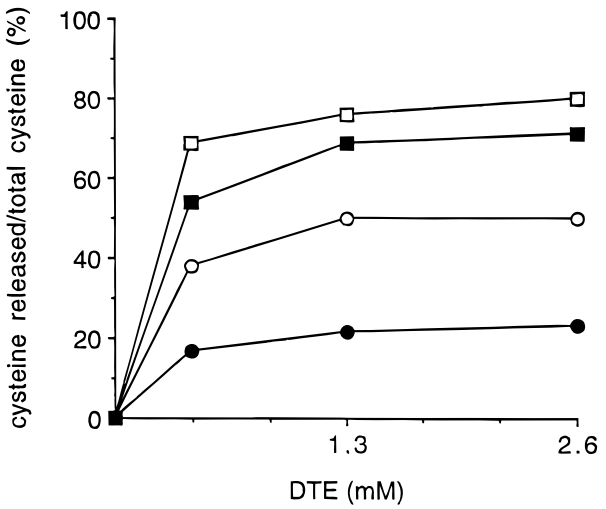

Figure 2. Disulfide reduction in proteolytic digests of whey protein extracts from raw (□), pasteurized (■), UHT-treated (○), and sterilized (●) milk samples by dithioerythritol (DTE). The values are the mean of four replicates (variability coefficient, <6%).

These major factors lower disulfide reactivity in whey proteins from raw to sterilized milk samples. However, in whey from UHT-treated and sterilized milk samples, we cannot rule out the presence of some cysteine that is unreactive because it is in oxidation states higher than disulfides, such as cysteic acid, and therefore does not participate in the reaction with DTNB. The mechanism responsible for the differences in disulfide reactivity between whole milk and whey proteins, as well as among whey proteins from differently thermal-treated milks, is being clarified by characterization of the proteolytic digestion products of whole milk samples and whey proteins.

The results of this study provide some evidence for the chemical reactivity of disulfides in proteolytic digests of milk and whey proteins as a possible analytical marker to evaluate the severity of thermal treatment of milk . Further studies are in progress to further validate this method.

In a recent study on disulfide reactivity in legume proteins, we found that the percentage of chemically reduced disulfides could be indicative of the in vivo availability of this amino acid (Carbonaro et al., 1992; Marletta et al., 1992). If this is the case for milk, too,

the measure of this index may give additional valuable information about the nutritional quality of milk proteins.

LITERATURE CITED

Andrews, G. R. Distinguishing Pasteurized, UHT and Sterilized Milks by their Lactulose Content. *J. Soc. Dairy Technol.* **1984**, *37*, 92−95.

Anfinsen, C. B.; Scheraga, H. A. Experimental and Theoretical Aspects of Protein Folding. *Adv. Protein Chem*. **1975**, *29*, 205−300.

AOAC, *Official Methods of Analysis*, 15th ed.; Association of Official Analytical Chemists: Arlington, VA, 1990.

Bodwell, C. E.; Satterlee, L. D.; Hackler, L. R. Protein Digestibility of the Same Protein Preparations by Human and Rat Assays and by In Vitro Enzymic Digestion Methods. *Am. J. Clin. Nutr*. **1980**, *33*, 677−686.

Boonvisut, S.; Whitaker, J. R. Effect of Heat, Amylase, and Disulfide Bond Cleavage on the In Vitro Digestibility of Soybean Proteins. *J. Agric. Food Chem*. **1976**, *24*, 1130−1135.

Burton, H. *Ultra-High Temperature Processing of Milk and Milk Products*; Elsevier Appl. Science: London, 1988.

Buser, W.; Erbersdobler, H. Determination of Furosine by Gas-Liquid Chromatography. *J. Chromatogr*. **1985**, *346*, 363−366.

Carbonaro, M.; Bonomi, F.; Iametti, S.; Carnovale, E. Modifications in Disulfide Reactivity of Milk Induced by Different Pasteurization Conditions. *J. Food Sci*. **1996**, *61*, 495−500.

Carbonaro, M.; Marletta, L; Carnovale, E. Factors Affecting Cystine Reactivity in Proteolytic Digests of *Phaseolus vulgaris*. *J. Agric. Food Chem*. **1992**, *40*, 169−174.

Carnovale, E.; Cappelloni, M.; Ibba, P. Digeribilità Proteica In Vitro, Disponibilità della Lisina e Lisinoalanina in Latte Trattato Termicamente. In *Effetti Bionutrizionali del Trattamento UHT sul Latte Alimentare*; Monacelli, R., Ed.; Soc. Ed. Alimenti, Alimentazione e Nutrizione: Roma, 1982; pp 229−234.

Dalgleish, D. G. Denaturation and Aggregation of Serum Proteins and Caseins in Heated Milk. *J. Agric. Food Chem*. **1990**, *38*, 1995−1999.

De Wit, J. N.; Klarenbeek, G. Effects of Various Heat Treatments on Structure and Solubility of Whey Proteins. *J. Dairy Sci*. **1984**, *67*, 2701−2710.

De Wit, J. N.; Swinkels, G. A. M. A Differential Scanning Calorimetric Study of the Thermal Denaturation of Bovine β-Lactoglobulin. Thermal Behavior at Temperatures up to 100 °C. *Biochim. Biophys. Acta* **1980**, *624*, 40−50.

Douglas, F. W.; Greenberg, R.; Farrell, H. M.; Edmondson, L. F. Effects of Ultra-High Temperature Pasteurization on Milk Proteins. *J. Agric. Food Chem*. **1981**, *29*, 11−15.

Eggum, B. O.; Hansen, I.; Larsen, T. Protein Quality and Digestible Energy of Selected Foods Determined in Balance Trials with Rats. *Plant Foods Hum. Nutr*. **1989**, *39*, 13−21.

Finley, J. W. Environmental Effects on Protein Quality. In *Chemical Changes in Food During Processing*; Richardson, T., Finley, J. W., Eds.; AVI: Westport, CT, 1985; pp 443-482.

Greenberg, R.; Dower, H. J. Detection of Added Whey Protein Concentrate in Nonfat Dry Milk by Amino Acid Analysis. *J. Agric. Food Chem*. **1986**, *34*, 30−32.

Guingamp, M. F.; Humbert, G; Linden, G. Determination of Sulfhydryl Groups in Milk Using Ellman's Procedure and Clarifying Reagent. *J. Dairy Sci*. **1993**, *76*, 2152−2155.

Haque, Z.; Kristjansson, M. M.; Kinsella, J. E. Interaction Between *k*-Casein and β-Lactoglobulin: Possible Mechanism. *J. Agric. Food Chem*. **1987**, *35*, 644−649.

Hiraoka, Y.; Segawa, T.; Kuwajima, K.; Sugai, S.; Murai, N. 1980. α-Lactalbumin: a Calcium Metalloprotein. *Biochim. Biophys. Acta* **1980**, *95*, 1098−1104.

Kinsella, J. E.; Whitehead, D. M. Proteins in Whey: Chemical, Physical and Functional Properties. *Adv. Food Nutr. Res*. **1989**, *33*, 343−438.

Lyster, R. L. J. Milk and Dairy Products. In *Effects of Heating on Foodstuffs*; Priestley, R. J., Ed.; Applied Science: London, 1979; pp 353−372.

MacDonald, J. L.; Krueger, M. W.; Keller, J. H. Oxidation and Hydrolysis Determination of Sulfur Amino Acids in Food and Feed Ingredients: Collaborative Study. *J. Assoc. Offic. Anal. Chem*. **1985**, *68*, 826−829.

Marletta, L.; Carbonaro, M; Carnovale, E. In-Vitro Protein and Sulphur Amino Acid Availability as Measure of Bean Protein Quality. *J. Sci. Food Agric*. **1992**, *59*, 497−504.

Moore, S.; Spackman, D. M.; Stein, W. H. Chromatography of Amino Acids on Sulphonated Polistyrene Resins. *Anal. Chem*. **1958**, *30*, 1185−1190.

Pagliarini, E.; Iametti, S.; Peri, C.; Bonomi, F. An Analytical Approach to the Evaluation of Heat Damage in Commercial Milks. *J. Dairy Sci*. **1990**, *73*, 41−44.

Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findley, J. B. C.; Sivaprasada Rao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The Structure of β-Lactoglobulin and its Similarity to Plasma Retinol-Binding Protein. *Nature* (London) **1986**, *324*, 383−385.

Parnell-Clunies, E.; Kakuda, Y.; Irvine, D.; Mullen, K. Heat-Induced Protein Changes in Milk Processed by Vat and Continuous Heating Systems. *J. Dairy Sci*. **1988**, *71*, 1472−1483.

Pieniazek, D.; Grabarek, Z.; Rakowska, M. Quantitative Determination of the Content of Available Methionine and Cysteine in Food Proteins. *Nutr. Metab*. **1975**, *18*, 16−22.

Privalov, P. L. Stability of Proteins. *Adv. Protein Chem*. **1979**, *33*, 167−241.

Reddy, I. M.; Kella, N. K. D.; Kinsella, J. E. Structural and Conformational Basis of the Resistance of β-Lactoglobulin to Peptic and Chymotryptic Digestion. *J. Agric. Food Chem*. **1988**, *36*, 737−741.

Resmini, P.; Pellegrino, L.; Hagenboom, J. A.; Andreini, R. Thermal Denaturation of Whey Protein in Pasteurized Milk. Fast Evaluation by HPLC. *Ital. J. Food Sci*. **1989**, *1*, 51−62.

Resmini, P.; Tripiciano, C.; Rampilli, M.; Lodi, R. Alcuni Aspetti del Controllo di Qualità del Latte al Consumo. *Riv. Soc. Ital. Sci. Aliment*. **1985**, *3*, 187−196.

Schram, E.; Moore, S.; Bigwood, E. J. Chromatographic Determination of Cystine as Cysteic Acid. *Biochem. J*. **1954**, *57*, 33−37.

Tripiciano, C.; Resmini, P.; Rampilli, M. Il Controllo del Trattamento Termico sul Latte Alimentare Pastorizzato come Approccio per una Classificazione Merceologica. *Riv. Soc. Ital. Sci. Aliment*. **1986**, *15*, 235−242.

Zahler, W. L.; Cleland, W. W. A Specific and Sensitive Assay for Disulfides. *J. Biol. Chem*. **1968**, *243*, 716−719.

Received for review December 19, 1995. Revised manuscript received August 30, 1996. Accepted September 3, 1996.[⊗] The research was supported by the National Research Council of Italy, Special Project "Ricerche Avanzate per l'Innovazione nel Sistema Agricolo (RAISA)", Subproject No. 4, Paper No. 2681.

JF950828I

[⊗] Abstract published in *Advance ACS Abstracts*, November 1, 1996.