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The influence of oxidation on proteolysis in raw milk

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The link between oxidation and increased proteolysis in raw milk was studied. To accelerate oxidation, H_2O_2 (1 mM) was added to raw milk, resulting in enhanced proteolysis by up to 11·2% after 24 h incubation at 5 °C. Addition of Cu^{2+} (10 μM) to milk or exposure of milk to light (60 min) likewise increased proteolysis. To explain the mechanism responsible for increased proteolysis as a result of oxidation, the effect of lipid oxidation products on plasmin-induced proteolysis was tested. Addition of malondialdehyde to skim milk increased the formation of γ-caseins, a proteolysis product from plasmin hydrolysis of β-casein. The same observation was made in a model system containing 4·5 g β-casein/l sodium tetraborate buffer at pH 8 and plasmin. Addition of a plasmin inhibitor blocked the formation of γ-casein. The results indicate that aldehydes accumulated from lipid oxidation can modify β-casein and thereby increase susceptibility of the proteins to proteolysis. Furthermore, the data suggest that proteolysis in raw milk may be connected to oxidative processes.

Keywords: Oxidation, proteolysis, plasmin, γ-caseins, storage.

Enhancement of proteolysis in raw milk may cause reduced clotting properties of the curd and reduced cheese yield (Bastian & Brown, 1996; Mara et al. 1998) as well as giving rise to astringent off-flavour in dairy products (Harwalkar et al. 1993). During storage of raw milk, the proteins are exposed to proteolysis, primarily by plasmin (fibrinolysin, E.C. 3.4 21.7) and bacterial proteases (Humbert et al. 1990; Guinot-Thomas et al. 1995; Celestino et al. 1996; Wiking et al. 2002). Grieve & Kitchen (1985) reported that psychrotrophic bacteria are unlikely to release extracellular proteases until their numbers have reached at least 10^7 cfu/ml. Therefore, the plasmin/plasminogen-system is probably responsible for the majority of the proteolysis during the first days after milking, depending on the milk quality. Plasmin mainly hydrolyses β-casein and α_{s2}-casein and, to a slower extent, α_{s1}-casein, while κ-casein is quite resistant to hydrolysis by plasmin (Verdi et al. 1987; Recio et al. 1997). γ-Casein is the primary proteolytic product from plasmin-catalysed proteolysis of β-casein. Plasminogen is the inactive precursor for plasmin in milk, and the conversation of plasminogen to active plasmin by a series of proteolytic cleavages is affected by plasminogen activators (Richardson, 1983; Saint-Denis et al. 2001). Rollema et al. (1983) reported that the ratio of plasminogen to plasmin varies from 4·8:1 to 37:1 in milk from individual cows. Plasminogen activator activity increases with high somatic cell counts, and it is found that somatic cell count is correlated with plasmin activity (Zachos et al. 1992; Le Roux et al. 1995). A system of plasmin inhibitors is also present in milk (Precetti et al. 1997). Furthermore, Igarashi (1990) reported that proteolysis is increased in unheated skim milk by mild oxidation initiated by addition of H_2O_2, ascorbic acid or by exposure to light, and that a higher challenge of oxidation inhibits or has less effect on the proteolysis, and explained the increased proteolysis by oxidative damage of plasmin inhibitors. The same effect on proteolysis has been observed in mammalian cells, where mild oxidative stress increases proteolysis caused by multicatalytic proteinase complex proteasome (Grune et al. 1995, 1996; Ullrich & Grune, 2001).

Oxidation takes place throughout storage of milk. Factors including fatty acid composition, content of low molecular anti-oxidants, pro- and anti-oxidative enzyme systems, transition metal ions, exposure to light and oxygen are known to influence the oxidation, which gives rise to oxidised flavours and changes in the functionality of milk components (Nielsen et al. 2002). The aim of the present work was to study the mechanisms responsible for increased proteolysis in oxidised raw milk.

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Materials and Methods

Materials

Plasmin from bovine plasma was obtained from Boehringer Mannheim (GmbH, D-68305 Mannheim, Germany). Bovine β-casein, bovine lung Aprotinin and 1,1,3,3-tetraethoxypropane (TEP) were obtained from Sigma (St Louis, MO 63178, USA). Fluorescamine was obtained from ACROS Organics (B-2440 Geel, Belgium) and the chromogenic substrate S-2403 (pyroGlu-Phe-Lys-pNA.HCl) from Chromogenix Instrumentation Laboratory S.p.A. (20128 Milano, Italy). All laboratory chemicals used were of analytical grade, and water was purified through an Elgastat Maxima unit (Bucks HP14 3JH, UK) prior to use.

Oxidation and storage of raw milk

Raw bulk milk was obtained from a herd of Danish Holstein production at the Research Centre Foulum. Oxidation of the milk was initiated by addition of H_2O_2, $CuSO_4$ or by exposure to light from TLD-18W/96 tubes (Philips, NL-5600 JM Eindhoven, The Netherlands) at 10 °C. The pro-oxidants were added 2–3 h after milking. Samples of 25 ml milk were stored for 24 h in PE bottles at 5 °C with 5 min stirring every hour after addition of pro-oxidants or light exposure.

Model system to detect proteolysis of β-casein

The β-casein model system contained 4·5 g β-casein/l sodium tetraborate buffer at pH 8 and plasmin (6·75 mg/l), in the presence or absence of the plasmin inhibitor aprotonin (3·23 mg/l). The samples were incubated for 24 h at 20 °C after addition of 1 mM TEP.

Proteolysis

The extent of proteolysis was measured by the Fluorescamine method described by Wiking et al. (2002). The assay is based on a reaction of primary amino groups of trichloroacetic acid-soluble peptides and amino acids with fluorescamine ($C_{17}H_{10}O_4$).

The milk samples were mixed with an equal volume of 240 g trichloroacetic acid/l (TCA) and held on ice for 30 min before centrifugation (16 060 × ***g***, 20 min). Supernatant (25 µl) was mixed with 750 µl 0·1 M-sodium tetraborate (pH 8), 250 µl fluorescamine in acetone (200 mg/l) was added, and the fluorescence (excitation wavelength, 390 nm and emission wavelength 480 nm) was measured after 18 min in a Luminescence spectrometer (Perkin Elmer LS 50 B, Shelton, CT 06487-4794, USA). The extent of proteolysis was expressed as equivalence (M) Leucine. Five replicates were performed.

Plasmin activity

Plasmin activity was determined by a modification of the method of Rollema et al. (1983). This method involved measuring the rate of hydrolysis of the chromogenic substrate S-2403 (pyroGlu-Phe-Lys-pNA.HCl). Raw skim (50 µl) milk was added to 100 µl PBS-EDTA buffer, pH 7·4 in a micro well plate (NUNC A/S, DK-4000 Roskilde, Denmark). A solution of 5·9 mM S-2403 (50 µl) was added just before the measurement of absorbance started at a Powerwave$_x$ microplate spectrophotometer (Bio-tek Instrument, Winooske, VT 05404, USA). The aborbance was measured every minute for 45 min The change in absorbance (dA_{405}/dt) was taken as a measure of plasmin activity.

Generation of malondialdehyde

In order to generate the lipid oxidation product malondialdehyde, 0·01, 0·1, 1, 10 and 40 mM-TEP, was added to raw skim milk. The method to measure proteolysis in milk was changed due to the present of aldehydes. The Fluorescamine method is based on a reaction of primary amino groups with fluorescamine. Aldehydes are able to react with primary amino groups and form Schiff bases, resulting in lower fluorescence response of amino terminals. Consequently, we decided on a method to measure the formation of γ-caseins as the primary proteolytic products derived from β-casein.

Quantitative determination of γ-casein by FPLC

The amount of γ-casein was determined by a method modified according to Igarashi (1989). The method is based on a separation of a γ-casein abundant fraction from skim milk applied to a DEAE Sephacel™ column (Amersham Pharmacia Biotek AB, SE-751 84 Uppsala, Sweden). A skim milk sample (3·0 ml) or a model system sample was mixed with 1 ml 4 M-Na_2SCN and 4 ml ethanol followed by 2 ml 0·75 M-$CaCl_2$ (in 50% (v/v) ethanol). The solution was held on ice for 30 min before centrifugation (1500 × ***g*** at 5 °C for 10 min). The supernatant (8·5 ml) was transferred into a new tube and mixed with an equal volume of 40 g/l (TCA). After 30 min at room temperature, followed by centrifugation (1500 × ***g*** at 5 °C for 15 min), the precipitates were washed with 2 ml 20 g TCA/l and then dissolved in 0·02 M-Tris–HCl, 6 M-Urea, pH 9·5. The protein solution was sterile-filtrated before it was injected into the Äkta FPLC-system (Amersham Pharmacia Biotek AB, SE-751 84 Uppsala). A gradient of 0 to 1·0 M-NaCl in 0·02 M-Tris–HCl, 6 M-Urea, pH 8 (flow: 0·2 ml/min for 45 min) was used The absorbance was measured at 280 nm.

Schiff bases

The formation of Schiff bases was detected by fluorescence spectroscopy (Perkin Elmer LS 50 B) in the β-casein model system without plasmin. The excitation wavelength was 381 nm and the emission wavelength 438 nm, as described by Trombly & Tappel (1975).

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Table 1. Effect of addition of pro-oxidants on proteolysis in raw milk after 24 h storage at 5 °C, measured by the Fluorescamine method. The control was raw milk

Values are means ($n=5$). CV<9·5%

Treatment	Proteolysis, % of control
1 mM H_2O_2	111·56*
1·5 mM H_2O_2	92·71
10 μM Cu	113·09*
100 μM Cu	102·58
Light 1 h	113·56*
Light 1·5 h	106·96

*Samples significantly different from control evaluated by *t* test, $P<0·05$

Table 2. Plasmin activity in raw milk stored for 24 h at 5 °C after addition of H_2O_2 or Cu^{2+}, measured as rate of hydrolysis of the chromogenic substrate S-2403 (pyroGlu-Phe-Lys-pNA.HCl) in PBS-EDTA buffer, pH 7·4. The change in absorbance (dA_{405}/dt) was taken as a measure of plasmin activity

Values are means ± SD for $n=5$

Treatment	Δ abs/min per ml milk
Control	0·1041 ± 0·0057
10 μM-Cu	0·1085 ± 0·0155
100 μM-Cu	0·1160 ± 0·0206
0·5 mM-H_2O_2	0·1078 ± 0·0069
1 mM-H_2O_2	0·1051 ± 0·0117
1·5 mM-H_2O_2	0·1134 ± 0·0112
40 mM-TEP	0·1097 ± 0·0071

No significance between raw milk and treatments indicated by *t* test
TEP: 1,1,3,3-Tetraethoxypropane

Results

The effects of oxidation initiated/propagated by H_2O_2, Cu^{2+} and light on proteolysis in raw milk stored for 24 h at 5 °C are shown in Table 1. Addition of 1 mM-H_2O_2, 10 μM-Cu^{2+} or light exposure (1 h) significantly ($P<0·05$) increased the proteolysis in the milk, by 11, 13 and 14%, respectively. A higher level of oxidising agent either inhibited proteolysis or enhanced proteolysis only little. Igarashi (1990) suggested that the enhancement of proteolysis is caused by oxidative damage to plasmin inhibitors. In order to test this hypothesis, we measured plasmin activities in milk to which was added H_2O_2 or Cu^{2+} to propagate oxidation. If the inhibitors were oxidatively damaged, the plasmin activities were expected to increase. However, addition of pro-oxidants to milk showed no significant effects on plasmin activities in raw milk (Table 2). These results indicate that the inhibitor/activator system of plasmin was not involved in the increase in proteolysis in oxidised milk.

Acceleration of lipid oxidation of milk by addition of pro-oxidants will increase the accumulation of lipid-derived aldehydes. Aldehydes are very reactive and able to react with milk proteins and form Schiff bases, affecting the structure and functionality of proteins (Leaver et al. 1999). In order to investigate how lipid-derived aldehydes in milk affect proteolysis, a series of experiments was conducted, where proteolysis was measured in the presence of aldehydes. Increasing concentrations of TEP added to raw skim milk in order to generate the lipid oxidation product malondialdehyde, resulted in enhanced formation of the proteolysis product γ-casein (Fig. 1). The effect of adding a large excess of plasmin to raw skim milk was only small, likewise the effect of adding plasmin to raw skim milk containing malondialdehyde was small compared with raw skim milk with only malondialdehyde added (Table 3). To confirm that the increased proteolysis originates from higher susceptibility of plasmin action due to aldehyde modification of β-casein, proteolysis in a model system containing β-casein as the only substrate protein was evaluated. Addition of aldehyde to the model system enhanced the formation of γ-caseins, and further addition of a plasmin inhibitor (aprotinin) blocked the formation of γ-caseins (Table 4). To investigate the responsible mechanism involved in the increased proteolytic activity, the formation of Schiff bases was studied in a model system containing β-casein and malondialdehyde, and it was found to increased during 24 h incubation (Table 5) indicating that β-casein is modified by malondialdehyde, giving rise to Schiff bases.

Table 3. Formation of γ-caseins during 24 h storage at 5 °C in raw skim milk with addition of 1 mM-1,1,3,3-tetraethoxypropane (TEP), plasmin (6·75 mg/l) or both, measured by FPLC

Treatment	γ-caseins (area)
Raw skim milk	618·8 ± 33·3
Raw skim milk+TEP	1404·9 ± 106·0
Raw skim milk+plasmin	661·6 ± 53·3
Raw skim milk+plasmin+TEP	1587·0 ± 47·6

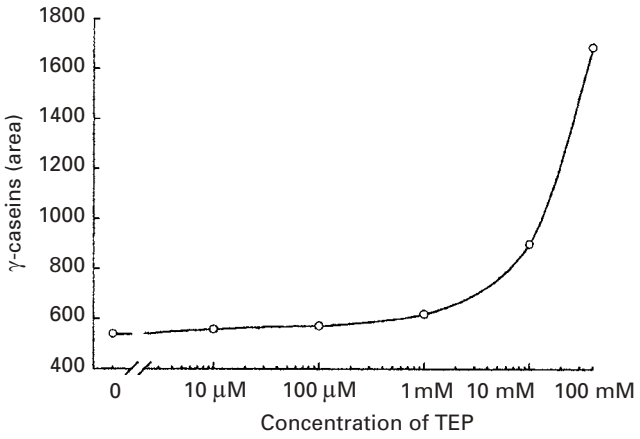

Fig. 1. Formation of γ-caseins during 24 h storage at 5 °C with increasing level of the malondialdehyde generator 1,1,3,3-tetraethoxypropane (TEP) in raw skim milk, measured by FPLC.

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Table 4. Formation of γ-caseins during 24 h at 20 °C in 0·1 M-sodium tetraborate buffer, pH 8, containing 4·5 mg β-casein/ml and plasmin. The concentration of 1,1,3,3-tetraethoxypropane (TEP) was 1 mM, and the plasmin inhibitor was aprotinin

Values are means ± SD for $n=2$

Treatment	γ-caseins (area)
Control	151·3 ± 9·3
+TEP	206·8 ± 21·6
+TEP+inhibitor	25·9 ± 8·9

Table 5. Formation of Schiff bases at 20 °C in 0·1 M-sodium tetraborate buffer, pH 8 containing 4·5 mg β-casein/ml

Values are means ± SD for triplicate measurements, fluorescent intensity at excitation wavelength 381 nm and emission wavelength 438 nm. MDA: Malondialdehyde

	0 h	24 h
β-casein	17·38 ± 0·62	16·87 ± 0·42
β-casein+MDA	20·25 ± 2·29	34·18 ± 0·48

Discussion

Three different pro-oxidants were added to raw milk. Hydrogen peroxide initiates oxidation through activation of the lactoperoxidase system and the transition metal ion, Cu^{2+}, propagates lipid oxidation. Exposure to light causes lipid and protein oxidation through excitation of the singlet sensitizer riboflavin found in milk. The present study showed that oxidative changes in milk caused by the pro-oxidants 1 mM-H_2O_2, 10 μM-Cu^{2+} or 1 h light exposure (Table 1) all enhanced proteolysis. These observations are in agreement with the results of Igarashi (1990), who found that proteolysis in raw milk increased by 28% after addition of H_2O_2 (15 mg/l) and that proteolysis declined toward the level of control sample at higher pro-oxidant concentrations. In the present study, proteolysis likewise decreased at severe level of oxidation. Furthermore, our observations confirm those of a previous study of another biological system that showed higher endogenous proteolysis in human leukaemia cells by action of proteosome after a 0·4 mM-H_2O_2 challenge, in comparison with a 1 mM-H_2O_2 treatment or control (Ullrich & Grune, 2001). These results clearly indicate that a slight oxidation increases the susceptibility of proteolytic substrates to the proteosome enzymes, but that more severe oxidation causes further conformational changes that decrease the protein susceptibility. Previous work has found that, due to non-enzymatic photochemical degradation, β-lactoglobulin and α-lactalbumin in model systems degrade when they are exposed to light for days in the presence of riboflavin (Gilmore & Dimick, 1979). In the present study, the milk samples were only illuminated for maximum of 90 min, and therefore the contribution of non-enzymatic photochemical degradation was negligible. However, the observation in the current study of inhibited proteolysis in milk at severe extent of oxidation could be caused by an oxidative conformational change due to cross-linking of proteins or peptide chains e.g. through the formation of dityrosine (Østdal, 2000), resulting in lower susceptibility of the oxidised proteins to plasmin action.

Kelly & Foley (1997) found both an increased plasmin activity in whole milk oxidised by potassium iodate (KIO_3) before UHT treatment and a higher proteolysis during subsequent storage. They suggested that KIO_3 protected plasmin from inactivation through thiol-disulphide interchange with β-lactoglobulin caused by heat denaturation, and the results are therefore not comparable with the present study. In the present experiment, plasmin activities were not affected by addition of pro-oxidants. Based on the present observations we suggest that the increased proteolysis is not a result of damaging inhibitors as suggested by Igarashi (1990) but is more likely to be caused by higher susceptibility of β-casein to act as substrate for plasmin action. Moreover, the observed inhibition of proteolysis by the higher oxidative challenge also indicate that the changes occur to the substrate protein, as plasmin activity was not affected by higher oxidative challenge (Table 2). Consequently, the suggestion of Igarashi (1990) that the increased proteolysis is derived from oxidative damage to plasmin inhibitors may be incorrect. Furthermore, the present findings of no effect on plasmin activity at the highest concentrations of H_2O_2 and Cu^{2+} indicate that plasmin is not damaged by oxidation. The current observation of no effect on plasmin activity in raw milk upon addition of TEP (Table 2) point out that neither TEP nor malondialdehyde are activators of plasmin.

To investigate the possibility of higher proteolytic susceptibility of β-casein to plasmin action in oxidised milk the interaction of lipid oxidation products with milk proteins were investigated. Recently published studies of the degradation of bovine serum albumin (BSA) by trypsin and other digestive proteases indicate that BSA modified with 4,5(E)-epoxy-2(E)-heptanal was less easily degrade than the native protein (Zamora & Hidalgo, 2001). These results contrast with our observations, which indicate that the increase or decrease of protolysis upon aldehyde-modification may be enzyme-specific. Malondialdehyde is able to cross-link to proteins (Gardner, 1979). The present results show that aldehydes accumulated from lipid oxidation can modify β-casein by forming Schiff bases, and thereby increase the susceptibility of protein to proteolysis. This increased susceptibility may be caused by a change in hydrophobicity. The data suggest that the mechanism of the increase in proteolysis is one whereby pro-oxidants like H_2O_2, Cu^{2+} and light initiate lipid oxidation, resulting in formation of aldehydes. These aldehydes cross-link to the caseins and thereby increase the susceptibility of protein to proteolysis by plasmin. However, the observed inhibition of proteolysis at higher oxidative challenges compared with the observed enhanced proteolysis on increasing addition of malondialdehyde could be explained by a hasher extent of protein oxidation, causing aggregation and thereby difficulties for plasmin actions. In

contrast, Grune et al. (1995) observed increased degradation of protein in liver cells after incubation with 10 μM-malondialdehyde, but proteolysis declined toward the level of control sample when malondialdehyde concentration reached 100 μM. The impact of different levels of oxidative agents and aldehydes on proteolysis are likely to be affected by the antioxidative status in the milk or other systems, thus making it difficult to compare with the precise level of slight and severe oxidation between different milks.

The present findings suggest that some of the off-flavours normally connected to oxidation in milk may originate from increased proteolysis in milk. The processes giving rise to off-flavours seem to be more complex than just one deterioration process affecting one sensory attribute. The increased proteolysis was observed at a storage temperature of only 5 °C, which is a very relevant temperature for dairy farms and industry. Summarising the result of this study, proteolysis in raw milk was increased by mild oxidation, while severe oxidation inhibited or had no effect on proteolysis. Moreover, the results strongly suggested that it is cross-linking between aldehydes accumulated from the lipid oxidation and β-casein that causes a higher susceptibility to plasmin actions, resulting in enhanced proteolysis.

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