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Effects of lactoperoxidase and hydrogen peroxide on rheological properties of yoghurt

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The effects of activation of the lactoperoxidase (LPO) system by H_2O_2–NaSCN and hydrogen peroxide (H_2O_2) on the accessibility of sulphydryl groups (SH) in skimmed milk, and on the dynamic rheological properties of the resulting yoghurt were investigated. Four different concentrations of each reagent (20–80 mg H_2O_2–NaSCN/kg milk and 100–400 mg H_2O_2/kg milk) were compared. Clear negative correlations were noted between the accessibility of SH groups and both LPO activation rate and H_2O_2 concentration. Also the native PAGE pattern of the heat-treated samples showed that with increase in the H_2O_2–NaSCN and H_2O_2 concentrations, the level of interaction between β-lactoglobulin (β-lg) and κ-casein (κ-CN) decreased. The complex modulus (G*) of skimmed milk yoghurts declined gradually with the decrease in the concentration of accessible SH groups accordingly. Tan δ values of yoghurt samples were found to be different from the control, but close to each other, indicating that protein interaction forces taking place in the formation of gel networks of treated yoghurts were different from the control.

Keywords: Lactoperoxidase, yoghurt, rheology, sulphydryl groups, hydrogen peroxide.

There are several methods, other than refrigeration, for retarding bacterial growth in raw milk during collection and transportation. The use of chemical preservatives such as hydrogen peroxide (H_2O_2), alkaline solutions etc, and activation of natural antibacterial enzymes are common practices in developing countries under tropical and subtropical conditions, where refrigeration is not practical (Özer et al. 2000). FAO/WHO strongly discourage the preservation of milk by chemical means, except the application of H_2O_2 at moderate levels (i.e. 100–300 mg/kg) and activation of the native lactoperoxidase (LPO) system. In the case of the use of H_2O_2, this chemical must be completely destroyed before consumption either by heat treatment or by means of catalase.

Previous studies demonstrated that H_2O_2 concentrations lower than 100 mg/kg had no significant effect on the bacterial growth and development of acidity (Özer & Atamer, 1999). In these studies, H_2O_2 concentrations ranging from 20 to 140 mg/kg were tested, and it was found that concentrations lower than 100 mg/kg were not sufficient to prevent milk from turning sour after 6 h at 30 °C (Özer & Atamer, 1999). The maximum permitted level for H_2O_2 is 800 mg/kg and above 600 mg/kg, LPO is completely destroyed (Björck, 1987). For liquid consumption, the H_2O_2 concentrations should be in the range 100–400 mg/kg (Björck, 1987).

Lactoperoxidase (E.C.1.11.1.7; LPO) is a haemoprotein present in milk whose molecular weight has been variously reported to be in the range 76–92·7 kDa (e.g. Evans, 1980; Sievers, 1981). In the presence of H_2O_2 and thiocyanate, the enzyme has an antibacterial effect against Gram-negative bacteria (Reiter, 1985).

Many attempts have been made to use the LPO system to preserve raw milk (Björck et al. 1979; Kamau & Kroger, 1984) and to manufacture products from such preserved milk (Zall et al. 1983; Mehenna & Hefnawy, 1988; Kumar & Mathur, 1989; Atamer et al. 1995; Hirano et al. 1998a, b; Özer & Atamer, 1999). It has been reported that yoghurts produced from milk preserved either by H_2O_2 or by the LPO system had smoother texture and softer body leading to whey separation during storage (Atamer et al. 1995; Hirano et al. 1998a, b).

Yoghurt has a weak gel structure formed from a 3-dimensional network, which immobilises the liquid phase (Lankes et al. 1998). The formation of this network is

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mainly determined by specific sulphydryl-disulphide (SH/S-S) exchange reactions triggered by heating above 70–75 °C. Although many factors affect the rheological properties of yoghurt, the interaction between κ-casein (κ-CN) and β-lactoglobulin (β-lg) via SH/S-S exchange is the primary factor determining the rheological, textural and organoleptic properties of yoghurt. On heating, SH groups of milk proteins become accessible and liable to interaction, and the degree of interaction is dependent mainly on heat intensity. Any factor disturbing total SH groups and/or reactivation process of these groups may directly influence the physical quality of the final product.

The objectives of the present study were i) to monitor the variations in total and accessible SH groups in milks treated with H_2O_2, and in which the LPO system was activated by H_2O_2–NaSCN at varying reagent concentrations; and ii) to investigate the effects of H_2O_2 treatment and LPO activation on the rheology of resulting yoghurt.

Materials and Methods

After removal of milk fat by means of a cream separator, activation of LPO system was achieved by means of a H_2O_2–NaSCN mixture. All chemicals were supplied by Sigma Aldrich Co. Ltd. (Gillingham, Dorset, SP8 4XT).

A batch of raw skimmed cows' milk (CEDAR, Reading, RG2 9HX) was divided as follows:

- Control sample – subdivided into 5 replicate samples (3 litres each).
- H_2O_2 containing samples (100, 200, 300 and 400 mg/kg milk) subdivided into 5 replicate samples (3 l each) at each concentration.
- H_2O_2–NaSCN containing samples (20, 40, 60 and 80 mg of both H_2O_2 and NaSCN/kg milk) subdivided into 5 replicate samples (3 l each) at each concentration.

H_2O_2 and H_2O_2–NaSCN mixtures at selected concentrations were added to the milks, and samples were stored at 25 °C for 6 h. Control samples were stored at 4 °C for 6 h. Preliminary studies showed that the titratable acidity of unpreserved milk kept at 25 °C increased from 0·163% to 0·203% lactic acid within 6 h. This level of acidity carries the risk of coagulation during heating in yoghurt-making. The marginal change in acidity was attributed to the poor microbiological quality of raw milk. In most developing countries the average cell count is well over 10^6 cfu/ml (Metin, 1999). Similar findings were obtained by Özer & Atamer (1999). Therefore, it was decided to keep control milk at 4 °C to prevent bacteriological deterioration.

After 6 h storage at 25 °C (or 4 °C) samples were converted to yoghurt according to the procedure of Tamime & Robinson (1985). Skim milk samples were heat treated at 85 °C for 20 min, prior to cooling to 42 °C for inoculation with a culture of *Streptococcus thermophilus* and *Lactobacillus dellbrueckii* subsp. *bulgaricus* (1 : 1) (CH-1; Chr. Hansen, Hungerford, RG17 OYT) at a rate of 20 kg/g. The inoculated milks were then incubated at 43 °C until pH 4·3 was reached. Yoghurt samples were refrigerated overnight and then subjected to rheological and electrophoretic analyses.

Determination of total and accessible SH groups

Samples (1 l) of milk were retained post-storage (6 h, 25 or 4 °C) period and post-heat treatment (85 °C, 20 min) for determination of total and accessible SH groups as described by Gaffar (1987) as follows:

Total SH groups. Samples of raw and heated milks (1 ml) were purged with nitrogen gas for 10 s and then mixed with 9·0 ml 8 M-urea to expose buried thiol groups, 4·9 ml phosphate buffer (0·1 M-$Na_2HPO_4 . 2H_2O$, pH 9·2–9·3) to bring the final pH to 8·0, and finally 0·1 ml DTNB (dithiobisnitrobenzoic acid) reagent (20 mg DTNB in 10 ml 0·1 M-sodium phosphate buffer, pH 7·0). Colour development was allowed to proceed for 5 min at room temperature. Samples were centrifuged at 15 000 ***g*** at 4 °C for 30 min, and supernatant was filtered through Whatman GF/F glass fibre paper. The absorbance of thiophenol specifically formed by reaction with SH groups was read at 412 nm and the concentration in that solution was calculated using molar absorption coefficient of 13 600 litre $mole^{-1}$ cm^{-1}.

Accessible SH groups. Heated milk (5 ml) was purged with nitrogen gas for 10 s and then diluted to 8·0 ml phosphate buffer (0·1 M-$Na_2HPO_4 . 2H_2O$, pH 9·2–9·3) to bring the pH to 8·0. To this solution was added 0·1 ml DTNB reagent, and colour development was allowed to proceed for 5 min at room temperature, then 6·9 ml saturated ammonium sulphate were added. This solution was centrifuged at 15 000 ***g*** at 4 °C for 30 minutes. The supernatant was then filtered through Whatman GF/F glass fibre filter paper. The absorbance of the filtrate was read at 412 nm and the concentration was calculated as above.

The concentrations of total and accessible available SH groups were calculated by using the following equation:

$$C = A_{412} D / \varepsilon$$

where: C, SH concentration (M); A_{412}, absorbance at 412 nm; ε, molar absorption coefficient (13 600 litre $mole^{-1}$ cm^{-1}) (Ellmann, 1959); D, dilution factor (total volume/sample volume).

Electrophoresis studies. Native PAGE (30% acrylamide; 2·67% bis acrylamide) was carried out on milk and yoghurt samples as described by Özer (1997). Sample

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Table 1. Effects of (a) H_2O_2 and (b) lactoperoxidase (LPO) activation on total SH groups of raw and heat-treated milks and accessibility of SH groups

Values are means ± SEM for $n=5$

		H_2O_2 concentrations (mg/kg)			
(a) Addition of H_2O_2	Control	100	200	300	400
Total SH_{raw}†	3·93 ± 0·09	3·37 ± 0·10	2·79 ± 0·15	2·33 ± 0·11	2·08 ± 0·08
Total SH_{heated}†	4·54 ± 0·08	3·71 ± 0·09	3·43 ± 0·11	3·24 ± 0·14	2·85 ± 0·11
$SH_{accessible}$†	3·29 ± 0·06	2·09 ± 0·02	1·83 ± 0·07	1·65 ± 0·04	1·34 ± 0·10
%R-$S_{remaining}$‡	—	81·7	75·6	71·4	62·8
		H_2O_2 and NaSCN concentrations (mg/kg)			
(b) LP activation	Control	20	40	60	80
Total SH_{raw}†	3·93 ± 0·09	3·76 ± 0·03	3·34 ± 0·09	3·08 ± 0·12	2·62 ± 0·07
Total SH_{heated}†	4·54 ± 0·08	4·15 ± 0·07	3·86 ± 0·10	3·34 ± 0·10	2·92 ± 0·04
$SH_{accessible}$†	3·29 ± 0·06	2·12 ± 0·06	1·91 ± 0·04	1·34 ± 0·07	1·06 ± 0·13
%R-$S_{remaining}$ ¶	—	91·4	85·0	73·6	64·3

† Total SH groups of raw and heat-treated milks and accessible SH groups after heat treatment at 85 °C for 20 mins (μmol SH/g protein)
‡ [Total SH_{heated}+H_2O_2]/[Total SH_{heated}] − 100
¶ [Total SH_{heated}+H_2O_2-NaSCN]/[Total SH_{heated}] − 100

loading volume, concentrations of separating and stacking gels were 20 μl, 12·5% and 4·0%, respectively. The gel was run at the constant voltage setting of 200 V until the bromphenol blue marker almost reached the bottom of the gel. The current was set to 60 mA initially, and dropped gradually to 40–45 mA during running. All procedures were completed within 1 h.

Dynamic rheological measurements. The dynamic rheological profiles (small deformation) of the samples were monitored using a Rheo Tech International (Camtel Ltd., Royston, SG8 9AZ) controlled-stress rheometer. The rheometer was set up with parallel plate geometry (10 mm radius chamber and 1 mm gap), and the temperature of the samples was maintained at 25 °C using a circulating water system. The complex modulus $[G^*=(G'^2+G''^2)^{1/2}]$ and loss tangent ($\tan\delta=G''/G'$) were determined as amplitude sweeps within a torque range 2×10^{-3} to 2×10^{-2} mNm at 0·25 Hz. The samples were transferred into the rheometer as the set gel. Each sample was loaded into the measuring system and allowed to relax for 1 min. Stirring was avoided before the dynamic measurements, so that the effects of physical disturbance on the rheology of yoghurts were eliminated. All measurements were taken in the linear viscoelastic region (LVE) in order to keep the structural integrity of the samples unchanged, and no slipping was observed in the measuring system.

Residual H_2O_2 was determined according to the method proposed by Asai et al. (1982).

The complete examination was carried out five times and differences between the samples were determined by the LSD test (Steel & Torries, 1980).

Statistical evaluations were made using a statistics software program (SPSS© UK Ltd. Woking, GU21 1EB).

Results

The effects of H_2O_2 and H_2O_2–NaSCN on total thiol groups before and after heat treatment are presented in Tables 1a & b, respectively. The heated milks displayed higher concentrations of SH groups in both the control and the chemically-treated milks. This is surprising as the SH groups of β-lg exposed by heating would be expected to form S–S bonds by interaction with β-lg or κ-CN. The mechanism is not clear and could arise through increased exposure of SH or reduction of S–S bonds. However, Patrick & Swaisgood (1975) also reported that heated milk had higher concentrations of SH than raw milk. Additions of H_2O_2 gave rise to significant reductions in the concentration of total SH groups in both raw and heated milks ($P<0·05$). The reduction was approximately linear with increasing H_2O_2 levels giving approximately 60% reduction in SH groups at the highest concentration of H_2O_2 tested. Similar results were obtained from LPO activated milks (Table 1b), with increasing thiocyanate concentrations producing significant reductions in total SH groups in almost linear fashion in both raw and heated milks ($P<0·05$). A slightly greater effect was seen with H_2O_2 alone than with the combined H_2O_2–NaSCN. The effects of H_2O_2 and H_2O_2–NaSCN treatments on the accessibility of the SH groups are presented in Tables 1a & b, respectively. For additions of H_2O_2 and H_2O_2–NaSCN to the samples, the amount of accessible-SH groups and the percentage of remaining R-S after oxidation by H_2O_2 decreased with the increase in the H_2O_2 and H_2O_2–NaSCN concentrations accordingly. Activation of the LPO system by H_2O_2–NaSCN at lower concentrations (i.e. 20–40 mg/kg) had less effect on the remaining SH groups than H_2O_2 treatment alone. This may be due

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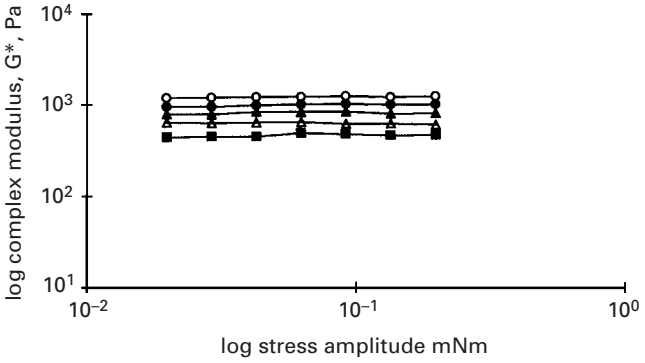

Fig. 1. Effect of H_2O_2 treatment on the rheological properties of yoghurts ($n=5$). (○) Control, (●) 100 mg H_2O_2/kg, (▲) 200 mg H_2O_2/kg, (△) 300 mg H_2O_2/kg, (■) 400 mg H_2O_2/kg.

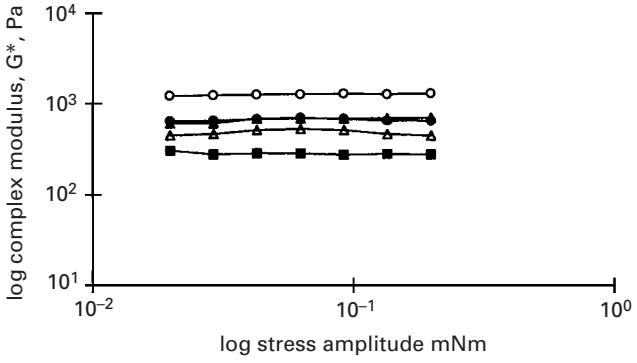

Fig. 2. Effect of H_2O_2/NaSCN treatment on the rheological properties of yoghurts ($n=5$). (○) Control, (●) 20 mg H_2O_2–NaSCN/kg, (▲) 40 mg H_2O_2–NaSCN/kg, (△) 60 mg H_2O_2–NaSCN/kg, (■) 80 mg H_2O_2–NaSCN/kg.

to the stronger oxidative effect of H_2O_2 at higher concentrations.

Dynamic measurements of yoghurt gels. The complex moduli (G*) of the gels were compared as a function of amplitude sweep (Figs. 1 & 2). The G* of the samples showed an inverse relationship with the concentration of H_2O_2 and H_2O_2–NaSCN to yoghurt milk. In general, the higher the concentrations of H_2O_2 and H_2O_2–NaSCN, the weaker the structure. Very highly significant relationships were found between concentration of accessible SH groups and G* in both the samples with added H_2O_2 and H_2O_2–NaSCN (Table 2). None of the samples showed structural breakdown as the stress increased.

The treated samples had higher tan δ values than the control (Table 3) indicating that the nature of the bonds contributing to the formation of gel structure may have been different from the control. On the other hand, in the treated samples, the tan δ values were close to each other. This may indicate that SH/S-S bonds were adversely affected by addition of H_2O_2 and H_2O_2–NaSCN, and this led to a weaker body. The incubation period of the samples (time to reach pH 4·3) differed significantly (Table 3). Overall, the higher the H_2O_2 or H_2O_2–NaSCN the longer the incubation time. Although, no residual H_2O_2 was detected after heat treatment, it was thought that the longer incubation time was caused by the reactivation of LPO during the incubation. High correlations were noted between incubation time and accessible SH groups (Table 2).

PAGE of milk and yoghurts. The native PAGE patterns of heat treated milks are shown in Fig. 3. Neither the H_2O_2 nor H_2O_2–NaSCN additions had a marked effect on protein fractions of raw milks (not shown). However, following heat treatment, whilst the bands corresponding to β-lg A, B and α-lactalbumin disappeared in the control, or with the 100 and 200 mg/kg H_2O_2 containing samples, the other samples had a band representing β-lg. This shows that interactions between κ-CN and β-lg were, to some extent, hindered by the H_2O_2 and H_2O_2–NaSCN treatments.

Discussion

Yoghurt gel is a heat-induced acid casein gel, and many factors are involved in the formation of the 3-dimensional gel network, at different levels depending upon environmental conditions. The involvement of SH/S-S interchange reactions (Tamime & Robinson, 1985; Hashizumo & Sato, 1988; Shimada & Cheftel, 1989), hydrophobic effects (Knoop & Peters, 1975; Heertje et al. 1985; Roefs & van Vliet, 1990), electrostatic interactions and salt bridges (Knoop & Peters, 1975; Rasic & Kurmann, 1978) in the formation of yoghurt gel have been well-established, as well as the importance of van der Waals interaction forces and steric effects (Roefs & van Vliet, 1990). Although, undoubtedly, the combination of all these interaction forces determines the characteristics of yoghurt gel, the major role of SH/S-S interchange reactions is beyond question. It is well established that whey protein–κ-CN interactions occur primarily via SH/S-S exchange reactions (Doi et al. 1983; Hill, 1989; Shimada & Cheftel, 1989; Özer, 1997). Heat treatment of milk results in denaturation of whey proteins, leading to interactions between κ-CN and β-lg especially. Any factor interfering with accessibility of SH groups (or more specifically efficiency of SH/S-S interactions) has a direct effect on gelation capacity. Both H_2O_2, and hypothiocyanate ions ($OSCN^-$) produced by LPO system, have an oxidative effect on SH groups (Hirano et al. 1998a, b). β-Lg possesses free SH groups, which are exposed as the molecule is denatured (Björck et al. 1979). $OSCN^-$ not only oxidises the SH groups, but also increases protein hydrophobicity leading to the enhanced gelation (Hirano et al. 1998a). Nevertheless, Hirano et al. (1998a) found that in parallel with the increase in hydrophobicity in LPO activated yoghurts, the degree of hardness was reduced. The reason for this contradiction could be the decrease in the reactivity of SH groups. It is well known that

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Table 2. Multiple correlation of samples†

	H_2O_2 added samples			LP activated samples		
	G*	tan δ	$SH_{accessible}$	G*	tan δ	$SH_{accessible}$
tan δ	−0·836			−0·925		
$SH_{accessible}$	0·940	−0·853		0·988	−0·886	
Incubation time (min)	−0·907	0·924	−0·828	−0·911	0·896	−0·921

†Values are correlation coefficients, level of significance at $P<0·001$

Table 3. Average tan δ values and incubation periods of test samples ($n=5$)

		H_2O_2 added samples (mg/kg)				H_2O_2/NaSCN added samples (mg/kg)			
	Control	100	200	300	400	20	40	60	80
tan δ	$0·309^a$	$0·349^b$	$0·341^c$	$0·350^b$	$0·400^d$	$0·340^a$	$0·338^a$	$0·338^a$	$0·364^b$
Incubation time (min)	200^a	237^b	256^c	318^d	400^e	243^a	260^b	295^c	364^d

Samples having a common superscript do not differ significantly ($P>0·05$)

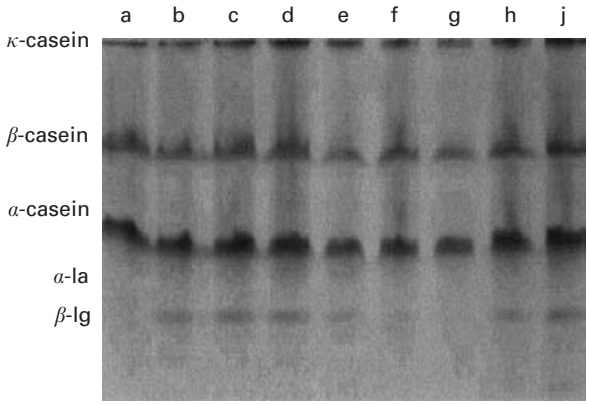

Fig. 3. Native P.A.G.E. pattern of heat treated milk samples. (a) Control, (b) 20 mg H_2O_2–NaSCN/kg, (c) 40 mg H_2O_2–NaSCN/kg, (d) 60 mg H_2O_2–NaSCN/kg, (e) 80 mg H_2O_2–NaSCN/kg, (f) 100 mg H_2O_2/kg, (g) 200 mg H_2O_2/kg, (h) 300 mg H_2O_2/kg, (j) 400 mg H_2O_2/kg.

thermophilic microorganisms are able to produce H_2O_2, and in the presence of thiocyanate the H_2O_2 produced may reactivate the LPO (Guirguis & Hickey, 1987; Kumar & Mathur, 1989). It is known that LPO is completely inactivated at temperatures greater than 85 °C (Metin, 1999). Therefore, in addition to the possible reactivation of LPO, it may also be speculated that as a result of SH oxidation by the addition of H_2O_2 and LPO activation, the gelation kinetics of the samples would have changed, leading to a longer incubation period.

Although the exact mechanism is not clear yet, both H_2O_2 and LPO cause a decrease in the accessibility of SH groups in milk proteins (Hirano et al. 1998a). In this experiment, there was a clear correlation between the PAGE pattern and the number of accessible SH groups of milk samples. It was noted that keeping milk at 25 °C for 6 h in the presence of either H_2O_2 or H_2O_2–NaSCN did not affect whey protein and casein fractions. However, following heat treatment, an interaction between whey proteins and κ-CN would be expected. Figure 3 showed that the band corresponding to β-lg did not completely disappear in the heat treated milks containing H_2O_2–NaSCN at all levels applied, or with H_2O_2 alone at 300 mg/kg and 400 mg/kg. This indicates that the heat-induced interaction between β-lg and κ-CN was prevented to some extent at these concentrations.

Addition of H_2O_2 and H_2O_2–NaSCN to yoghurt milk resulted in a decrease in complex moduli (G*) (Figs. 1 & 2). Although the G* of the samples were significantly different, the tan δ values of treated samples, which give information about the type of bonding taking place in gel-forming, were not much different. This indicates that, in all samples tested, similar types of bonds were formed, but at different levels. Considering the tan δ values of the samples, it could be said that the modification of micelle was limited. The oxidation by H_2O_2 may have produced a small amount of β-lg associated to the casein micelles and a great quantitiy of β-lg–β-lg.

The rheological properties of a yoghurt gel strongly depend upon the macromolecule concentration and upon the enthalpic/entropic nature and strength of the inter-particle forces during and following gelation (Dickinson & McClement, 1996). Also, the size, shape and spatial distribution of the basic elements forming the casein network and the character of junction points are among the main features for the interpretation of the physical properties of a gel (Roefs, 1986). In the present study, the concentrations of total solids and proteins were identical; therefore, the H_2O_2 and H_2O_2–NaSCN dependency of dynamic rheology could be explained by oxidation of SH groups by these chemicals. Since none of the samples were broken down at any point of amplitude sweep, it may be speculated that the decrease in the number of cross-links

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resulted in a decrease in dynamic moduli. If the strength of cross-links changed, the amplitude dependency of G* varied. Similar results were reported by Hirano et al. (1998a).

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