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Inhibition of the Antibacterial Lactoperoxidase-Thiocyanate-Hydrogen Peroxide System by Heat-Treated Milk

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ABSTRACT

The antibacterial system, lactoperoxidase-H_2O_2-SCN^- was affected by the presence of heated milk or skim milk reconstituted from powders having received severe heat treatment. This inhibitory effect was related to the increase in exposed sulfhydryl groups and to the redistribution of protein between micellar and whey phases. Chromatographic analyses of heat-treated milk showed that the inhibitory factor was associated with the casein micelle fraction. The inhibition, however, was overcome by addition of unheated skim milk.

Milk is not only an essential nutrient for the young animal, but it also contains several minute constituents with specialized functions. Bovine milk contains specific and nonspecific antibacterial factors which may play a role in protection of the neonate from enteric infections (*13*). One of the most potent antibacterial factors of milk is the lactoperoxidase (LP)-system. The LP-system consists of the enzyme lactoperoxidase (EC 1.11.1.7) and the substrates thiocyanate and hydrogen peroxide. This system may be activated for use in milk preservation (*3*) and to improve performance of calves fed on raw milk (*14*) and reconstituted calf milk replacers (*19-21*). Activation of the LP-system results in generation of intermediate oxidation products of thiocyanate, including the hypothiocyanite ion (*1*), which are bactericidal to many gram-negative bacteria, for example strains of *Escherichia coli* and *Salmonella typhimurium* which have been implicated in incidents of calf scouring (*8*).

Preliminary details concerning the production of milk powders containing lactoperoxidase activity have been reported (*11,12*). Powders for use in calf milk replacers designed to exploit the bactericidal activity of the LP-system must contain lactoperoxidase activity but should not contain inhibitors of the antibacterial activity of this enzyme system.

Severe heating of the milk will cause aggregation between the casein micelles and the whey proteins, the exposure and oxidation of sulfhydryl groups and even protein denaturation (*4*). During our studies on development of lactoperoxidase-containing milk replacers, it was observed that skim milk powder, which had received a high heat treatment, contained substances inhibitory to the antibacterial activity of the LP-system. In this communication the effects of heat treatment on lactoperoxidase activity in skim milk and on the antibacterial activity of the LP-system in heated milk are presented. Attempts are made to identify the inhibitory agents by fractionating heated milk by gel filtration chromatography and testing the fractions for inhibitory activity.

MATERIALS AND METHODS

Milk samples

Milk from the college herd was defatted by heating at 37°C for 30 min followed by centrifugation at a minimal centrifugal force of $2000 \times g$ for 30 min. The milk was heated in 10-ml portions in a waterbath at temperatures mentioned for each experiment. A heating time of 5-7 min was necessary to raise the actual temperature in the sample (as monitored by a thermometer immersed in one of the portions) from ambient to the experimental value. This time is included in the times given in the tables and diagrams.

Skim milk powders spray dried at high or low temperatures were kindly provided by the Scottish Milk Marketing Board. These powders were analysed for non-protein nitrogen (NPN) and LP activity.

Chemicals

All chemicals were of analytical grade, purchased from either Sigma or British Drug House. Lactoperoxidase was obtained as a lyophilized powder (Sigma, St. Louis, MO, USA) or as a suspension in 3.5 *M* $(NH_4)_2SO_4$ (Boehringer, Mannheim, West Germany). β-Lactoglobulin was bought from Sigma as a lyophilized powder.

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Organisms

Bacterial strains used were *Escherichia coli* 904 and *E. coli* NCD 2328(9703). They were maintained on slopes of nutrient agar and grown in sterile milk over night at 37°C before inoculation.

Determination of the antibacterial effect

Bacterial growth in the presence of the LP system was determined in two ways: bacterial acid production as indicated by the decrease in pH, and bacterial number by a plate-counting method.

The sample solutions contained various combinations of heated and unheated skim milk, or 4 ml of heated skim milk mixed with 6 ml of 0.01 *M* potassium phosphate buffer (pH = 6.83), or a solution of 0.5 g β-lactoglobulin in the same buffer used directly for the bacterial growth test.

The LP system consisted of 0.25 m*M* SCN^-, 0.25 m*M* H_2O_2, and when LP was added, 10 μg of enzyme/ml. To inhibit the LP system, cysteine was added to a final concentration of 1.0 m*M*. Bacterial acid production was measured as the pH decrease after incubation at 37°C for 6 h. The viable count was determined by the standard plate-counting method. Ten ml of the sample was inoculated with *E. coli* (2%, or ca. 10^7 cfu/ml), and incubated for 2 h at 37°C. Serial dilutions were made in 25% Ringer solutions and suitable dilutions were surface-plated on Violet Red Bile Agar (Oxoid) and incubated at 37°C for 18 h. The experiments were repeated three times, and mean values are presented in tables and diagrams.

Gel filtration chromatography

Skim milk was fractionated by gel filtration chromatography performed in a 25 × 400-mm glass column packed with Sephadex G-200 (Pharmacia, Uppsala, Sweden) and equilibrated with salt solution simulating milk ultrafiltrate (SMUF) following Jenness and Koops (*6*). Absorbance at 280 and 320 nm was measured on a Pye Unicam double beam spectrophotometer. Protein was determined according to Lowry et al. (*7*).

Lactoperoxidase assay

2,2'-Azino-di(3-ethylbenzthiazoline-6- sulfonic acid) (ABTS) was used as a chromogenic substrate for lactoperoxidase (*15*). Three ml of 1 m*M* ABTS in 0.1 *M* sodium acetate buffer (pH = 4.4) was mixed with 0.1 ml sample and 0.1 ml of 3.2 m*M* H_2O_2 and the absorbance was measured at 412 nm for 3 min. The activity was determined from the slope of the initial linear portion of the curve (molar absorption coefficient 32400 M^{-1} cm^{-1}). One unit of enzyme will form 1 μmole of oxidized ABTS per min at 20°C.

Sulfhydryl group determination

The concentration of sulfhydryl groups was determined by using Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB) according to the modified method by Beveridge et al. (*2*). One-tenth to 0.5 ml of sample was mixed with 2.5 ml of 0.1 *M* tris-glycine buffer (pH = 8.0). Sulfhydryl groups accessible after urea-treatment were measured with buffer containing 8 *M* urea. Twenty μl of reagent solution (4 mf of DTNB/ml in tris-glycine buffer) was added and the absorbance was measured at 412 nm (molar absorption coefficient 13600 M^{-1} cm^{-1}). The initial absorbances of both the sample and the reagent were subtracted.

RESULTS AND DISCUSSION

Two different strains of *E. coli*, 904 and 2328, were used in this work. *E. coli* 904 turned out to be the more resistant against the LP-system, and it was predominantly used when acid-production was measured as a pH difference. The plate-counting method was the most suitable method for a susceptible strain, such as *E. coli* 2328.

The effect of heated milk on the antibacterial properties of the LP-system is shown in Fig. 1. Skim milk was heated to 80°C for different times and inoculated with *E. coli* 904 (10^7cfu/ml). The pH difference after 6 h indicated the bacterial growth. There is no information however, about the extent of the bactericidal effect, because a small change in pH only implicates a bacteriostatic situation. The decrease in pH followed the decrease in lactoperoxidase activity. No enzyme was added in this experiment. The SH-concentration was measured with and without urea present. After 10 min of heat treatment, the -SH with urea decreased, but the content of directly ac-

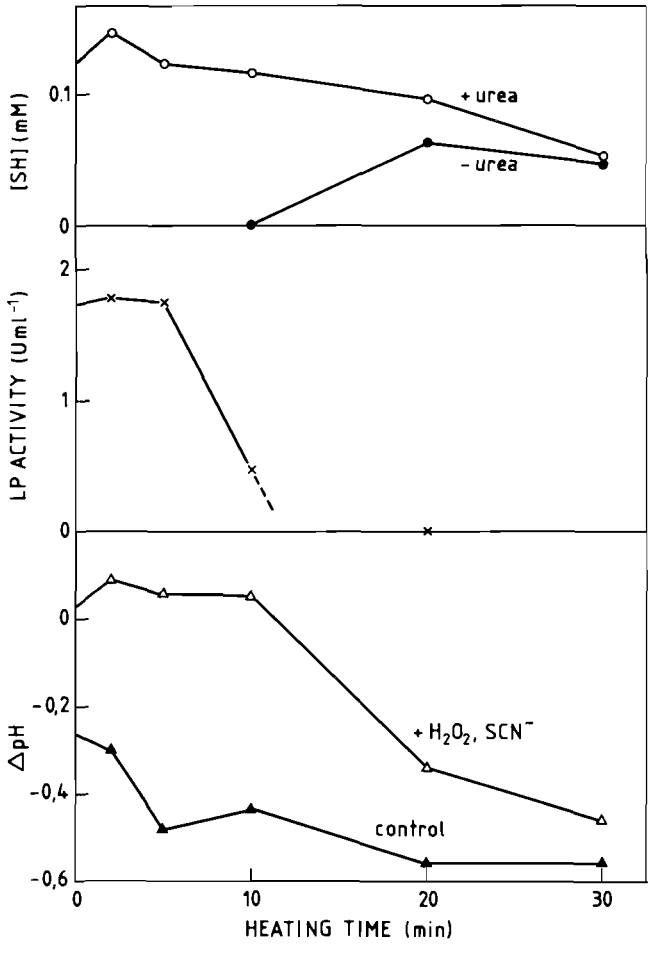

Figure 1. *The effect of heating skim milk on the LP-system, dependent on intrinsic LP activity. The skim milk was heated at 80°C and samples were taken at various times, supplied with* SCN^- *and* H_2O_2 *and inoculated with* E. coli *904 at 37°C for 6 h (control with no* SCN^-, H_2O_2*).* -○-○-, *[SH] with urea;* -●-●-, *[SH] without urea;* -x-x-, *LP activity,* -▲-▲-, *ΔpH (control);* -Δ-Δ-, *ΔpH (+*H_2O_2, SCN^-*).*

cessible SH-groups started to increase. After 20 min, both concentrations decreased and also the difference between them, implicating that all SH-groups were exposed to the reagent DTNB.

Heat denaturation of lactoperoxidase is also shown in Fig. 1. The effect of the LP-system disappears as the LP-activity decreases. Beside this decline in intrinsic LP-activity, heated milk also inhibited the LP–system with added enzyme. This inhibitory effect is shown in Table 1 (see also Fig. 3 below); skim milk was heated to 80°C for 0, 15 and 45 min. If the LP–system was added, the pH measurement indicated that there was no bacterial acid production in unheated milk, but in heated milk the LP-system was inhibited and the bacteria produced acid. If heated and unheated milk were mixed, the LP–system was functioning gradually more efficiently dependent on the amount of untreated milk (Table 2). Obviously there was something in unheated milk, that counteracted the inhibition caused by heated milk.

In Table 3, the effect of heated milk on the enzymic activity of LP is shown. When heated milk was added to the enzyme assay, no inhibition of the ABTS oxidation could be seen. The inhibitory effect of heated milk seems not to be dependent upon any change in the enzyme activity itself.

In a separate experiment low-heat skim milk powder was reconstituted in distilled water (10% w/v) and one part was heated to 85°C for 30 min. If unheated skim milk was added to a concentration of 20% in heated milk, the LP-system worked (ΔpH = -0.09). This means that an enzyme content of 2-3 μg/ml is sufficient for a bacteriostatic effect if unheated milk is present. But when 10 μg of pure lactoperoxidase/ml was added to the heated milk with no unheated milk present, the LP-system did not work (ΔpH = -0.90). Evidently, the products of thiocyanate oxidation react with something in heated milk, which is deactivated by unheated milk.

The effect of the LP-system is supposed to be connected with the oxidation of sulfhydryl groups in proteins essential to the metabolism of the attacked organism (*1,18*). Other sulfhydryl-containing substances may therefore inhibit the antibacterial effect of the LP-system (*18*).

TABLE 1. *Inhibition of the LP-system by heated skim milk (85°C, 30 min)*[a].

Sample	log N/N_o (2 h)	ΔpH (6 h)
Growth controls		
Skim milk	0.62	−0.32
Heated skim milk	2.31	−0.65
+ H_2O_2, SCN^-		
Skim milk	−3.31	−0.15
Heated skim milk	1.71	−0.58
+ LP, H_2O_2, SCN^-		
Skim milk	−2.99	±0
Heated skim milk	1.06	−0.25

[a]Concentration of added LP: ca 20 μg/ml; substrate concentrations: 0.25 m*M* H_2O_2, 0.25 m*M* SCN^-. Inoculation with *E. coli* 2328 (10^7 cfu/ml). N_o and N represent the bacterial count after 0 and 2 h of incubation, respectively.

TABLE 2. *The inhibitory effect of mixtures of heated and unheated skim milk (85°C, 30 min) on the antibacterial properties of the LP-system on* E. coli *2328*[a].

Sample	log N/N_o (2 h)	ΔpH (6 h)
Growth controls		
heated skim milk (%)		
0	0.62	−0.07
20	1.37	−0.24
40	1.39	−0.45
60	1.20	−0.60
+ LP-system		
heated skim milk (%)		
0	−3.44	−0.05
20	−1.68	−0.06
40	−0.45	−0.09
60	0.20	−0.11

[a]Enzyme (ca 10 μg/ml), 0.25 m*M* H_2O_2, 0.25 m*M* SCN^-. For details of sample preparation, see Materials and Methods. Inoculation with *E. coli* 2328 (10^7 cfu/ml).

TABLE 3. *Effect of heated skim milk on LP activity*[a].

Sample	Activity (U/ml)
Enzyme	0.19
Heated skim milk	0
Enz. + heated skim milk (10 μl)	0.22
Enz. + heated skim milk (20 μl)	0.22
Enz. + heated skim milk (30 μl)	0.22

[a]The enzyme sample contained ca 6 μg of lactoperoxidase/ml, and 10, 20 and 30 μl of skim milk heated at 80°C for 30 min was added to 3 ml of the 1 m*M* ABTS-solution in the lactoperoxidase assay (see Materials and Methods).

Under the heating conditions presented in Fig. 1 and 3 the SH-groups accessible without urea increased, but the SH-groups accessible with urea decreased. Heating of the skim milk causes an exposure of SH-groups, as well as an oxidation of them (*16*). So heated skim milk inhibited the antibacterial effect of the LP-system and showed an increased exposure of SH-groups.

Of the major milk proteins, β-lactoglobulin is the only one with an exposed SH-group. The effect of heat treatment on milk SH-groups should therefore be mimicked by a β-lactoglobulin solution. That heated β-lactoglobulin solutions were inhibitory to the LP-system was shown by inoculating β-lactoglobulin solutions, heated to 85°C for different periods, with *E. coli* 2328 (Fig. 2).

Skim milk showed a similar effect (Fig. 3). Four ml of skim milk heated to 85°C for different times was mixed with 6 ml of 0.01 *M* potassium phosphate buffer (pH = 6.8), and these mixtures were inoculated with *E. coli* 2328 (10^7 cfu/ml). (The enzyme concentration was ca. 10 μg/ml, as for the β-lactoglobulin experiments). The amount of urea-accessible SH-groups decreased, but the exposed SH-groups increased, so that nearly 85% of all SH-groups were accessible for DTNB. But inhibition of the system took place even with milk heated to 85°C only for 15 min, actually before any measurable increase in amount of exposed SH-groups appeared. This could

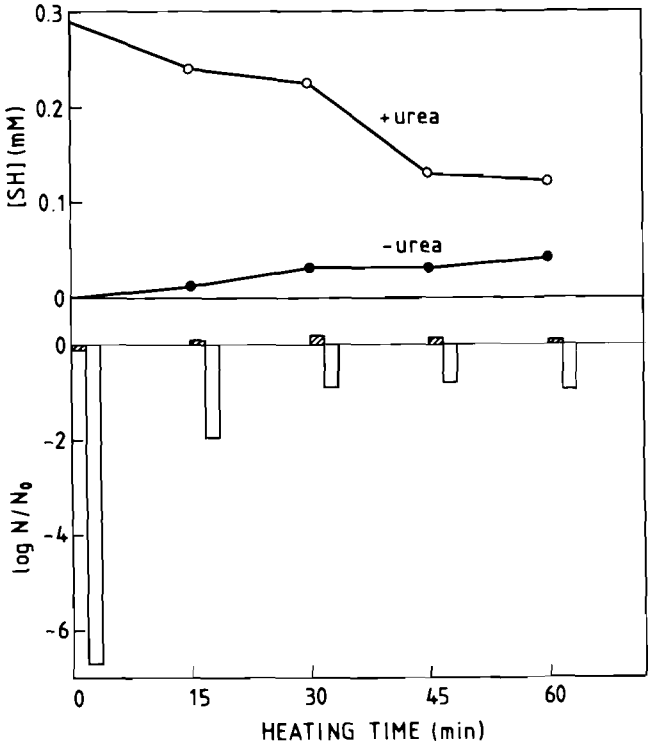

Figure 2. *The effect of heat treatment of* β-*lactoglobulin on its inhibition of the LP-system. A solution of* β-*lactoglobulin (0.5 g/100 ml) was heated at 85°C. Two samples of 3 ml were taken at each time and diluted with 7 ml 0.01* M *potassium phosphate buffer (pH=6.8), one of which was used as a reference for bacterial growth, the other was supplied with LP (10* μg *ml*$^{-1}$*), SCN*$^-$ *(0.25 m*M*) and* H_2O_2 *(0.25 m*M*). After incubation with* E. coli *2328 (10*^{7} *cfu/ml) at 37°C for 2 h the bacterial number and SH-group concentration were determined.*-o-o-, *[SH] with urea;* -●-●-, *[SH] without urea. Staple diagram:* ▨, *control;* □□, *with LP–system.*

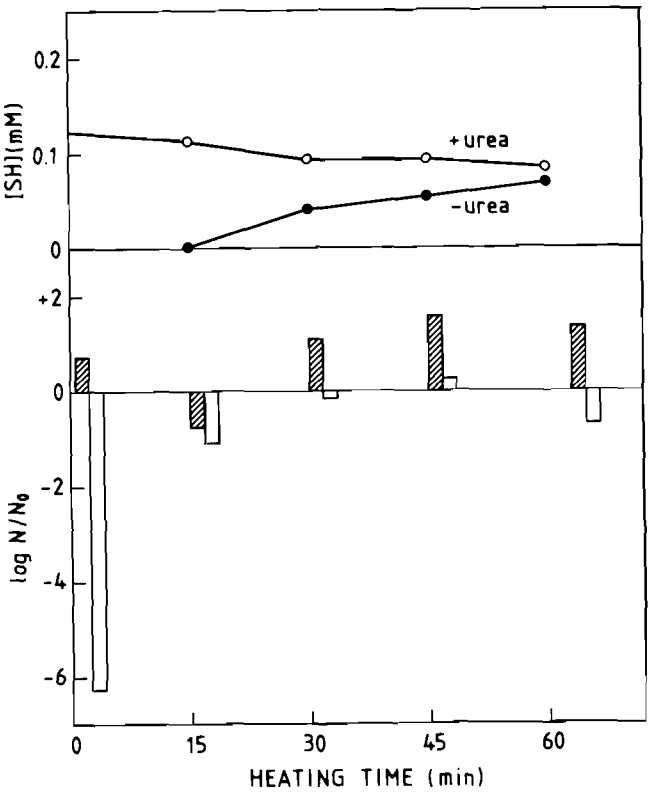

Figure 3. *The effect of heated skim milk on the LP-system with added LP. Skim milk was heated at 85°C. The samples were treated and analysed in the same way as for Fig. 2.* -o-o-, *[SH] with urea;* -●-●-, *[SH] without urea. Staple diagram:* ▨, *control;* □□, *with LP-system.*

be due to a difference in penetrability between DTNB and $OSCN^-$. It could also be caused by a reducing effect associated with other changes in the heated milk rather than the increase in exposed SH-groups (see below), or finally some quite different type of inhibitory mechanism might take place in heated milk.

To find out which fraction was inhibitory, casein and whey fractions were prepared from heat-treated skim milk and a reconstituted heat treated skim milk powder (with no LP-activity). Casein was precipitated by acidifying reconstituted skim milk to pH=4.6. After centrifugation, the pH of whey was adjusted to 6.8 with NaOH. Measurements of the ΔpH after inoculation with *E. coli* 904 for 6 h indicated that whey did not inhibit the LP system (ΔpH-values for control, control+LP-system and control+LP-system+cysteine were -0.13, -0.07 and -0.52, respectively).

In chromatography on Sephadex G-200 (Fig. 4a, b), the differences between native and heated skim milk could be seen. After heat treatment there was a decrease in one of the whey peaks and an increase in the casein micelle fraction (*5,8*). This is thought to be the result of β-lactoglobulin (*9*) associating with the casein micelles and perhaps also aggregation of other whey components. When skim milk was heated to 85°C for 45 min, the main whey protein peak decreased substantially (Fig. 4b). There was no increase in the low molecular weight peak after heat treatment that would indicate thermal disintegration of the proteins to peptides. Because of the dilution of the sample caused by the chromatography, it was difficult to measure the sulfhydryl content in eluted fractions, but for fresh milk a small peak could be detected in the β-lactoglobulin region. When heated milk was analysed, the only detectable sulfhydryl content was associated with the casein micelle peak.

The whey and casein fractions indicated in Fig. 4 were dialysed, lyophilized and dissolved in 0.1 *M* K-phosphate buffer (pH=7). The casein fraction from high heat-treated skim milk turned out to decrease the effect of the LP-system when enzyme and substrate were added (Table 4). The corresponding fractions from unheated skim milk did not affect the LP-system. When pasteurized milk is sonicated, the casein fraction exhibits antioxidant activity not connected with the sulfhydryl content (*17*).

As a result of this study, it is clear that the only detectable inhibition in heat-treated skim milk is connected with the casein fraction. This might be due to either denaturation of the milk proteins or aggregation between the casein micelles and serum constitutents caused by the relatively severe heat treatment necessary to produce this inhibitory effect.

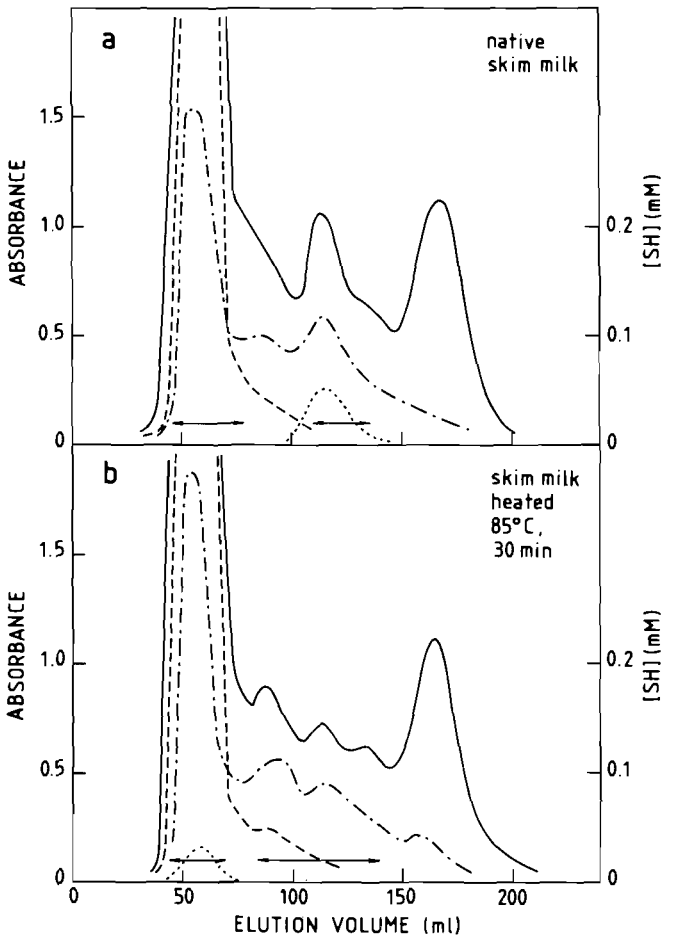

Figure 4. Chromatography of skim milk on Sephadex G-200. Buffer: SMUF. Column: 25×400 mm. Sample volume: 6 ml. Flow rate: 12 ml h^{-1}. Fraction time: 20 min. (a) *Native skim milk;* (b) *skim milk heated at 85°C for 30 min;* ———, A_{280}; -----, A_{320}; -·-·, A_{750} *(protein according to Lowry et al., 1951);* ·····, *SH with urea. Fractions pooled for test of the LP-system (Table 4) indicated by lines at the bottom of the figure.*

TABLE 4. *Identification of the inhibitory fraction in skim milk after chromatography on Sephadex G-200*[a].

Sample	Experimental log N/N_o (4 h)	Control log N/N_o (4 h)
Fresh skim milk		
Casein	−5.89	−1.15
Whey	−6.90	−0.63
Low mol. weight fraction	−5.80	−0.58
Heat treated skim milk		
Casein	−3.46	−0.59
Whey	−6.43	−0.56
Low mol. weight fraction	−6.27	−0.35

[a]Pooled fractions (shown in Fig. 4) were dialysed against 0.1 *M* potassium phosphate buffer (pH = 6.7), lyophilized and dissolved in buffer to the original concentration. After inoculation with *E. coli* 2328 (10^8 cfu/ml) and addition of LP, NaSCN and H_2O_2, samples were incubated at 37°C at 4 h. (The low mol. weight fractions represent the last peak to be eluted in Fig. 4 a and b).

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