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Antibacterial Activity of the Lactoperoxidase System in Milk Against Pseudomonads and Other Gram-Negative Bacteria

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Products of thiocyanate oxidation by lactoperoxidase inhibit gram-positive bacteria that produce peroxide. We found these products to be bactericidal for such gram-negative bacteria as *Pseudomonas* species and *Escherichia coli*, provided peroxide is supplied exogenously by glucose oxidase and glucose. By the use of immobilized glucose oxidase the bactericidal agent was shown to be dialyzable, destroyed by heat and counteracted, or destroyed by reducing agents. Because the system is active against a number of gram-negative bacteria isolated from milk, it may possibly be exploited to increase the keeping quality of raw milk.

It has been known for a long time that bovine milk can inhibit the growth of certain bacteria. Hansen (2) suggested in 1924 that this could partly be due to an oxidizing enzyme. Since then several antibacterial systems have been described (for review see reference 13). Wright and Tramer (18) found a high correlation between antistreptococcal activity of milk and its lactoperoxidase content. These findings were confirmed by Portmann and Auclair (12), who used partially purified lactoperoxidase. The involvement of hydrogen peroxide was demonstrated by Jago and Morrison (4) and Reiter et al. (15) showed that the inhibitory system, consisting of lactoperoxidase and peroxide, required thiocyanate or another oxidizable substrate (14). Further studies (3, 9, 10, 16) have contributed to the understanding of this inhibitory system in milk, but the exact mechanism is still obscure. Since the end products of SCN^- oxidation were found to be inactive, it has been suggested that the antibacterial action is due to intermediate oxidation products such as $S(CN)_2$ (10) or HO_2SCN, HO_3SCN (3).

Of the components of the system, lactoperoxidase and thiocynate occur naturally at concentrations in milk which are adequate for inhibition. The concentration of lactoperoxidase is about 30 mg/liter (11) and varies according to lactation, the highest values being present 3 to 4 days post partum, after which there is a gradual decline (5). The thiocyanate concentration varies considerably because it depends on the feeding regime of the cows. According to Virtanen (19) it is derived by enzymatic hydrolysis of glucosides in brassicaceae and raphani and detoxification of cyanide. Levels reported varied between 0.017 to 0.26 mM (1, 7, 19).

The third component, hydrogen peroxide, is not known to occur naturally in bacteria-free milk. However, catalase-negative bacteria, e.g., lactic acid bacteria, generate peroxide metabolically and can then be inhibited by the completed system in milk. Alternatively, an exogenous supply of H_2O_2 can be used to complete the system (6, 9). The lactoperoxidase-thiocyanate-peroxide system (LP-SCN^--H_2O_2) has hitherto only been found to be inhibitory for certain gram-positive organisms (3, 6, 9, 10, 12, 14-16, 18) and a strain of *Escherichia coli* (ATCC 11775) (6). The effect of the LP-SCN^--H_2O_2 system against gram-negative organisms of medical interest, e.g., enteropathogenic serotypes of *E. coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*, will be reported elsewhere (B. Reiter, V. Marshall, L. Björck, and C-G. Rosén, submitted for publication).

The purpose of the present paper was to study the antibacterial effect of this system against milk spoilage organisms, such as *Pseudomonas fluorescens*, and other gram-negative rods isolated from raw milk when an enzymatic generation of peroxide was used.

MATERIALS AND METHODS

Cultures. *P. fluorescens* EF 1998 was obtained from the Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden. Also used were *E. coli* NCTC 9703 (0 111) and a number of gram-negative bacteria isolated from raw milk and identified as being *Pseudomonas alicaligenes-P. fluorescens* spp. (aR 3, aR 10, aR 13, aR 19, aR 21, aR

24, aR 31, and aR 38), isolated in the Bacteriology Department, National Institute for Research in Dairying, Shinfield, Reading, England. All cultures were maintained on agar slopes (plate count agar, Difco) and, when required, transferred to tryptic soy broth (Difco).

Inhibitory test. Milk, whey, or a synthetic medium was used for the tests. Milk was obtained aseptically by cannulation from bacteria-free udders and stored at −20 C. The thiocyanate content was adjusted by addition of NaSCN as stated in the individual experiments. Whey was prepared by adding 0.4 ml of commercial rennet per liter to fresh, pooled milk. The clotted milk was centrifuged at about 2,000 × *g*, and the resulting whey was sterilized by filtering through a 0.2-μm filter. The synthetic medium had the following composition: glucose, 3.0 g; NH_4Cl, 0.5 g; $Na_2HOP_4 \cdot H_2O$, 1.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 4.7 g; tap water, 1.0 liter; pH adjusted to 6.7.

The milk, whey, or medium was inoculated with an overnight culture to a concentration of 10^6 to 10^7 organisms ml and incubated at 30 C. At zero hour and at certain intervals thereafter, samples were withdrawn, and serial dilutions were made in quarter-strength Ringer solution. Suitable dilutions were surface plated in duplicate on predried agar (8) (plate count agar, Difco) and incubated at 30 C for 48 h or at 37 C for 24 h for *E. coli*.

Chemicals. Chemicals of analytical grade were used. Glucose oxidase (EC 1.1.3.4) grade I (210 U/ml) and grade III (20 U/ml) were purchased from Boehringer Mannheim Corp., Mannheim, West Germany; lactoperoxidase and horseradish peroxidase (EC 1.11.1.7) were from Sigma Chemical Co., St. Louis, Mo.

Immobilization of glucose oxidase. Glucose oxidase (grade III) was immobilized by using CNBr-activated Sepharose 4B purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The coupling was performed according to the manufacturer's directions. To remove non-covalently bound enzyme the gel was first washed several times on a glass filter alternately with 0.1 M borate buffer (pH 8.5) containing 1 M NaCl and 0.1 M acetate buffer (pH 4.0) containing 1 M NaCl.

The gel was thereafter packed in a small column (26 mm in diameter) and washed with the same buffers for 24 h each at a flow rate of 2 ml/min and finally with a 0.5 M phosphate buffer, pH 6.7, for 12 h.

Thiocyanate determination. Thiocyanate was determined using the Fe^{3+} reagent of Sörbo (17). The milk proteins were precipitated with 20% (wt/vol) trichloroacetic acid (1). The colored complex was measured at 460 nm, and the concentration of SCN^- was calculated from a standard curve.

Hydrogen peroxide determination. Hydrogen peroxide was determined enzymically with horseradish peroxidase and *o*-tolidine. To 3.0 ml of a 0.1 M phosphate buffer, pH 7.0, containing 0.1 mg of *o*-tolidine and 5 μg of horseradish peroxidase per ml, 250 μl of the sample was added. The absorbance at 436 nm was read after 10 min of incubation at room temperature (20 to 22 C). The concentration of H_2O_2 was calculated from a standard curve.

RESULTS

Effect of the LP-SCN^--H_2O_2 system against P. fluorescens EF 1998 in raw milk. Raw milk, containing 0.085 mM SCN^-, was supplemented to contain 0.3% glucose and 0.1 U of glucose oxidase (grade I) per ml and in certain cases thiocyanate to give a final concentration of 0.17 mM SCN^-. The milk then was inoculated with *P. fluorescens* EF 1998 and incubated at 30 C. In the absence of glucose and glucose oxidase (control) the organisms multiplied (Fig. 1), but failed to do so in the presence of the enzyme system and 0.085 mM SCN^- up to 10 h; after that time multiplication took place at the same rate as the control. At the higher concentration of SCN (0.17 mM), the number of organisms declined rapidly in the first 4 h of incubation and remained nearly static in the next 6 h, but increased from 10 h onwards at approximately the same rate as the control.

Relationship between H_2O_2 production, SCN^- oxidation, and bactericidal effect. The synthetic medium containing 0.3% glucose, 0.05 U of glucose oxidase (grade I) per ml, 0.26 mM SCN^-, and 1.5 U of lactoperoxidase per ml was inoculated with *P. fluorescens* EF 1998 and incubated at 30 C. The concentration of H_2O_2 and SCN^- (Fig. 2) and bacterial numbers (Fig. 3) were assayed at regular intervals. The thiocyanate concentration decreased to 0.02 mM in about 3 h. Little peroxide could be detected while the thiocyanate concentration was above 0.02 mM but thereafter rapidly accumu-

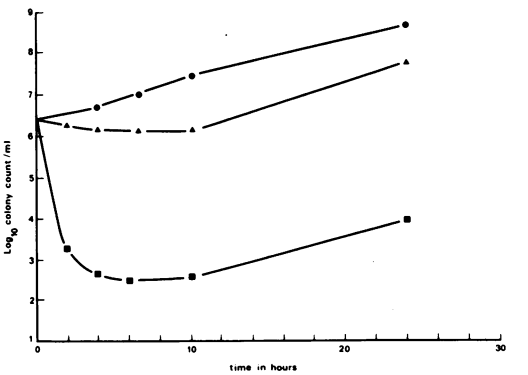

FIG. 1. *P. fluorescens EF 1998 in raw milk containing 0.085 mM SCN^-. Supplements: ●, none; ▲, 0.1 U of glucose oxidase per ml and 0.3% glucose; ■, 0.1 U of glucose oxidase per ml, 0.3% glucose, and 0.085 mM SCN^-.*

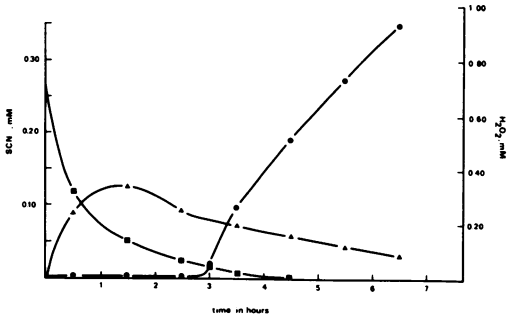

FIG. 2. *Relationship between H_2O_2 production and SCN^- oxidation in a glucose-salts medium (0.3% glucose, pH 6.7) supplemented with 0.05 U of glucose oxidase per ml, 0.26 mM SCN^-, and 1.5 U of lactoperoxidase per ml and inoculated with P fluorescens EF 1998. Symbols: ●, H_2O_2 concentration; ■, SCN^- concentration; ▲, H_2O_2 concentration in the absence of lactoperoxidase and SCN^-.*

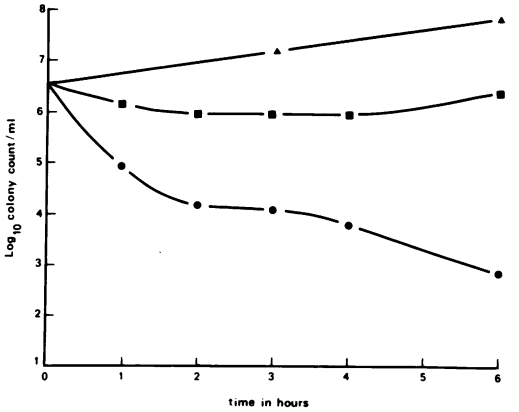

FIG. 3. *P. fluorescens EF 1998 in a glucose-salts medium (0.3% glucose, pH 6.7) at 30 C. Supplements: ▲ none; ■, 0.05 U of glucose oxidase per ml; ●, 0.05 U of glucose oxidase per ml, 0.26 mM SCN^-, and 1.5 U of lactoperoxidase per ml.*

lated in the medium. When lactoperoxidase and SCN^- were omitted the accumulation of peroxide started immediately, but probably due to bacterial catalase production the build up ceased after about 1.5 h and thereafter the concentration decreased continuously. When the LP-SCN^--H_2O_2 system was complete, the number of organisms was substantially reduced at the time when thiocyanate was oxidized (Fig. 3; cf. Fig. 2), and when peroxide started to accumulate in the medium a further decrease in number took place. The addition of glucose oxidase only (without LP and SCN^-) (Fig. 2) initially gave a small decrease in bacterial numbers compared with the control, but after a few hours the bacteria started to multiply.

Effect of the LP-SCN^--H_2O_2 system against some gram-negative organisms from raw milk. Whey containing 0.26 mM SCN^-, 0.3% glucose, and 0.05 U of glucose oxidase (grade I) per ml was inoculated with 2×10^6 to 4×10^6 organisms/ml and incubated at 30 C. The number of organisms was assayed at 0 and 4 h. Table 1 shows that every isolate tested was sensitive to the system, at least 91% of the inoculum being killed after 4 h of incubation.

Physical separation of peroxide production and antibacterial system. Thus far, soluble glucose oxidase had been used, and it was shown that peroxide accumulated when SCN^- was depleted (Fig. 2). It was therefore thought that by using immobilized glucose oxidase the accumulation of peroxide would be avoided and thus the antibacterial effect due to the oxidation of thiocyanate would be separated from the effect of peroxide. Whey containing 0.3% glucose and 0.26 mM SCN^- was passed once through a column packed with 7 g of Sepharose 4B to which glucose oxidase had been coupled. With a flow rate of 2 ml/min at room temperature (20 to 22 C), the SCN^- concentration decreased to 0.20 mM during the passage and was thereafter constant during the experiments. No peroxide could be detected. The eluate was kept at 10 C until enough whey had been collected (usually about 50 ml) for the experiment. It was then sterilized by passing through a 0.45-μm membrane filter (Millipore Corp.), inoculated with the bacteria to be used, and incubated at 30 C.

Whey treated in this way was bactericidal for *P. fluorescens* EF 1998 and *E. coli* 9703 (Fig. 4). The *E. coli* strain was killed more efficiently, albeit slower, than the *P. fluorescens*. The antibacterial effect lasted for about 4 h, after which the remaining viable bacteria started to multiply. The bactericidal effect could be completely reversed by heating the whey at 60 C for 15 min and by the addition of 0.5 mM sodium

TABLE 1. *Effect of the LP-SCN^--H_2O_2 system against some gram-negative rods isolated from raw milk at 30 C*

Isolates	% killed after 4 h
aR 3	91.2
aR 10	>99.98
aR 13	96.9
aR 19	>99.98
aR 21	99.1
aR 24	>99.98
aR 31	91.8
aR 38	98.3

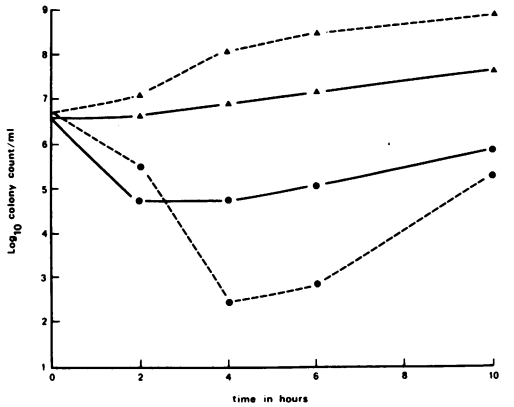

FIG. 4 *Effect of whey containing 0.3% glucose and 0.26 mM SCN⁻ treated with immobilized glucose oxidase against E. coli 9703 (-----) and P. fluorescens EF 1998 (——) at 30 C. Symbols: ▲, untreated control; ●, treated with immobilized glucose oxidase.*

hydrosulfite or 1 mM cysteine hydrochloride (Table 2) prior to the inoculation.

To study the stability of the bactericidal effect, whey treated with immobilized glucose oxidase as described earlier was stored at 30 C for 1 and 4 h or at 5 C for 1, 4, and 24 h before being inoculated with *P. fluorescens* EF 1998 and incubated at 30 C. The bactericidal effect decreased rapidly when stored at 30 C and had disappeared after 4 h (Table 3). At 5 C (Table 3) the decrease was much slower, and the whey still had a bactericidal effect after 4 h, which had, however, disappeared after 24 h of storage. To investigate whether the bactericidal effect was due to a dialyzable factor, 10 ml of whey (supplemented with 0.3% glucose and 0.26 mM SCN^-) was dialyzed at 2 C for 2 h against 90 ml of whey treated with immobilized glucose oxidase. As a control, 10 ml of supplemented whey was dialyzed against 90 ml of untreated whey. The dialyzed whey was then filter sterilized (0.45 μm; Millipore Corp.), inoculated with *E. coli* 9703, and incubated at 30 C. Bacterial numbers were assayed at 0 and 4 h. The whey became bactericidal against *E. coli* 9703 after being dialyzed against the whey treated with immobilized glucose oxidase, whereas the bacteria grew normally in the whey dialyzed against the untreated whey (Table 4).

TABLE 2. *Influence of heat or reducing agents on the bactericidal effect of whey containing 0.3% glucose and 0.26 mM SCN^- and treated with immobilized glucose oxidase against P. fluorescens EF 1998 at 30 C*

Supplements	Colony counts/ml × 10^{-6}	
	0 h	2 h
None	3.5	0.06
Sodium hydrosulfite, 0.5 mM	3.4	3.6
Cysteine hydrochloride, 1.0 mM	3.5	3.6
Heating 60 C, 15 min	3.5	3.7

TABLE 3. *Influence of storage at 30 and 5 C on the bactericidal effect of whey containing 0.3% glucose and 0.26 mM SCN^- and treated with immobilized glucose oxidase against P. fluorescens EF 1998 at 30 C*

Storage temp (C)	Storage time (h)	Colony counts/ml × 10^{-6}		% killed after 2 h
		0 h	2 h	
30	0	3.8	0.05	98.5
	1	4.9	2.0	59.0
	4	4.9	4.9	0
5	0	3.8	0.05	98.5
	1	4.2	0.15	96.5
	4	4.2	1.4	67.0
	24	4.9	5.6	0

TABLE 4. *E. coli 9703 in whey dialyzed at 2 C for 2 h against whey treated with immobilized glucose oxidase*[a]

E. coli 9703	Colony counts/ml × 10^{-6}	
	0 h	4 h
Control (dialyzed against untreated whey)	2.5	35.0
Whey (dialyzed against whey treated with immobilized glucose oxidase	2.8	0.002

[a] All whey contained 0.3% glucose and 0.26 mM SCN^-.

DISCUSSION

The present work shows that the LP-SCN^--H_2O_2 is bactericidal for several gram-negative bacteria. The bactericidal effect is closely related to the oxidation of thiocyanate. Both in a synthetic medium and in milk the decrease in bacterial numbers closely follows the decrease of thiocyanate, and in milk the remaining bacteria start to multiply when thiocyanate is depleted. The large increase in bactericidal effect in milk noted when the thiocyanate concentration is increased from 0.085 to 0.17 mM is however, not understood.

When 0.3% glucose and 0.05 to 0.1 U of

glucose oxidase per ml are used as a peroxide-generating system, little peroxide can be detected as long as enough thiocyanate is present, but when the SCN^- has been oxidized sufficient peroxide accumulates to exert an antibacterial effect. This may be avoided by using immobilized glucose oxidase, and the antibacterial effect of the lactoperoxidase system may be studied without the interference of significant amounts of peroxide.

Whey, supplemented with 0.3% glucose and 0.26 mM SCN^-, becomes bactericidal for *P. fluorescens* EF 1998 and *E. coli* 9703 when treated with immobilized glucose oxidase. The bactericidal effect was less pronounced than it was with soluble glucose oxidase, probably due to the fact that less thiocyanate was oxidized, (i.e., less antibacterial agent was formed). The bactericidal effect of the treated whey decreases rapidly at 30 C and disappears after 4 h of storage; consequently, the remaining bacteria start to multiply after about 4 h when incubated at 30 C. At 5 C the bactericidal effect decreases more slowly, and the whey still showed bactericidal activity after 4 h. Even at this temperature it has, however, disappeared after 24 h. This is not in agreement with the observations of Hogg and Jago (3), who found in their study of the inhibition of streptococci by the lactoperoxidase system that the antibacterial substance was stable for several days at low temperature. However, since they used a synthetic medium and removed lactoperoxidase from the solution, it is not possible to make an exact comparison.

The bactericidal effect can be abolished by the treatments previously shown by Tramer (18) and Hogg and Jago (3) to reverse the bacteriostatic action of the lactoperoxidase system against certain streptococci, i.e., heating at 60 C, the addition of reducing agents such as sodium hydrosulfite and cysteine, and storage at 5 and 30 C.

Oram and Reiter (10) and Hogg and Jago (3) have concluded that the inhibitory agent for streptococci is a low-molecular-weight compound, but this has only recently been confirmed. (H. Hoogendoorn, Ph.D. thesis, Technische Hogeschool, Delft, The Netherlands, 1974). Earlier attempts have failed, probably due to the fact that the antibacterial action has been studied at 30 C, at which the agent is unstable. However, by separating the dialysis and the bacterial test, the dialysis can be carried out at a low temperature, which makes it possible to extend the dialysis time for a fairly long period without extensive decomposition of the antibacterial substance. Using this technique we could directly demonstrate that the bactericidal effect is due to a low-molecular-weight substance.

The results reported here show that the bactericidal effect of the lactoperoxidase-catalyzed oxidation of thiocyanate is due to a low-molecular-weight substance that is easily destroyed by heat and reducing agents, such as cysteine and sodium hydrosulfite. Both the identity of this agent and its mode of action is still obscure, but the properties demonstrated in this study indicate that it is identical to the agent previously shown to be bacteriostatic to streptococci (3, 9, 10, 18).

The fact that the LP-SCN^--H_2O_2 system is bactericidal for several gram-negative bacteria, as shown in this paper and to be reported elsewhere, and bacteriostatic for several gram-positive organisms (3, 9, 13, 15, 16) occurring in milk suggests that it may be used to improve the bacterial quality of raw milk. Preliminary experiments have shown that by use of an enzymatic generation of peroxide to complete the lactoperoxidase system it is possible to extend the storage period of raw milk containing a mixed flora considerably without deterioration due to bacterial growth.

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