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FREE-RADICAL MECHANISM OF THE ANTIMICROBIAL ACTION OF XANTHINE OXIDASE AND LACTOPEROXIDASE

D. V. Al'perovich, É. V. Kesel'man, A. P. Shepelev, and L. N. Chernavskaya

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Human and animal milk contains a group of antimicrobial factors of specific (immune) and nonspecific nature, intended to protect the newly born offspring against bacteria and viral infections [4, 10]. Among the nonspecific antimicrobial factors of milk an important role is played by the enzymes xanthine oxidase (XO) and lactoperoxidase (LP), which are involved in the generation and utilization of active forms of oxygen.

The mechanism of action of LP is based on oxidation of the thiocyanate ion, which is constantly present in milk, with the aid of hydrogen peroxide, with the formation of bactericidal intermediates [7, 10]. XO in milk catalyzes oxidation of xanthine and other purines to uric acid, with the formation of the superoxide anion-radical $O_2^{\cdot-}$. The antimicrobial action of LP [3, 5, 8, 9] and XO [1, 11] on different species of microorganisms has been demonstrated. The possibility of including XO and LP in a single common system, on functional grounds, and of their interaction with antioxidant systems of the pathogenic agents of intestinal infections has not previously been considered.

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The aim of this investigation was to study the mechanism of interaction of XO and LP in milk with the antioxidative enzyme of microorganisms – superoxide dismutase (SOD), and to study the antimicrobial action of a system of these enzymes.

EXPERIMENTAL METHOD

Model Experiment. Chromatographically and electrophoretically pure enzymes were used: XO from butter, LP from bovine colostrum, and erythrocytic SOD was produced by Rostov Bioengineering Center. The model system in vitro consisted of incubation medium (0.1 M Na-phosphate buffer, pH 7.4), to which the following components were added consecutively (here and subsequently, final concentrations are shown): XO ($1.3 \cdot 10^{-4}$ IU/ml, SOD (3.3 IU/ml), xanthine or hypoxanthine (0.1 mM). The mixture was incubated for 2 min at 30°C to generate hydrogen peroxide in the reactions:

$$O_2 \xrightarrow{\text{XO, xanthine}} O_2^- \xrightarrow{\text{SOD}} H_2O_2,$$

after which LP ($0.9 \cdot 10^{-2}$ IU/ml) and its substrate o-tolidine (0.1 mg/ml) were added to the system. The velocity of the peroxidase reaction was determined spectrophotometrically on an SF-46 instrument at 630 nm, measuring accumulation of the colored oxidation product of o-tolidine [2].

Determination of SOD Activity of *Escherichia coli.* Activity of periplasmatic SOD in intact *E. coli* 0-111 cells was determined by the chemiluminescence quenching method (a modified method in [9]) on the PKhL-1 chemiluminometer. The following components were added consecutively to the chemiluminometer cuvette: 0.05 M Na-phosphate buffer, pH 7.8, EDTA ($1 \cdot 10^{-4}$ M), hypoxanthine ($5 \cdot 10^{-5}$ M), and luminol (1 mM). The reaction was triggered by the addition of XO ($9 \cdot 10^{-3}$ IU/ml) and the flash of chemiluminescence was recorded. A solution of a culture of *E. coli* cells ($5 \cdot 10^5$ cells/ml) was added to the cuvette 30 sec after addition of XO and the fall of the chemiluminescence level was recorded. SOD activity of the cells was determined by comparison with quenching of chemiluminescence in the control after the addition of SOD in known concentrations.

The Antimicrobial Action of XO and LP on *E. coli.* The antimicrobial action of the enzymes XO and LP, and of a system of both enzymes (XO + LP) on a 24-h culture of the conditionally pathogenic strain *E. coli* 0-111, at a final concentration of the culture of $1 \cdot 10^3$ cells/ml and with physiological concentrations of the enzymes and their substrates, namely hypoxanthine (XO), KCNS, and H_2O_2 (LP). To the enzyme system (or control), with a volume of 1 ml, 1 ml of a suspension of the *E. coli* culture was added and the mixture incubated at 37°C. The incubation mixture was seeded in a volume of 0.1 ml on Petri dishes with Endo's agar after an interval of 30 min or 1, 3, 6, and 24 h. The number of viable cells was counted as the number of colonies growing on the dishes after 24 h. The antimicrobial effect was calculated as the ratio, in per cent, of the number of surviving cells to the control for each incubation time. In the XO + LP experiment, H_2O_2 was not added to the reaction mixture, on the grounds that it would be produced in the reaction of utilization of O_2^- generated by XO with the aid of periplasmatic SOD of the bacterial cells.

EXPERIMENTAL RESULTS

The model experiment in vitro revealed accumulation of the product of the lactoperoxidase reaction despite initial absence of its substrate (exogenous H_2O_2) in the medium. It is evident that H_2O_2 is produced endogenously as a result of a sequence of XO and SOD reactions from oxygen dissolved in the incubation medium (Fig. 1a). LP, in a final concentration of $0.9 \cdot 10^{-2}$ IU/ml, revealed activity in the experiments corresponding to $(0.65 \pm 0.03) \cdot 10^{-2}$ IU/ml, i.e., 72 ± 3% of the theoretically possible level. This demonstrates the high degree of coupling of the three enzymic reactions and the potential possibility of such coupling taking place in biological systems.

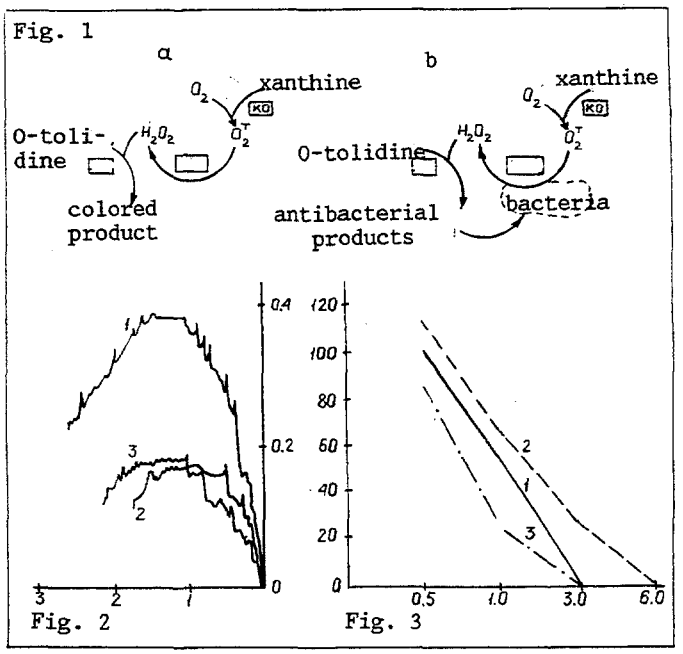

Fig. 1. Mechanism of action of antimicrobial system XO–SOD–LP: a) plan of experiment in vitro, b) plan of action of system in vitro on living objects.

Fig. 2. Extinction of chemiluminescence flash induced by O_2^- generation by XO-system. 1) Control, 2) extinction by enzyme SOD, 3) extinction by culture of *E. coli*. Abscissa, time (in min); ordinate, chemiluminescence (in light-sum units).

Fig. 3. Antimicrobial action of enzymes XO (1), LP (2), and a combination of both (XO + LP; 3) on E. coli 0-111. Abscissa, time (in h); ordinate, number of viable cells (in percent of control).

The chemiluminescence quenching method showed that intact *E. coli* cells in a final concentration of $5 \cdot 10^5$ cells/ml quenches by 50% the chemiluminescent flash induced by production of O_2^- by the xanthine oxidase–hypoxanthine system. This corresponds to SOD activity of 200 IU/ml (final concentration) determined in the control experiments with the pure enzyme (Fig. 2). Thus the presence of significant SOD activity in intact *E. coli* cells was proved. Gregory and co-workers [6] showed that *E. coli* possesses not only cytoplasmic, but also periplasmatic SOD, which is intended for antioxidant protection against exogenous O_2^-. It is evidently this SOD that formed the SOD-activity of intact *E. coli* cells in our experiments.

A study of the action of XO, LP, and a combination of both on the conventional pathogenic strain *E. coli* 0-111 showed (Fig. 3) that all three enzyme systems have an antimicrobial effect. A significant decrease in the number of surviving cells was observed immediately after the first hour of incubation of the culture with the enzyme systems at 37°C. Thus the number of cells surviving after incubation for 1 h with the LP-system was 67% compared with the control (a culture incubated without the enzyme system). With the same duration of incubation with the XO-system and the combined XO + LP enzyme system the number of viable cells was only 56 and 23% respectively.

After incubation of the culture of *E. coli* with the LP-system for 3 h only 25% of viable cells still remained, but the XO-system and the combined XO + LP system had a total bactericidal effect, corresponding to the time interval between feeds of the newly born offspring. A total bactericidal effect in the case of incubation of the culture with the LP-system was found after incubation for 6 h at 37°C.

If the enzyme systems are arranged in order of diminishing power of their antimicrobial action, the sequence is as follows: XO + LP ← XO ← LP.

The XO + LP system had maximal antimicrobial effect, significantly stronger than the effect of the XO-system, despite the initial absence of substrate for LP, namely H_2O_2, in the incubation medium. In our opinion this is proof of the role of LP in the antimicrobial action of the XO + LP system and, consequently, of the appearance of endogenous H_2O_2 in the incubation medium. The most likely supplier of this H_2O_2 is the periplasmatic SOD of *E. coli*, which converts $O_2^{\div}$ (a product of the XO reaction) into H_2O_2 (substrate for LP) (Fig. 1b).

Thus the model of the mechanism of action of nonspecific antimicrobial factors of milk (XO and LP) was confirmed experimentally in a model system in vitro and on a conditionally pathogenic strain of *E. coli*, the agent of intestinal infections. In our opinion, the enzymes XO and LP, which are constantly entering the newborn organism with milk, combine into an antimicrobial system in response to the entry of SOD-positive agents of intestinal infections into the gastrointestinal tract, and they utilize the antioxidant defenses of bacteria in order to produce bactericidal products. The bacterial SOD in this case behaves as a system-forming factor.

It seems likely that milk possesses its own antimicrobial system with a free-radical mechanism of action, similar to the universal biological protective mechanism involving NADPH-oxidase and myeloperoxidase.

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