

## Research Note

# Effect of the Lactoperoxidase System against Three Major Causal Agents of Disease in Mangoes

DOAN DUY LE NGUYEN,<sup>1</sup> MARIE-NOELLE DUCAMP,<sup>2\*</sup> MANUEL DORNIER,<sup>2,3</sup> DIDIER MONTET,<sup>2</sup> AND GÉRARD LOISEAU<sup>2,3</sup>

<sup>1</sup>Cantho University, Faculty of Agriculture, Food Technology Department, Cantho, Vietnam; <sup>2</sup>Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Tropical Fruits Department (FLHOR), and Advanced Methods for Innovation in Science Department (AMIS), avenue Agropolis, TA 50/PS4, 340398 Montpellier cedex 5, France; and <sup>3</sup>Ecole Nationale Supérieure des Industries Alimentaires (ENSIA), Tropical Food Department (SIARC), 1101 avenue Agropolis, CS 24501, 34093 Montpellier cedex 5, France

MS 04-434: Received 14 September 2004/Accepted 8 January 2005

### ABSTRACT

The antibacterial activity of the lactoperoxidase system (LPS) on the growth of *Xanthomonas campestris*, the causal agent of bacterial black spot in mangoes, *Botryodiplodia theobromae*, the causal agent of stem-end rot disease in mangoes, and *Colletotrichum gloeosporioides*, the causal agent of anthracnose disease in mangoes, was determined during culture at 30°C and at several pH values (4.5, 5.5, and 6.5). When the results of using the LPS were compared with those from control cultures without the LPS reagents, the growth of the three microorganisms was totally inhibited in all of the conditions tested. Viability tests enumerating cultivable cells of *X. campestris* showed that the LPS had a bactericidal effect, whatever the pH value. This effect is faster at pH 5.5, corroborating the results reported in the literature (optimal pH for the LPS efficiency). Further, we proved that hydrogen peroxide alone had little inhibition effect on the growth of the microorganisms studied. This compound is essentially used to convert thiocyanate into hypothiocyanate during the lactoperoxidase reaction. The potential of the LPS for the postharvest treatment of the fruits for controlling microbial diseases was thus demonstrated. Nevertheless, further studies are needed on fresh fruits before envisaging any application.

The lactoperoxidase system (LPS) is a natural system that was first discovered in raw milk. The antibacterial activity of the LPS is well documented. The hypothiocyanate ion (OSCN<sup>-</sup>), the major intermediary product of LPS reactions, oxidizes essential sulfhydryl groups in proteins, altering cellular system functions that result in the inhibition of growth or the death of microorganisms. The hypothiocyanate ion is formed by the reaction of hydrogen peroxide on thiocyanate catalyzed by lactoperoxidase (4, 14). The LPS mechanism can be schematically described in three steps: (i) hydrogen peroxide generation (H<sub>2</sub>O<sub>2</sub> may be also added directly); (ii) enzymatic reaction wherein the conversion of thiocyanate to hypothiocyanate (OSCN<sup>-</sup>) is catalyzed by lactoperoxidase using hydrogen peroxide as a cosubstrate; and (iii) inactivation of the microorganisms by hypothiocyanate.

Hypothiocyanate has a bacteriostatic and bactericidal effect on a wide range of microorganisms. The LPS has a bactericidal effect against *Escherichia coli* in a semisynthetic medium (1). It was found to have both bacteriostatic and bactericidal activities against several strains of *Salmonella* Typhimurium (15). The authors believed that the bactericidal activity was clearly dependent on the permeability of the bacterial cell envelope. The LPS was investigated for

its activity against *Salmonella* in vivo and in vitro (17). In acidified raw milk, in which the LPS was supplemented with an exogenous supply of hydrogen peroxide, the number of *Salmonella* cells decreased rapidly. The activity of the LPS on *Listeria monocytogenes* strains in raw milkfat at different refrigeration temperatures was also studied (5), and the system exhibited a bactericidal activity at 4 and 8°C. Another study showed that the LPS inhibited the growth of *L. monocytogenes* and *Staphylococcus aureus* at 35 and 37°C, respectively (8). The bactericidal effect of the LPS against strains of *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry has also been demonstrated (3).

The possible role of peroxidase systems in defense against filamentous fungi and yeast has been investigated by studying the antifungal activity of the myeloperoxidase–hydrogen peroxide–thiocyanate system. Strong inhibition of *Saccharomyces* spp., *Geotrichum* spp., *Rhodotorula* spp., and *Aspergillus fumigatus* was reported (9).

The goal of our investigation was to examine the potential of the LPS for the inhibition of some of the microorganisms that cause postharvest diseases on mango, such as *Xanthomonas campestris*, the causal agent of bacterial black spot in mangoes (11), *Botryodiplodia theobromae*, the causal agent of stem-end rot disease in mangoes (12), and *Colletotrichum gloeosporioides*, the causal agent of anthracnose disease in mangoes (6, 7, 10).

\* Author for correspondence. Tel: +33/4-67615557; Fax: +33/4-67614433; E-mail: ducamp@cirad.fr.

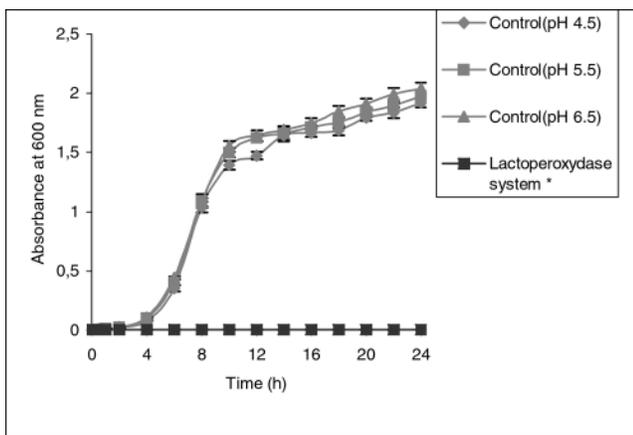


FIGURE 1. Effect of the LPS on the growth of *Xanthomonas campestris* in a liquid culture medium at 30°C and pH 4.5, 5.5, and 6.5 (turbidity of the culture medium evaluated using absorbance at 600 nm). Vertical bars indicate the standard deviation of three replicates. \*, same curves at the three pH values tested.

## MATERIALS AND METHODS

**The lactoperoxidase system.** Lactoperoxidase (400 U/mg) was donated by Bio serae (Bram, France). Sodium percarbonate ( $\text{CH}_3\text{Na}_2\text{O}_6$ ), used as a peroxide generator, and sodium thiocyanate ( $\text{NaSCN}$ ) were purchased from Aldrich (Milwaukee, Wis.).

**Microorganisms and inoculum preparation.** *X. campestris* and *B. theobromae* were isolated from contaminated mangoes from Réunion Island. *C. gloeosporioides* was isolated from contaminated mangoes in Guadeloupe. All of the culture media were purchased from BioRad (Hercules, Calif.).

An overnight preculture at 28°C in a medium consisting of 5 g of yeast extract per liter, 5 g of glucose per liter, 0.5 g of  $(\text{NH}_4)_2\text{SO}_4$  per liter, and 0.2 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per liter was used as the inoculum for *X. campestris* ( $7 \times 10^9$  CFU/ml). For *B. theobromae* and *C. gloeosporioides*, spore suspensions were chosen as the inoculum. These fungi were grown at 28°C for at least 14 days on potato dextrose agar plates, and the spores were harvested by suspension in 9 ml of 0.85% NaCl solution supplemented with Tween 80 at a concentration of  $100 \mu\text{l} \cdot \text{liter}^{-1}$ . Suspensions with  $7 \times 10^5$  spores per ml and  $9 \times 10^5$  spores per ml for *B. theobromae* and *C. gloeosporioides*, respectively, were then obtained (enumeration was conducted with a Malassez hemacytometer). They were stored at 4°C in tubes until they were used during the same day.

**Cultures and evaluation of the antimicrobial activity of the LPS.** Cultures were maintained in 250-ml Erlenmeyer flasks with 25 ml of culture media (same medium as for preculture for *X. campestris* and potato dextrose broth for the fungi). After adjusting the pH with a pH meter to 5.5, 6.5, or 7.5 using phosphate buffer (0.2 M  $\text{NaH}_2\text{O}_4 \cdot \text{H}_2\text{O} / \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ), the flasks were sealed with cotton wads and aluminum foil and then sterilized at 120°C for 15 min. For the inoculation,  $100 \mu\text{l}$  of the preculture of *X. campestris* and 0.75 ml of the spore suspensions of the fungi were used. To evaluate the antimicrobial activity of the LPS, the LPS reagents were added aseptically after filtration through a sterile filter with a pore diameter of  $0.45 \mu\text{m}$  (lactoperoxidase at  $25 \text{ mg} \cdot \text{liter}^{-1}$  of final concentration, sodium thiocyanate at  $100 \text{ mg} \cdot \text{liter}^{-1}$ , and sodium percarbonate at  $50 \text{ mg} \cdot \text{liter}^{-1}$ ). The results were compared in all the cases with a control culture using the same medium at the same pH but without the LPS reagents. Im-

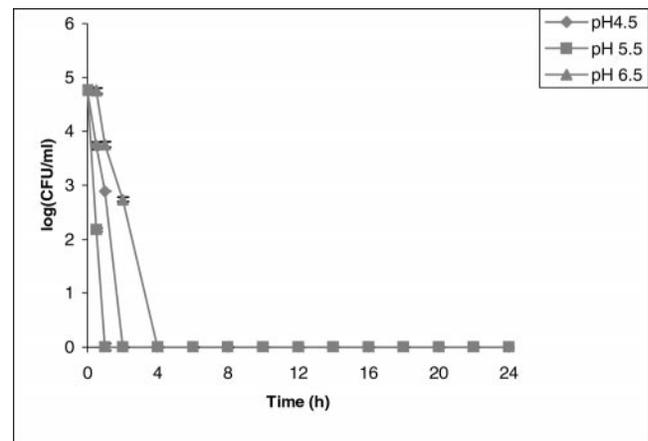


FIGURE 2. Effect of the LPS on the number of viable cells of *Xanthomonas campestris* in a liquid culture medium at 30°C and pH 4.5, 5.5, and 6.5 (enumeration on plate count agar after incubation at 28°C for 24 h). Vertical bars indicate the standard deviation of three replicates.

mediately after the inoculation and addition of the LPS reagents for the test culture, the flasks were placed on a rotary shaker set at 180 rpm and 30°C. Samples were collected after 30 min, 1 h, and 2 h and then every 2 h until 24 h. Each test was repeated three times to evaluate the standard deviation.

The development of *X. campestris* was studied by measuring the turbidity of the medium. The absorbance of the samples was measured with a double-beam spectrophotometer (Unikon C33, Kontron Instrument, Zurich, Switzerland) at 600 nm. To determine the viability of *X. campestris*, an enumeration of viable cells was performed in triplicate by drop plating on plate count agar and subsequently incubating at 28°C. Development of the fungi was determined by the increase in biomass. In that case, the contents of the cultures in Erlenmeyer flasks were filtered on  $0.45 \mu\text{m}$  of dried membranes. After drying at 105°C for 24 h, they were weighed until constant mass ( $\pm 0.5$  mg).

## RESULTS AND DISCUSSION

**Antimicrobial activity against *X. campestris*.** At each of the three pH values tested, the effect of the LPS was very significant (Fig. 1). The absorbance of the culture treated with the LPS stayed constant, while that of the controls increased rapidly. These results demonstrate the antimicrobial effect of the LPS on *X. campestris*, because it clearly inhibited the growth of this bacteria. During these tests, the effect of the pH on the LPS efficiency was not evident.

To better evaluate the effect of the LPS on the viability of *X. campestris*, complementary trials were carried out by enumerating viable cells on plate count agar (28°C, 24 h). At the three pH values tested, the number of cultivable cells decreased strongly during the first 4 h (Fig. 2). Under these conditions, the effect of the LPS appeared bactericidal. The inhibition efficiency depended on the pH of the culture medium. It was maximal at pH 5.5. At this pH, there were no more viable cells within 1 h of treatment. This result corroborated the results reported in the literature, because it corresponded to the optimum pH of the LPS. At this pH value, it is known that SCN products exist half in the form of  $\text{OSCN}^-$  and half in the form of  $\text{HOSCN}$ . The uncharged

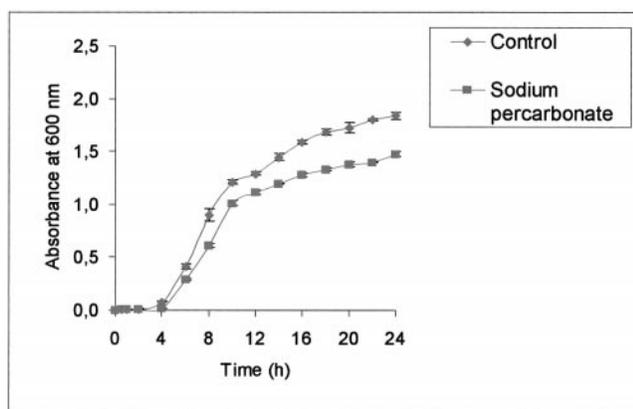


FIGURE 3. Effect of sodium percarbonate at  $100 \text{ mg}\cdot\text{liter}^{-1}$  on the growth of *Xanthomonas campestris* in a liquid culture medium at  $30^\circ\text{C}$  and pH 5.5 (turbidity of the culture medium evaluated using absorbance at 600 nm). Vertical bars indicate standard deviation of three replicates. \*, same curves for *B. theobromae* and *C. gloeosporioides*.

HOSCN appears to have a more bactericidal effect than OSCN<sup>-</sup> (16).

Until now, to our knowledge, no research has been mentioned in the literature on the effect of the LPS on *X. campestris*. Some studies, however, have shown that the LPS has a bacteriostatic effect on gram-positive bacteria and a bactericidal effect on gram-negative bacteria (1, 2). *X. campestris* is an aerobic gram-negative bacteria, and our results confirmed that the LPS can totally inhibit the growth of this bacterium.

The hydrogen peroxide released by sodium percarbonate may itself have an antimicrobial activity. To evaluate this activity within the concentrations of sodium percarbonate used in the experiments, a culture of *X. campestris* was prepared under the same conditions, and the LPS reagents were replaced by sodium percarbonate at  $100 \text{ mg}\cdot\text{liter}^{-1}$  (pH 5.5). The growth of *X. campestris* obtained with sodium percarbonate alone was lower than that of the control (Fig. 3). This means that the hydrogen peroxide released by sodium percarbonate had only a small inhibiting effect on the development of *X. campestris*. To exhibit a strong antimicrobial activity, hydrogen peroxide must be present in larger amounts. This result showed that, in the LPS, the primary purpose of hydrogen peroxide was to catalytically aid lactoperoxidase in the conversion of thiocyanate into hypothiocyanate.

**Antimicrobial activity against *B. theobromae* and *C. gloeosporioides*.** The results of the study of the influence of the LPS against *B. theobromae* and *C. gloeosporioides* at pH 5.5 are presented in Figure 4. In both experiments, there was a difference between the biomass of the culture treated with the LPS and that of the control. After 24 h of culture, the biomass of the control increased to  $0.5 \text{ g}\cdot\text{liter}^{-1}$  for *B. theobromae* and to  $0.6 \text{ g}\cdot\text{liter}^{-1}$  for *C. gloeosporioides*. On the contrary, in the culture medium treated with the LPS, the biomass remained fairly constant for both fungi. These results clearly showed that the LPS efficiently inhibited the development of these two microorganisms.

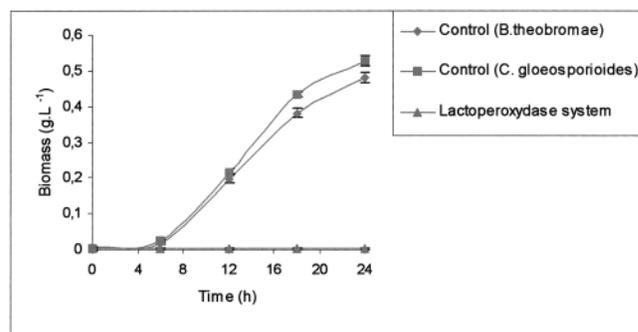


FIGURE 4. Effect of the LPS on the growth of *Botrydiplodia theobromae* and *Colletotrichum gloeosporioides* in a liquid culture medium at  $30^\circ\text{C}$  and pH 5.5. Vertical bars indicate the standard deviation of three replicates.

The literature is sparse with respect to studies that document the effect of the LPS against yeasts and fungi, except for the study of Popper and Knorr (13), who showed that the LPS had an antimicrobial effect against *Rhodotorula rubra*, *Saccharomyces cerevisiae*, *Mucor rouxii*, *Aspergillus niger*, and *Byssoschlamys fulva*.

As with *Xanthomonas*, we wanted to confirm whether the concentration of peroxide hydrogen released by sodium percarbonate had an effect on the development of *B. theobromae* and *C. gloeosporioides*. For this study, a culture of *B. theobromae* and a culture of *C. gloeosporioides* were prepared under the same conditions as before, but the LPS was replaced by sodium percarbonate alone ( $100 \text{ mg}\cdot\text{liter}^{-1}$ , pH 5.5). For both fungi, the biomass of the culture treated with sodium percarbonate increased with time (Figs. 5 and 6). Hydrogen peroxide alone did not have a significant antimicrobial effect on the two fungi, and so it was primarily used to catalytically convert thiocyanate to hypothiocyanate in the LPS reactions.

Our experiments carried out in vitro showed that the LPS had an important antimicrobial effect against *X. campestris*, *B. theobromae*, and *C. gloeosporioides*. The growth of these three microorganisms was strongly inhibited in all

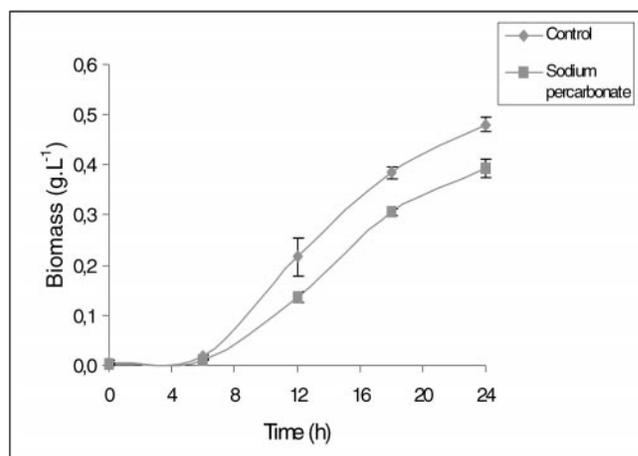


FIGURE 5. Effect of sodium percarbonate at  $100 \text{ mg}\cdot\text{liter}^{-1}$  on the growth of *Botrydiplodia theobromae* in a liquid culture medium at  $30^\circ\text{C}$  at pH 5.5. Vertical bars indicate the standard deviation of three replicates.

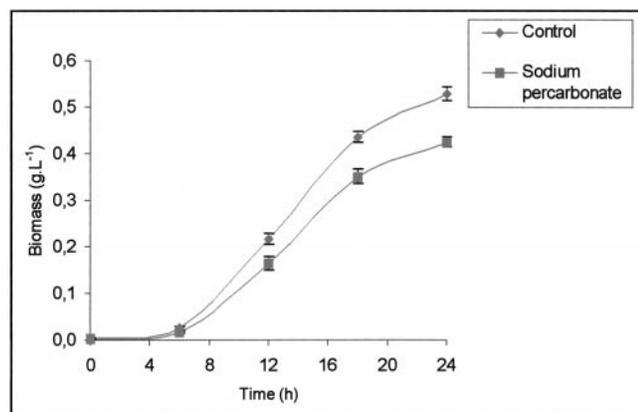


FIGURE 6. Effect of sodium percarbonate at  $100 \text{ mg-liter}^{-1}$  on the growth of *Colletotrichum gloeosporioides* in a liquid culture medium at  $30^\circ\text{C}$  at pH 5.5. Vertical bars indicate the standard deviation of three replicates.

of the tests. An optimal bactericidal effect was clearly evident at pH 5.5 on *Xanthomonas*. The antimicrobial action of the LPS is due to the hypothiocyanate generated by lactoperoxidase, because sodium percarbonate (used for peroxide generation) by itself did not have significant antimicrobial properties.

The potential of the LPS for the treatment in mangoes after harvesting was demonstrated. The LPS treatment can inhibit the growth of three pathogens that are responsible for serious diseases on this fruit, causing considerable loss during storage. Nevertheless, to envisage the LPS as an alternative to the traditional chemicals used in fruit disinfection, further studies should be carried out on mangoes in vivo.

## REFERENCES

1. Björck, L., and O. Claesson. 1980. Correlation between concentration of hypothiocyanate and antibacterial effect of the lactoperoxidase system against *Escherichia coli*. *J. Dairy Sci.* 63:919–922.
2. Björck, L., C. G. Rosen, V. M. Marshall, and B. Reiter. 1975. Antibacterial activity of the lactoperoxidase system in milk against

3. *Pseudomonas* and other gram-negative bacteria. *Appl. Microbiol.* 30:199–204.
3. Borch, E., C. Wallein, M. Rosen, and L. Björck. 1989. Antibacterial effect of the lactoperoxidase/thiocyanate/hydrogen peroxide system against strains of *Campylobacter* isolated from poultry. *J. Food Prot.* 52:638–641.
4. Ekstrand, B. 1994. Lactoperoxidase and lactoferrin, p. 15–63. In V. M. Dillon and R. G. Board (ed.), *Natural antimicrobial systems and food preservation*. CAB International, Wallingford, UK.
5. Gaya, P., M. Medina, and M. Nunez. 1991. Effect of the lactoperoxidase system on *Listeria monocytogenes* behavior in raw milk at refrigeration temperatures. *Appl. Environ. Microbiol.* 57:3355–3360.
6. Jacobs, C. J., H. J. Brodrick, H. D. Swarts, and N. J. Mulder. 1973. Control of postharvest decay of mango fruit in South Africa. *Plant Dis. Rep.* 57:173–176.
7. Johnson, G. I., and L. M. Coates. 1993. Postharvest disease of mango. *Postharvest News Inf.* 4:27N–34N.
8. Kamau, D. N., S. Doores, and K. M. Pruitt. 1990. Antibacterial activity of the lactoperoxidase system against *Listeria monocytogenes* and *Staphylococcus aureus* in milk. *J. Food Prot.* 53:1010–1014.
9. Lehrer, R. I. 1969. The antifungal of the myeloperoxidase-hydrogen peroxide-thiocyanate-system (MPO-S). *J. Bacteriol.* 99:361–365.
10. Mc Millan, R. J. 1984. Control of mango anthracnose with foliar sprays. *Proc. Fla. State Hort. Soc.* 97:344–345.
11. Nakasone, H. Y., and R. E. Paull. 1998. *Tropical fruits*. CAB International, New York.
12. Pathak, V. N., and D. N. Srivastava. 1969. Epidemiology and prevention of *Diplodia* stem end rot of mango fruit. *Phytopathology* 65:165–175.
13. Popper, L., and D. Knorr. 1997. Inactivation of yeast and filamentous fungi by the lactoperoxidase-hydrogen peroxide-thiocyanate system. *Nahrung* 41:29–33.
14. Pruitt, K. M., and B. Reiter. 1985. Biochemistry of peroxidase system: antimicrobial effect, p. 144–178. In K. M. Pruitt and J. Tenovuo (ed.), *The lactoperoxidase system: chemistry and biological significance*. Marcel Dekker, New York.
15. Purdy, M. A., J. Tenovuo, K. M. Pruitt, and W. E. White. 1983. Effect of growth phase and cell envelope structure on susceptibility of *Salmonella typhimurium* to the lactoperoxidase-thiocyanate-hydrogen peroxide system. *Infect. Immun.* 39:1187–1195.
16. Thomas, E. L., K. A. Pera, K. W. Smith, and A. K. Chwang. 1983. Inhibition of *Streptococcus mutans* by the lactoperoxidase antimicrobial system. *Infect. Immun.* 39:767–778.
17. Wray, C., and I. McLaren. 1987. A note on the effect of the lactoperoxidase systems on *Salmonellae* in vitro and in vivo. *J. Appl. Bacteriol.* 62:115–118.