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Effects of Esterified Lactoferrin and Lactoferrin on Control of Postharvest Blue Mold of Apple Fruit and Their Possible Mechanisms of Action

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ABSTRACT: The effects of esterified lactoferrin (ELF) and lactoferrin (LF) on blue mold caused by *Penicillium expansum* in apple fruit stored at 25 °C were investigated. Both ELF and LF provided an effective control and strongly inhibited spore germination and germ tube elongation of *P. expansum* in vitro. Assessment by propidium iodide staining combined with fluorescent microscopy revealed that the plasma membrane of *P. expansum* spores was damaged more seriously by ELF than by LF treatment, and the leakage of protein and sugar was higher from ELF-treated mycelia. Interestingly, ELF treatment induced a significant increase in the activities of chitinase, β-1,3-glucanase, and peroxidase in apple fruit, whereas both LF treatment and the control showed no obvious difference. These findings indicated that the effects of ELF on blue mold in apple fruit might be associated with the direct fungitoxic property against the pathogens and the elicitation of defense-related enzymes in fruit.

KEYWORDS: *esterified lactoferrin, lactoferrin, blue mold, apple fruit, physiological properties*

INTRODUCTION

Apple fruit can be infected by many fungal pathogens.[1] Among them, *Penicillium expansum* Link is one of the most destructive pathogens causing production losses during postharvest handling of the fruit.[2] Although current control of this disease still relies mainly on the use of synthetic chemical fungicides, public concern over the potential impact of fungicides on human health and the environment[3] and the development of pathogen resistance to fungicides[4] have created interest in new strategies for disease control.

Components of milk and whey, particularly lactoferrin and lactoperoxidase, have been extensively researched as antimicrobial agents for use in human medicine and food preservation.[5,6] Bettiol et al.[7] found that lactoferrin, an antimicrobial component of milk, was active in the control of various pathogens, and the inhibitory effects were also related to increased production of free radicals. Despite its high biological properties, native lactoferrin is not hydrolyzed easily by means of digestion enzymes such as pepsin and trypsin, due to disulfide bonds in the protein molecules. The poor digestibility of whey proteins is considered to be the reason for their allergenicity.[8] Therefore, modification of lactoferrin to enhance or alter their biological and functional properties may increase its applications, which can be accomplished by chemical, enzymatic, or physical techniques.[9,10]

Esterification is an important and easy tool for the modification of proteins. Esterification blocks free carboxyl groups, thus elevating the net positive charge and rendering more basic the modified protein.[11,12] In general, increasing the positive charge on the protein and peptide molecules enhances their antimicrobial and, more specifically, antibacterial effects. Amidation, which also increases the positive charges on the modified protein molecules, was found to improve the effectiveness of bovine lactoferrin (LF) against a range of Gram-positive and Gram-negative bacteria.[13,14] Moreover, the modified lactoferrin has been widely used in clinical trials against human immunodeficiency virus.[13] However, they all focused on the antimicrobial and antiviral activities of modified whey proteins in human, but the systemic esterified lactoferrin (ELF) responses and the underlying mechanism of the ELF-mediated disease resistance against plant pathogen have not been elucidated.

The objectives of this study were to investigate the effects of ELF on the control of blue mold caused by *P. expansum* in apple fruit stored at 25 °C, as well as to evaluate antifungal activity of ELF against *P. expansum* in vitro and elicitation of defense enzymes, including polyphenoloxidase, peroxidase, chitinase, and β-1,3-glucanase by ELF in fruit.

MATERIALS AND METHODS

Fruit. 'Golden Delicious' apples (*Malus* × *domestica* Borkh.) were harvested at commercial maturity. Fruit without wounds or rot were selected on the basis of uniformity of size, disinfected with 2% (v/v) sodium hypochlorite for 2 min, rinsed with tap water, and dried in air according to the method of Yao et al.[15] These fruits were used in a subsequent in vivo experiment.

Pathogen. *P. expansum* Link was isolated from infected apple fruit showing typical blue rot symptom and maintained on potato dextrose agar (PDA) (Oxoid, U.K.) at 4 °C. The single spore isolate was obtained by picking a single-spore colony 2 days after the diluted spore suspension had been spread on the PDA medium (containing the extract of 200 g of boiled potato, 20 g of glucose, and 20 g of agar in 1 L of distilled water) and then was confirmed by examining the growth features and the morphological characteristic of its conidiophore and conidium under a microscope (Olympus BH2).

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The pathogen was inoculated into apple fruit wounds and reisolated onto PDA prior to use. A spore suspension was obtained from 2-week-old cultures on PDA at 25 °C. Spores of *P. expansum* do not germinate in water; thus, their inoculum was prepared by washing 7-day-old cultures with sterile apple juice. The juice was pressed from Jonagold apples, sterilized, and used either directly or diluted 4 times with distilled water. The number of spores was calculated with an automated cell counter, Cellometer Vision (Nexcelom Bioscience, USA), and the spore concentration was adjusted with the aid of a hemocytometer prior to use.

Preparation of Esterified Lactoferrin and Lactoferrin. ELF was prepared according to the methods of Sitohy et al.[10] using 99.5% methyl alcohol, at 4 °C for 10 h. The color reaction using hydroxylamine hydrochloride was used according to the method of Bertrand-Harb et al.[16] to quantify the extent of esterification of lactoferrin. Lactoferrin was 100% esterified by this procedure, as assessed by the study of Sitohy et al.[17] Lactoferrin (95% protein, LF) was obtained from commercial sources (Sigma-Aldrich, St. Louis, MO, USA). All other chemicals used in this study were of analytical grade.

Measurements of Spore Germination and Germ Tube Elongation of *P. expansum* in Vitro. The effects of ELF and LF on spore germination and germ tube elongation of *P. expansum* were assayed according to the method of Liu et al.[18] Aliquots of 100 μL of spore suspension at 5×10^5 spores/mL were plated on Petri dishes (90 mm in diameter) with 20 mL of PDA, which contained different concentrations of ELF and LF (0, 0. 1, 0. 3, 0. 5, 0. 7, 1.0 mg/mL). Each plate was incubated at 25 °C for 12 h. Spore germination and germ tube elongation was determined in three microscopic fields using 10 × 40 ocular micrometers. Approximately 200 spores within each replicate were observed. For the individual spores' germination to be examined, a *z*-motorized microscope (Olympus, BX61, Tokyo, Japan) was used, which was operated in phase contrast mode with a 40× objective (Olympus). A high-resolution device camera (Olympus DP71) was mounted on the microscope to snap images, which were further analyzed by the image analysis software Image-Pro Plus version 6.3 (Media Cybernetics Inc., Bethesda, MD, USA). A spore was scored as germinated when the germ tube extended to at least twice the length of the spore itself.[19] Germination and germ tube elongation were assessed microscopically (×400) with an ocular micrometer at various concentrations of tested chemicals compared with control treatment. There were three replicates in each treatment, and each experiment was repeated three times.

Assay of Plasma Membrane Integrity of *P. expansum* Spores. Membrane integrity was assayed following the method of Liu et al.[18] Aliquots of 100 μL of spore suspensions of *P. expansum* (5×10^5 spores/mL) were treated with 0.5 mg/mL ELF and LF in potato dextrose broth (PDB) medium. PDB without ELF and LF served as the control. After 0, 2, 4, and 6 h of incubation (prior to germination) at 200 rpm at 25 °C,[18,20] spores were centrifuged at 8000*g* for 10 min, stained with 10 μg/mL propidium iodide (PI) for 5 min at 30 °C, and then collected by centrifugation and washed twice with 50 mM of sodium phosphate buffer (pH 7.0) to remove residual dye.[20] The spores were observed with a Zeiss Axioskop 40 microscope (Carl Zeiss, Oberkochen, Germany) equipped with an individual fluorescein rhodamine filter set (Zeiss no. 15: excitation BP 546/12 nm, emission LP 590 nm). Three fields of view from each slide (at least 200 spores) were chosen randomly, and the number of spores in bright field was defined as the total number. Membrane integrity was calculated according to the following formula:

$$\text{membrane integrity (\%)} = \left[1 - \left(\frac{\text{no. of stained spores}}{\text{no. of total spores}}\right)\right] \times 100$$

Determination of Protein and Sugar Leakage. The intracellular leakage of mycelia was determined according to the method of Liu et al.[21] *P. expansum* was grown in PDB medium at 25 °C on a rotary shaker at 100 rpm, and mycelia were harvested after 3 days of incubation. After being pooled and washed with sterile distilled water, the mycelia were resuspended in 100 mL of sterile distilled water containing ELF and LF [0 (control), 0.5 mg/mL] and incubated on a rotary shaker at 25 °C for 1, 2, 3, and 4 h. Then, the mycelia were removed by filtration through a 0.2 μm pore size membrane, and the filtrate was collected for determining the leakage of intracellular content by the assays of total soluble proteins and total soluble sugars.

Soluble protein content was determined according to the method of Bradford[22] with bovine serum albumin (Sigma-Aldrich, Shanghai, China) as a standard, and soluble sugar content was estimated by the phenol−sulfuric acid method of Dubois et al.[23] using glucose (Sigma-Aldrich, Shanghai, China) as the standard. The protein or sugar leakage was expressed as milligrams per gram of wet weight of mycelia. Each treatment contained three replicates, and the experiment was repeated three times.

Effects of ELF or LF on Blue Mold of Apple Fruit. According to the results of the effects of ELF and LF on *P. expansum* in vitro and our preliminary unreported data in vivo, 0.5, 0.7, and 1.0 mg/mL ELF and LF were chosen as the optimal concentrations to assay the effects on control blue mold of apple fruit. Three wounds (4 mm deep × 3 mm wide) were made on the equator of each fruit with a sterile nail. Twenty microliters of ELF and LF at the desired concentration (0.5, 0.7, or 1.0 mg/mL) was applied to each wound. Sterile distilled water served as a control. After fruits were air-dried for 2 h, 5 μL of *P. expansum* suspension (5×10^5 spores/mL) was inoculated into each wound. Treated fruits were placed in a covered plastic food tray, covered with a polyethylene bag, and stored at 25 °C. Disease incidence and lesion diameter of apple fruit caused by *P. expansum* were determined after 4 days. Each treatment contained three replicates with 10 fruits per replicate, and the experiment was repeated three times. Incidence represented the percentage of fruit displaying rot, whereas lesion diameter was measured only on those wounds that were infected.

Determinations of Defense-Related Enzymes in Apple Fruit. For enzyme assays, apple fruits were wounded and 20 μL of ELF or LF at 0.5 mg/mL was added to each wound as described above; fruit inoculated with sterile water in the wound served as control. After treatment, the fruits were stored at 25 °C. Flesh samples surrounding the wounds of 10 fruits were taken at 0, 1, 2, and 3 days. Each treatment contained three replicates, and the experiment was repeated three times.

Polyphenol oxidase (PPO) and peroxidase (POD) were extracted accordig to the method of Chen et al.,[24] with some modifications. Tissue samples (10 g) of each treatment were homogenized with 30 mL of 100 mM sodium phosphate buffer (pH 6.4) containing 0.2 g of polyvinyl polypyrrolidone and ground at 4 °C. The homogenate was centrifuged at 15000*g* for 30 min at 4 °C, and the supernatant was used for the enzyme assay.

PPO activity was determined by adding 0.1 mL of enzyme preparation to 3.0 mL of catechol substrate (500 mM, in 100 mM sodium phosphate buffer, pH 6.4), and the increase in absorbance at 398 nm was measured immediately.

POD activity was determined using guaiacol as substrate.[25] The reaction mixture consisted of 0.1 mL of crude extract, 2 mL of guaiacol (8 mM, in 100 mM sodium phosphate buffer, pH 6.4), incubated for 30 min at 30 °C. The increase in absorbance at 470 nm was measured after 1 mL H_2O_2 (24 mM) was added. The activity of PPO and POD were expressed as units per microgram of total protein (U mg^{-1} protein), where one unit was defined as the change in absorbance per minute.

For chitinase and β-1,3-glucanase activity assay, a fresh sample of apple fruit (10 g) was mixed with 20 mL of sodium acetate buffer (50 mM, pH 5.0) and ground thoroughly at 4 °C. The homogenate was centrifuged at 17000*g* for 30 min at 4 °C, and the supernatant was used for the enzyme assay. Protein content was determined according to the method of Bradford[22] with bovine serum albumin (Sigma-Aldrich, Shanghai, China) as the standard. The activity of β-1,3-glucanase was determined by measuring the amount of reducing sugar released from the substrate (laminarin) by using the dinitrosalicylate method,[26] and the amount of reducing sugars was measured at 500 nm using a UV-160 spectrophotometer (Shimadzu, Japan). Chitinase was measured according to the method of Wirth and Wolf[27] with carboxymethyl chitin as substrate. The absorbance of the supernatant

was measured at 550 nm. The specific activity of enzymes was expressed as units per milligram of protein, where 1 unit was defined as 1 μmol of product h^{-1} mg^{-1} protein for chitinase and β-1,3-glucanase, respectively.

Statistical Analysis. All statistical analyses were performed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was carried out to determine the effects of the treatments, and those means were compared by Duncan's multiple-range tests ($P < 0.05$). Analysis between ELF and LF treatment groups was performed with a Student's t test, and differences were considered to be significant at $P \leq 0.05$ or $P \leq 0.01$. Data presented in this paper were pooled across three independent repeated experiments.

RESULTS

Effects of ELF on Spore Germination and Germ Tube Elongation of *P. expansum*. The inhibitory effect of ELF or LF on spore germination and germ tube elongation of *P. expansum* was positively related to the concentration used. Spore germination of *P. expansum* was significantly inhibited when the concentration of ELF or LF was >0.5 mg/mL, whereas the obvious inhibition of germ tube elongation was obtained at >0.3 mg/mL (Figure 1) ($P < 0.05$). The inhibitory effect of ELF on spore germination of *P. expansum* was already significantly higher than that of LF when the concentration reached 0.7 mg/mL, whereas germ tube elongation of *P. expansum* was more obviously inhibited by ELF than by LF at 0.5 mg/mL ($P < 0.05$). This indicated that spore germination was less sensitive to ELF or LF than germ tube elongation. The half-maximal inhibitory concentration (IC_{50}) of ELF or LF on spore germination was 0.37 or 0.51 mg/mL, respectively (data not shown), confirming that the antifungal effect of ELF was stronger than that of LF.

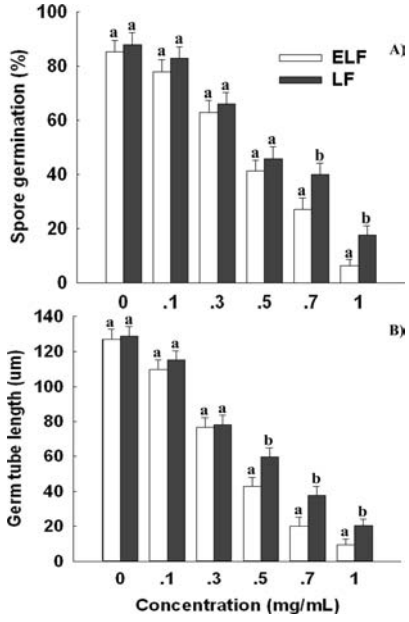

Figure 1. Effects of ELF and LF on spore germination (A) and germ tube elongation (B) of *P. expansum* 12 h after incubation at 25 °C (mean of nine replicates ± standard deviation). Columns with different letters at each concentration are significantly different according to Student's t test at $P < 0.05$.

Effects of ELF and LF on Plasma Membrane Integrity of *P. expansum*. The plasma membranes of *P. expansum* were markedly damaged by ELF and LF ($P < 0.05$) (Figure 2A). Membrane integrity (MI) of *P. expansum* spores declined with increasing incubation time in PDB containing ELF and LF (Figure 2B). However, MI of control spores was maintained at a relatively high level (>90%). That of *P. expansum* incubated in PDB containing 0.5 mg/mL ELF and LF was 56.5 and 80.4%, respectively, whereas that of control spores was 96% after 2 h of incubation.

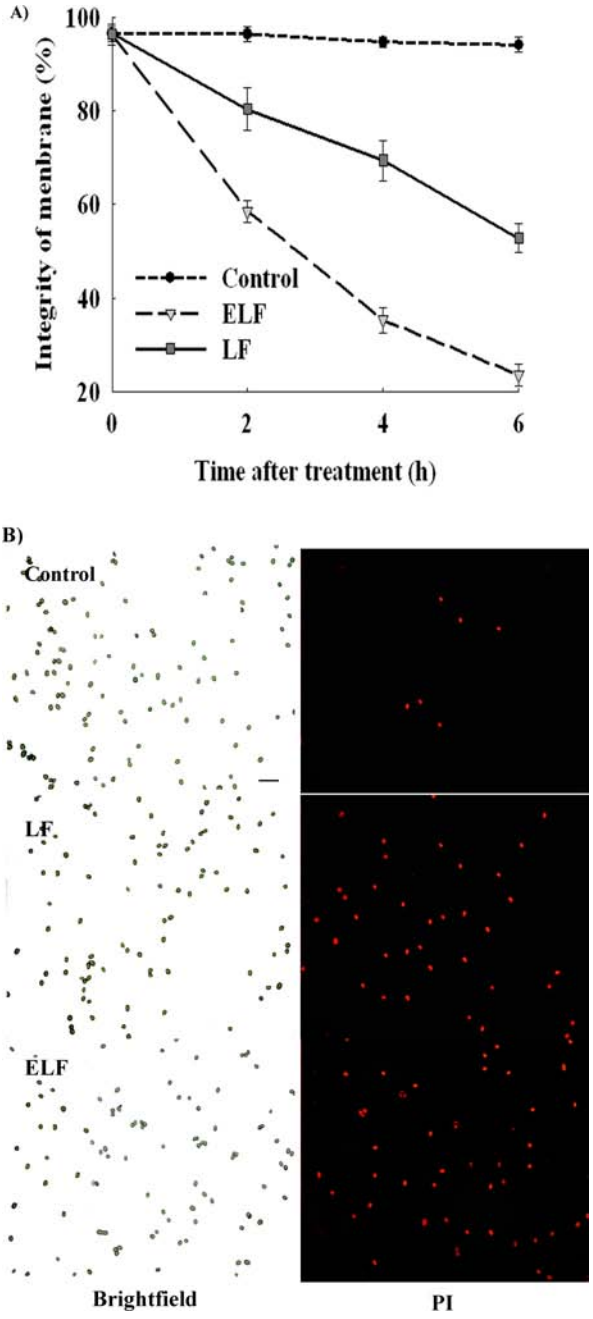

Figure 2. Effects of ELF and LF at 0.5 mg/mL on plasma membrane integrity of *P. expansum* spores cultured in PDB at 25 °C (mean of nine replicates ± standard deviation): (A) percentage of plasma membrane integrity of *P. expansum* spores; (B) microscopy images of *P. expansum* spores after 2 h of incubation. Bar represents 20 μm.

Determination of Protein and Sugar Leakage. Leakage of protein and sugar in *P. expansum* mycelia was detected markedly in the ELF and LF treatments and increased in a time-dependent manner (Figure 3) ($P < 0.05$). After the first 1 h, proteins leaked markedly in the ELF or LF treatment, and leakage increased gradually with treatment duration. Moreover,

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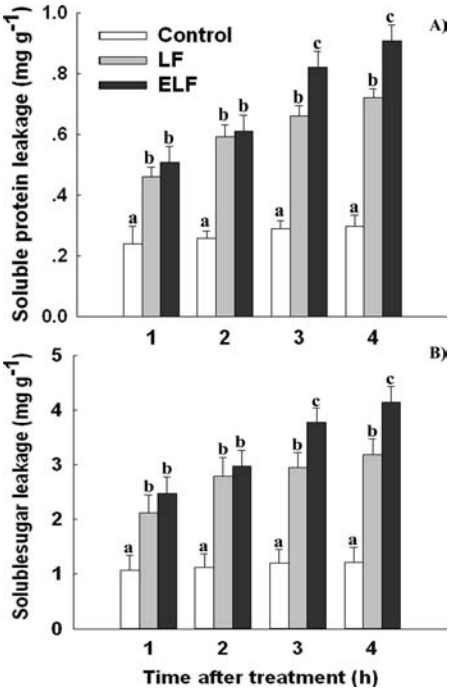

Figure 3. Effects of ELF and LF at 0.5 mg/mL on protein (A) and sugar (B) leakage of *P. expansum* mycelia cultured in sterile double-distilled water at 25 °C (mean of nine replicates ± standard deviation). Columns with different letters at each time point are significantly different according to Duncan's multiple-range test at $P < 0.05$.

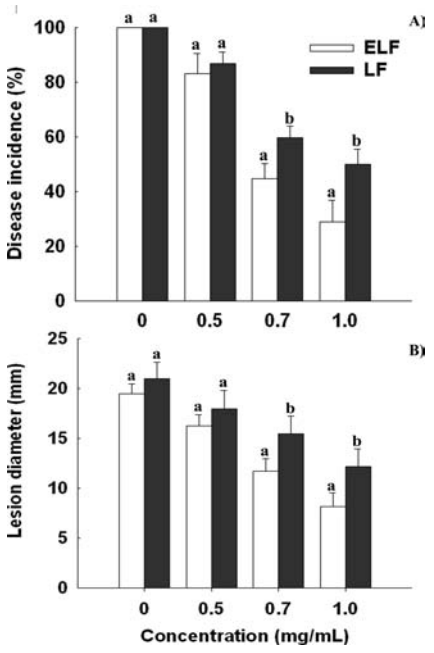

Figure 4. Effects of ELF and LF on disease incidence (A) and lesion diameter (B) of blue mold caused by *P. expansum* in apple fruit (mean of nine replicates ± standard deviation). Columns with different letters at each concentration are significantly different according to Student's *t* test at $P < 0.05$.

leakage of sugar exhibited a trend similar to that of protein. However, there was no difference obtained between the ELF and LF treatments before 2 h of incubation ($P < 0.05$). The leakage of protein and sugar stayed at very low levels in control mycelia incubated in sterilized double-distilled water without ELF or LF during the experiment.

Control Effects of ELF and LF on Blue Mold in Apple Fruit. Treatments with ELF and LF significantly reduced the disease incidence caused by *P. expansum* and inhibited the lesion expansion of blue mold in a concentration-dependent manner in apple fruit stored at 25 °C (Figure 4) ($P < 0.05$). When the disease incidence of control fruit reached 100%, the disease incidences of 0.5 mg/mL ELF or LF treated fruit were 83.2 and 86.8%, respectively. However, there was significant difference obtained in disease incidence between the ELF or LF treatment at >0.7 mg/mL (Figure 4A). Similarly, the reduced lesion diameter exhibited a similar trend (Figure 4B).

Elicitation of Defense-Related Enzyme Activities in Fruit by ELF Treatment. Chitinase and β-1,3-glucanase are two kinds of pathogenesis-related protein. Chitinase and β-1,3-glucanase activities in the flesh around wounds of control apple fruit increased with storage time, whereas ELF inoculation obviously enhanced the activities of both enzymes (Figure 5A,B). Furthermore, ELF inoculation was more effective than LF, whereas both LF-treated fruit and the control fruit showed no obvious difference. For example, the activities of chitinase and β-1,3-glucanase in ELF-treated fruit after 2 days were almost 1.6-fold those in control fruit.

Peroxidase and polyphenol oxidase are two kinds redox enzymes. POD activity in ELF-treated fruit increased continuously, reached the highest value at 2 days, and then decreased a little afterward. However, obvious difference was obtained after 2 days between the ELF and LF treatments ($P <$ 0.05), and the level in ELF-treated fruit was almost 1.5-fold that in LF treatment at 2 days (Figure 5C). PPO exhibited higher activity in ELF-treated fruit at 3 days, although decreased a little at 2 days (Figure 5D). PPO activity reached its highest value at 3 days in ELF-treated fruit, and the level was almost 1.3-fold that LF-treated fruit at the same time. Moreover, the activity of PPO was enhanced more markedly than the LF treatment and control during the experiment ($P < 0.05$).

DISCUSSION

In the present study, both ELF and LF had an effect on controlling blue mold caused by *P. expansum* in apple fruit stored at 25 °C. The activity of ELF and LF in controlling postharvest diseases of fruit is considered to involve several mechanisms.[28−31] It may involve a direct fungitoxic property and elicitation effect on fruit hosts. Bettiol[28] demonstrated that the effectiveness of whey against powdery mildew (*Podosphaera xanthii*) of cucumber and zucchini squash was dose- and concentration-dependent. Milk applied twice a week at concentrations of 10% and higher controlled powdery mildew at least as effectively as the conventional fungicides, fenarimol and benomyl. Moreover, Crisp et al.[31] found that conidia and hyphae of *Erysiphe (Uncinula) necator* were significantly damaged and collapsed by lactoferrin until 48 h after treatment. Our results indicated that ELF and LF were effective in inhibiting the growth of *P. expansum* in vitro, and the inhibitory effect was positively correlated with the concentration of ELF and LF (Figure 1). The results confirmed previous findings on the antifungal activity of LF against the fruit fungal species. However, several other studies reported that raw cow's milk did not have direct antifungal activity against *Sclerospora graminicola*,[28] *Tilletia caries*,[32] *Macrophomina phaseolina*, and *Fusarium* spp.[33] The difference in direct antifungal effect of raw cow's milk or whey ptotein (LF) may be associated with the

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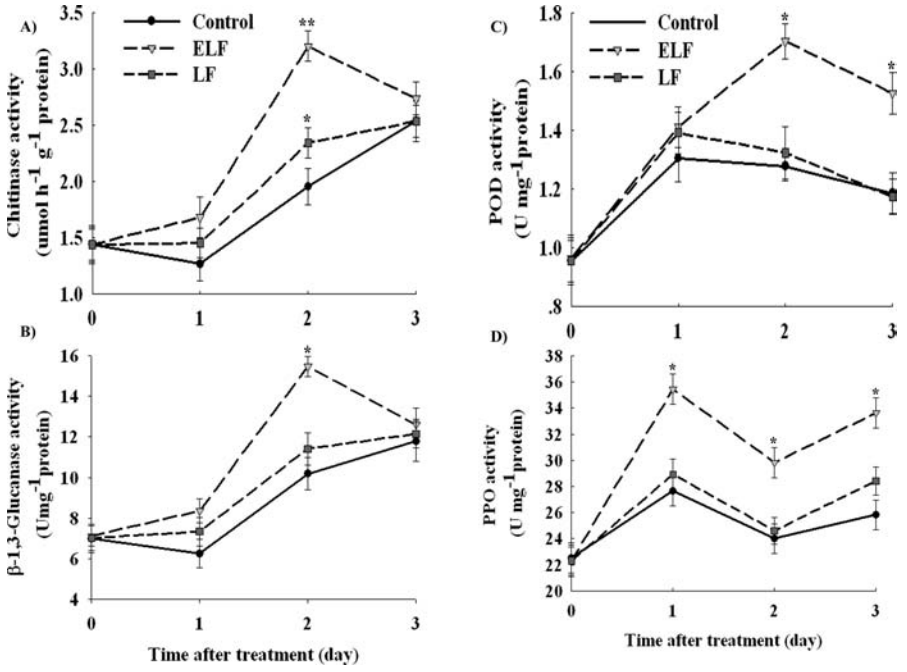

Figure 5. Effects of ELF or LF on activities of chitinase (A), β-1,3-glucanase (B), POD (C), and PPO (D) of apple fruit (mean of nine replicates ± standard deviation). ∗, $P \leq 0.05$, and ∗∗, $P \leq 0.01$, by t test denote significant differences between the control and treated apple fruits.

different sensitivities of various fungal species and the active compounds and concentration used.

Lactoferrin, an 80 kDa iron-binding glycoprotein, binds to the membranes of various bacteria and fungi, causing damage to membranes and loss of cytoplasmic fluids.[5] The antifungal mode action of lactoferrin was proposed to be due to cell wall perturbation, as confirmed by cryo-scanning electron microscopy, which revealed drastic changes to the cell wall, resulting in surface blebs, swelling, and cell collapse.[34] However, research has focused largely on Gram-negative bacteria and other species primarily involved in food spoilage and on fungi related to human health, especially *Candida* spp.[34] To investigate the mechanisms whereby ELF or LF caused fungal death, we first detected the integrity of the plasma membrane of *P. expansum*. The plasma membrane plays a crucial role in maintaining cell viability, and membrane damage could result in the loss of osmotic balance and influx of fluids and ions, as well as loss of proteins and ribonucleic acids, eventually leading to the onset of cell death.[35] Among them, proteins and sugars are two basic and functional constituents.[36] Correspondingly, compared with the control, the leakage of proteins and sugars in mycelia was more significant in the ELF and LF treatments, especially in the ELF treatment (Figure 3). Furthermore, milk exposed to the ultraviolet radiation in sunlight results in the photogeneration of superoxide anions[29] and oxygen radicals that interfere with the cell membranes of *Phytophthora infestans*.[30] It is well recognized that oxygen radicals can rapidly lead to disintegration of biological membranes, resulting in cell death. The production of free radicals when milk, whey, and whey protein have been exposed to natural light has been shown to control powdery mildew.[31] Our results with PI staining showed that the plasma membrane integrity of *P. expansum* spores clearly declined in PDB containing ELF or LF with the increase of incubation time (Figure 2). We also analyzed the reactive oxygen species (ROS) production in *P. expansum* exposed to ELF or LF using the oxidant-sensitive probe DCHF-DA.[5] As expected, more cells were stained with the dye, indicating that more ROS were produced in ELF-treated cells than in LF-treated cells. The difference may be associated with esterification increasing the net positive charge, which is thought to be related to interference with negatively charged macromolecules exposed on the fungal cell surface[29] and enhancing the production of free radicals, thereby changing the permeability of the plasma membrane.[31]

Changes in the functionality of the plasma membrane of *P. expansum* by the presence of ELF and LF were also investigated. This study focused on the changes in external minimum medium pH, membrane potential, potassium efflux, and determination of membrane phospholipids and proteins and of the H^+-ATPase enzymatic kinetic activity. These results indicated that the damage to the plasma membrane of *P. expansum* by the presence of ELF and LF alters the H^+-ATPase, affecting the physiological and metabolic functions of this phytopathogen fungus. Total H^+-ATPase activity and potassium efflux were affected by ELF, whereas there was no change in the external minimum medium pH and membrane potential compared with the control, which will be published elsewhere with the generation of H_2O_2 (under review). These results all indicated that plasma damage and cytoplasm leakage caused by ELF and LF contributed to the inhibitory effect on *P. expansum*.

In addition to antifungal activity, ELF may also have the potential for inducing defense-related responses in plant hosts. Chitinase, β-1,3-glucanase, and peroxidase have been suggested to be the crucial enzymes of plant hosts against fungal infection.[23] Chitin, as an essential component of the cell wall of many fungal pathogens, can be degraded by chitinase. β-1,3-Glucanase is one of the most fully characterized pathogenesis-related (PR) proteins, and it can act directly by degrading cell walls of pathogens or indirectly by releasing oligosaccharide and eliciting defense reactions. Both of these processes are potential defense mechanisms against fungal infection.[37] Additionally, peroxidase catalyzes the last step of lignin biosynthesis, and lignin plays an important role in the defense mechanism of plant hosts.[38] In this study, we found ELF to be more effective

at eliciting the activities of PPO, POD, chitinase, and β-1,3-glucanase than LF (Figure 5). Moreover, the activities of chitinase and β-1,3-glucanase in ELF-treated fruit at 2 days reached the maximum. These results might imply that ELF not only had a preventive action in delaying the onset of the disease at the early stage of the infection but also offered an enduring resistance or a curative effectiveness. Similarly, in our previous study, compared with LF, ELF showed a long-term protection against tobacco mosaic virus. Moreover, ELF acted as a potential elicitor in inducing chemical defense against tobacco mosaic virus in tobacco seedlings. The application of ELF significantly increased the transcript levels of defense-related enzymes (phenylalanine ammonia-lyase and lipoxygenase), stimulated an oxidation burst, and induced the accumulation of antiviral phenolic compounds.[39] Such different effectiveness may be associated with the extent of esterification and the plant species. This may confirm that esterification is a potent tool that introduces this antifungal activity into native proteins. It also showed that esterified whey proteins might also indirectly affect *P. expansum* by inducing systemic resistance.

In conclusion, the present study reported the different effects of ELF and LF on developmental inhibition of *P. expansum* and on inductive physiological and biochemical change of the host. ELF and LF treatments were effective in controlling bule mold caused by *P. expansum* in apple fruit stored at 25 °C, but ELF had better control effect than LF. Furthermore, ELF treatment had a more obvious effect on improving resistance against blue mold by enhancing activities of PPO, POD, chitinase, and β-1,3-glucanase in apple fruit. However, further study on the mechanism of ELF against fungal pathogens at the molecular level is needed. In addition, an integrated program, where ELF is synergistic with biocontrol agents or other modest fungal inhibitors or inducers of host resistance to control the postharvest disease of fruit and vegetables, should be considered.

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Author Contributions

‖Both authors made the same contributions to this paper.

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Notes

The authors declare no competing financial interest.

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