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Short Communication

Electrochemical Studies on Bactericidal Mechanism of Human Lactoferrin and Its Demand for Microenvironment

Junyi Huang[1,2,*], *Tao Liu*[2,**], *Jie Cao*[2], *Zhengjun Wu*[1], *Xian-yan Liao*[2], *Hong-sheng Song*[2,*]

[1] State Key Laboratory of Dairy Biotechnology, Bright Dairy & Food Co. Ltd., Shanghai 200072, China;
[2] Key Laboratory of Food Nutrition and Function, School of Life Sciences, Shanghai University, Shanghai, 200444, China
[**]Co-first author: the contribution for this article is equal to Junyi Huang.
[*]E-mail: jy-huang@i.shu.edu.cn, hssong@staff.shu.edu.cn

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The bactericidal mechanism of human lactoferrin (LF) was analyzed by employing bioelectrochemistry technique. The result showed that LF bound to lipopolysaccharides (LPS) and caused release of LPS, resulting in formation of ion channels in the mimic biomembrane. Furthermore, a survey of microenvironment factors for bactericidal activity of LF was conducted. In weakly alkaline condition of pH 8, LF exhibited maximally bactericidal activity. The bactericidal activity of LF was closely related to its concentration. In addition, the bactericidal activity would decrease with a higher concentration of metal ions. Divalent cations showed greater influence on the bactericidal activity than that of monovalent cations.

Keywords: Lactoferrin; Bactericidal mechanism; Bioelectrochemistry; Microenvironment

1. INTRODUCTION

Lactoferrin (LF), which is an iron binding glycoprotein (MW 80 kDa), is synthesized and secreted by mammary epithelial cells and polymorphonuclear leukocytes. Besides human and domestic animal milk, LF is also found in tears, saliva, vaginal fluids, semen, nasal and bronchial secretions, bile, gastrointestinal fluids and urine [1-3]. LF plays many important roles including broad-spectrum antibacterial, anti-inflammatory, inhibition of tumor cell growth and regulation of immune response, and so on [4-6]. LF is considered to be a novel antibacterial and anticancer drug, showing great development potential in the food, cosmetic and feed additives [7].

Among these functions, its antibacterial activity was the first to be researched [8], and then was further focused by Arnold *et al*. [9]. LF has been demonstrated to inhibit the growth of a number of

bacteria [10]. This antibacterial activity of LF is merely bacteriostatic by iron deprivation, because bacterial growth can be completely restored after iron ions are supplied again [11]. The mechanism was initially proposed to be solely bacteriostatic according to its iron-withholding ability.

However, a bactericidal activity of LF, which was distinct from its iron-withholding activity [12, 13], was reported by Arnold *et al*. [9]. Experimental results implied that direct binding of LFs to bacteria is involved [14-16]. The bactericidal activity of LF appears to be quite similar for either Gram-negative or Gram-positive bacteria.

It was found that LF binds to porins presented on the outer membrane of Gram-negative bacteria [17] and induces the rapid release of LPS which can enhance bacterial susceptibility to osmotic shock [18]. The cluster with high density of surface positive charges located in the N-terminal region of the LF molecule [19] can bind to the lipid A part of LPS presented on the outer membrane of bacterial species [20, 21]. Ellison *et al*. [22] tested the ability of LF to release LPS, their result showed that LF could damage the outer membrane of Gram-negative bacteria and then cause death. Appelmelk [20] found LF bound directly to intact LPS, indicating that LF was a LPS-binding protein. The result of Rossi *et al*. [23] proved that Lf can also bind Ca^{2+}, and affect release of LPS from Gram-negative bacteria.

By directly binding to LPS embedded in the membrane surface of bacteria, Lf promotes the release of LPS and the destabilization and permeability of the bacterial outer membrane. This destabilization will further cause death of entire bacterial [24-26]. To date, there are no other reports to further validate this bactericidal mechanism.

Taking advantage of simplicity, rapidness, and high sensitivity, bio-electrochemical technique can judge the change of tiny current on a PG electrode by the formation of ion-channel in mimic biomembrane. In the previous study, authors had published an article about porin Annexin V [27], which is a better foundation for this experiment. In addition, the use of self-assembly technique to build biomembrane was performed [28, 29]. Therefore, bactericidal mechanism of LF and its demand for microenvironment was analyzed by using biological electrochemical techniques in the current study.

2. EXPERIMENTAL

2.1.Materials

Human lactoferrins (iron saturated and iron unsaturated) were purchased from Sigma and directly used without further treatment. Phosphatidylcholine (PC), bovine serum albumin (BSA) and LPS were also purchased from Sigma. Other chemicals were of analytical reagent grade. Water was obtained from a Milli-Q purification system (Barnstead, MA) to a specific resistance (>18 MΩ cm^{-1}).

2.2. Preparation of PG electrodes

The pyrolytic graphite (PG) working electrode was treated according to the previous report [27]. The PG electrode surface was modified only with 20 μL of 2 mM PC aqueous solution (by

ultrasonicated more than 4 hours) or with a mixed solution containing 10 μL of 2 mM PC aqueous solution and 10 μL of 0.25% LPS solution, and then dried for overnight to form a uniform PC or PC-LPS mimic biomembrane. The modified PG electrodes was immersed into 100μL LF solution of certain pH to react for 30 minutes and washed with ultrapure water. The range of LF concentration was designed from 0 to 1.5 mg/mL in phosphate buffer (PB). BSA concentration was 1mg/mL in same PB solution.

2.3. Electrochemical determination

Electrochemical experiments were analyzed using a CHI 660C electrochemical analyzer (CH Instruments). A three-electrode configuration was employed. A glass sample cell of 10 ml, consisting of a modified PG working electrode, a saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode, was used for the determination. All CV experiments were carried out in 0.1 M PB (pH 7.4) containing 5 mM $Fe(CN)_6^{3-/4-}$ (1:1) at room temperature. Scan rate: 100 mV s^{-1}, scan range: 0.6V～-0.2V.

3. RESULTS AND DISCUSSION

3.1. Electrochemical response

The bactericidal activity of LF was measured through the changes of redox peak current on the PG electrode. As shown in Figure 1, a pair of well-defined peaks at the bare PG electrode (curve a) was obtained, suggesting there was a reversible electron transfer process. But no obvious peak current was observed (curve b) when the electrode was modified with PC+LPS mimic biomembrane. The result indicated that the electron transfer between the probe and PG electrode was significantly inhibited by the mimic biomembrane. When the PC+LPS modified electrode was immersed into LF solution at room temperature for 30 min and tested in the same way, a relatively obvious peak current was observed (curve c), though it was lower than that on the bare PG electrode. However, under the same condition, no obvious difference was found between PC-LPS/BSA electrode and PC+LPS modified electrode (curve d), when the PC+LPS modified electrode was immersed into BSA solution and tested. These results suggested that the electrode current response (curve c) was due to the processing of LF. It was proved that LF did damage the lipid membrane, result in the formation of ion permeable channels in the solid mimic biomembrane, and make the redox probe easily reach the electrode surface.

In addition, no changes happened between iron unsaturated LF and iron saturated LF, suggesting that the bactericidal activity of LF is irrelevant with iron binding capacity (date not shown).

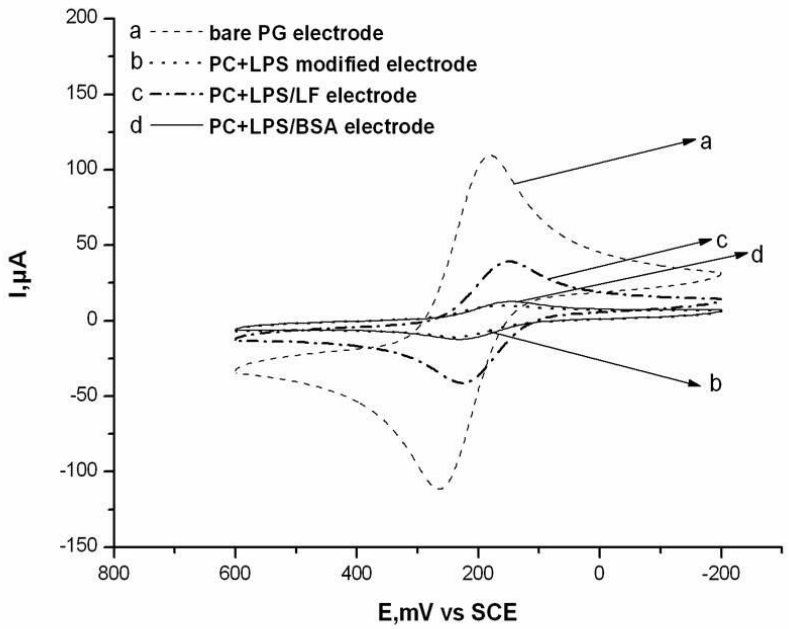

Figure 1. Cyclic voltammograms of 5 mM $Fe(CN)_6^{3-/4-}$ (1:1) solution obtained on electrodes under different processing. (a) bare PG electrode, (b) PC+LPS modified electrode, (c) PC+LPS modified electrode dealt with LF (1 mg/mL), and (d) PC+LPS modified electrode dealt with BSA (1 mg/mL). Support solution: 0.1 M PB (pH 7.4); Scan rate: 100 mV s^{-1}. Scan range: 0.6V ～-0.2V.

To further prove that these ion channels were due to LF interacting with LPS, we removed LPS from the mimic biomembrane. The result showed that no obvious changes happened (data not shown), indicating that LF can indeed bind to LPS, cause the structure changes of LPS and then produce a current response.

It is well known that, the formation of Lf-LPS complexes occurs through electrostatic interactions. Binding saturation of Lf to Lipid A promotes the conversion of the molecular shape of lipid A from a conical form (active) into a cylindrical form (inactive) [30], in accordance with previous studies suggesting that the conical shape of lipid A is a prerequisite for its endotoxic activity [31-32].

This opinion is agreement with that of Ellison *et al.*[22] and ours results.

3.2. Effect of pH on the bactericidal activity of LF

The effect of pH on the antibacterial activity of LF was examined. The pH gradient was designed from 3 to 10 in Britton-Robinson buffer. As shown in **Fig.2**, the electrode anodic peak current gradually increased with pH increasing from 3 to 8. The result showed the high level of pH help LF bind to LPS and pull LPS out of the biomembrane, and then lead the formation of ion-channel, suggesting a good antibacterial activity of LF is around pH8.0. However, when the pH exceed 8.0, peak current of the electrode decreased sharply, suggesting a higher alkaline environment was not conducive for LF to combine with LPS, or affect the structure of active site of LF.

Thus, LF demands a weakly alkaline condition for fully exerting its bactericidal function. This result was agreement with the result of Lu *et al*. [33], who found that pH values can affect the inhibition capability of bacteria, while the optimum antibacterial activity are at pH 7.5～8.0.

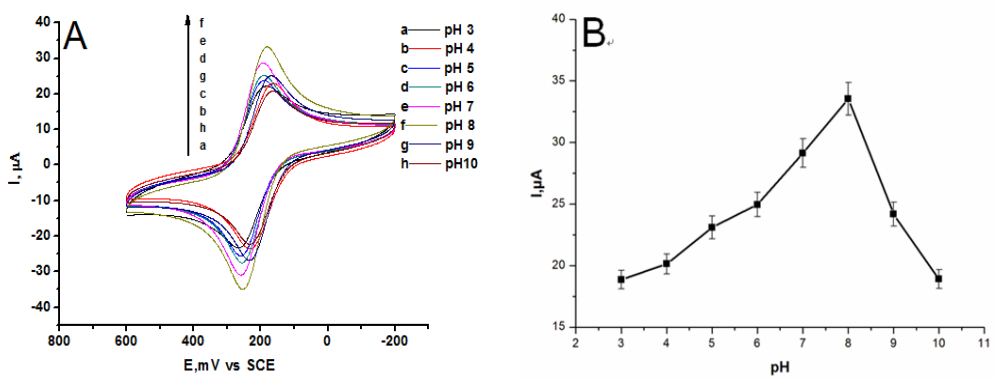

Figure 2. Effect of pH on the cathode peak current. A, Cyclic voltammograms; B, The average values of cathode peak current from three different experiments. Support solution: 0.1 M Britton-Robinson buffer (pH from 3 to 10) with 5 mM $Fe(CN)_6^{3-/4-}$ (1:1) solution; Other conditions are same as in **Figure** 1.

3.3. Effect of LF concentration on the bactericidal activity

The effect of LF concentration on antibacterial ability was examined. LF concentration gradient was 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5 mg/mL. The results showed that the reduction peak current gradually increased with the increasing of LF concentration (Figure 3). When LF concentration was higher than 1.0 mg/mL, the peak current of redox curve stopped increasing. It may be inferred that there is an important effect of the content of LPS in mimic biomembrane on peak current of redox curve.

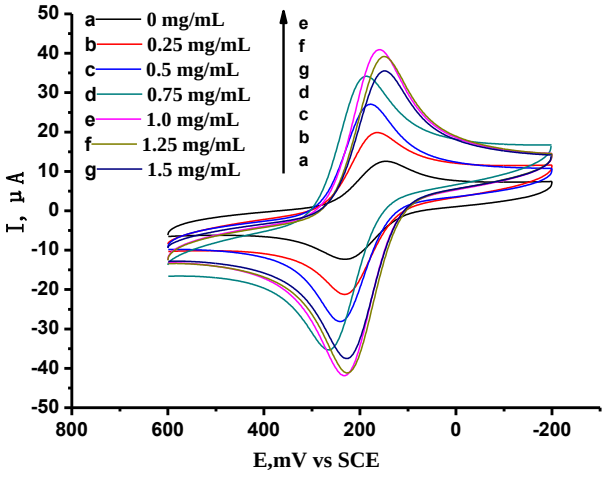

Figure 3. Effect of LF concentration on the cathode peak current. LF concentration was from 0.25 to 1.5 mg/mL. Support solution: 0.1 M PB (pH 7.4) with 5 mM $Fe(CN)_6^{3-/4-}$ (1:1) solution; Scan rate: 100 mV s^{-1}. Other conditions are same as in Figure 1.

3.4. Effects of metal ions concentration on the bactericidal activity of LF

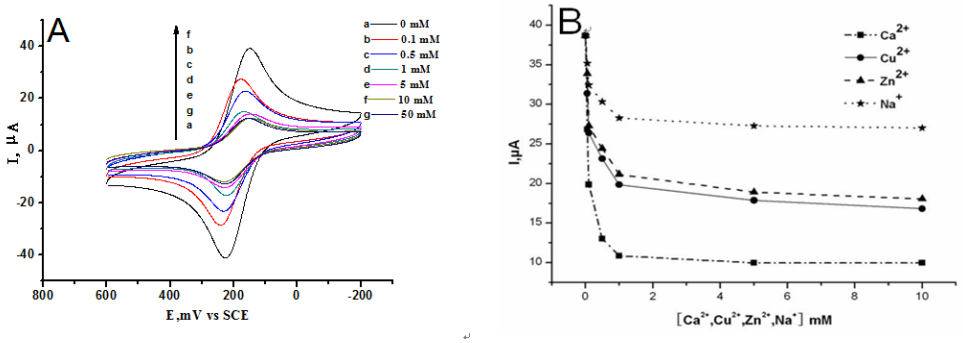

Figure 4. Effect of metal ions on the cathode peak current of $Fe(CN)_6^{3-/4-}$ on the modified electrode. A, Cyclic voltammograms of Ca^{2+}; B, The average values of cathode peak current from three different experiments. Metal ions concentration was designed to be 0, 0.05, 0.1, 0.5, 1, 5 and 10 mM. Other conditions are same as in Figure 1.

Besides of binding Fe^{3+}, LF can also bind Cu^{2+}, Zn^{2+}, Ca^{2+}, Na^{+}, etc [34]. All experimental metal ions could affect the interaction between LF and mimic biomembrane, and the electrode reduction peak current decreased in different extent as increasing in the concentration of metal ions (Figure 4). Obviously, divalent cations, especially Ca^{2+} (Figure 4 A) showed greater effect than that of monovalent cations. It maybe because Ca^{2+} is much easier to bind to LF and affect the structure of active site, thus reducing LF in combination with LPS, resulting in a decline in its bactericidal activity. The result of Rossi *et al* [23] also showed that Ca^{2+} can affect LF release LPS from Gram-negative bacteria. Therefore, these results implied that the concentration of cationic in the food system using LF as an antimicrobial agent, should be properly controlled.

4. CONCLUSION

This study validated the bactericidal mechanism of LF by the bio-electrochemistry technique. The results showed that LF binds directly to LPS embedded in the mimic bio-membrane and promotes it release and concomitantly the destabilization and permeability of the bacterial outer membrane. It can be inferred that this destabilization and permeability will further cause the death of entire bacterial. This result is basically consistent with the previous report [24].

Furthermore, we also made a survey for the microenvironment factors of bactericidal capacity of LF. It was found that LF can fully exert its bactericidal function in the weakly alkaline conditions of vicinity of pH 8.0; the bactericidal activity of LF is closely related to its concentration; the bactericidal activity of LF will decrease with the rising in concentration of metal ions. Divalent cations show greater effect than that of monovalent cations.

The experimental results suggest that biological electrochemical method is a simple, convenient and practical technique and provide a new way for the associated biochemical experiment.

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Conflict of interest
There is no conflict of interest.

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