

Anti-bacterial properties of lactoferrin immobilized wool fabric

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A new biological cross-linker, microbial transglutaminase (mTGase), has been used to catalyze the immobilization of lactoferrin onto the wool fabrics, and the antibacterial properties of immobilized wool on both Gram-negative and Gram-positive bacteria are studied. It is found that the minimal inhibitory concentration of lactoferrin against *S.aureus* and *E.coli* is 0.5mg/mL and 0.25mg/mL respectively. As compared to the control sample, the amount of lactoferrin adhered onto the wool fabric improves from 4.87 mg(g fabric)⁻¹ to 12.96 mg(g fabric)⁻¹, indicating that the crosslinking reaction initiated by mTGase can increase the amount of lactoferrin fixed onto wool fabric obviously. The ratios of bacteriostasis to *S.aureus* and *E.coli* of wool fabrics immobilized with lactoferrin are bound to be 57.95% and 69.96% respectively, showing good antibacterial property.

Keywords: Antibacterial properties, Lactoferrin, Transglutaminase, Wool

1 Introduction

Lactoferrin is an iron-binding glycoprotein transferrin and its relative molecular weight ranges from 70,000 to 80,000. In 1960, lactoferrin was first separated from milk¹. Research shows that lactoferrin has extensive biological activity including broad-spectrum antimicrobial function². Till date, lactoferrin has been widely used in food^{3,4}, cosmetics and food additives⁵. However, although lactoferrin is well known for its bacteriostasis, the antibacterial mechanism is not precisely known. In general, its antibacterial mechanism is thought to be the combination of direct interaction of 'iron deprivation' and indirect interaction of 'membrane permeation'. Interaction of 'iron deprivation' can be attributed to the fact that lactoferrin is an iron-binding glycoprotein and hence can combine with iron ions, thus inhibiting the growth of pathogenic microorganism. The pathogenic microorganism dies due to lack of iron ions which they need for growth. 'membrane permeation' is ascribed to strong cationic amino-terminal of lactoferrin. The membrane permeation of bacteria is enhanced, leading to lipopolysaccharide extravasation which contributes to the death of bacteria⁶⁻⁸.

Microbial transglutaminases (mTGase, EC 2.3.2.13) are a group of enzymes capable of catalyzing the acyl

transfer reaction between the γ -carboxamide groups in Gln residues of peptides or proteins and ϵ -amino groups in Lys residues, resulting in the formation of ϵ -(γ -glutamyl) lysine linkages and the release of ammonia. In this reaction, the γ -carboxamide group of glutamine and the ϵ -amino group of lysine function as the acyl donor and the acceptor respectively⁹. In the textile industry, mTGase treatment of proteinous can improve shrink resistance properties of wool fabrics¹⁰ and crease resistance properties of silk fabrics¹¹. Previous study showed that the incorporation of primary amine molecules into wool with mTGase to alters wool functionality, was demonstrated using a fluorescent primary amine (fluorescein cadaverine)¹². In our earlier study¹³, lactoferrin was successfully grafted onto wool fabric catalyzed by mTGase. Based on the same mechanism, the reaction scheme for the current system is illustrated in Fig. 1. In this study, *Escherichia coli* (Gram-negative bacteria) and *Staphylococcus aureus* (Gram-positive bacteria) were chosen for further assessing the antibacterial activities of wool fabrics immobilized with lactoferrin via mTGase.

2 Materials and Methods

2.1 Materials

Worsted wool fabric (220 g/m², 2/1 twill, 32s, 410 ends/10 cm×250 picks/10 cm) was supplied from Wuxi Xiexin Group (China) and used in all the experiments. Lactoferrin (LF) was purchased from

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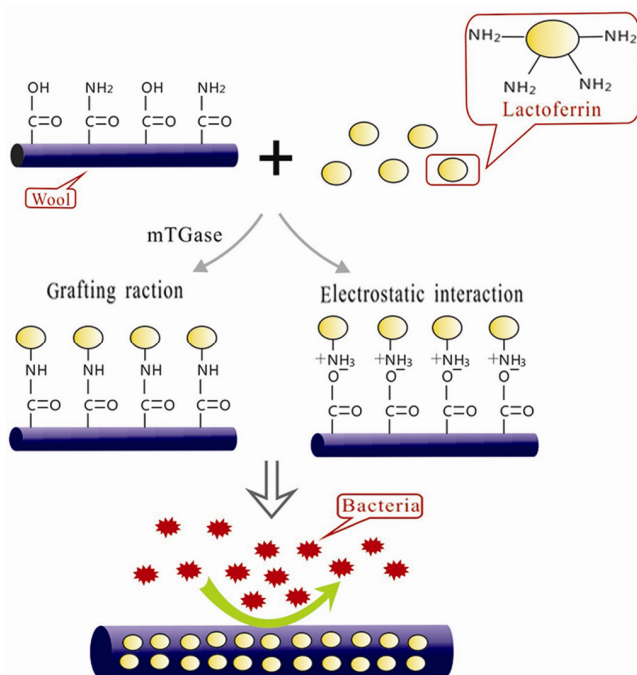


Fig. 1—Possible model of wool fabrics immobilized with lactoferrin via mTGase

Nanjing Tianchun Trading Co. Ltd. mTGase isolated from *Streptomyces mobaraense* with an activity of 0.1U/mg was purchased from Yiming Biological Products Co. Ltd. (China).

2.2 Pretreatment of Wool Samples

Wool fabrics were pretreated with 4% (owf) potassium permanganate in a solution with a liquid-to-fabric ratio of 20:1 at pH 4 and 40 °C for 30 min. After the reaction, wool samples were neutralized in a solution containing 2% (owf) sodium bicarbonate at 45 °C for 10 min followed by a decolorization treatment in a solution containing 6% (owf) sodium bisulfite and 1% (v/v) acetic acid with a liquid-to-fabric ratio of 20:1 at 40 °C for 30 min. Then, the treated samples were thoroughly washed with distilled water and air dried.

2.3 mTGase-Catalyzed Grafting of Lactoferrin on Wool Samples

Pretreated wool fabrics were incubated in 0.01 M phosphate buffer solutions (pH 7.0) containing 5 mg/mL lactoferrin and 30 U/g mTGase with a liquid-to-fabric ratio of 30:1. The incubation was carried out at 40 °C for 3 h. The wool samples were completely rinsed with deionized water and air dried at 50 °C.

2.4 Determination of Minimal Inhibitory Concentration of Lactoferrin

Minimal inhibitory concentration (MIC) of lactoferrin was measured by a tube dilution method. The sterile capped test tubes were numbered 1-7 and following steps were carried out using aseptic technique. One milliliter of sterile broth was added to all tubes, then 1.0 mL of lactoferrin solution (8 mg/mL) was added to the first tube and mixed the contents well. Then one milliliter solution from the first tube was transferred to the second tube. Another 1.0 mL solution from the second tube was transferred to the third one with a separate pipette. The dilutions were continued in this manner up to the sixth tube. The pipettes used between tubes were changed to prevent the carryover of the solutions on the external surface of the pipettes. One milliliter solution was removed from the sixth tube and discarded. The seventh tube, which served as a control, received nothing. The concentrations of lactoferrin from tube 1 to tube 6 were kept 4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL respectively.

The tested bacteria solution of 1mL was added to above seven tubes respectively, and cultured for 18h at ambient temperature. The visible bacterial growth was observed and counted. The experiment was repeated for three times for accuracy. MICs were recorded as the lowest concentrations under which no visible bacteria colony growth on the plates is observed.

2.5 Scanning Electron Microscopy

The surface morphological characterization of wool samples was performed by a Quanta-200 scanning electron microscope (FEI Company, The Netherlands).

2.6 Determination of Amount of Lactoferrin Immobilized onto Wool

The ultraviolet absorbance of lactoferrin solution was determined and the concentration of lactoferrin was calculated in terms of ultraviolet absorbance of lactoferrin at 280 nm (ref. 14).

The amount of immobilized lactoferrin on the surface of wool was calculated using the formula as follows:

$$m = (\Delta C \times V) / m_0 \quad \dots (1)$$

Here m is the amount of lactoferrin immobilized onto wool; m_0 , the mass of wool; ΔC , the concentration difference of lactoferrin solution; and V , the volume of lactoferrin solution.

2.7 Evaluation on Antibacterial Activity

Staphylococcus aureus and *Escherichia coli* were selected as the experimental bacteria for antibacterial tests. The antibacterial activities of the wool samples were measured by a shake flask test and assessed in terms of the ratio of bacteriostasis (R) (ref. 9). R was calculated using following equation:

$$R(\%) = 100 \times (A - B) / A \quad \dots(2)$$

where A and B are the mean numbers of bacteria colonies on the wool samples before and after shake flask tests respectively.

3 Results and Discussion

3.1 Antibacterial Activity of Lactoferrin

Lactoferrin is antibacterial to Gram-negative and Gram-positive bacteria, showing a broad spectrum of antibacterial activity. *Staphylococcus aureus* belonging to Gram-positive bacteria and *Escherichia coli* belonging to Gram-negative bacteria were chosen for the experiment.

The MIC of lactoferrin was measured by a tube dilution method. Table 1 shows that lactoferrin has obvious antibacterial property to *Staphylococcus aureus* when the concentration of lactoferrin is higher than 0.5 mg/mL. Bacteriostasis to *Escherichia coli* is also observed at the concentration of lactoferrin higher than 0.25mg/mL. Therefore, the MICs of lactoferrin against *Staphylococcus aureus* and *Escherichia coli* are 0.5 mg/mL and 0.25mg/mL respectively.

3.2 Grafting of Lactoferrin on Wool

Lactoferrin was attached onto the wool fabric via interactions of electrostatic adsorption alone and also via interactions of electrostatic adsorption plus catalytic crosslinking catalyzed by mTGase. The graft yields of lactoferrin on wool as a function of the incubation time are shown in Fig. 2.

As shown in Fig. 2, the graft yield of lactoferrin onto wool by means of electrostatic adsorption alone increases with the increase in incubation time up to 1h. As the time increases, the immobilized

lactoferrin on the wool is maintained approximately at $5 \text{ mg}(\text{g fabric})^{-1}$ (Sample A), while the amount of immobilized lactoferrin on the wool via dual actions of electrostatic adsorption + enzymatic crosslinking with mTGase continuously increases and the final concentration is maintained approximately at $13 \text{ mg}(\text{g fabric})^{-1}$ up to the incubation time 2h (Sample B). It means that $8 \text{ mg}(\text{g fabric})^{-1}$ of lactoferrin has been immobilized on the wool with the help of catalytic crosslinking of mTGase. The occurrence of grafting reaction could be proved by the obvious increment of the maximum amount of immobilized lactoferrin. This enhanced amount could be ascribed to the mTGase-catalyzed coupling via acyl transfer reaction between the γ -carboxamide group of the wool and the amino group of lactoferrin.

3.3 Characterization of LF Immobilized Wool Surface

Figure 3 shows the SEM images of the wool samples. It can be seen that the surface of untreated wool fabric is covered by shell-like scale structures. Scale edges of the control wool has been weakened and blunted to some extent due to the oxidation of potassium permanganate. The pretreatment before enzymatic grafting is necessary in order to partially dislodge or damage the hydrophobic scales of wool so as to enhance the accessibility of enzymes to the substrates on the wool surface. The scales of wool adsorbing lactoferrin are hardly to be observed due to the coating of adsorbed lactoferrin on the surface of the wool (Fig.3c). The lactoferrin adhered to wool via electrostatic

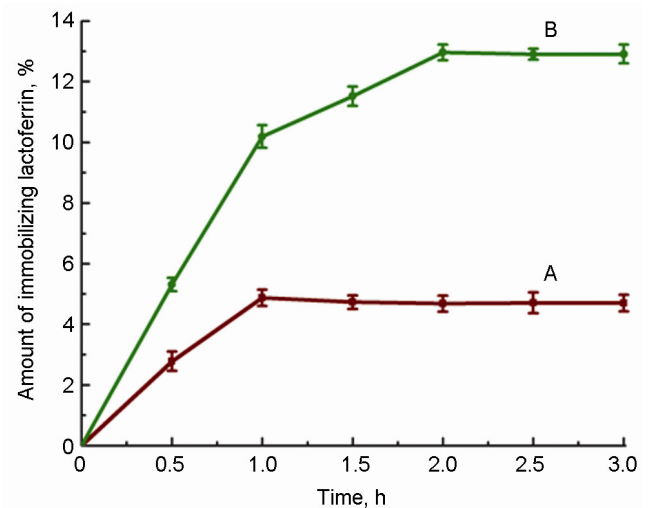


Fig. 2—Amount of immobilizing lactoferrin on wool treated with (A) lactoferrin alone and (B) lactoferrin and mTGase

Table 1—Antibacterial activity of lactoferrin

Bacteria	Lactoferrin concentration, mg mL^{-1}					
	4	2	1	0.5	0.25	0.125
<i>S.aureus</i>	×	×	×	×	√	√
<i>E. coli</i>	×	×	×	×	×	√

√—Growth with bacteria. ×—Growth without bacteria.

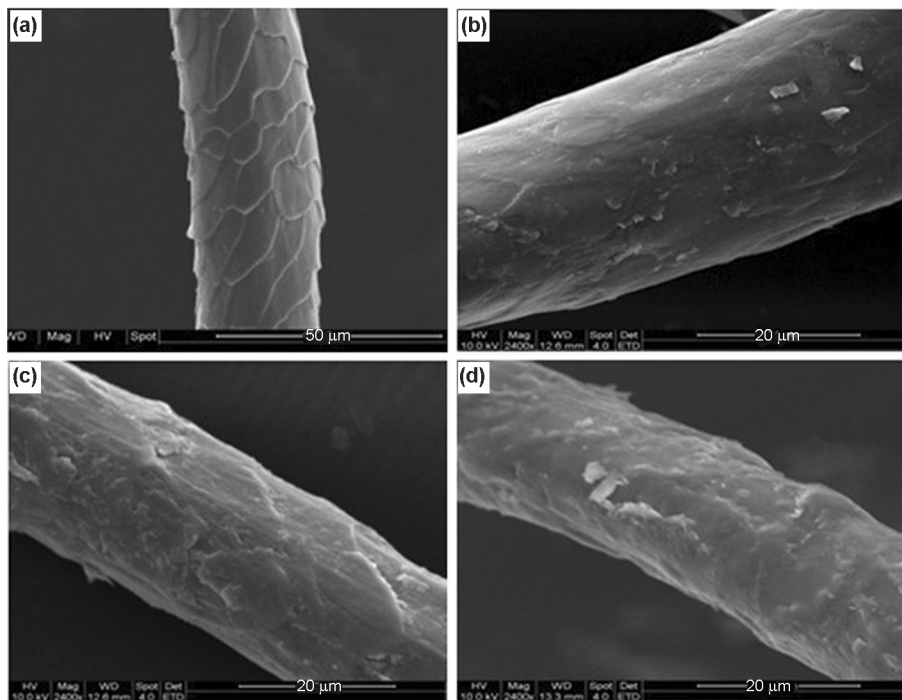


Fig. 3—SEM images of wool samples (a) untreated wool, (b) potassium permanganate-pretreated wool, (c) wool adsorbing LF, and (d) wool treated with mTGase and LF

adsorption + catalytic crosslinking of mTGase reaches the highest among all the samples. The scales are completely covered due to the mTGase-catalyzed grafting of lactoferrin on the wool, except in the electrostatic adsorption alone of lactoferrin. On the whole, the increased amount of the coated substance is also related with the amount of immobilized lactoferrin as shown in Fig. 2.

3.4 Antibacterial Measurement

Table 2 shows that the wool immobilized with lactoferrin has less amounts of two kinds of bacterial colonies than untreated wool. For the *S.aureus*, the ratios of bacteriostasis are 57.95, 29.54 and 7.95% for the wool treated with mTGase/LF, LF alone, and blank respectively. For the *E.coli*, the ratios of bacteriostasis are 69.96, 34.08 and 0% for the wool treated with mTGase/LF, LF alone, and blank respectively. This result reveals that the wool bonding lactoferrin via either individual electrostatic adsorption or via combined interactions of electrostatic adsorption + enzymatic grafting could greatly improve the antibacterial abilities as compared to untreated wool. This means that the wool fabric grafted with lactoferrin catalyzed by mTGase shows good antibacterial

Table 2—Antibacterial assays

Parameter	<i>Staphylococcus aureus</i>		<i>Escherichia coli</i>	
	Amount of colony	Ratio of bacteriostasis %	Amount of colony	Ratio of bacteriostasis %
Initial colony	88	—	223	—
Untreated wool fabric	81	7.95	267	0
Wool fabric adsorbing LF	62	29.54	147	34.08
Wool fabric immobilizing LF	37	57.95	67	69.96

activities. In addition, the modified wool has better antibacterial activities against *E.coli* than against *S.aureus*.

3.5 Laundering Durability of Antibacterial Effect

The antibacterial durability of the wool immobilized with lactoferrin via dual actions of electrostatic adsorption + mTGase-catalyzed crosslinking is assessed by washing the specimens for given cycles (Fig. 4).

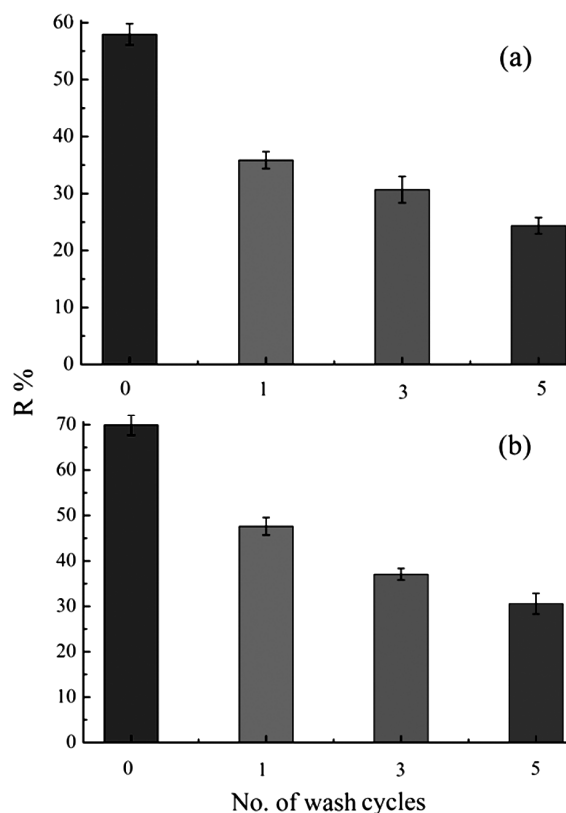


Fig. 4—Antibacterial durability against (a) *S.aureus* and (b) *E.coli* of the wool fabric immobilized with lactoferrin

Figure 4 shows that the ratio of bacteriostasis for *S.aureus* of the LF immobilized wool fabric sharply decreases after one wash cycle. The loss in bacterial activity could be attributed to the lactoferrin based on adsorption. The ratio of bacteriostasis (R) decreases to 30.68% after three wash cycles but the wool still shows antibacterial activity. However the wool fabric has lower antibacterial activity after five wash cycles. On the other hand, antibacterial activity for *E.coli*, the ratio of bacteriostasis of the wool fabric, treated by lactoferrin using combined interactions, is bound to be 69.96%. The ratio of bacteriostasis after 1, 3 and 5 wash cycles decreases to 47.62, 37.06 and 30.58% respectively. It can be found that the antibacterial ratio of the washed specimen decreases with wash cycle.

4 Conclusion

4.1 Wool fabric immobilized with lactoferrin by catalytic crosslinking of mTGase exhibits satisfactory antibacterial activities. This enzymatic method has no harm to human and provides an environmental friendly treatment of textiles for antimicrobial finishing.

4.2 The lowest concentrations of lactoferrin toward *E.coli* and *S.aureus* are 0.25mg/mL and 0.5mg/mL respectively.

4.3 Microbial transglutaminases show a capability of catalytic crosslinking that immobilizes lactoferrin onto the wool fabric. As compared to control sample, the amount of lactoferrin adhered onto wool fabric improves from about 5 mg(g fabric)⁻¹ to about 13 mg(g fabric)⁻¹. That means the crosslinking reaction catalyzed by mTGase can improve the amount of lactoferrin immobilized onto wool fabric obviously.

4.4 Lactoferrin is grafted onto wool fabric by catalytic crosslinking of mTGase, which endows the wool fabric with antimicrobial activity. The ratios of bacteriostasis for *S.aureus* and *E.coli* are bound to be 57.95% and 69.96% respectively. The antibacterial effect is found to be durable for five laundering cycles; however, the effectiveness decreases with increase in wash cycles.

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