Appl Biochem Biotechnol (2010) 160:2486–2497
DOI 10.1007/s12010-009-8708-7

Antibacterial Functionalization of Wool via mTGase-Catalyzed Grafting of ε-Poly-L-lysine

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Received: 19 April 2009 / Accepted: 5 July 2009 /
Published online: 1 August 2009

Abstract ε-Poly-L-lysine (ε-PL), a natural biomacromolecule having a broad spectrum of antibacterial activity, was grafted on the wool fiber via the acyl transfer reaction catalyzed by microbial transglutaminase (mTGase) to develop a new strategy for antibacterial functionalization of proteinous materials. The effects of the concentrations of ε-PLs and mTGases on the graft yields were investigated. A coating of ε-PL that almost completely covered the scale profile on the wool surface was visualized by scanning electron microscopy (SEM) and further demonstrated in terms of Allwörden's reaction characteristic of wool. Identifiable differences in lysine content and color depth among the stained wool samples reveal the changes in the surface composition and polarity caused by the incorporation of ε-PL onto the wool substrate, respectively. The ratio of bacteriostasis to *Escherichia coli* of the wool fabric grafting ε-PL reached 96.6 %, indicating an excellent antibacterial effect. The application of ε-PL and corresponding mTGase-catalyzed grafting reaction would provide a worthwhile reference for antibacterial functionalization of proteinous materials in various forms.

Keywords Microbial transglutaminase (mTGase) · Antibacterial functionalization · Enzyme-catalyzed grafting · ε-Poly-L-lysine · Wool

Introduction

Wool fibers are used for the high-grade textiles due to their lightness, warmness, softness, and smoothness. However, as a protein fiber, the wool fiber easily suffers degradation, skin irritation, and infection due to the generation and propagation of microorganisms under certain temperature and humidity. To overcome this weakness, some inorganic and organic antibacterial agents have been applied on wool goods, and positive results have been achieved [1–5].

In recent years, greater attention has been given to enzymatic processes of wool textiles as effective alternatives to conventional chemical treatments because of the nontoxic and

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eco-friendly characteristics of enzymes as well as the increasingly important requirement for reducing pollution in textile production [6]. To date, different types of enzymes—including proteases [7–9], lipases [10], laccases [11], peroxidase [12], tyrosinases [12, 13], and transglutaminases [14–17]—have been used for improving the qualities of wool fabrics or providing new functions to wool. However, as far as our knowledge goes, no report has so far involved the enzymatically antibacterial functionalization of wool textiles. In this regard, transglutaminases might be candidate enzymes with great potential.

Transglutaminases (TGase, EC 2.3.2.13) are a group of enzymes capable of catalyzing the acyl transfer reaction between the γ-carboxyamide groups in Gln residues of peptide or protein and ε-amino groups in Lys residues, resulting in the formation of ε-(γ-glutamyl) lysine linkages and the release of ammonia [18]. In this reaction, the γ-carboxyamide group of glutamine and the ε-amino group of lysine function are the acyl donor and the acceptor, respectively [19]. Since wool fiber contains Gln (0.45 mmol/g) and Lys (0.25 mmol/g) residues in its peptide chain [20], several pioneering applications of TGases in the modification of wool textiles have been reported. It has been proven that TGases could catalyze the intermolecular cross-linking of the wool macromolecules [14, 17], which decreased the propensity of wool fabrics to shrink and remedied the strength loss caused by chemically or enzymatically shrink-resistant treatments. Furthermore, to improve physical and mechanical properties of wool fabrics, two types of regenerative proteins, silk proteins (sericin and commercial silk digest) and wool keratins, were grafted on the wool substrates using TGases, which serve as protein cross-linking enzymes [15, 16].

ε-Poly-L-lysine (ε-PL), which is now industrially produced by *Streptomyces albulus*, is a homo-polypeptide of L-lysine with the amide linkages between the ε-amino and carboxyl groups. Positively charged ε-PL molecules generally inhibit the proliferation of micro-organisms including yeasts, fungi, and gram-positive or gram-negative bacterial species [21]. As a biomacromolecule with the advantages such as water solubility, nontoxicology, biodegradability, and antibacterial activities in a broad spectrum, novel applications of ε-PL have been developed in the fields of medicine, food, environment, agriculture, and electronics in the past decades [22, 23]. In particular, ε-PL has already been used generally as a natural toxicologically safe food additive because it meets FAO/WHO standards and is certified as a generally recognized as safe material of FDA in the USA [24].

Considering that wool and ε-PL could be used as the acyl donor and the acyl acceptor, respectively, in this work, we primarily studied the antibacterial functionality of wool via mTGase-catalyzed grafting of ε-PL on the wool surface. Compared to traditional chemical grafting, mTGase-catalyzed grafting is a site-specific reaction, which is illustrated in Fig. 1. The effects of ε-PL and mTGase concentrations on the graft yields and the surface morphologies and polarities as well as the antibacterial effectiveness of the grafted wool fibers were investigated. Moreover, the washing resistance of the modified wool was also examined.

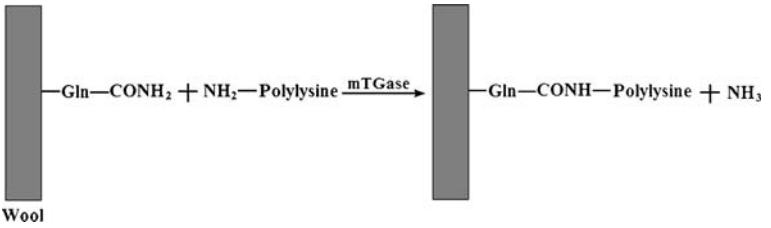

Fig. 1 Scheme of mTGase-catalyzed grafting of ε-PL on wool

Materials and Methods

Materials

Worsted wool fabric (220 g/m^2, 2/1 twill, 32^s, 410 ends/10 cm×250 picks/10 cm) was obtained from Wuxi Xiexin Group (China). The mTGase isolated from *Streptomyces mobaraense* with an activity of 100 U/g was purchased from Yiming Biological Products Co., Ltd. (China). ε-Poly-L-lysine (ε-PL) was purchased from Handary Bio-Engineering B.V. (The Netherlands). Direct Turquoise Blue GL was obtained from Qingdao Chuanlin Dye Industrial Co., Ltd. (China). *Escherichia coli* (AATCC25922) was provided by the School of Food Science, Jiangnan University. Other chemicals used in this work were all of chemical grade without exception explanation.

Pretreatment of Wool Samples

The wool fabrics were pretreated with 4% on weight of fabric (owf) potassium permanganate in a solution with a liquid-to-fabric of 20:1 at pH 4 and 40 °C for 30 min. After the reaction, the wool samples were neutralized in a solution consisting of 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 at 40 °C for 30 min. The treated samples were completely rinsed with deionized water and air-dried.

mTGase-Catalyzed Grafting of ε-PL on Wool Samples

Pretreated wool fabrics were incubated in 0.01 M phosphate buffer solutions (pH 8.0) containing ε-PLs and mTGases with a liquid-to-fabric ratio of 30:1. The incubation was carried out at 50 °C for 2 h. The wool samples were then washed with distilled water five times (20 min per time) and dried at 50 °C. Control samples were treated with the same buffer solution using the same liquid-to-fabric ratio but either without adding any enzyme dose or without ε-PL. The samples subjected to incubation in phosphate buffers without ε-PL and mTGase served as blank. The experimental conditions except the dosages of the enzymes and substrates were referred to the optimal activity range provided by the enzyme manufacturer.

Determination of Graft Yields

The graft yields of ε-PLs on the wool fibers were calculated in terms of the changes in the concentrations of ε-PLs in treatment bath during the enzymatic grafting reaction. The concentration of ε-PL solution was determined by a modified Itzhaki colorimetric method [25].

Determination of ε-PL Concentrations

The ε-PL could stoichiometrically react with methyl orange (MO), which results in a decrease in absorbance of MO solution due to the formation of ε-PL-MO precipitation. Therefore, the concentrations of ε-PLs can be obtained according to the decrease in absorbance of MO solution at its λmax (464 nm). The standard curve of absorbance at 464 nm as a function of ε-PL concentration was made according to the following procedure: 3 ml of ε-PL solution ranging from 0 to 0.16 mg/ml and 7 ml of 1 mM MO solution were mixed well in a 50-ml glass tube and then incubated in a vibrating bath

(Rapid Precion Machinery Co., Ltd., Xiamen, China) with a speed of 260 rpm at 30 °C for 30 min. After this, the solution was centrifuged at a speed of 4,000 rpm for 15 min. A supernatant of 5 ml was removed into a 50-ml measuring flask and then diluted with 0.01 M phosphate buffer solution to 50 ml. The absorbance of the test solution against the blank was measured in a 2802 S UV/Vis spectrophotometer (Unico Instruments Co., Ltd., Shanghai, China). As shown in Fig. 2, the absorbance is inversely proportional to the concentration of ε-PL.

Procedure and Determination of Graft Yields of ε-PLs on Wool

The residual reaction solutions and the washing solutions collected from five-cycle washings at 50 °C were mixed and diluted to 100 ml. The concentration of ε-PL solution was obtained according to the above standard curve. Considering that ε-PLs also could be adsorbed onto the wool surfaces via electrostatic interactions, the graft yields of ε-PLs were calculated in terms of the difference of ε-PL concentrations in the residual bath without and with the addition of TGase. It should be noted that the addition of TGase into the treatment bath ranging from 0 to 0.8 mg/ml had no effects on this method (data not shown).

Scanning Electron Microscopy

The surface morphologies of the wool samples were visualized using a FEI Quanta-200 scanning electron microscope (FEI Company, The Netherlands), operating at a typical accelerating voltage of 50 kV with a magnification of ×2,000. The samples were mounted on an aluminum stub and sputter-coated with a layer of gold prior to the observation.

Allwörden's Reaction

The wool fiber samples were spotted with bromine water at room temperature for 2 min, and the images were recorded with a DZ3 Ultra-High-Magnification Zoom Microscope (Union Optical Co., Ltd., Japan) at a magnification of ×1,400.

Determination of Lysine Content

The wool samples were hydrolyzed with 6 M HCl at 110 °C for 24 h. Then, the hydrolysates were derivatized with *o*-phthaldialdehyde and then analyzed using an Agilent

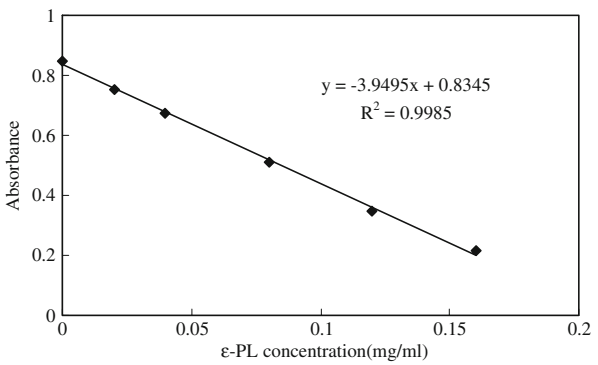

Fig. 2 The standard curve of the absorbance at 464 nm of methyl orange solution as a function of ε-PL concentration

1100 Series LC system (Agilent Technologies, Inc., USA). All samples were analyzed in duplicate with errors of less than 2%. The lysine content was determined using external standard calibration.

Staining Test

All wool samples were stained with 2% owf Direct Turquoise Blue GL in a solution with a liquid-to-fabric ratio of 20:1 at pH 7 and 20 °C for 5 min, followed by washing with distilled water and drying at 50 °C. Images of the stained wool fabrics were recorded with a digital camera. The relative color depth of stained fabrics, expressed as K/S, was measured by the light reflectance technique using the Kubelka–Munk equation.

$$K/S = (1 - R)^2/2R$$

The reflectance (R) of dyed fabrics was measured at the maximum absorbance wavelength of the dye solution on a Color-Eye 7000A spectrophotometer (GretagMacbeth, USA).

Antibacterial Activity Evaluation

The antibacterial activities of the wool samples against *E. coli* were measured by shake-flask test. After contacting with the wool samples at 37 °C for 18 h under continuous shaking, the bacterial suspensions were diluted and inoculated on agar plates. The plates were kept at 37 °C, and the colonies were counted after 24 h. The antibacterial effect was assessed in terms of the ratio of bacteriostasis, which was calculated using the following equation:

$$\text{Ratio of bacteriostasis}\,(\%) = 100 \times (A - B)/A$$

Where A and B denote the mean numbers of bacteria in the suspensions before and after shake-flask test, respectively.

The durability of antibacterial wool fibers was tested as follows: the wool samples were washed in a solution containing 2 g/l Zhengzhang wool detergent with a liquid ratio of 30:1 at 40 °C for 5 min. Then, the washed samples were rinsed twice with deionized water at room temperature. Each rinsing procedure included 2 min of rinsing and 0.5 min of centrifugal dehydration. Finally, the samples were dried at 50 °C.

Results and Discussion

Effect of the Concentrations of ε-PL and TGase on Graft Yields

Figure 3a reveals the effect of ε-PL concentration in the treatment bath on graft yields of ε-PL. As shown, the maximum graft yield of ε-PL on wool was reached when 1 mg/ml ε-PLs were added into the treatment bath. The graft yields decreased and tended to level off as the concentrations of ε-PLs further increased. This phenomenon might be attributed to the competition between the electrostatic adsorption and enzymatic grafting.

The wool fibers and ε-PL molecules would carry negative charges and positive charges, respectively, under the treatment condition (pH 8.0) because their isoelectric points are 4.2–

4.8 and approximately 9.0, respectively. Therefore, electrostatic adsorption of ε-PLs on wool would occur simultaneously with enzymatic grafting reaction. When fewer ε-PLs were added in the treatment bath, there are sufficient sites on the wool surface to covalently and electrovalently bond the ε-PL molecules. As the concentrations of ε-PLs were increased, more ε-PL molecules bonded on the wool surface, which would not only cover partial grafting sites but also produce stronger electrostatic repulsion to free ε-PL molecules.

As shown in Fig. 3b, the graft yields of ε-PL gradually increase with increasing TGase concentrations and improve less when TGase concentration is close to 1 U/ml. This result could be ascribed to the saturation of grafting sites resulting from the depletion of grafting groups (γ-carboxyamide group in Gln residues) on the wool surfaces.

To ensure the occurrence of grafting reaction, an abundant setup of processing parameters was made in the following experiments: pretreated wool fabrics were incubated in 0.01 M phosphate buffer solutions (pH 8.0) containing 2 mg/ml ε-PLs and 2 U/ml mTGases with a liquid-to-fabric ratio of 30:1. The incubation was carried out at 37 °C for 12 h. The wool samples were then washed with distilled water five times (20 min per time) and dried at 50 °C.

SEM Surface Analysis

Figure 4 shows the SEM micrographs of the wool samples. The scale structures on the surfaces of the blank (Fig. 4a) and mTGase-treated wool fiber (Fig. 4b) were still relatively

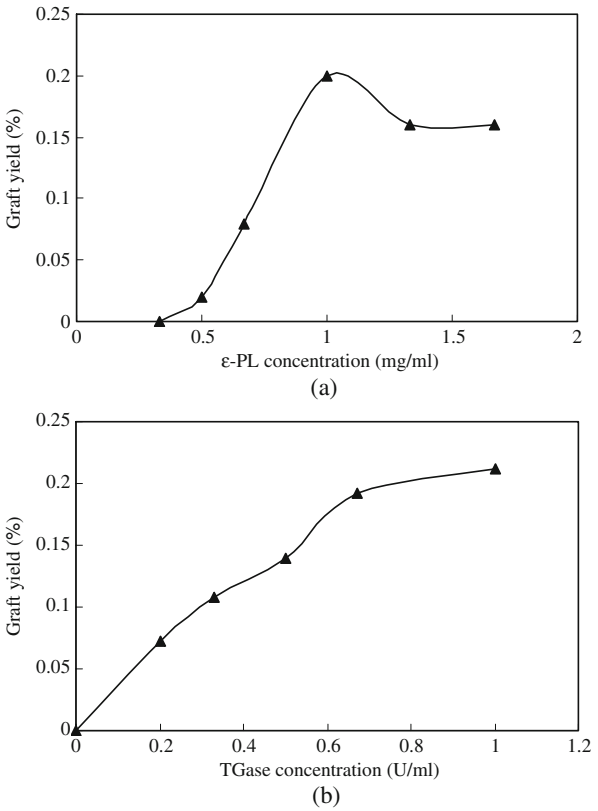

Fig. 3 Effects of the concentrations of **a** ε-PLs and **b** mTGases on the graft yields of ε-PLs on wool fibers

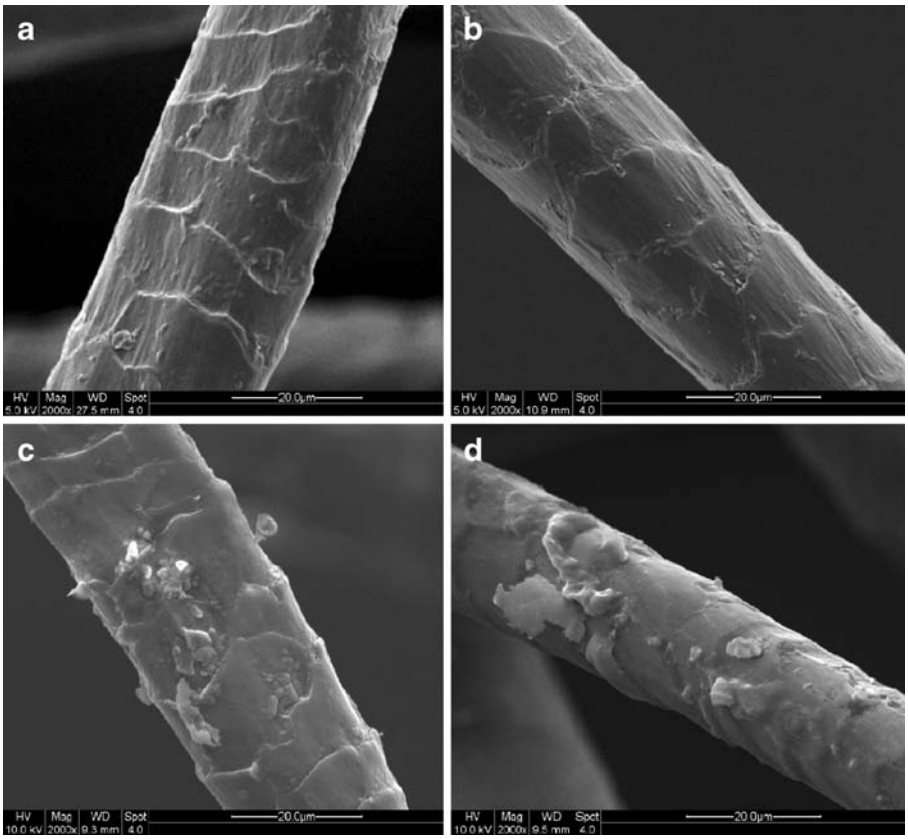

Fig. 4 SEM morphology of wool fibers treated with **a** buffer (blank), **b** mTGase, **c** ε-PL, and **d** mTGase and ε-PL

intact, but the edges of the wool scales had been weakened and blunted to some extent due to the oxidation of potassium permanganate. mTGase treatment could catalyze the intermolecular cross-linking of wool molecules, which leads to a compensation of strength loss, but it had fewer effects on the surface morphology of the wool samples. The sample treated with ε-PL alone presents more blurred scale edges and some agglomerates on the wool surface (Fig. 4c), which could be attributed to the adsorption of ε-PL synthetically caused by coulombic forces, van der Waals forces, and hydrogen bond interactions. As shown in Fig. 4d, the profile of the scale structure of mTGase/ε-PL-treated wool is almost undetectable, indicating that more polymers (ε-PLs) were coated on the wool surface compared with the wool sample treated with ε-PL alone. This enhanced coating could be ascribed to the mTGase-catalyzed grafting of ε-PL on the wool fiber via acyl transfer reaction.

Allwörden's Reaction

Allwörden's reaction is a microscopic test for the scale structure of chemically modified wool [26]. The wool fiber with intact scales would present strings of bead-like sacs on the fiber surface when it is immersed in the aqueous of chlorine or bromine. Chlorine or

bromine could diffuse through the epicutic1e and degrade the underlying layer (peptides in the exocuticle of wool). As water-soluble high molecular peptide fragments cannot pass the epicuticle, which functions as a semipermeable membrane, they would lead to osmotic swelling of the scale cells as soon as the fiber is immersed in water. The osmotic pressure generated thereby could cause distention of the membrane, forming special Allwörden sacs [27]. Although this reaction is used for the detection of the damage of wool scales, it is conversely postulated that the degree of the coating of wool could also be assessed indirectly. More coatings of polymers on wool scales would repress the formation of Allwörden sacs theoretically. Figure 5 shows the images of Allwörden's reaction of the wool fibers treated with different methods. Compared to the blank, which presents a characteristic image of Allwörden's reaction (Fig. 5a), the samples treated with mTGase or ε-PL show suppressed Allwörden effects to varying degrees (Figs. 5b–d). Among them, mTGase/ε-PL-treated wool presents the weakest Allwörden effect due to the fact that more polymers were enzymatically grafted on the wool surface, which was in accordance with the corresponding SEM image shown in Fig. 5d.

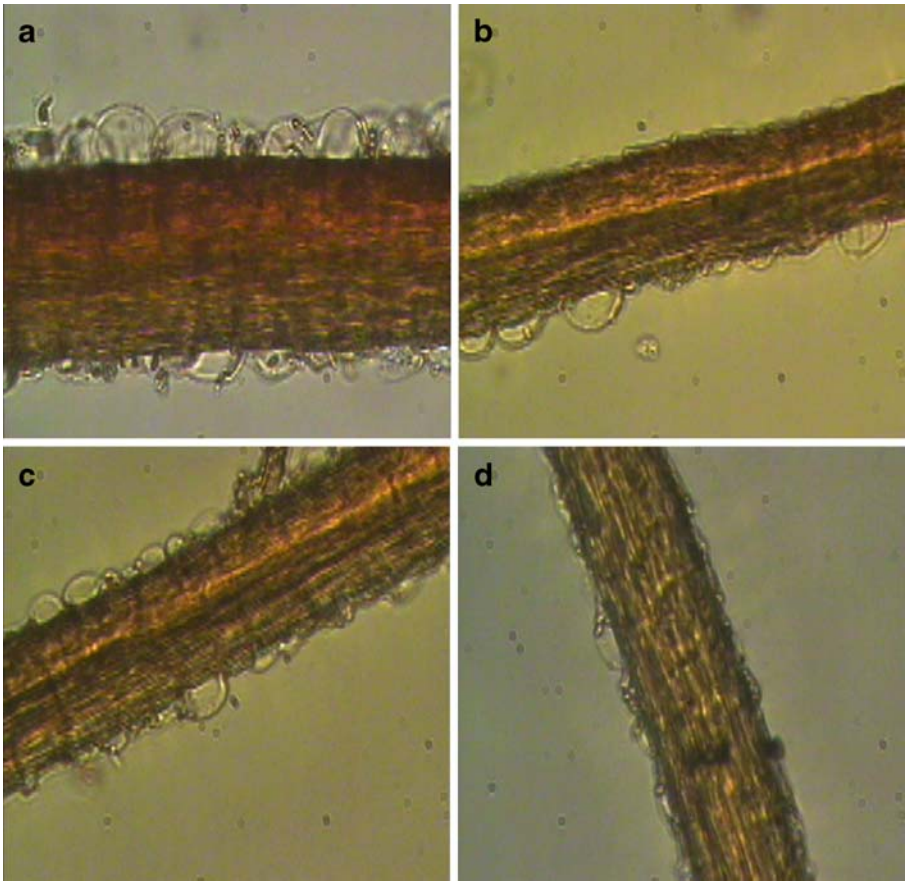

Fig. 5 Allwörden's reaction of wool fibers treated with **a** buffer (blank), **b** mTGase, **c** ε-PL, and **d** mTGase and ε-PL

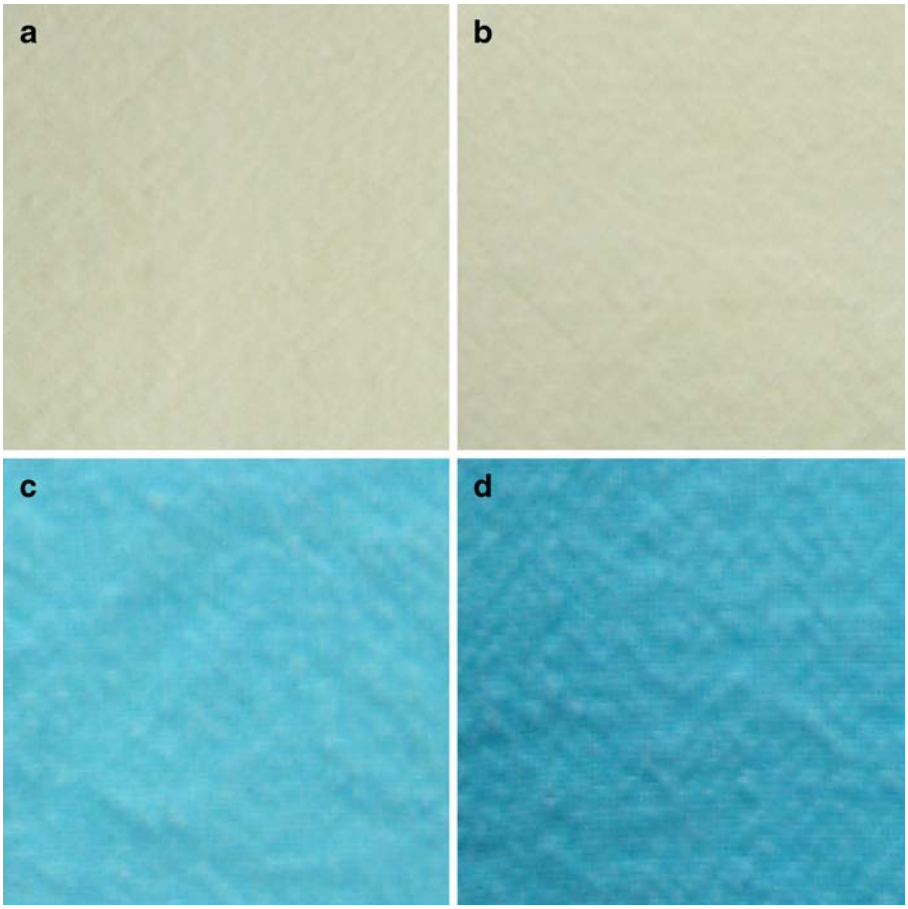

Fig. 6 Staining of wool fibers treated with **a** buffer (blank), **b** mTGase, **c** ε-PL, and **d** mTGase and ε-PL

Determination of Lysine Content

When ε-PL was grafted on the wool fiber, the relative content of lysine in the wool fiber should be increased in theory. The result shows that the wool samples treated with ε-PL alone and mTGase/ε-PL contain 4.05% and 4.27% of lysine, respectively, indicating identifiable increases in lysine content as compared to 3.58% of the blank. For the wool sample treated with ε-PL alone, an increase of 13.13% in lysine content as compared to the blank could be ascribed to the adsorption of ε-PL caused by Coulombic forces, van der Waals forces, and hydrogen bond interactions as discussed above. These interactions produced strong combination between the wool and ε-PL, which adsorbed some ε-PLs onto the wool surface. The wool sample treated with mTGase/ε-PL presents a further increase in the content of lysine, i.e., an increase of 19.27% as compared to the blank, revealing that more ε-PLs were enzymatically grafted on the wool via acyl transfer reaction. This result is in accordance with the proofs of SEM and Allwörden's reaction. It should be noted that the grafting of ε-PLs on the wool is difficult to demonstrate directly except the amino acid content test. Since both ε-PLs and wool are protein molecules, Fourier transform infrared

cannot identify the characteristic adsorption of amide bonds. The products of the acyl transfer reaction, ammonia gasses, are difficult to determine due to its small amount and possible adsorption by the wool fibers. This could be demonstrated by several previous reports involving TGase-catalyzed modification of wool [14–17], which did not determine the formation of ammonia to verify the grafting but used some indirect methods such as the changes in mechanical properties of wool. In addition, it seems that the graft yield of ε-PLs is very low. This might be due to the following reasons. On one hand, electrostatic adsorption of ε-PLs would compete the limited Gln residues on the wool surfaces with enzymatic grafting. On the other hand, as biomolecules, the ε-PLs that have bonded on the wool surfaces in the initial stage would form steric effect to the free ε-PLs, thus restraining the grafting reaction.

Staining Property

The wool samples were stained with a water-soluble anionic dye, Direct Turquoise Blue GL, in order to further prove the grafting reaction through the change of the wool surface. The pictures of the stained specimens are shown in Fig. 6. As show in Fig. 6a, b, both the blank and mTGase-treated wool samples were not stained due to the coulombic repulsions between the wool molecules and the dyes caused by their surface electric properties. This also reveals that almost no mTGase was adsorbed on the wool surface. However, the coulombic repulsion between the wool and the anionic dye would decrease when some ε-PL molecules were coated on the wool surface because ε-PL has an isoelectric point of approximately 9.0. The coating of ε-PL resulted in the existence of remarkably visual blue on the wool surfaces (Fig. 6c, d). In accordance with the above-mentioned SEM observation, Allwörden's reaction, and lysine analysis, the wool sample grafted with ε-PL via the catalysis of mTGase presents the deepest blue (Fig. 6d). In order to quantitatively evaluate the staining difference, the color depth of the surface of the stained wool was further determined (Fig. 7).

Antibacterial Measurement

The antibacterial activities of the wool fabrics with and without the coating of ε-PL were determined by using shake-flask test. The ratio of bacteriostasis to *E. coli* was up to 96.6%, 95.8%, and 0% for the wool samples treated with mTGase/ε-PL, ε-PL alone, and the blank, respectively. This result reveals that the wool fibers bonding PLs via either individual electrostatic adsorption or combined interactions of electrostatic adsorption and enzymatic

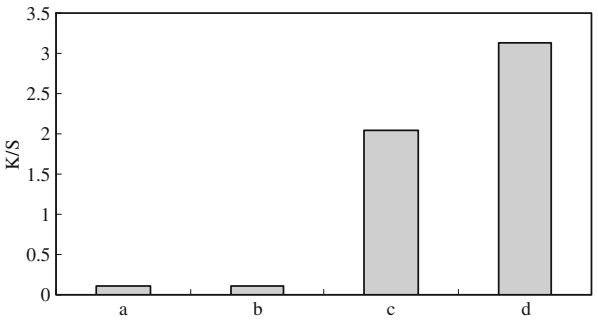

Fig. 7 Color depth of the wool samples treated with (*a*) buffer (blank), (*b*) mTGase, (*c*) ε-PL, and (*d*) mTGase and ε-PL

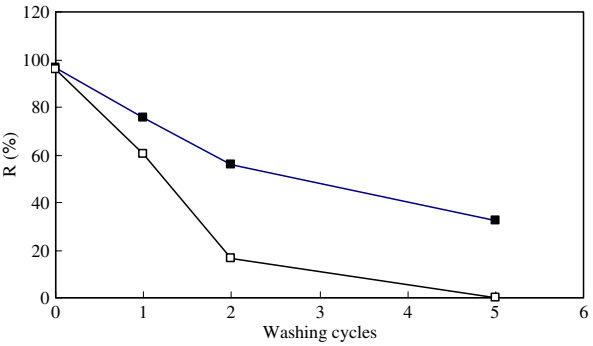

Fig. 8 Antibacterial testing of the wool samples treated with (*empty squares*) ε-PL and (*filled squares*) mTGase and ε-PL. (a) Bacterial solution without sample at 0 h, (b) bacterial solution without sample after 18 h; (c) buffer-treated wool (blank), and (d) ε-PL-grafted wool

grafting could greatly improve the antibacterial abilities compared to nonfunctionalized wool (blank sample). The durability of antibacterial wool was assessed by washing the specimen for given cycles. Figure 8 further reveals the effect of washing cycles on the ratio of bacteriostasis. It can be found that the antibacterial ratio of the washed specimen decreased with the increase of washing cycles in general. However, the wool samples treated with ε-PL alone present a more remarkable decrease than those treated with mTGase/ε-PL. The latter retained approximately 32.1% of its antibacterial activity after five cycles of use, while the former did not show any antibacterial activity. This could be attributed to the additional grafting of ε-PLs based on adsorption. The fact that the enzymatic grafting could improve the durability of antibacterial-functionalized wool shows that further work involving wool pretreatment or precoating to increase the grafting site (Gln) is interesting and worthwhile. The ε-PL coated on the wool surface can damage the cell membrane structures of the microorganisms via electrostatic interaction between the cationic ε-PL and anionic microbial cells, which could interrupt the transfer of substance, energy, and information of the cells and lead to the abnormal distribution of the cytoplasm, resulting in the death of microbial cells [28, 29].

Conclusion

To expand the applications of antibacterial biopeptides for textile use, we explored a novel method for site-specific functionalization of the protein fibers using a substrate peptide (ε-PL) and mTGase. According to the results reported in this work, it can be concluded that ε-PL could be enzymatically grafted on the wool surface, which endued the wool substrate (and possibly other proteinous materials) with better antibacterial effects. The grafting of ε-PL on wool fibers was visualized by using SEM, Allwörden's reaction characteristic of wool and the staining test, which was quantitatively proven by the increase in lysine content of the wool fiber. A higher ratio of bacteriostasis to *E. coli* of the ε-PL-grafted wool reveals the application potential of antibacterial functionalization of the wool fabric based on mTGase-catalyzed acyl transfer reaction. Further work focused on improving the grafting efficiency is ongoing.

Acknowledgments This work was sponsored by the National High Technology Research and Development Program of China (2008AA02Z203), Undergraduate Innovation Program, and Qing Lan Project.

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