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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

A novel "trifunctional protease" with reducibility, hydrolysis, and localization used for wool anti-felting treatment

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Abstract

Proteases can cause unacceptable fiber damage when they are singly applied to wool anti-felting treatment which can make wool textiles machine-washable. Even if protease is attached by synthetic polymers, the modified protease plays a limited role in the degradation of keratin with dense structure consisting of disulfide bonds in the scales. Here, to obtain "machine-washable" wool textiles, a novel "trifunctional protease" with reducibility, hydrolysis, and localization is developed by means of covalent bonding of protease molecules with poly (ethylene glycol) bis (carboxymethyl) ether (HOOC-PEG-COOH) and L-cysteine using carbodiimide/N-hydroxysuccinimide (EDC/NHS) coupling, aiming at selectively degrading the scales on the surface of wool. The formation of polymer is confirmed with size exclusion chromatography (SEC) and Fourier transform infrared spectroscopy (FT-IR). Ellman's test and fluorescence microscopy reveal that the modified protease can reduce disulfide bonds and restrict hydrolysis of peptide bonds on the wool scales. Furthermore, when applied to wool fabrics, the modified protease reach better treatment effects considering dimensional stability to felting (6.12%), strength loss (11.7%) and scale dislodgement proved by scanning electron microscopy (SEM), alkali solubility, wettability, and dyeability. This multifunctional enzyme is well-designed according to the requirement of the modification of wool surface, showing great potential for eco-friendly functionalization of keratin fibers rich in disulfide linkage.

Keywords Protease modification PEG wool anti-felting

Introduction

It is well known that wool fabrics have the tendency to felt and shrink during washing due to the existence of the cuticle scales on the surface of wool fiber (Fu et al. 2015). The conventional anti-felting treatments like chlorination to remove scales or polymer deposition to cover scales are widely used to overcome this problem. However, severe health and ecological hazards from adsorbable organic halides (AOX) caused by chlorination and deterioration in inherent characteristic properties of wool like handle, comfort properties, and strength (Schroeder et al. 2009; Shen et al. 2017; Smith and Shen 2011) have always bothered wool anti-felting process for years.

Protease treatment has been tentatively used for wool anti-felting as a potential alternative for chlorination due to process eco-friendly and milder conditions (Kaur et al. 2016). However, owing to the wool scales mainly consisting of keratin, proteases preferentially attack the non-keratinizing and highly swellable cell membrane complex (CMC), by penetrating between cuticular and cortical cells, resulting in deterioration of the wool fibers (Gaffar Hossain et al. 2009; Vílchez et al. 2010). Therefore, how to restrict the action range of the proteases to the scale rather than CMC has received great interest. Increasing the size of proteases by bonding synthetic polymers was once used to realize this aim (Jus et al. 2007). To date, varied synthetic polymers such as soluble polymer PEG (Jus et al. 2007; Schroeder et al. 2009; Silva et al. 2005), Eudragit S100(Shen et al. 2007; Silva et al. 2006a, b), Eudragit L100 (Smith et al. 2009), enteric polymer (Kollicoat MAE 100P, HPMCP-55, CAP, HPMCAS-AQOAT) (Smith et al. 2010b), chitosan (Chen et al. 2012), and dextran(Zhu et al. 2011) have been covalently bonded with proteases successfully. However, another barrier still exists even if

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the modified protease localizes on scales of wool through chemically enlarging its molecular weight and volume. Modified protease can merely hydrolyze peptide bonds due to its inherent specificity, which plays a limited role in the degradation of scale keratins rich in disulfide bond cross-linking (Ibrahim et al. 2012). Various pretreatments such as hydrogen peroxide (H_2O_2) (Liu et al. 2013), potassium permanganate ($KMnO_4$) (Kantouch et al. 1978), dichloroisocyanuric acid (DCCA), sodium sulfite (Na_2SO_3) (Shen et al. 2017), cetyltrimethylammonium bromide(CTAB) (Smith et al. 2010a; Smith and Shen 2012), and magnesium hydroxide(Wang et al. 2013) have to be introduced for enhancing the effect of the modified protease.

In the present work, we prepared a "trifunctional protease" with reducibility for disulfide bond, localization on scale, and hydrolysis of peptide bond, aiming at better degrading wool scales without severe damages to fiber. On the one hand, the modified protease was endowed with reducing capacity to break up disulfide bonds in keratinized proteins; on the other hand, apart from the inherent hydrolysis of the peptide bond, polyethylene glycol (PEG) was covalent-bonded with protease to increase its size, thus restricting hydrolysis focus on the wool scales. This trifunctional protease was synthesized as Scheme 1 describes: L-cysteine (L-cys), a reagent reducing the disulfide bonds in keratin (Wang et al. 2016), was covalently linked to poly (ethylene glycol) bis (carboxymethyl) ether (HOOC-PEG-COOH) using carbodiimide/N-hydroxysuccinimide-mediated (EDC/NHS) coupling, and then the carboxyl groups (-COOH) on L-cysteine were coupled to the amino groups (-NH_2) on protease (Savinase) in the same way (Kynclova et al. 1996; Roberts et al. 2012). Size exclusion chromatography (SEC), Fourier transform infrared (FT-IR), Ellman's test, and fluorescence microscopy were carried out to characterize the structure of modified trifunctional protease followed by determining the properties of modified proteases. Furthermore, the modified protease was applied to the wool treatment to comprehensively assess its anti-felting effect by determining dimensional stability to felting, strength loss, and SEM.

Materials and methods

Materials

Savinase 16 L (EC 3.4.21.14), a subtilisin secreted by *Bacillus lentus*, was supplied by Novozymes (Beijing, China). Poly (ethylene glycol) bis (carboxymethyl) ether (HOOC-PEG-COOH, MW600), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 98%), N-Hydroxysuccinimide (NHS, 98%) and Morpholinoethanesulfonic acid (MES, 99%) were purchased from Aladdin Reagent Company (Shanghai, China). All other chemicals were from Sinopharm Chemical Reagent Co.,Ltd. Wool fabric (gabardine, 56 × 56/128 × 68, 230 g m^{-2}) and wool fibers (24 μm) were provided by Wuxi Xiexin Woolen Textile Co.,Ltd.

Synthesis and characterization of the protease conjugate

Savinase was purified with ethanol. Then the enzyme solution was washed using ultrafiltration over centrifuge and was converted to powder by means of freezing dryer. A stock solution of MES was prepared at the concentration of 50 mmol L^{-1} in water and was adjusted to pH 5.5 with diluted HCl. Stock solutions of EDC/NHS were prepared by mixing EDC solution in water and NHS solution in DMF in the molar ratio of 1:1 and used immediately (Kaisersberger-Vincek et al. 2016).

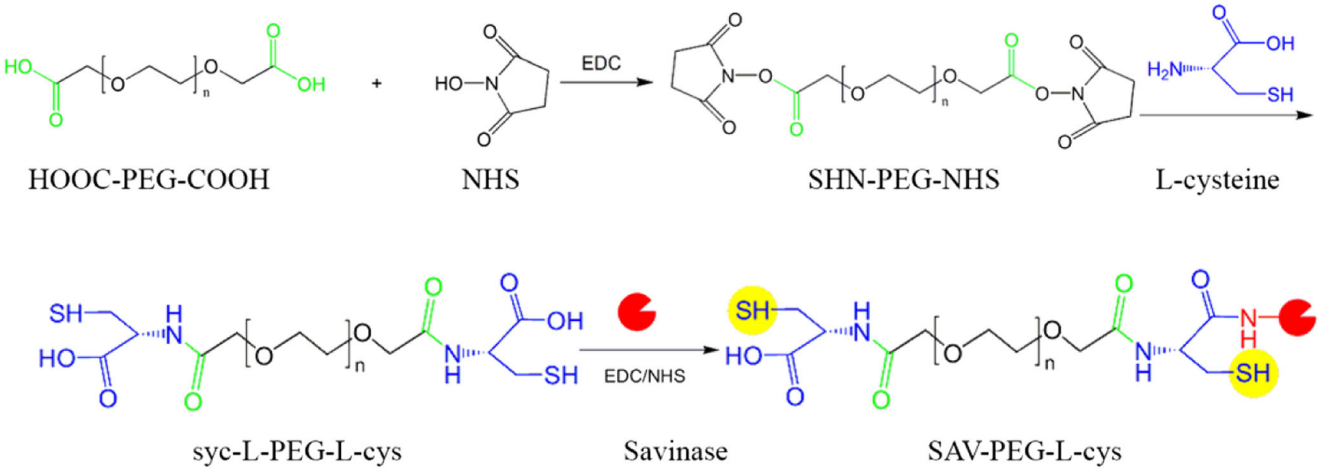
HOOC-PEG-COOH (1 mL) was added into the MES solution and then the EDC/NHS solution (4 M excess) was added and the mixture was magnetically stirred for 30 min at 37 °C. Afterwards, L-cysteine (3 M excess) was added and the mixed solution was magnetically stirred for 5 h at 37 °C in the dark. After that, Savinase (mole ratio (SAV:PEG) = 1:200) was put into the reaction mixture and was incubated for 16 h at 4 °C in the dark. The final product (SAV-PEG-L-cys) was obtained by repeatedly using ultrafiltration over centrifuge and the filtrate collected from the ultrafiltration tube was detected by color reaction to confirm that the HOOC-PEG-COOH and L-cysteine remained. In addition, the product (SAV+PEG+L-cys) was acquired with the above process without EDC/NHS in order to compare with the SAV-PEG-L-cys.

Size exclusion chromatography

Size exclusion chromatography (SEC) was used to determine the molecular weight distribution of the Savinase, SAV-PEG-L-cys, and SAV+PEG+L-cys. These proteases were filtered and investigated with an Ultrahydrogel™ Linear SEC column (Waters, USA). Buffer solution (pH 7.0) containing 50 mM sodium phosphate and 0.3 M NaCl was used as the eluent, with a flow rate of 0.5 mL min^{-1}. The size exclusion chromatography (SEC) profiles of the eluting proteins were determined at 214 nm with an UV–Vis detector connected to the SEC system (Waters).

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy of Savinase, SAV-PEG-L-cys, and SAV+PEG+L-cys was determined with a Nicolet IS 10 infrared spectrophotometer (Thermo Nicolet, USA) to identify changes in the structure of the protease. The spectra were collected over a range from 4000 to 500 cm^{-1}, at a resolution of 4 cm^{-1} and 32 scans per sample.



Scheme 1 The modification of protease with HOOC-PEG-COOH and L-cysteine

L-Cysteine quantification with Ellman's test

The quantification of L-cysteine in the modified protease was tested using Ellman's test (Moser et al. 2015): 500 μL of the corresponding samples (10 mg mL^{-1}) were merged with 5 mL of phosphate buffer (0.1 mol L^{-1} with 1 mmol L^{-1} EDTA, pH 8.0) and 100 μL of the Ellman's stock solution (4 mg mL^{-1}; phosphate buffer). The absorption spectrum was measured at hourly intervals with a UV-1800 Ultraviolet Spectrophotometer (Shimadzu, Japan) at room temperature in quartz cuvettes. The L-cysteine concentration was determined at the absorbance 410 nm. Stock solutions of the Ellman's reagent and L-cysteine were stable at least for 1 day and always freshly prepared.

Circular dichroism spectra

The secondary structures of Savinase and SAV-PEG-L-cys were assessed by a MOS-450 Circular Dichroism chromatograph (Biologic, France) with the condition that the scanning scope was 190–250 nm and the sweep bandwidth was 1 nm. Moreover, the relevant helix, strand, turns, and unordered were calculated according to the circular dichroism spectra.

Fluorescence spectrum

Tryptophan fluorescence was measured on a F4600 Fluorescence Spectrometer (Hitachi, Japan). The excitation wavelength was 280 nm. All experiments were carried out at 25 °C with protein concentration of 0.1 mg mL^{-1} in 0.1 M phosphate buffer.

Enzyme assay and kinetics of enzymatic reactions

The proteolytic activities of the native protease and modified protease were determined using casein as substrate. One unit of activity is defined as the amount of enzyme that hydrolyze casein to produce 1 μmol Tyrosine, per minute, at pH 8.0 and 40 °C. Sample (1 mL) was mixed with 1 mL of 1% casein in 40 mM Britton-Robinson buffer (Yu et al. 2009) (the mixture of phosphoric acid, acetic acid, and boric acid) adjusted to pH 8.0 by 0.2 M NaOH. After incubation for 10 min at 40 °C, the reaction was stopped by adding 2 mL 10% trichloroacetic acid and then centrifuged (10 min, 10,000 r min^{-1}). Supernatant (0.5 mL) was added to 2 mL of water and mixed. The absorbance at 275 nm was measured using a UV-1800 Ultraviolet Spectrophotometer (Shimadzu, Japan). Different pH, temperature, and concentrations of casein were used to investigate the effects of pH and temperature on enzymatic activity and kinetics of enzyme-catalyzed reactions.

Treatment of the wool fabrics

Before the treatment of wool fabrics, the activities of the different protease conjugates were determined against casein and then samples were diluted to give a final activity of 5 U mL^{-1} in the incubation. Untreated fabrics (12 × 12 cm, with the edgings cast off) were incubated in 0.1 M pH 8.0 Tris-HCl buffer at a fabric-to-solution weight ratio of 1:25 for 3 h at 40 °C. Finally, the enzyme in the incubation mixture was inactivated by raising the temperature to more than 85 °C for a further 10 min.

Dimensional stability to felting

The measurement of dimensional stability to washing of the enzymatic treated wool fabric was tested according to the Woolmark Test Method TM31: Washing of Wool Textile Products and new standard ISO 6330:2012. The samples were subjected to a 4G (corresponded to 7A in ISO 6330: 2000) wash cycle for relaxation shrinkage and 4 N (in accordance with 5A) wash cycles up to three times for felting shrinkage. Treatment and testing of the wool samples were performed in triplicate and the mean area after washing was calculated.

Tensile strength

The tensile strength of knitted wool fabric after enzyme treatment was measured using a HD026NS Electronic Fabric Strength Tester (Hongda, China). The procedure used was as described in BS EN ISO 13934-1:2013. The mean tensile strength was recorded to calculate the strength loss.

Scanning electron microscopy analysis

To determine the morphological change of wool samples, the scanning electron microscopy (SEM) micrographs were recorded using a SU1510 scanning electron microscope (Hitachi, Japan) under 5.00 kV at 3.0 K magnification. The surface of the wool fiber was coated with gold by vacuum evaporation.

Alkali solubility

The alkali solubility of wool fabric was evaluated using the standard method ASTM D1283-85 (Mojsov 2017). One gram of wool sample oven-dried at 105 °C for 15 min was weighed as original weight and then was incubated in a beaker containing 100 mL of 0.1 M sodium hydroxide at 65 °C for 1 h. The residues were filtered and rinsed six times with distilled water and dried in an oven at 105 °C for 1 h and weighted. Afterwards, the alkali solubility of the samples was calculated as a percentage of the original weight. Values are the average of three replicates.

Wettability

The wetting property of wool fabrics was evaluated by measuring the contact angles at room temperature, using a JC2000D4 contact angle meter (Zhongchen Digital Technic Co., China). A fixed steel needle injected a water droplet of 5 μL onto the wool fabrics' surface. The images were captured while the droplet fell on samples after 10 s by a SCA-20 real-time camera. Contact angles were measured at five different points for each sample and average reading was recorded.

Dyeability

Wool fabrics were immersed in 1% (o.w.f) Weak Acid Brilliant Red solution at pH 4.5 and a liquid ratio of 1:50 using a laboratory dyeing machine. The bath temperature was raised to 90 °C at an increase rate of 2 °C min^{-1} and held at this temperature for 1 h. The dyed fabrics were washed with distilled water and dried at 50 °C. The dye solutions before and after dyeing were diluted five times and their absorption spectra were measured with a UV-1800 Ultraviolet Spectrophotometer (Shimadzu, Japan) at room temperature in quartz cuvettes to calculate their percentage of dye uptake. The color parameters including color depth (K/S value), brightness (L*), red-green (a*) and yellow-blue (b*) for different dyed fabrics were determined using a Gretag Macbeth Color-Eye 7000A spectrophotometer (Datacolor, New Windsor, USA).

Tracking the protease with FITC

One milliliter of protease solution (30 mg mL^{-1}) was diluted in 10 mL 0.1 M pH 8.5 sodium borate buffer. After addition of 2 mL of fluorescein isothiocyanate (FITC) solution (5 mg mL^{-1}, dissolved in DMSO), the reaction mixture was shaken for 2 h in a water bath at 40 °C and 40 rpm. Unbound FITC was removed using a dialysis bag and ultrafiltration. Samples were frozen at − 20 °C overnight and freeze-dried for 48 h using a freeze dryer.

Wool fibers (20 mg) suspended in 10 mL 0.1 M pH 8.0 Tris-HCl buffer, were incubated with 1 mL of proteases labeled with FITC (2 mg mL^{-1}) and shaken for 3 h at 40 °C. After incubation, the fibers were embedded in a frozen section media and frozen. Then the frozen blocks were cut in slides of 10 μm thickness using a CM1950 microtome (Leica, Germany). Finally, the penetration of proteases into the cross-sectional samples was examined by an Axio Vert A1 fluorescence microscope (Zeiss, Germany).

Statistical analysis

All of the data are reported as mean ± standard deviation (SD). The statistical analysis was carried out using an Origin Pro 2016 software (Origin lab, USA) and statistical significance was considered at $p < 0.05$.

Results

Synthesis of the modified protease

Size exclusion chromatography analysis (detection at 214 nm) of the modified and native Savinase was performed in order to confirm that covalent coupling occurred. The elution profiles of different sample solutions are shown in Fig. 1. For the untreated Savinase, an individual peak fraction corresponding to an elution time of 19.93 min was detected, similar to the control group (SAV+PEG+L-cys) at 19.98 min. A slight shift to an earlier time was detected for the modified Savinase (SAV-PEG-L-cys, 19.49 min).

FT-IR spectroscopy was used to characterize the chemical structures of Savinase, the modified Savinase (SAV-PEG-L-cys) and the control group (SAV+PEG+L-cys) (Fig. 2). The spectra show characteristic absorption bands ascribed to the amide (–CONH) which have been labeled as amide A, amide I, amide II, and amide III bands. For the native Savinase, there

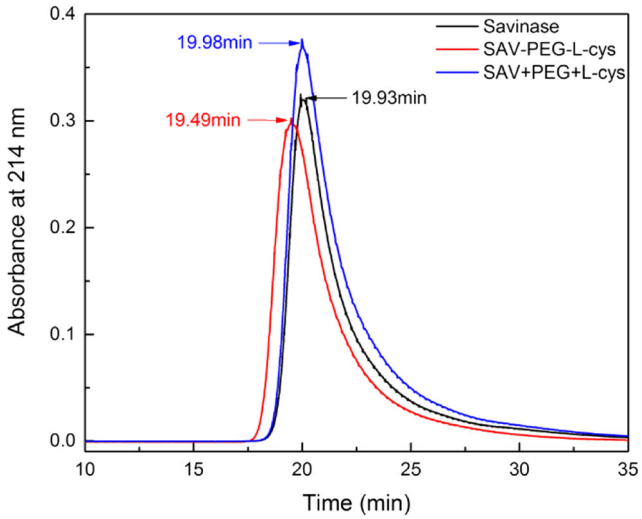


Fig. 1 Size exclusion chromatography of Savinase, the modified Savinase (SAV-PEG-L-cys) and the control group (SAV+PEG+L-cys)

are three characteristic peaks observed in the spectrum, that is, the amide I, II, and III peaks at 1652 cm^{-1}, 1537 cm^{-1}, and 1240 cm^{-1}. The control group (SAV+PEG+L-cys) appears a new peak at 1102 cm^{-1} and a slight blue shift of the amide I to 1656 cm^{-1}, while the modified protease (SAV-PEG-L-cys) not only displays the new peaks at 2361 cm^{-1} and 1105 cm^{-1}, but also slightly transfers the wavenumbers of amide I, II, and O-H stretching vibration peaks to 1659 cm^{-1}, 1543 cm^{-1}, and 3300 cm^{-1} compared with native protease.

Variation in structure of protease

The circular dichroism spectra were measured to observe whether the secondary structure of Savinase had been changed during the modification. Absorption in this region (240 nm and below) is due principally to the peptide bond; there is a weak but broad $n \rightarrow \pi^*$ transition centered around 220 nm and a more intense $\pi \rightarrow \pi^*$ transition around 190 nm (Kelly et al. 2005) (see Fig. S1). The circular dichroism (CD) spectra of native and modified Savinase both display an identical minimum value at 221 nm. The content of helix increases from 51.8 to 67.1%, while unordered slightly falls to 20.4% and the content of strand and turns also correspondingly decrease during modification, shown in Table S1.

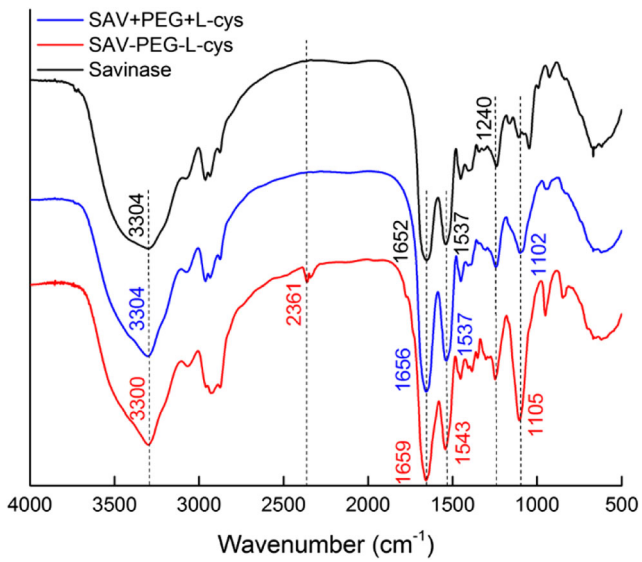


Fig. 2 FT-IR spectra of Savinase, modified Savinase (SAV-PEG-L-cys), and the control group (SAV+PEG+L-cys)

Protein containing aromatic amino acid (tryptophan, tyrosine, and phenylalanine) residues can generate fluorescence under the excitation light of 280 or 295 nm. Therefore, according to this feature, the tertiary structure changes of Savinase before and after modification can be judged by the spectral characteristics of these aromatic amino acids (Hosseinkhani et al. 2004). As illustrated in Fig. S2, the maximum emission wavelength of SAV-PEG-L-cys migrates to 347 nm in comparison with that of Savinase (344 nm) when the excited wavelength was 280 nm. Moreover, the maximum fluorescence intensity of native Savinase (2683) is higher than that of the modified Savinase (2386).

Characterization of enzymatic properties

The Michaelis constant (K_m) is a critical kinetic parameter for the evaluation of enzymatic reaction, which can indicate the affinity between enzyme and the substrate. The K_m value of Savinase increased from 15.21 to 40.23 g L^{-1} after modification and the maximum speed of enzymatic catalytic reaction (V_{max}) of SAV-PEG-L-cys (5.28×10^{-3} g L^{-1} S^{-1}) was about 2.5 times than that of native Savinase (2.18×10^{-3} g L^{-1} S^{-1}).

The influence of pH values on the activities of Savinase and SAV-PEG-L-cys was investigated by using Britton-Robinson buffer ranging from 2.0 to 11.0 (Fig. 3a). The activity curves of Savinase and SAV-PEG-L-cys both have a bell-type trend under the change of pH value. Furthermore, no matter whether modified or not, Savinase has good alkali resistance and a maximum enzymatic activity at pH 8.0. As shown in Fig. 3b, the enzymatic activity curves of Savinase and SAV-PEG-L-cys present bell-type trends as the temperature goes up, and reach the maximum value at 40 °C.

Reducibility and localization of the modified protease

The reducibility of modified protease was tested with Ellman's reagent shown in Fig. 4. The newly formed absorption band at 410 nm originates from the release of the 2-nitro-5-thiobenzoate anion (NTB^{2-}) upon reaction of the Ellman's reagent ($DTNB^{2-}$) with the thiolate anion of L-cysteine (Moser et al. 2015). There are a few absorptions of Savinase and the control group (SAV+PEG+L-cys) at 410 nm, while a sharp increase of absorption intensity at 410 nm of the modified Savinase (SAV-PEG-L-cys) occurs. Nevertheless, the

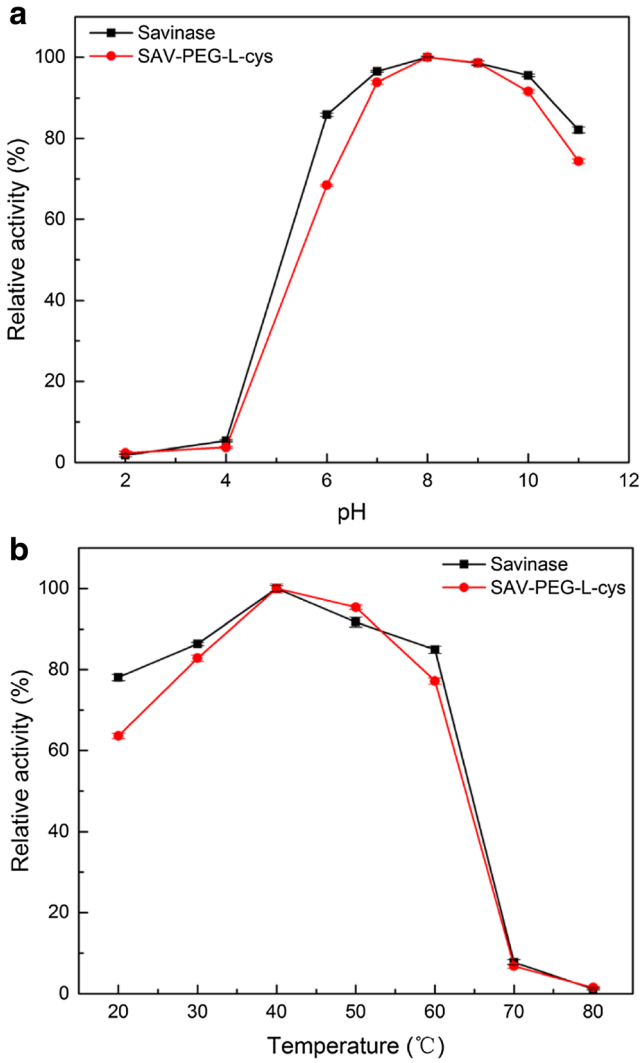


Fig. 3 Influence of pH and temperature on the activity and stability of proteases. **a** pH-activity profile of native and modified Savinase. The maximum activity at individual pH was taken as 100%. **b** Temperature-activity profile of native and modified Savinase. The original activity without incubation was taken as 100%. The residual activity was measured under assay condition. The given values are mean values of three independent determinations

content of L-cysteine in the modified Savinase (SAV-PEG-L-cys) is 4.09 mg g^{-1} according to the calibration curve of the Ellman's reagent for L-cysteine at 410 nm (Fig. 4a).

The proteases labeled with FITC were tracked and photographed (Fig. 5) using a fluorescence microscope to observe the localization of proteases. The original wool fibers (Fig. 5a) have weak fluorescence as a consequence of the aromatic amino acid (tryptophan, tyrosine, and phenylalanine). While, the wool fibers treated with the FITC-labeled proteases show strong fluorescence. The wool fibers treated with inactivated proteases (Fig. 5b, c) were taken to observe the location of non-hydrolysis proteases. The inactivated Savinase (Fig. 5b) can diffuse into wool fibers through CMC, while the inactivated modified Savinase (Fig. 5c) appears to remain on the surface of the fibers. Moreover, the wool fibers treated with activated proteases (Fig. 5d, e) were taken to observe the degree of damage causing by protease. Figure 5d (Savinase) presents fluorescence both outside and inside the wool fibers, instead, Fig. 5e (SAV-PEG-L-cys) exhibits local fluorescence on the surface of fibers.

Wool treatment

The SEM images displayed in Fig. 6 show to observe the influence of different treatments on the surface feature of wool fiber. The scales of original sample (Fig. 6a) are complete and the outline of the edge is very clear. In respect of control sample (Fig. 6b), the scales of the fibers do not change much and are merely slightly damaged on the edge, indicating that the alkaline buffer causes less harm to the wool which is consistent with the conclusions of above strength experiments. Parts of the scale margins are tilted and the wool fibers suffer damage after being treated with Savinase (Fig. 6c) and L-cys + Savinase (Fig. 6d). However, the surface of the fiber treated by the modified Savinase (Fig. 6e) is smooth and some scales are obviously removed.

The dimensional stability to felting is an important index to evaluate the effect of the anti-felting finishing. Figure 7 presents that the fabric treated with SAV-PEG-L-cys (6.12%) for 3 h obtains a best anti-shrinking effect. The dimensional stability to felting of the fabric treated with Savinase (9.3%) is less than that of control fabric (10.97%), while higher than that of the fabric treated with L-cys + Savinase (8.76%).

Tensile strength loss (Fig. 7) and alkali solubility (Table 1) of wool fabrics were tested to estimate the damage to the main part of wool fiber caused by different treatments. The control fabric causes 7.14% strength loss shown in Fig. 7. However, the strength loss of the fabric treated with SAV-PEG-L-cys (11.7%) is lower than that of the fabric treated with Savinase (14.35%) and L-cys + Savinase (18.55%). The alkali solubility exhibits the same tendency presented in Table 1.

The contact angle of original sample is 118.9 ° presented in Table 1, indicating that the wool surface is hydrophobic due to the existence of the hydrophobic lipid layer on the surface of wool fiber. The slight decrease in the contact angle of the control sample was due to the alkaline buffer solution (pH 8.0) having a certain dissolution effect to the lipid layer. The contact angle of the fabric treated with Savinase is similar to that of the control sample. However, the contact angle of L-cys + Savinase and SAV-PEG-L-cys decreased significantly to 101.6 ° and 108.2 °, respectively.

The percentage of dye uptake was calculated according to the absorbance (Fig. S4) and shown in Table 2. Meanwhile, other dyeing properties including K/S value, brightness (L*), red-green (a*) and yellow-blue (b*) were also tested and listed in Table 2. It is obvious that the percentage of dye

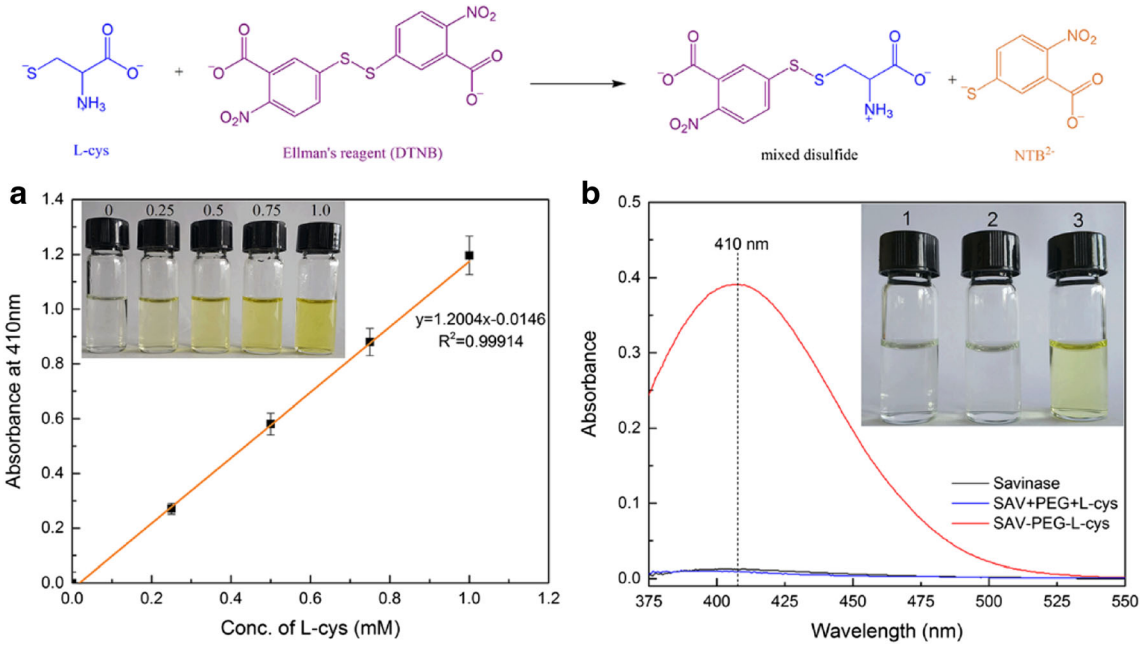


Fig. 4 Above: possible reactions of the Ellman's test with L-cysteine. **a** The calibration curve of the Ellman's reagent for L-cysteine at 410 nm. Inset: the solution of L-cysteine with different concentration reacted with Ellman's reagent (DTNB). **b** Spectrum: the wavelength–absorbance of proteases reacted with Ellman's reagent. Inset: the solution of proteases reacted with Ellman's reagent (DTNB): 1 Savinase; 2 the control group (SAV+PEG+L-cys); 3 the modified Savinase (SAV-PEG-L-cys)

uptake of the fabric treated with L-cys + Savinase is the maximum, while the percentage of dye uptake of the fabric treated with SAV-PEG-L-cys is higher than that of Savinase, and K/S value also follows the same trend.

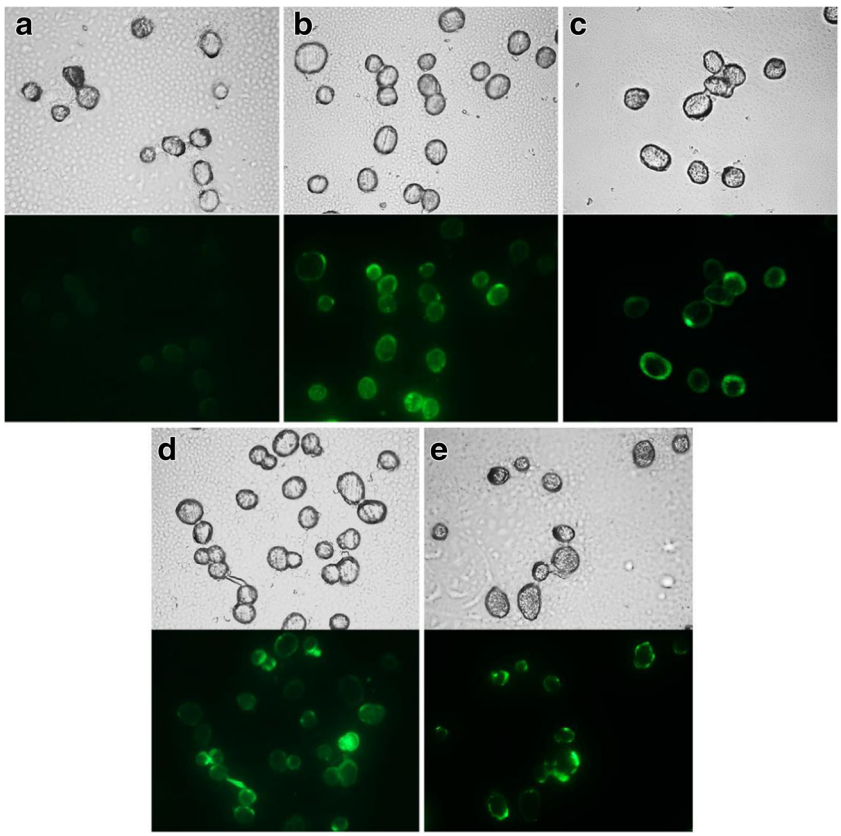


Fig. 5 Fluorescence microscopy images of cross sections of wool fibers treated with FITC-labeled Savinase. **a** Without Savinase (original wool); **b** inactivated Savinase; **c** inactivated modified Savinase; **d** Savinase; **e** modified Savinase. Proteases were inactivated under 80 °C for 10 min

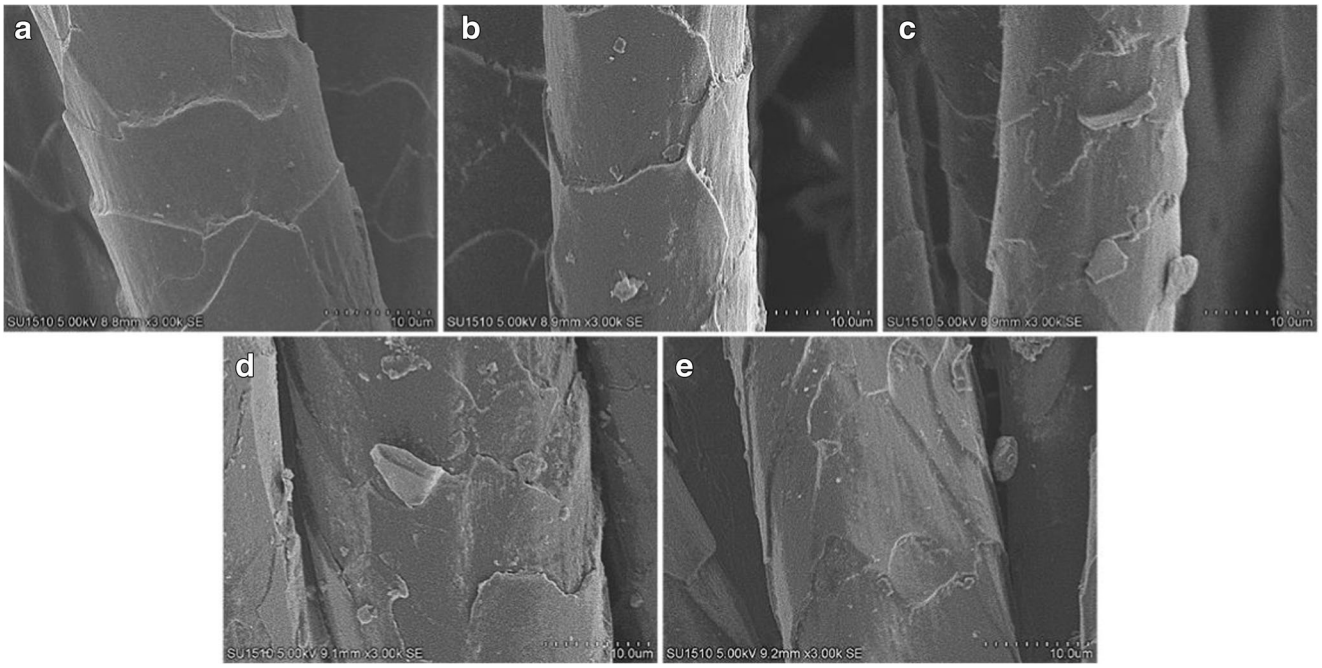


Fig. 6 SEM images of wool fabrics after different treatments for 3 h. **a** Original sample; **b** control sample (without Savinase); **c** Savinase; **d** L-cys + Savinase (mix L-cysteine equivalent to SAV-PEG-L-cys with Savinase); **e** modified Savinase (SAV-PEG-L-cys)

Discussion

A slight shift to an earlier time at 19.49 min in Fig. 1 was detected for the modified Savinase. The higher molecular weight of the protease, the earlier it elutes, indicating the generation of cross-linked conjugates of protein and HOOC-PEG-COOH. As the spectra shown in Fig. 2, an absorption peak at around 3303 cm^{-1} is assigned to N–H stretching (amide A). A strong absorption peak at 1651–1659 cm^{-1} should be ascribed to the C=O (amide I). A medium strong peak is observed at 1536–1543 cm^{-1} and assigned to C–N stretching and N–H in-plane bending vibrations (amide II), while the weak band at 1239 cm^{-1} is related to the C–N and C–O stretching vibrations (amide III) (Wang et al. 2016). In addition, the wavenumber at 2361 cm^{-1} and 1100 cm^{-1} represent S–H stretching vibration and -C-O-C- stretching vibration, respectively. The control group (SAV+PEG+L-cys) appears a new peak at 1102 cm^{-1} and a slight blue shift of the amide I to 1656 cm^{-1} in contrast with Savinase, resulting from a small quantity of HOOC-PEG-COOH adsorbed on Savinase by hydrogen bonds. However, SAV-PEG-L-cys not only displays the new peaks at 2361 cm^{-1} and 1105 cm^{-1}, but also slightly transfers the wavenumbers of amide I, II, and O–H stretching vibration peaks to 1659 cm^{-1}, 1543 cm^{-1}, and 3300 cm^{-1} compared with native protease, which demonstrates that the Savinase was successfully linked to HOOC-PEG-COOH and L-cysteine through covalent bonding.

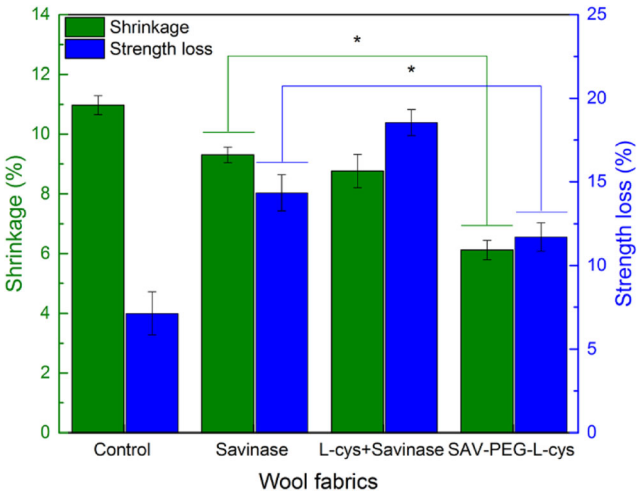
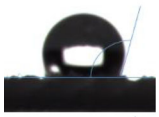
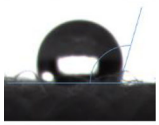


Fig. 7 Dimensional stability to felting after three washing cycles and strength loss of wool fabrics. The fabrics were incubated in pH 8.0 Tris-HCl buffer with different Savinase for 3 h. Control (without Savinase); Savinase; L-cys + Savinase (mix L-cysteine equivalent to SAV-PEG-L-cys with Savinase); modified Savinase (SAV-PEG-L-cys). The given values are mean values of three independent determinations (*asterisk* indicates significant difference comparing Savinase with SAV-PEG-L-cys, $p < 0.05$)

As illustrated in Fig. S1 and Table S1, there is a little difference between Savinase and modified Savinase (SAV-PEG-L-cys). This fact suggests that the secondary structure of Savinase had minor change after modification. The reason why the maximum emission wavelength and fluorescence intensity of Savinase change after modification presented in Fig. S2 could be that the fluorescent aromatic amino acids are partly embedded due to the closer structure of the protease modified by the HOOC-PEG-COOH and L-cysteine. In summary, the secondary and tertiary structure of Savinase changed less after modification with HOOC-PEG-COOH and L-cysteine.

Table 1 The alkali solubility and wettability of wool fabrics treated with different treatments

Samples	Alkali solubility (%)	Contact angle (°)	
Original sample	4.60 ± 0.12	118.9 ± 1.1	
Control sample	4.81 ± 0.09	115.4 ± 1.7	
Savinase	5.53 ± 0.07	112.6 ± 1.3	
L-cys+Savinase	5.83 ± 0.08	101.6 ± 2.1	
SAV-PEG-L-cys	5.34 ± 0.11	108.2 ± 1.0	

The K_m values show that the affinity of Savinase for the substrate decreased after modification, probably causing by the more compact structure of Savinase after connected with L-cysteine and HOOC-PEG-COOH, which may prevent the active center of Savinase from the substrate under the steric hindrance. The modified Savinase with the same protein concentration still remained 90.82% of the enzymatic activity under the optimum temperature (40 °C and pH 8.0), which was superior to the reported modified protease (Smith et al. 2010b; Chen et al. 2012; Zhu et al. 2011). The above tests show that there was no prominent change of the enzymatic properties during modification and the modified protease still maintained good hydrolysis of peptide bonds.

As shown in Fig. 4, L-cysteine can react with DTNB by reducing its disulfide bonds to generate yellow NTB^{2-} with maximum absorption at 410 nm. Moreover, the greater the concentration of L-cysteine is, the deeper the color of the solution becomes and the greater the absorbance is. However, only did the modified protease represent obvious characteristic color (yellow) and maximum absorption after the three proteases reacted with DTNB in Fig. 4b, making it clear that L-cysteine was covalently linked to Savinase to endow the modified protease with "reducibility."

The wool fibers treated with inactivated proteases (Fig. 5b, c) were taken to observe the location of non-hydrolysis proteases. The inactivated Savinase (Fig. 5b) can diffuse into wool fibers through CMC due to their relatively small size (approximately 3.6 nm according to Fig. S3), while the inactivated modified Savinase (Fig. 5c) appears to remain on the surface of the fibers owing to its treble size of native protease proved by grain-size graph presented in supplementary material. Therefore, Fig. 5d (Savinase) presents fluorescence both outside and inside the wool fibers because Savinase can penetrate fiber cortex causing the degradation of the external and internal parts of wool structure. Figure 5e exhibits local fluorescence, revealing the fact that the modified Savinase can selectively hydrolyze the wool scales and cause little damage to interior fiber because it focused on the scale rich in keratin by means of L-cysteine reducing disulfide bonds and protease hydrolyzing peptide bonds simultaneously. A similar effect of limiting the hydrolysis focus on the wool surface scales had been reported before (Araújo et al. 2009; Schroeder et al. 2009). Above all, it can be concluded from the above results that the modified protease possessed "reducibility" for disulfide bonds and "localization" that was limiting the hydrolysis only at the wool scales.

The SEM images in Fig. 6 show that the scales of the fiber treated with Savinase (Fig. 6c, d) are tilted and turn up but do not fall off, while those treated with the modified protease (Fig. 6e) are not turned up but passivated. It is because that protease would preferentially attack cell membrane complex (CMC), the cementing substance between the scale, and cortex of wool fiber, while the modified protease could not access CMC and was restricted on the surface of scale on account of enlarged steric hindrance (Schroeder et al. 2006) and larger volume of protease as the result of the incorporation of PEG

Table 2 The dyeability of wool fabrics undergoing different treatments

Samples	Percentage of dye uptake (%)	L*	a*	b*	K/S value	Color
Original sample	43.63 ± 0.51	47.6	54.92	0.04	7.79 ± 0.22	
Control sample	49.07 ± 0.65	46.28	54.51	0.5	8.68 ± 0.13	
Savinase	53.51 ± 1.07	44.68	55.44	1.25	10.19 ± 0.17	
L-cys+Savinase	60.80 ± 0.93	43.98	55.39	1.59	10.77 ± 0.24	
SAV-PEG-L-cys	57.08 ± 0.81	44.41	55.26	1.38	10.42 ± 0.19	

molecules. The result further demonstrated that modified protease had "localization" focusing on the scales of wool. Moreover, the scales of wool fiber treated with SAV-PEG-L-cys present passivated edges and become smoother than other treatments to acquire lower coefficients of friction, resulting in its preferable anti-felting effect.

Figure 7 presents that the fabric treated with SAV-PEG-L-cys (6.12%) for 3 h obtains a best anti-shrinking effect and passes the Woolmark machine-washable standard in comparison with other wool samples. Though other samples do not come up to the standard, the dimensional stability to felting of the fabric treated with Savinase (9.3%) is less than that of control fabric (10.97%) because proteases can destroy the wool fibers to a certain extent. While, the fabric treated with L-cys + Savinase (8.76%) acquires a better anti-felting effect than that with Savinase, leading to more serious damage to wool fibers. Besides, the strength loss of the fabric treated by the mixture of L-cysteine and Savinase is the highest among these fabrics. It is due to the fact that L-cysteine can break up disulfide bonds in keratinized proteins to help Savinase further remove the scales so that the joint effect was stronger than single Savinase. Nevertheless, contrasting the fabric treated by SAV-PEG-L-cys with L-cys + Savinase under the same content of L-cysteine and Savinase, a conclusion could be drawn that the damage caused by SAV-PEG-L-cys was restrained on account of enlarged steric hindrance and size of protease due to the incorporation of PEG molecular. The alkali solubility of wool fabrics undergoing different treatments in Table 1 draws the same conclusion as above-mentioned strength loss. It further confirmed that the modified protease caused less damage to the main part of wool fibers than Savinase and L-cys + Savinase.

Wool is a kind of protein fiber with strong hydrophobicity that is determined by the fiber surface properties (hydrophobic lipid). Therefore, the wettability (see Table 1) of wool fabrics was measured to know how serious the damage to the lipid layer and scale structure is. The contact angle of the fabric treated with Savinase is similar to that of the control sample. This was due to the fact that the protease had no destructive effect on the lipid layer, and the presence of the lipid layer also hindered the action of the protease on the wool scale. The contact angle of the modified protease decreased significantly to 108.2°, because L-cysteine can break the disulfide bond (-S-

S-) in the scale layer and protease can hydrolyze peptide bonds to degrade the scales as well as a part of the lipid layer.

The scale layer has some hindrance to dye when wool dyes, so the dyeability of wool fabrics can also reveal the destruction of the scales. It is distinct from Table 2 that the percentage of dye uptake of the fabric treated with L-cys + Savinase is the maximum, while the percentage of dye uptake of the fabric treated with SAV-PEG-L-cys is higher than that of Savinase, and K/S value also follows the same trend. This demonstrated that the damage to scale caused by the modified protease is superior to that of native protease because protease would preferentially attack CMC or penetrate to hydrolyze cortex, while the modified protease just focused on hydrolyzing scale, supporting by Fig. 5. Moreover, though the fabric treated with L-cys + Savinase has preferable wettability and dyeability, in fact, the mixture of L-cysteine and Savinase caused larger damage to the scales and cortex, leading to higher dimensional stability to felting and strength loss than SAV-PEG-L-cys, based on the above-mentioned results. Therefore, we can conclude from the above experimental results that the modified protease focused on hydrolyzing wool scale, caused less damage to cortex and acquired better anti-felting effect than native protease. In addition, the anti-felting effect merely treated by SAV-PEG-L-cys, without pretreatment process, was equivalent to that of the modified protease in reported studies (Silva et al. 2005; Schroeder et al. 2009; Araújo et al. 2009; Smith et al. 2010b) after comprehensive evaluation.

Proteases can play an important part in the development of novel technologies for wool anti-felting treatment. Especially, modified proteases have been described as a promising alternative for the conventional chlorination used in wool anti-felting finishing, because they can effectively remove wool scales and can be an ecological and gentle treatment. In this study, we have developed a "trifunctional protease" with reducibility, localization, and hydrolysis, which makes a breakthrough in decomposing the keratin, and promises as an ecological alternative to the traditional highly polluting chlorination, used in textile industry. In addition, this protease has great potential value for other fabrics rich in keratin like feather, camel hair, and rabbit hair.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

Araújo, R., Silva, C., Machado, R., Casal, M., Cunha, A. M (2009) Proteolytic enzyme engineering: a tool for wool. Biomacromolecules 10(6):1655–1661 doi: https://doi.org/10.1021/bm9002943

Chen R, Yuan J, Yu Y, Fan X, Wang Q, Zhu Y (2012) Preparation of chitosan conjugated protease used for shrink resistance of wool and its properties. J food Sci Biotechnolo 31(5):63–68. https://doi.org/10.13560/j.cnki.biotech.bull.1985.2012.02.030

Fu J, Su J, Wang P, Yu Y, Wang Q, Cavaco-Paulo A (2015) Enzymatic processing of protein-based fibers. Appl Microbiol Biotechnol 99(24):10387–10397. https://doi.org/10.1007/s00253-015-6970-x

Gaffar Hossain KM, Juan AR, Tzanov T (2009) Simultaneous protease and transglutaminase treatment for shrink resistance of wool. Biocatal Biotransform 26(5):405–411. https://doi.org/10.1080/10242420802364940

Hosseinkhani S, Ranjbar B, Naderi-Manesh H, Nemat-Gorgani M (2004) Chemical modification of glucose oxidase: possible formation of molten globule-like intermediate structure. FEBS Lett 561(1–3): 213–216. https://doi.org/10.1016/s0014-5793(04)00134-6

Ibrahim NA, El-Shafei HA, Abdel-Aziz MS, Ghaly MF, Eid BM, Hamed AA (2012) The potential use of alkaline protease from *Streptomyces albidoflavus* as an eco-friendly wool modifier. J Text Inst 103(5): 490–498. https://doi.org/10.1080/00405000.2011.588417

Jus S, Schroeder M, Guebitz GM, Heine E, Kokol V (2007) The influence of enzymatic treatment on wool fibre properties using PEG-modified proteases. Enzyme Microb Technol 40(7):1705–1711. https://doi.org/10.1016/j.enzmictec.2006.10.001

Mojsov K (2017) Enzymatic treatment of wool fabrics - opportunity of the improvement on some physical and chemical properties of the fabrics. J Text Inst 108(7):1136–1143. https://doi.org/10.1080/00405000.2016.1222856

Kaisersberger-Vincek M, Štrancar J, Kokol V (2016) Antibacterial activity of chemically versus enzymatic functionalized wool with ε-poly-L-lysine. Textile Res J 87(13):1604–1619. https://doi.org/10.1177/0040517516657060

Kaur A, Chakraborty JN, Dubey KK (2016) Enzymatic functionalization of wool for felting shrink-resistance. J Nat Fibers 13(4):437–450. https://doi.org/10.1080/15440478.2015.1043686

Kelly SM, Jess TJ, Price NC (2005) How to study proteins by circular dichroism. Biochim Biophys Acta 1751(2):119–139. https://doi.org/10.1016/j.bbapap.2005.06.005

Kynclova E, Elsner E, Kopf A, Hawa G, Schalkhammer T, Fritz P (1996) Novel method for coupling of poly(ethyleneglycol) to carboxylic acid moieties of proteins. J Mol Recognit 9(5–6):644–651. https://doi.org/10.1002/(SICI)1099-1352(199634/12)9:5/6<644::AID-JMR314>3.0.CO;2-7

Kantouch A, Bendak A, Sadek M (1978) Studies on the shrink resist treatment of wool with potassium permanganate. Text Res J 48(11):619–624. https://doi.org/10.1177/004051757804801101

Liu B, Zhang J, Li B, Liao X, Du G, Chen J (2013) Expression and characterization of extreme alkaline, oxidation-resistant keratinase from *Bacillus licheniformis* in recombinant *Bacillus subtilis* WB600 expression system and its application in wool fiber processing. World J Microb Biot 29(5):825–832. https://doi.org/10.1007/s11274-012-1237-5

Moser M, Behnke T, Hamers-Allin C, Klein-Hartwig K, Falkenhagen J, Resch-Genger U (2015) Quantification of PEG-maleimide ligands and coupling efficiencies on nanoparticles with Ellman's reagent. Anal Chem 87(18):9376–9383. https://doi.org/10.1021/acs.analchem.5b02173

Roberts MJ, Bentley MD, Harris JM (2012) Chemistry for peptide and protein PEGylation. Adv Drug Deliver Rev 64:116–127. https://doi.org/10.1016/j.addr.2012.09.025

Schroeder M, Lenting HB, Kandelbauer A, Silva CJ, Cavaco-Paulo A, Gubitz GM (2006) Restricting detergent protease action to surface of protein fibres by chemical modification. Appl Microbiol Biotechnol 72(4):738–744. https://doi.org/10.1007/s00253-006-0352-3

Schroeder M, Schweitzer M, Lenting HBM, Guebitz GM (2009) Chemical modification of proteases for wool cuticle scale removal. Biocatal Biotransfor 22(5–6):299–305. https://doi.org/10.1080/10242420400025794

Shen J, Rushforth M, Cavaco-Paulo A, Guebitz G, Lenting H (2007) Development and industrialisation of enzymatic shrink-resist process based on modified proteases for wool machine washability. Enzyme Microb Tech 40(7):1656–1661. https://doi.org/10.1016/j.enzmictec.2006.07.034

Shen J, Smith E, Chizyuka M, Prajapati C (2017) Development of durable shrink-resist coating of wool with sol-gel polymer processing. Fiber Polym 18(9):1769–1779. https://doi.org/10.1007/s12221-017-7294-5

Silva CJSM, Gübitz G, Cavaco-Paulo A (2006a) Optimisation of a serine protease coupling to Eudragit S-100 by experimental design techniques. J Chem Technol Biot 81(1):8–16. https://doi.org/10.1002/jctb.1350

Silva CJSM, Prabaharan M, Gübitz G, Cavaco-Paulo A (2005) Treatment of wool fibres with subtilisin and subtilisin-PEG. Enzyme Microb Tech 36(7):917–922. https://doi.org/10.1016/j.enzmictec.2005.01.017

Silva CJSM, Zhang Q, Shen J, Cavaco-Paulo A (2006b) Immobilization of proteases with a water soluble–insoluble reversible polymer for treatment of wool. Enzyme Microb Tech 39(4):634–640. https://doi.org/10.1016/j.enzmictec.2005.11.016

Smith E, Farrand B, Shen J (2010a) The removal of lipid from the surface of wool to promote the subsequent enzymatic process with modified protease for wool shrink resistance. Biocatal Biotransfor 28(5–6):329–338. https://doi.org/10.3109/10242422.2010.530543

Smith E, Schroeder M, Guebitz G, Shen J (2010b) Covalent bonding of protease to different sized enteric polymers and their potential use in wool processing. Enzyme Microb Tech 47(3):105–111. https://doi.org/10.1016/j.enzmictec.2010.05.011

Smith E, Shen J (2011) Surface modification of wool with protease extracted polypeptides. J Biotechnol 156(2):134–140. https://doi.org/10.1016/j.jbiotec.2011.08.012

Smith E, Shen J (2012) Enzymatic treatment of wool pre-treated with cetyltrimethylammonium bromide to achieve machine washability. Biocatal Biotransfor 30(1):38–47. https://doi.org/10.3109/10242422.2012.644445

Smith E, Zhang Q, Shen J, Schroeder M, Silva C (2009) Modification of Esperase® by covalent bonding to Eudragit® polymers L 100 and S 100 for wool fibre surface treatment. Biocatal Biotransfor 26(5):391–398. https://doi.org/10.1080/10242420802249521

Vílchez S, Jovančić P, Erra P (2010) Influence of chitosan on the effects of proteases on wool fibers. Fiber Polym 11(1):28–35. https://doi.org/10.1007/s12221-010-0028-6

Wang K, Li R, Ma JH, Jian YK, Che JN (2016) Extracting keratin from wool by using L-cysteine. Green Chem 18(2):476–481. https://doi.org/10.1039/c5gc01254f

Wang P, Yuan J, Ren X, Cui L, Wang Q, Fan X (2013) Bio-antifelting of wool based on mild methanolic potassium hydroxide pretreatment. Eng Life Sci 13(1):102–108. https://doi.org/10.1002/elsc.201200015

Yu F, Du P, Lei X, Zhang S (2009) Investigation of voltammetric enzyme-linked immunoassay system based on *N*-heterocyclic substrate of 2,3-diaminopyridine. Talanta 78(4–5):1395–1400. https://doi.org/10.1016/j.talanta.2009.02.031

Zhu Y, Fan X, Wang Q, Chen R, Yu Y, Yuan J (2011) Chemical modification of Savinase by dextran and its properties. China Biotechnol 31(10):45–49 doi: https://doi.org/10.13523/j.cb.20111009