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Collagen and Leather

RESEARCH

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Effects of bactericide–protease interactions on the protease-assisted soaking performance

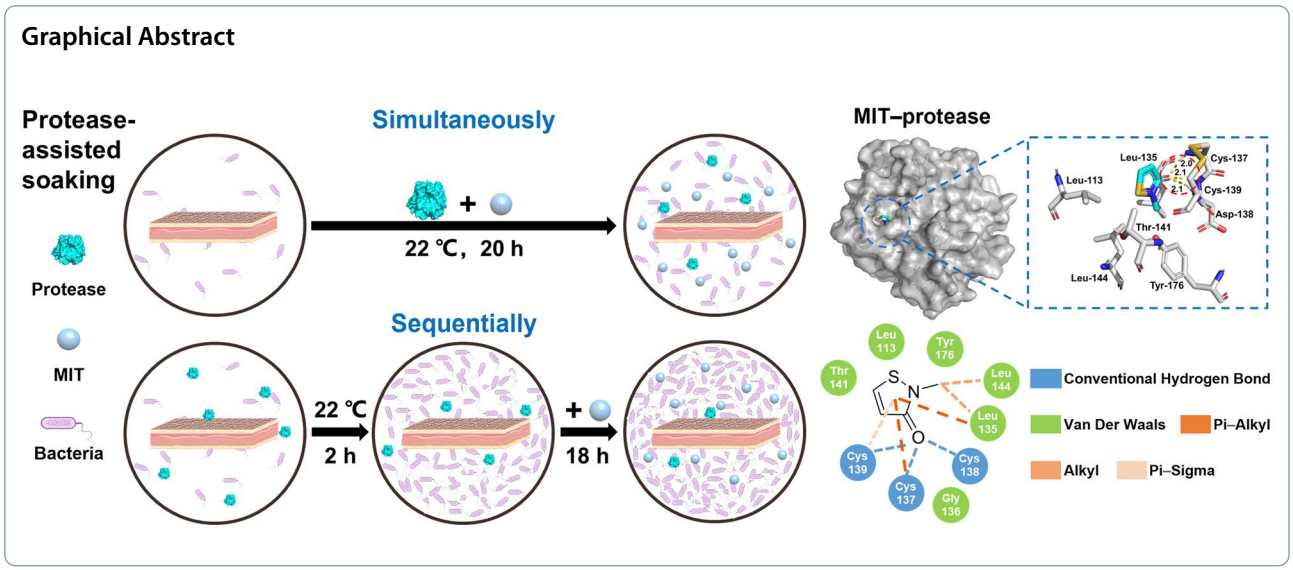
Hao Liu[1], Yahang Pan[2], Bi Shi[1,2] and Yunhang Zeng[1,2*]

Abstract

Protease-assisted soaking has received increasing attention in recent years. However, few reports have elaborated on the effect of bactericides, which are used to protect raw hides from microbial damage in the soaking process, on the performance of protease-assisted soaking. Here we investigate the effects of three bactericides, namely, 2-methyl-4-isothiazolin-3-one (MIT), sodium propyl 4-hydroxybenzoate (SPHB) and cetyl trimethyl ammonium bromide (CTAB), on the catalytic activity of protease. MIT and SPHB have little effect on the proteolytic activity, whilst CTAB has a negative effect. Fluorescence spectroscopy, synchronous fluorescence spectroscopy, molecular docking and molecular dynamics simulation were used to analyse the bactericide–protease interaction. The data reveal that MIT and SPHB are bound to the non-catalytic sites of protease, whilst CTAB affects the catalytic triad of protease. Furthermore, the protease and bactericides were used alone, simultaneously and sequentially in the soaking process, and their soaking performances were evaluated. The evaluation shows that the use of protease increases the microorganisms in the soaking float, and MIT exhibits the best bactericidal effect. The simultaneous use of protease and MIT effectively inhibits bacteria and scarcely affects the removal of unstructured proteins from hides and the attack on epidermis by protease. These findings contribute to a better understanding of the scientific use of protease with other auxiliaries in soaking.

Keywords Protease, Bactericide, Leather, Soaking

*Correspondence:
Yunhang Zeng
zengyunhang@scu.edu.cn
Full list of author information is available at the end of the article



1 Introduction

Soaking is an important precursor to the whole leather-making process [1, 2]. The main purpose of soaking is to clean the dirt and blood, rehydrate the raw hide and remove unstructured proteins [3]. However, the soaking procedure usually takes 12–48 h for ideal effectiveness. Therefore, auxiliaries, such as sodium carbonate, surfactants and enzymes, are often used to shorten the soaking time [4]. Moreover, bactericides are always used in the soaking to inhibit the growth of microorganisms [5]. The reason is that the raw hide, which is rich in protein and fat, is particularly susceptible to microbial damage [6–8]. This susceptibility will result in leathers with grain damage, loose grain or an empty handle.

Enzymes have received increased attention as environmentally friendly and efficient biocatalysts used for a clean leather-making process in recent years [9–11]. In the soaking process, proteases have also been gradually used to reduce the soaking time by accelerating the removal of unstructured proteins and attacking the epidermis of raw hides [12–14]. However, the protease is sensitive to environmental temperature, pH and some chemicals [15]. The soaking conditions, such as room temperature and pH 8–10 regulated by sodium carbonate, commonly have little effect on neutral and alkaline proteases that are chosen for soaking process. In addition, nonionic surfactants used for soaking barely affect the protease activity. However, the effect of bactericides that protect hides from microbial damage on the performance of protease-assisted soaking remains unclear. Tanners generally consider that bactericide is likely to affect the activity of protease and the performance of protease-assisted soaking given that bactericides can affect the structure and functions of microorganisms and inhibit their growth [16, 17]. Thus, they usually use protease and bactericide separately in the soaking. However, the interactions between bactericide and microorganisms are different from the interaction between bactericide and protease. Some bactericides may not affect the structure and activity of protease. Protease can accelerate the removal of unstructured proteins from raw hides and thus provide nutrients for the growth of microorganisms. Thus, using protease and bactericide separately may not be conducive to bacterial inhibition and is probably not the best way to perform the enzyme-assisted soaking. Therefore, understanding the effect of bactericides on the protease action on the hide and the performance of protease-assisted soaking is important for a rational design of the enzyme-assisted soaking operation.

Gram-positive bacteria play a major role in the putrefaction of raw hides [18]. 2-Methyl-4-isothiazolin-3-one (MIT), sodium propyl 4-hydroxybenzoate (SPHB) and cetyl trimethyl ammonium bromide (CTAB) have been reported to show inhibitory effects on the bacteria [19, 20]. The chemical structures of the three bactericides are shown in Additional file 1: Fig. S1. MIT, which is one of the main components of industrial bactericides, has the advantage of being broad-spectrum antibacterial and is widely used in the leather industry [21]. The electron-deficient N–S bond of MIT is susceptible to a nucleophilic reaction with sulfhydryl groups of microbial proteins, which can impair microbial functions and lead to cellular growth inhibition or cell death [22]. SPHB can inhibit bacteria, yeast and mould and is widely used in the food processing industry, because it can inhibit the synthesis of folic acid, which is necessary for DNA and

cell replication [23, 24]. CTAB is used as a bactericidal agent and an algaecide in the field of water treatment by compromising the integrity of the plasma membrane [24–26].

In this study, MIT, SPHB and CTAB were used to investigate the effects of bactericides on the properties of soaking protease such as catalytic activity, surface charge and practical size. Subsequently, the interactions between the three bactericides and protease were analysed by fluorescence spectroscopy, synchronous fluorescence spectroscopy, molecular docking and molecular dynamics (MD) simulation. Furthermore, the protease and bactericides were used alone, simultaneously and sequentially in the soaking process. Their soaking performances were evaluated by measuring the total microorganism number, the protein concentration and the hydroxyproline concentration of soaking float. The epidermis, collagen fibre and rehydration of soaked hides were also observed for this evaluation. Thus, this study provides scientific guidance for the design of reasonable enzyme-assisted soaking process.

2 Experimental

2.1 Materials

Protease from *Bacillus* sp. (serine protease; product number P0029) and MIT were purchased from Sigma–Aldrich Co., LLC (USA). SPHB was purchased from Shanghai Adamas-beta Reagent Co., Ltd. (China). CTAB was purchased from Chengdu Kelong Chemical Reagent Co., Ltd. (China). Salted cattle hide was presoaked and fleshed conventionally for main soaking trials. The chemicals used for analyses were of analytical grade, and those used for leather processing were of commercial grade.

2.2 Determination of protease properties in the presence of bactericide

2.2.1 Assay of protease activity and stability

Protease activities were assayed at different concentrations of bactericide. A protease solution, three MIT–protease mixture solutions, three SPHB–protease mixture solutions and three CTAB–protease mixture solutions were prepared using a phosphate buffer (pH 8.5). In all the solutions, protease had a concentration of 0.2 g/L. In the bactericide–protease mixture solutions, the mass ratios of bactericide to protease were 7.5:200, 15:200 and 75:200. All the solutions (1 mL) were incubated with a casein solution (1 mL, 10 g/L, pH 8.5) at 22 °C for 10 min. Then, their protease activities were determined by Folin phenol method [27]. The relative proteolytic activity is defined in Eq. (1):

$$\text{Relative proteolytic activity}(\%) = \frac{P_{\text{p-b}}}{P_{\text{p}}} \times 100\% \quad (1)$$

where $P_{\text{p-b}}$ is the proteolytic activity of bactericide–protease mixture solution; P_{p} is the proteolytic activity of the protease solution.

Protease activities were then assayed at different time points and a mass ratio of bactericide to protease 15:200. A protease solution, a MIT–protease mixture solution, a SPHB–protease mixture solution and a CTAB–protease mixture solution were prepared using a phosphate buffer (pH 8.5). In the four solutions, protease had a concentration of 0.2 g/L, and MIT, SPHB and CTAB had a concentration of 15 mg/L. The protease activities of the four solutions were determined after incubating with a casein solution (10 g/L, pH 8.5) at 22 ℃ for 10 min, 1 h, 2 h, 4 h and 8 h. The relative proteolytic activity is defined in Eq. (2):

$$\text{Relative proteolytic activity}(\%) = \frac{P_1}{P_0} \times 100\% \quad (2)$$

where P_1 is the proteolytic activity determined in all the experimental groups; P_0 is the proteolytic activity of the protease solution determined after incubating for 10 min.

2.2.2 Zeta potential and particle size of protease solution

A protease solution, three MIT–protease mixture solutions, three SPHB–protease mixture solutions and three CTAB–protease mixture solutions were prepared. In all the solutions, protease had a concentration of 1.0 g/L. In the bactericide–protease mixture solutions, the mass ratios of bactericide to protease were 7.5:200, 15:200 and 75:200. All the solutions were incubated at 22 °C for 5 min, and their zeta potentials in the range of pH 5–11 and their particle sizes at pH 8.5 were measured using a zeta potential and particle size analyser (Nano Brook Omni, Brookhaven, USA).

2.3 Interaction measurement

2.3.1 Fluorescence spectrum measurement

The solutions described in Sect. 2.2.2 were incubated at pH 8.5 and 22 ℃ for 30 min. Then, the fluorescence emission signals of the solutions were measured under a wavelength range of 300–500 nm using a fluorescence spectrophotometer (Lumina, Thermo Fisher, USA), where the fluorescence excitation wavelength was set at 276 nm. Moreover, the synchronous fluorescence spectra of the solutions were recorded at $\Delta\lambda = 15$ nm and $\Delta\lambda = 60$ nm. The scan range of emission wavelength was recorded from 200 to 500 nm, whereas the slit widths of excitation and emission spectra were 5 nm.

2.3.2 Molecular docking

The crystal structure of a serine protease (Enzyme Commission number 3.4.21.62) from *Bacillus* sp. was

downloaded from the Protein Data Bank (https://www.rcsb.org, PDB ID: 1DBI) [28]. The 3D structures of MIT, SPHB and CTAB were obtained by GaussianView 6 software. The programme AutoDock 4 was used to elucidate the docking of bactericides and protease. The grid box was defined as enclosing the active site of protease molecule, and the grid spacing was set at 0.0375 nm with dimensions of 7.0 nm×7.0 nm×7.0 nm. Semi-flexible docking and Lamarckian genetic algorithm were employed to obtain the possible binding mode. The docking model with the lowest energy and bonding condition was obtained.

2.3.3 MD simulation

GROMACS 2019 was used for MD simulation. The protein force field used amber99sb-ildnff, and the ligand force field was obtained by the acpype.py programme in Amber tools 2020. TIP3P was selected as the water model. The size of the cubic box followed the standard that the distance from the protein molecule to the edge of the box was larger than 2.0 nm, and the Na^+ and Cl^- ions were used to the neutralisation simulation systems [29]. The electrostatic interactions were calculated by the particle mesh Ewald algorithm. The van der Waals non-bonding interactions were calculated by Lennard–Jones model, and the cutoff distance was 1.0 nm. The linear constraint solver algorithm was used to fix the chemical bonds between protein atoms [30], and the Berendsen coupling algorithm was used to maintain the constant temperature and pressure of each component. Periodic boundary conditions were applied in all directions. After the energy minimisation, heating and equilibration processes [31], the dynamic simulation was run for 50 ns. The abovementioned process was repeated three times. Then, the results were evaluated with the analytical tools in GROMACS 2019.

2.4 Soaking trials and their performance evaluation

The bactericides that barely affected the protease properties were used for the following soaking trials. Eight groups of fleshed hides (Nos. 1–8) were soaked with 300% water, 0.5% sodium carbonate, protease and bactericide at 22 °C for 20 h. Here, the percentages were based on the weight of fleshed hides, and the dosages of protease and bactericides are shown in Table 1. After soaking, the total amounts of bacteria, yeasts and moulds in the floats were determined using mikrocount® dipslides (mikrocount® duo, Schülke, Germany) [32], and the concentrations of protein and hydroxyproline in the floats were determined as described in the documents [33, 34]. The soaked hides were sampled to observe the epidermis and collagen fibre by HE staining and evaluate the rehydration of hides by observing the longitudinal section using a stereo microscope (M205C, Leica, Germany).

3 Results and discussion

3.1 Effect of bactericide on protease properties

Given that this study aimed to investigate the effect of bactericides on the performance of protease-assisted soaking, the effects of MIT, SPHB and CTAB on the activity of protease were firstly analysed. As shown in Fig. 1a, when the mass ratio of bactericide to protease was lower than 75:200, MIT and SPHB barely affected the proteolytic activity, whilst CTAB had an inhibitory effect on the protease activity that increased with the rise in mass ratio. The soaking process generally takes a long time. Therefore, the protease activity should be maintained for a prolonged period. Figure 1b shows that the relative activity of protease in the absence and presence of bactericides all gradually decreased over time due to its self-hydrolysis. However, the decrements in the protease activity of the MIT and SPHB groups were similar to that of the blank group (in the absence of bactericide).

Table 1 Dosages of protease and bactericide in soaking trials

No	Dosages of protease and bactericide and operation
1	Run and stop for 20 h
2	Add 15 mg/L MIT and run and stop for 20 h
3	Add 15 mg/L SPHB and run and stop for 20 h
4	Add 200 mg/L protease[a] and run and stop for 20 h
5	Add 200 mg/L protease and 15 mg/L MIT and run and stop for 20 h
6	Add 200 mg/L protease and 15 mg/L SPHB and run and stop for 20 h
7	Add 200 mg/L protease and run for 2 h; add 15 mg/L MIT and run and stop for 18 h
8	Add 200 mg/L protease and run for 2 h; add 15 mg/L MIT and run and stop for 18 h

[a] Adding 200 mg/L protease is equivalent to using the protease of 20 U/g fleshed hide

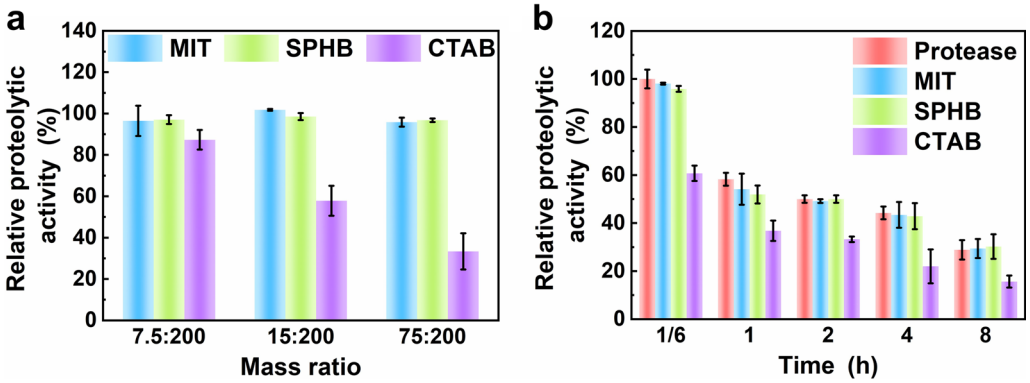


Fig. 1 Effects of bactericide concentration (**a**) and incubating time (**b**) on the protease activity. The proteolytic activity was 33,300 U/g at pH 8.5 and 22℃

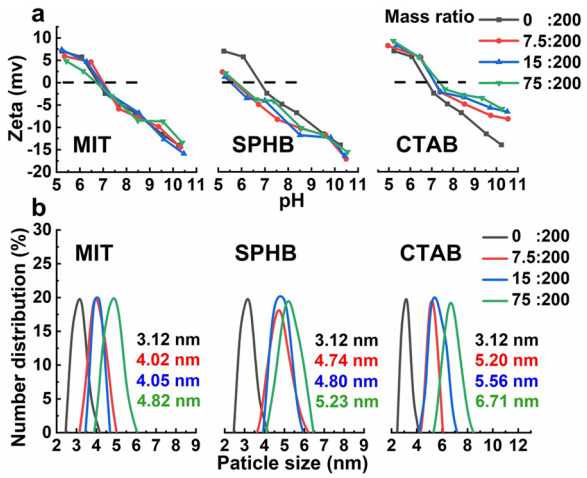


Fig. 2 Effects of bactericides on the zeta potential (**a**) and particle size distribution (**b**) of protease solution

The protease activity decreased more sharply in the CTAB group than in the other groups. These results indicated that MIT and SPHB had little effect on the protease activity, whilst CTAB had a negative effect.

The surface charge and particle size of protease play important roles in the mass transfer of protease into the hide and affect the enzymatic treatment of hide [35]. Therefore, the effects of bactericides on the surface charge and particle size of protease were also analysed. As shown in Fig. 2a, MIT nearly did not affect the zeta potential of protease solution, SPHB decreased the zeta potential of protease solution and CTAB increased the zeta potential of protease solution. These results indicated that MIT barely affected the surface charge of protease, whilst SPHB and CTAB increased negative and positive charges of the protease, respectively, because they can ionise anions/cations in the solution. Figure 2b shows that the addition of bactericides increased the effective diameter of protease solution, and the order of rising particle size was CTAB > SPHB > MIT. The particle size of protease solution became the largest after CTAB was added. The reason should be that CTAB ionised cations in the solution and reduced the total amount of negative surface charges at pH 8.5, which weakened the electrostatic repulsion between enzyme molecules and the stability of colloidal protease solution.

3.2 Interaction between bactericide and protease

3.2.1 Fluorescence spectra analysis

The catalytic activity of protease is closely related to its molecular structure. In this section, the effect of bactericide on the structure of protease was investigated by measuring the fluorescence spectra and synchronous fluorescence spectra of protease solution at different mass ratios of bactericide to protease. When the mass ratio of MIT to protease increased, the fluorescence intensities of protease solution (Fig. 3a), tyrosine residues (Fig. 3b) and tryptophan residues (Fig. 3c) decreased drastically, and a red shift of the emission band was observed (Figs. 3b and c). The results indicated that MIT reduced the hydrophobicity of tyrosine and tryptophan residues in the microenvironment. The intensities of fluorescence emission peaks of tyrosine and tryptophan residues decreased with the increase in SPHB concentration, which implies that SPHB changed the microenvironment in which tyrosine and tryptophan residues were located. When the mass ratio of CTAB to protease increased, a blue shift of the emission band for tyrosine residues (Fig. 3b) and a red shift of the emission band for tryptophan residues (Fig. 3c) were observed. The phenomena indicate that the hydrophobicity of tyrosine residues was enhanced, whilst that of tryptophan residues was weakened in the microenvironment. The fluorescence emission peak of protease solution showed a red shift in the presence of CTAB

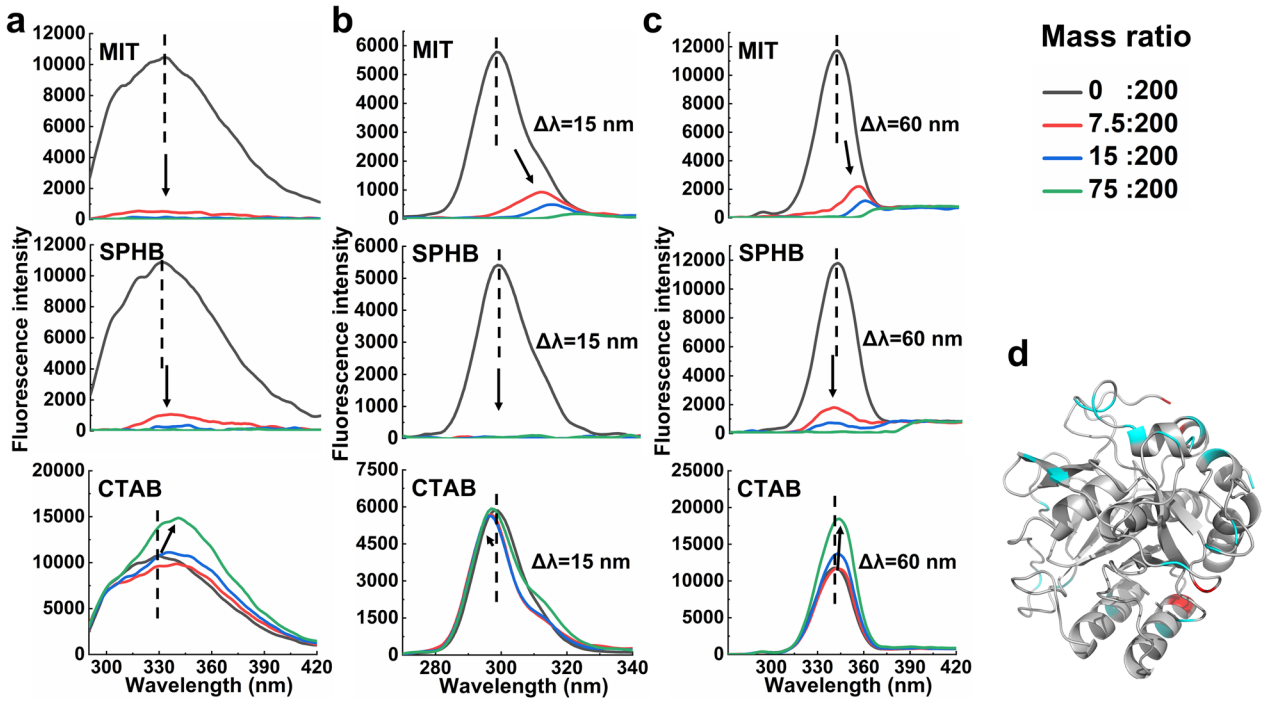


Fig. 3 Effects of bactericide concentrations on the fluorescencespectra and synchronous fluorescence spectra of protease: **a** Fluorescence spectra of protease; **b** Synchronous fluorescence spectra of protease (Δλ = 15 nm); **c** Synchronous fluorescence spectra of protease (Δλ = 60 nm); **d** Cartoon structure of protease: tyrosine(blue), tryptophan (red)

(Fig. 3a). This phenomenon suggests that the polarity of the fluorescent amino acid residues of protease molecules increased in the microenvironment, and the contribution of tryptophan residues to the fluorescence of protease was higher than that of tyrosine residues. The results confirmed that MIT, SPHB and CTAB all changed the conformation of protease to some extent. The bactericides easily interacted with tyrosine and tryptophan residues because these residues were distributed on the protease surface (Fig. 3d).

3.2.2 Molecular docking analysis

Molecular docking was used to predict the possible binding sites of MIT, SPHB and CTAB on protease molecules. As shown in Fig. 4, the MIT molecule inserted into the active pocket of the protease. Three hydrogen bonds formed between MIT and Cys137, Cys138 and Cys139 residues of the protease, as well as the hydrophobic interaction between MIT and Leu135, Cys137, Cys139 and Leu144 residues, were far from the catalytic centre of the protease. As a result, MIT barely affected the protease activity. The interaction between SPHB and protease was similar to that between MIT and protease. Thus, SPHB also had little effect on the catalytic activity of protease. The van der Waals force between CTAB and the residues His72, Leu109, Gly111, Leu113, Ile116, Leu135, Gly136, Cys137, Asp138, Cys139, Thr141, Leu144, Gly163, Asn164, Tyr176, Thr225 and Ser226 supported the spatial conformation of the CTAB–protease complex. The hydrophobic chain of CTAB was close to His72 and Ser226 residues that belonged to the catalytic triad of protease. This condition would affect the substrate binding, which would lead to the decrease in the protease activity.

3.2.3 MD simulation

The stabilities of protease, MIT–protease, SPHB–protease and CTAB–protease complexes were studied by MD simulation. The root mean square deviation (RMSD) and root mean square fluctuation (RMSF) were analysed to evaluate the tertiary structural stability and the changes in the flexibility and local structure of protease molecule, respectively [36, 37]. A high fluctuation value reflects the instability of protein structure. As shown in Fig. 5a, the RMSD value of MIT was obviously higher than that of native protease in the MD simulation of 50 ns, which suggests that MIT changed the native structure of protease and the structure of the MIT–protease complex was unstable. As shown in Fig. 5b, the high fluctuation regions of MIT–protease complex were in the residue ranges of 172–178 and 188–195; the high fluctuation regions

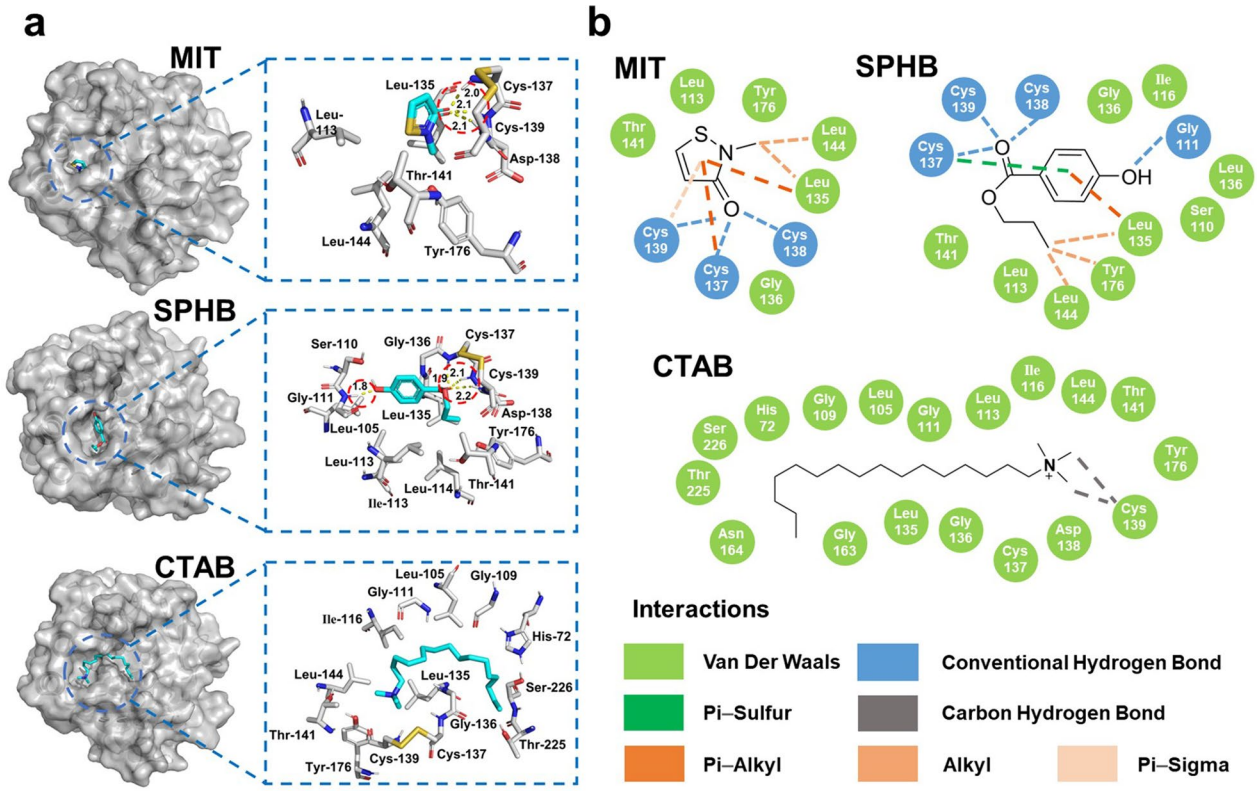


Fig. 4 Interactions between bactericides and protease: **a** Molecular docking model of bactericide and protease; **b** 2D diagram of the interaction between bactericide and protease processed by Discovery Studio Visualiser software

of SPHB–protease complex were in the residue ranges of 61–65, 105–113 and 172–178; the high fluctuation regions of CTAB–protease complex were in the residue ranges of 92–100 and 248–258. These results showed that some changes occurred in the above-mentioned regions of protease structure. The residues Asp40, His73, Leu136, Gly137, Ser224 and Ser227, which were the active sites of protease, were not in the high fluctuation regions of the bactericide–protease complexes. Thus, the complexes had little effect on the protease activity. Figure 5c represents the topological structures of amino acid residues of native protease, MIT–protease, SPHB–protease and CTAB–protease complexes, which were in the high fluctuation regions. The structures of bactericide–protease complexes nearly overlapped the native protease structure, which indicates that the bactericides changed the protease structure slightly. B-factor plots that directly reflect the conformational stability of the protein are shown in Fig. 5d. The blue area represents the structural stability, and the red area represents the structural instability. The results showed that part of the protease structure was distorted by the interactions between bactericides and protease.

In summary, the interaction between MIT/SPHB and protease had little effect on the catalytic activity of protease, whilst the interaction between CTAB and protease decreased the protease activity by forming a stable complex and occupying the catalytic site.

3.3 Effect of bactericide on the soaking performance of protease

According to the effects of the three bactericides on the properties of protease obtained above, MIT and SPHB were used to treat cattle hide with protease simultaneously or sequentially in the soaking process, and the soaking performances were evaluated to achieve an ideal protease-assisted soaking operation.

Bactericides are always used to prevent bacterial damage to hides when soaking for a long time. Thus, the total amount of microorganisms in the soaking float was measured, as shown in Fig. 6 and Additional file 1: Fig. S2 and Table 2. The results showed that MIT had a better germicidal efficacy than SPHB. This may be because the minimum inhibitory concentration of SPHB against bacteria is higher than that of MIT [20, 38]. Adding protease in the soaking process (control) greatly increased the total amount of bacteria, yeasts and moulds in soaking

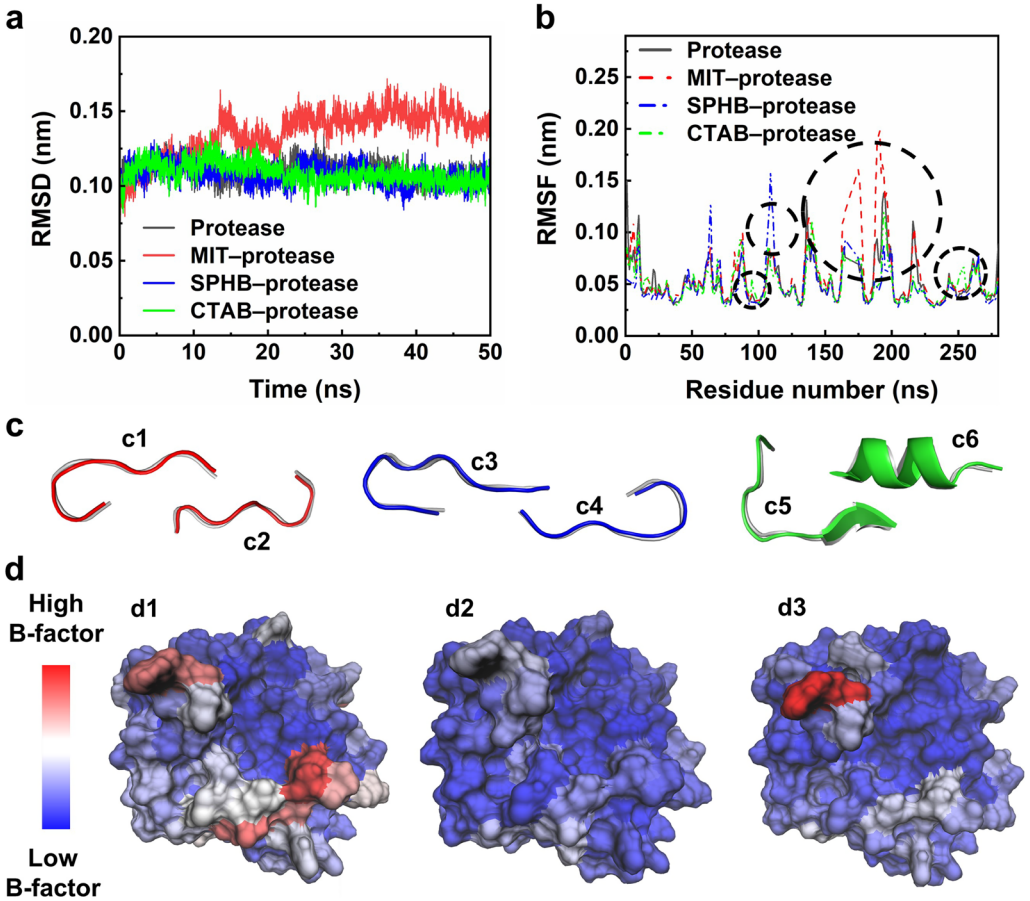


Fig. 5 Profiles of MD simulations: **a** Root mean square deviations (RMSD) of protease residues; **b** Root mean square fluctuation (RMSF) of protease residues; **c** Aligned structures of amino acids: (c1) 162–178, (c2) 188–195, (c3) 105–113, (c4) 162–178, (c5) 92–100 and (c6) 248–258, native protease (grey), MIT–protease complex (red), SPHB–protease complex (blue), CTAB–protease complex (green); **d** B-factor plots: (d1) MIT, (d2) SPHB and (d3) CTAB

float. The reason might be that hide proteins hydrolysed by the protease can provide nutrients for the growth of microorganisms. Fortunately, the simultaneous use of protease and MIT could create an environment that is not conducive to the reproduction and proliferation of microorganisms. However, the sequential use of protease and bactericide (using bactericide after adding protease for 2 h) could not limit the growth of microorganisms successfully. The reason might be that a large number of microorganisms proliferated within 2 h after protease was added, and a small amount of bactericide could not exhibit an effective germicidal efficacy. These results suggested that protease and bactericide should be used simultaneously in the soaking process to effectively prevent bacterial damage to hides.

A main purpose of soaking, especially protease-assisted soaking, is to remove unstructured proteins from hides and attack on the epidermis, which is essential to accelerate the soaking process and enable the rapid penetration of chemicals during the subsequent liming process. Thus, the removal of protein from soaked hides was evaluated by analysing the total protein concentration of soaking float, and the epidermis and collagen fibre of soaked hide were observed by HE staining.

As shown in Fig. 7, the protein concentration of soaking float followed the order using bactericide alone < using protease alone < using protease and bactericide simultaneously < using protease and bactericide sequentially. The higher protein concentration by using protease alone than those by using bactericide alone should be attributed to the added protease (which itself is also a protein), the hide proteins hydrolysed by protease and the increased microorganisms. The protein concentration by using protease and MIT simultaneously was higher than that by using protease alone, which might be due to that the effective germicidal efficacy of MIT could protect the protease from microorganisms and was helpful for the hydrolysis of hide proteins by protease [39, 40].

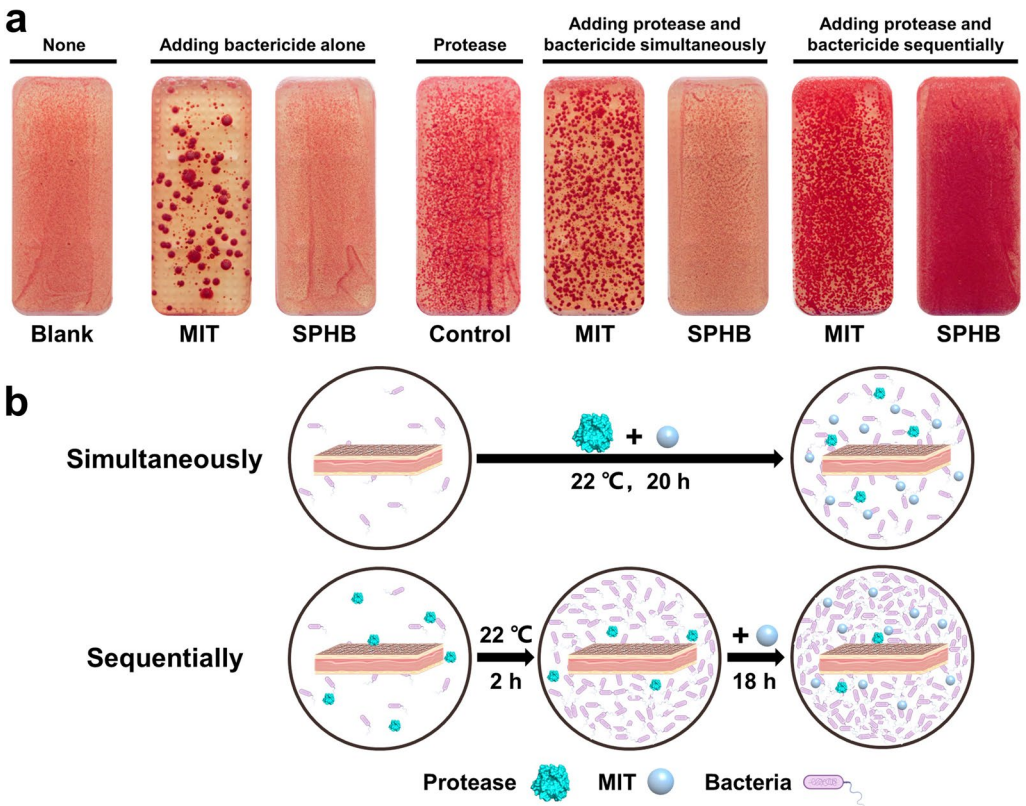


Fig. 6 Evaluation of the total amount of bacteria in the soaking float: **a** Images of mikrocount® dipslides; **b** Schematic of the total amount of bacteria in the soaking float with simultaneous and sequential use of protease and MIT

Table 2 Total amounts of bacteria, yeasts and moulds in the soaking float

No	Sample	Total amount of bacteria (CFU/mL)	Total amount of yeasts and moulds (CFU/mL)
1	Blank	10^7	10^3
2	MIT	10^4	10^2
3	SPHB	10^7	10^3
4	Control	10^7	10^4
5	Protease + MIT	10^6	10^2
6	Protease + SPHB	10^7	10^3
7	Protease (2 h) + MIT	10^7	10^2
8	Protease (2 h) + SPHB	10^7	10^4

In addition, the protein concentrations by using protease and bactericides sequentially were higher than those by using protease and bactericides simultaneously. The reason might be that the microorganisms containing protein multiplied rapidly within 2 h after protease was added and damaged some hide collagen (the hydroxyproline concentration of soaking float was the highest).

The HE stained images of longitudinal sections of soaked hide are shown in Fig. 8 and Additional file 1: Fig. S3. The epidermis of soaked hide by using bactericide alone was intact, and that by using protease alone was attacked, which should be attributed to the proteolysis of hide proteins. The epidermis was also attacked by using protease and bactericides simultaneously, which might be due to that the bactericides barely affected the protease activity and could protect the protease from microorganisms. Moreover, the rehydration of all the hides was similar (Additional file 1: Fig. S4), and the grain and flesh surfaces of all soaked hides were clean (Additional file 1: Figs. S5 and S6).

These results suggested that the addition of protease can improve soaking performance including removing unstructured proteins and attacking on the epidermis. In the meantime, the bactericides that barely affected the catalytic activity of protease should be used with the soaking protease simultaneously to reduce the microbial damage to hides.

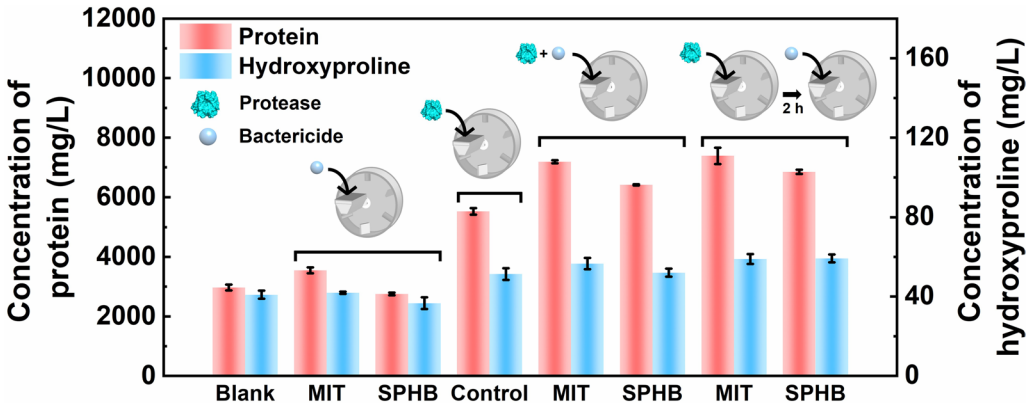


Fig. 7 Protein and hydroxyproline concentrations of the soaking float

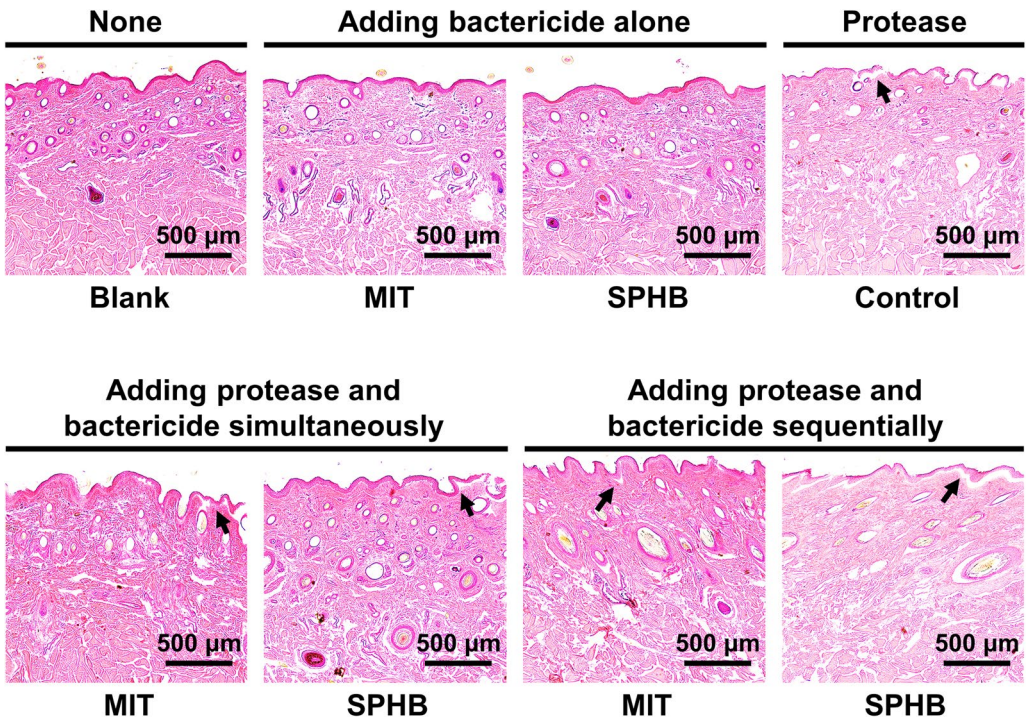


Fig. 8 Microscopic images of the longitudinal sections of soaked hides (HE staining)

4 Conclusion

The effects of three bactericides MIT, SPHB and CTAB on the protease properties and their interactions with the protease were analysed. The results indicated that MIT and SPHB barely affected the protease activity because the hydrogen bonds and hydrophobic interaction between MIT/SPHB and the protease were far from the catalytic site of protease. CTAB decreased the protease activity with the increase in concentration because it interacted with the catalytic triad of protease and interfered with the substrate binding. The experimental results for enzyme-assisted soaking showed that MIT had excellent germicidal efficacy in the soaking environment. Adding protease increased the microorganisms in the soaking float because protease can hydrolyse hide proteins and provide nutrients for the growth of microorganisms. The simultaneous addition of MIT and protease can protect protease and hides from microbial damage and promote the hydrolysis of unstructured hide proteins and the rehydration of raw hide. However, adding bactericides and protease sequentially had difficulty inhibiting the growth

of microorganisms. Overall, protease and bactericides that nearly do not affect the protease activity and have ideal germicidal efficacy are suggested to be used simultaneously in an enzyme-assisted soaking process to promote the soaking process and protect hides from microbial damage.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42825-023-00135-5.

Additional file 1: Fig. S1. The chemical structures of the three bactericides. **Fig. S2.** Images of mikrocount® dipslides for determining the total amount of yeasts and moulds in the soaking float. **Fig. S3.** Microscopic images of the longitudinal sections of soaked hides (HE staining). **Fig. S4.** Stereo microscopic images of longitudinal sections of soaked hides (bar: 5 mm). **Fig. S5.** Photos of grain surfaces of soaked hides. **Fig. S6.** Photos of flesh surfaces of soaked hides.

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Author contributions
HL: Investigation, Methodology, Visualization, Writing—original draft; YHP: Investigation, Formal analysis; BS: Supervision, Writing—review and editing; YHZ: Conceptualization, Methodology, Supervision, Writing—review and editing. All authors read and approved the final manuscript.

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Availability of data and materials
Data will be made available on request.

Declarations

Competing interests
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Author details
[1]National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, Chengdu 610065, China. [2]College of Biomass Science and Engineering, Sichuan University, Chengdu 610065, China.

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