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ORIGINAL ARTICLE

Chymotrypsin both directly modulates bacterial growth and asserts ampicillin degradation-mediated protective effect on bacteria

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Abstract Recent studies have shown that some serine protease family members may play an important role in antibacterial activity. Chymotrypsin, a major member of the serine protease family, was used in our study to investigate whether it has a similar function. Optical absorbance, broth microdilution and scanning electron microscopy (SEM) assays were carried out to investigate the direct effect of chymotrypsin on bacteria. A disk diffusion test and LC-MS spectrometry were then used to investigate the effect of chymotrypsin on antibacterial activity of ampicillin. Chymotrypsin exhibited potentially antimicrobial properties to two kinds of Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*). However, the existence of chymotrypsin could also hinder the antibacterial activity of ampicillin, in part because chymotrypsin could degrade ampicillin in a dose-dependent manner. These results could be helpful in guiding current commercial usages of chymotrypsin and devising better strategies of clinical applications.

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Introduction

Bacterial infections are mainly treated with antibiotics. Although antibiotics are highly effective in disease control, their use has been seriously hampered by the emergence of drug-resistant bacteria. New antibiotics have been constantly developed and put into use, but resistance emerges in a very short time. Therefore, the search for new antimicrobial agents is considered to be a good strategy to solve these problems (Guell et al. 2012).

Recently, several new antimicrobial agents have been found, such as serine protease. For example, ovochymase in amphioxus *Branchiostoma belcheri* is an ovary-specific trypsin-like serine protease with antibacterial activity (Gao and Zhang 2009). Another novel serine protease produced by *Sarcophaga peregrina* showed antibacterial activity against several bacteria (Tsuji et al. 1998). The serine protease homolog protein with antibacterial activity was identified from human granulocytes (Morgan et al. 1991). The antibacterial activities of these agents are generally broad in target spectrum, but the mechanisms have not been completely elucidated.

Based on their evolutionary relationships, serine proteases can be further grouped into 11 clans. Among these, chymotrypsin-like enzymes are considered as the main clan in the family of serine proteases. Chymotrypsin is a 25.8-kDa, water soluble enzyme with an ellipsoidal structure containing about 50 % β-sheets, 10 % α-helices, and the rest being relaxed and turn structures (Meersman et al. 2005). It can cleave peptides at the carboxyl side of tyrosine,

tryptophan, and phenylalanine by a hydrolysis reaction. Despite being studied for a long time, up to now, many aspects of chymotrypsin are still unclear. Inhibitors of trypsin and chymotrypsin have also been suggested to be correlated with plant resistance to pathogens (Woloshuk et al. 1991; Kim et al. 2005), whereas, in our laboratory, we occasionally found antimicrobial activity of ampicillin could be inhibited when it was mixed with chymotrypsin. This unexpected observation prompted us to investigate the potential mechanism of this phenomenon. This study had two major aims: (1) to investigate any possible direct effect on bacterial growth, and (2) to investigate any possible degradation effect on ampicillin.

In the present study, the growth of five bacterial strains treated with chymotrypsin at different concentrations were first analyzed by optical absorbance assay and broth microdilution method. In addition, scanning electron microscopy (SEM) was applied to reveal their diagnostic morphological characteristics. Then, a disk diffusion test was used to investigate whether antibacterial activity of ampicillin could be influenced with the addition of chymotrypsin. Finally, LC-MS spectrometry was used to investigate whether ampicillin can be directly degraded by chymotrypsin.

Materials and methods

Bacterial strains

Three Gram-negative (*Escherichia coli* BL21 (DE3), *E. coli* 25922 and *Klebsiella pneumoniae* 700603) and two Gram-positive (*Staphylococcus aureus* 25923 and *Enterococcus faecalis* 29212) bacterial strains were used in this study. *E. coli* BL21 (DE3) was purchased from Takara Chemical, and the other four bacterial strains were gifts from the First Affiliated Hospital of Nanjing Medical University.

Chemical

Bovine chymotrypsin, bovine albumin, N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and ampicillin were purchased from Sigma Chemical. Tris base used for buffer preparation was purchased from Aldrich. Methanol (LabScan), and formic acid (BDH) were obtained commercially and used according to instructions.

Effects of chymotrypsin on bacterial cell growth

Two methods were used to test the influence of chymotrypsin on bacterial growth:

Spectrophotometric and optical absorbance assay: Four microliter bacterial samples (OD_{600}=0.04) were aliquoted into each tube with a wide range of final concentrations (0, 0.05, 0.25, 0.5, 0.75, and 1 mM) of chymotrypsin separately (in triplicate for each concentration of chymotrypsin). Bacteria were cultured in a shaker incubator with constant shaking at 180 rpm at 37 °C. Absorbance at 600 nm was monitored every hour until the culture reached stationary phase. Growth curves were described as the mean absorbance values for triplicate assays minus the absorbance value for the blank [Luria Bertani (LB) medium without chymotrypsin] versus time.

Broth microdilution method: Chymotrypsin at given final concentrations, which showed significant biological effect based on the results of optical absorbance assay (*E. coli* BL21 (DE3) at 0.5, 1 mM, *E. coli* 25922 at 0.25, 1 mM, *K. pneumoniae* at 0.5, 1 mM, *S. aureus* at 0.5, 1 mM, and *E. faecalis* at 0.5, 1 mM), were added to the prepared bacterial samples (OD_{600}=0.04) before 4 h incubation with constant shaking of 180 rpm at 37 °C. To assure the observed positive effect on bacterial growth was not nutritional, we included two control proteins, albumin and heat-inactivated chymotrypsin, of the same concentration as the active chymotrypsin. A sample incubated with normal medium was set as blank control. After appropriate dilution steps with sterile LB medium, 100 μl of each sample was plated onto LB agar plates or blood agar plates. Colonies were counted after 18 h incubation at 37 °C and back-extrapolated to the original volume to determine colony-forming units (CFUs). Each condition was tested in triplicate.

SEM analysis of bacterial strains treated with chymotrypsin

The samples treated with or without chymotrypsin were prepared as described in broth microdilution assay. After 4 h immobilization by 0.2 M phosphate buffer (pH 7.2) including 4 % glutaraldehyde (w/v) at 4 °C, the bacteria were pelleted by brief centrifugation (1,500 rpm), and the supernatant was carefully removed and discarded. The fixed bacteria were then rinsed three times in 0.1 M phosphate buffer (pH 7.2), followed by a brief centrifugation to remove the buffer. Then, 1 % OsO_4 was added to the tube and these bacteria were post-fixed in the same buffer for 1 h at room temperature. A graded acetone series (30, 50, 70, 90, and 100 %) was used to dehydrate the bacteria, and each step was followed by a brief centrifugation to remove the liquid. Then, the bacteria were exchanged in an isoamyl acetate series, dried with an HCP-2 critical point dryer (Hitachi) for 7 h, mounted on aluminum stubs, coated with a gold-palladium mixture by an E1010 coater (Hitachi), and viewed with a scanning electron microscope (FEI QUANTA-200).

Disk diffusion test to determine the effects of chymotrypsin on ampicillin bactericidal activity

This assay was carried out in Petri dishes containing LB agar. Six sterile blank paper disks (6 mm diameter) were

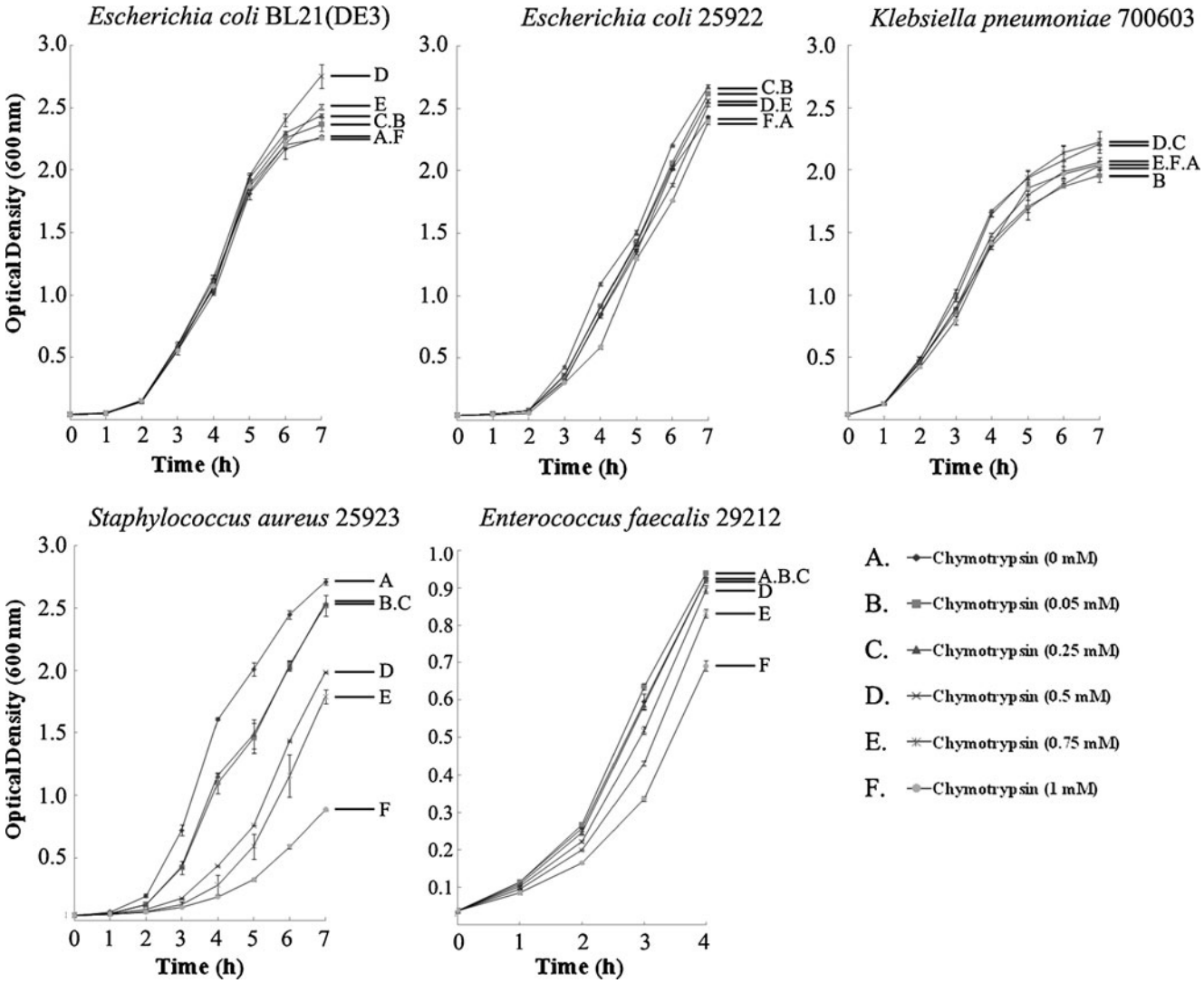

Fig. 1 Growth curves of five bacterial strains after exposure to different concentrations of chymotrypsin. Five bacteria (*E. coli* BL21 (DE3), *E. coli* 25922, *K. pneumoniae* 700603, *S. aureus* 25923 and *E. faecalis* 29212) were treated with chymotrypsin at different final concentrations (**a** 0 mM; **b** 0.05 mM; **c** 0.25 mM; **d** 0.5 mM; **e** 0.75 mM; **f** 1 mM). Absorbances at 600 nm of the bacterial cultures were measured at different intervals. Each point is expressed as mean ± standard deviation (SD) in triplicate from one representative experiment out of three

placed on the LB agar plates of growing bacteria. As an indicator of ampicillin bactericidal activity, we used *E. coli* BL21 (DE3) as a representative of ampicillin-susceptible bacteria. We expect chymotrypsin-mediated reduction in ampicillin activity to have similar indirect effects on different ampicillin-susceptible bacteria, although these bacteria might have different sensitivities to chymotrypsin because of their different sensitivities to ampicillin. The choice of *E. coli* BL21 (DE3) was mostly for safety concerns and ease in handling. First, the enzyme activity of chymotrypsin was removed by thermal denaturation in 58 °C water bath for 2 h. The irreversible heat-induced unfolding of chymotrypsin occurred at a temperature of 52.8 °C (Rezaei-Ghaleh et al. 2007), so chymotrypsin heated in 58 °C water beforehand will lose its enzymatic activity. Then, ampicillin was incubated for 60 min at 37 °C with thermally denatured or untreated chymotrypsin (1 mM). At the same time, ampicillin (3.5 mM, 7 mM, 14 mM) and 1 mM chymotrypsin were also incubated for 60 min at 37 °C. After all these samples were again heated in 58 °C water bath for 2 h, a 5-μl aliquot of each sample was added to each disk, and the plates were incubated at 37 °C for 72 h. Antibacterial activity was shown as a clear zone of growth inhibition. Each condition was tested in triplicate.

Chymotrypsin inhibition by TPCK

TPCK is an analog of chymotrypsin substrate and can irreversibly bind to and inhibit chymotrypsin enzymatic activity. TPCK treatment was carried out by mixing TPCK and chymotrypsin in 200 μl of Tris–HCl (pH 7.3) to the

final concentration of 0.5 mM for TPCK and 1 mM for chymotrypsin. The mixture was incubated for 30 min at 37 °C. Subsequently, ampicillin was mixed into each LB plate with or without chymotrypsin (treated with TPCK), chymotrypsin (untreated with TPCK), or TPCK. The final concentration of ampicillin was 14 mM. *E. coli* BL21 (DE3) was then plated at an appropriate concentration (OD_{600} =0.4) onto LB plates. All assays were performed in triplicate. Microbial growth was assessed by counting the colonies after 18 h incubation at 37 °C.

LC-MS spectrometry

Ampicillin was dissolved in Tris–HCl (pH 7.3) to obtain a solution of 0.5 μM. Then, 400 μl ampicillin solution (0.5 μM) was incubated at 37 °C for 90 min, mixed with 0 μM, 0.05 μM, 0.5 μM, 5 μM, and 50 μM bovine chymotrypsin or 0 μM, 0.05 μM, 0.5 μM, 5 μM, and 50 μM bovine albumin. After incubation, to remove the remaining protein, these samples were mixed with the same volume of methanol. After mixing, 800-μl aliquots were transferred into 2-ml Quick Seal centrifuge tubes and subjected to ultracentrifugation operated at 185,000 rpm for 10 min. After ultracentrifugation, a 500-μl aliquot of the clear supernatant was collected for analysis. For each reaction, 25 μl of clear supernatant for LC-MS analysis was immediately injected into high-performance liquid chromatography (Waters) with the effluent flowing directly into the MS - MS spectrometer (Thermo Finnegan), which was equipped with an electro spray ionization source and operated with a spray voltage of 5.0 kV and a capillary voltage of 15 V at 300 °C. The extract was separated on a C18 column (5 μM, 2.1 × 100 mm) (Waters), with separation performed at 0.25 ml/min at 25 °C with methanol (solution A) and 0.1 % formic acid (solution B) as the mobile phase. The separation was performed on a gradient that consisted of the following steps: (1) 20 % solution A: 80 % solution B for 4 min; (2) 90 % solution A: 10 % solution B for 2 min; (3) 90 % solution A: 10 % solution B for 0.1 min; and (4) 20 % solution A: 80 % solution B for 2 min. The runtime for each injection was 7 min. After each analyte was injected, collision energy was optimized for ampicillin. Detection of ampicillin was based on isolation of the protonated molecular ion, $[M+H]^+$ ion (m/z 350) and subsequent MS-MS fragmentation was carried out by monitoring the full scan product masses. Here, we chose specific product ions (m/z 160) derived from ampicillin by collision as the peak of ampicillin, so we used mass spectrometry as a quantitative tool to determine accurate amounts of the remaining ampicillin in each analyte. Standard curves were created from solutions of different concentrations of ampicillin (10–1,000 ng in Tris–HCl, pH 7.3) subjected to the same MS-MS analysis. The amounts of ampicillin were calculated from the areas of ampicillin peaks. For confirmation, ampicillin was monitored by parent ion (m/z 350) and two transitions (106 and 160) (Granelli and Branzell 2007).

Results

Effect of chymotrypsin on the growth of bacteria

As seen in Fig. 1, the amount of bacteria increased with the lapsing of time as expected, and the growth curve effectively reflected the lag phase, exponential phase, and stationary phase of each bacterial strain. The results showed that the growth of three Gram-negative bacteria plotted against chymotrypsin concentration formed similar parabolae. For *E. coli* BL21 (DE3), its growth in the presence of 0.25 and 0.5 mM chymotrypsin was accelerated compared with the control group with no chymotrypsin ($p<0.05$) over 3 h. Meanwhile, no significant effect was observed in other tested concentration groups. Similar effects were also observed in *E. coli* 25922 and *K. pneumoniae*, where 0.25 and 0.5 mM were the optimal concentrations for the growth acceleration, respectively ($p<0.05$). However, compared with the control, a significant decrease was found in the growth of Gram-positive bacterial strains (*S. aureus* and *E. faecalis*) cultured with chymotrypsin during the whole observation period ($p<0.05$), and *S. aureus* is more sensitive than *E. faecalis* to chymotrypsin.

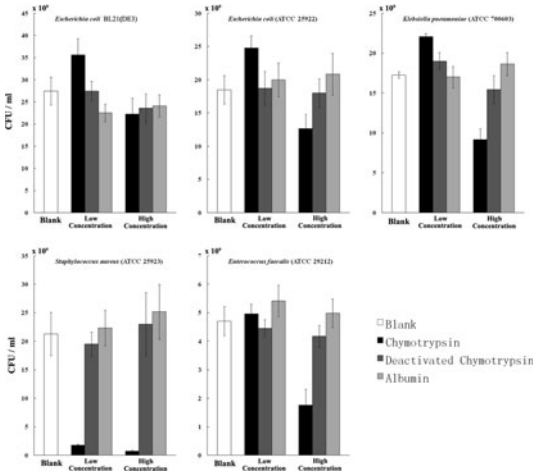

Fig. 2 Effect of chymotrypsin on bacterial growth. Survival of five bacteria without (□) or with active chymotrypsin (■) or with inactivated chymotrypsin (■) or with albumin (■) were assessed using the broth microdilution method. Chymotrypsin treatment groups were cultured at given concentrations (low concentration: 0.5 mM, *S. aureus* 25923 at 0.25 mM; high concentration: 1 mM). Inactivated chymotrypsin and albumin of the same concentration were used as controls. Results are expressed as mean ± standard deviation (SD) of triplicate plates for each condition

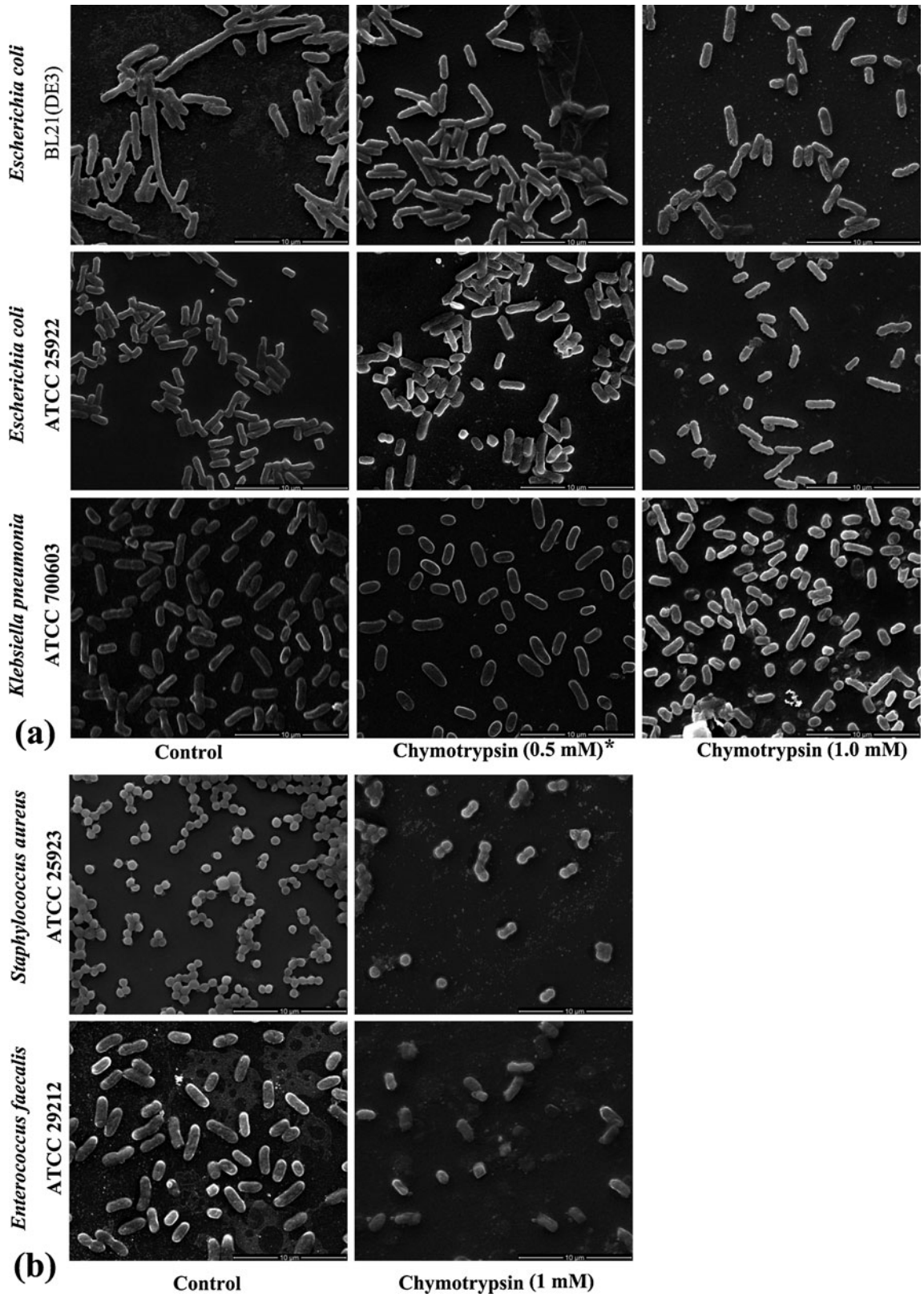

Fig. 3 Morphological details of five tested bacterial strains observed by scanning electron microscopy. **a** Three Gram-negative bacterial strains treated with or without 0.5 mM and 1 mM chymotrypsin. **b** Two Gram-positive bacteria strains treated with or without 1 mM chymotrypsin

One caveat of liquid bacterial culture assay is that both living and dead bacteria may contribute to optical absorbance. To further study the relationship between enzymatic activity and bacterial growth, broth microdilution assay was carried out to quantify living bacteria. Compared with the optical absorbance assay, similar effects of chymotrypsin on bacterial growth were observed in this experiment (Fig. 2). The results revealed that the growth of three Gram-negative bacterial strains (*E. coli* BL21 (DE3), *E. coli* 25922, and *K. pneumoniae*) was accelerated by the presence of chymotrypsin at the final concentrations from 0.25 to 0.5 mM, and inhibited at the final concentration of 1 mM ($p<0.05$). A significant negative effect at higher concentration (1 mM) was detected on the growth of two Gram-positive strains (*S. aureus* and *E. faecalis*) ($p<0.05$). For *S. aureus*, a significant inhibition was even observed at 0.5 mM chymotrypsin ($p<0.05$). In contrast, when bacteria were cultured with the inactivated chymotrypsin or albumin, no effect on growth was found ($p>0.05$).

SEM

The characteristic shapes and morphological details of each tested species were observed under SEM according to standard protocols as seen in Fig. 3. Bacteria existed as loosely packed aggregates or single cells with smooth surface and normal appearance in the untreated group, while distinct morphological features were clearly evident from micrographs when the bacteria were exposed to chymotrypsin. For Gram-negative bacteria, chymotrypsin increased the surface area/volume ratio significantly in all tested concentration groups, causing these cells to be shortened. Under lower concentrations (0.5 mM for *E. coli* BL21 (DE3), *K. pneumoniae* and 0.25 mM for *E. coli* 25922) of chymotrypsin, the number of dividing cells increased, and their shapes were generally preserved while the deformations of the cell surface were almost absent in the fields compared with control group. However, rough cell surfaces and membrane indentations were detected at a high concentration of chymotrypsin (1 mM for three Gram-negative bacteria). As for two Gram-positive bacterial strains, fewer morphological variations of *S. aureus* were observed upon exposure to chymotrypsin, while the normal shape of *E. faecalis* was lost and most of the cells had collapsed.

Effects of chymotrypsin on ampicillin bactericidal activity

Here, we studied the influence of chymotrypsin on antibacterial activity of ampicillin by a disk diffusion test. According to Table 1, antibacterial activity of ampicillin (14 mM) treated with thermally denatured chymotrypsin was similar to untreated ampicillin (14 mM), while the antibacterial activity of ampicillin (14 mM) treated with native chymotrypsin was reduced to be equivalent to ampicillin between 7 mM and 3.5 mM. These data indicated that chymotrypsin could reduce the antibacterial activity of ampicillin. In contrast, heat-inactivated chymotrypsin did not inhibit antibacterial activity of ampicillin efficiently.

Table 1 Diameter of inhibition zone of chymotrypsin and ampicillin exhibited against *E. coli* BL21 (DE3)

Sample	Inhibition zone (mm)
Chymotrypsin (1 mM)	0
Ampicillin (3.5 mM)	12.25±0.35
Ampicillin (7 mM)	15.50±0.71
Ampicillin (14 mM)	22.75±1.06
Ampicillin (14 mM)+thermally denatured chymotrypsin (1 mM)	19.60±0.57
Ampicillin (14 mM)+chymotrypsin (1 mM)	13.45±0.64

As shown in Fig. 4, in the presence of ampicillin, a significant ($p<0.05$) increase in bacterial growth was observed with native chymotrypsin (1,092.71%), while a relative increase was observed with TPCK inactivated chymotrypsin (81.25%) and with TPCK (33.33%) compared to the control (ampicillin). These data indicate that chymotrypsin can reduce the antibacterial activity of ampicillin, and that this effect can be inhibited by chymotrypsin inhibitor or heat-denaturation.

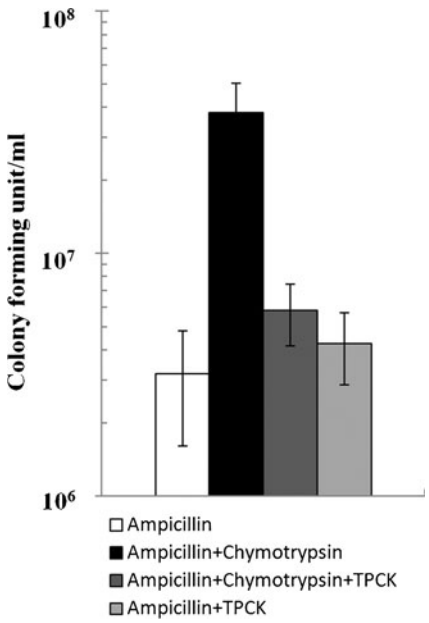

Fig. 4 Effect of chymotrypsin and TPCK on ampicillin. Ampicilin was mixed into each LB plate alone (□), or with untreated chymotrypsin (■), or with TPCK-treated chymotrypsin Tris–HCl, pH 7.3 (■), or TPCK (■). All assays were performed in triplicate

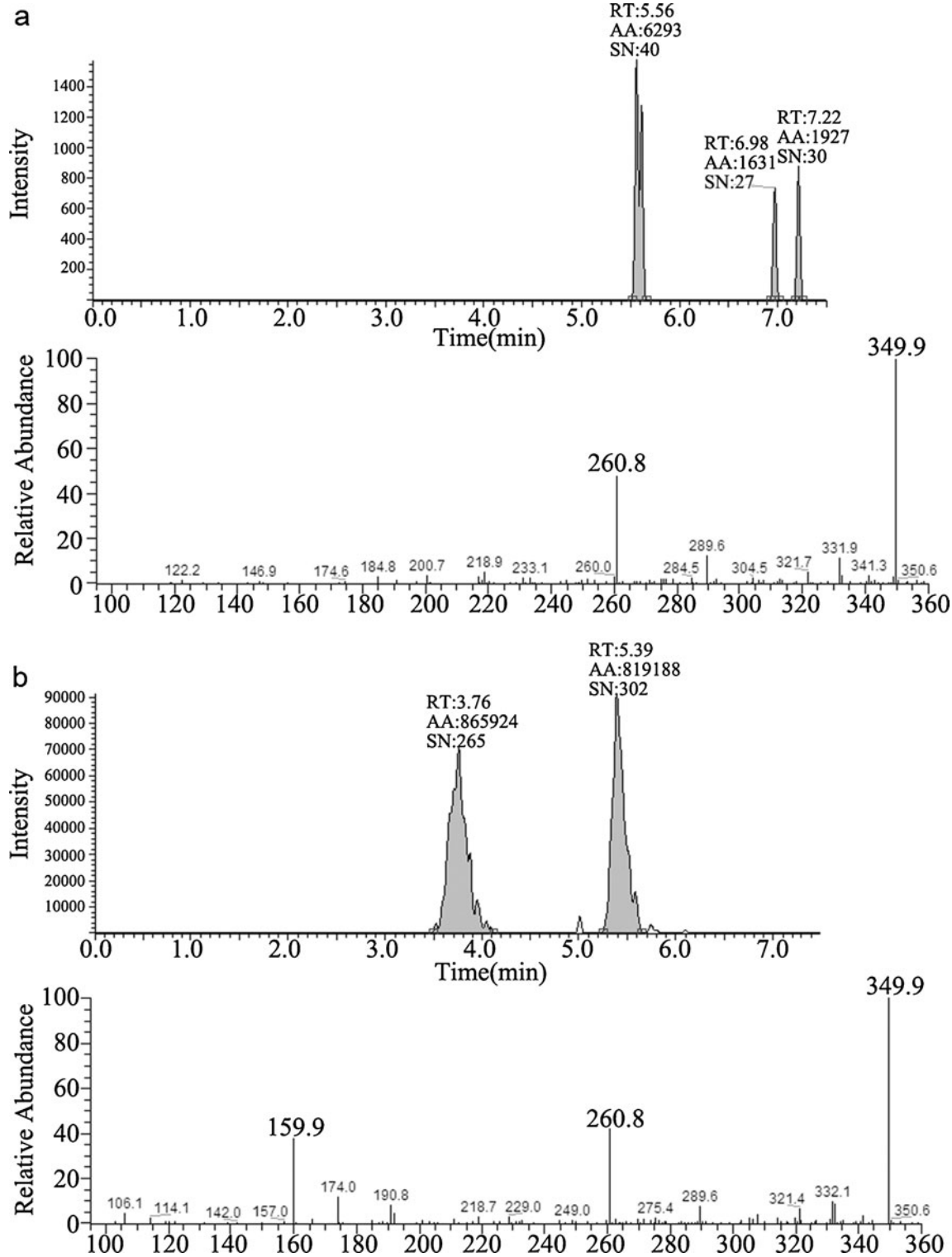

Fig. 5 **a** Chromatograms and MS-MS spectrum of blank; **b** chromatograms and MS-MS spectrum of ampicillin

Analysis of ampicillin degraded by chymotrypsin by LC-MS spectrometry

Two peaks (the retention times of standard ampicillin were 3.72 and 5.25 min) were detected in LC. Subsequent MS-MS analysis revealed that both peaks represented ampicillin. For each LC peak, $[M + H]^+$ ion equal to 350 and specific product ions equal to 106 and 160 were detected. These MS-MS fragment peaks were characteristic of mass spectrometry of ampicillin (Fig. 5). According to these data, we

measured the linear regression equation of the quantity of ampicillin (X) and the area of peak (Y): Y=16,163X, R^2= 0.9982, linear range is 10–800 ng/ml (Fig. 6). With the same manipulation, samples were identified according to the MS-MS fragment peaks and quantified according to the combined areas of both LC peaks. We calculated the remaining quantity of ampicillin by the linear regression equation. The results (Table 2) demonstrated that chymotrypsin degraded ampicillin, and the degradation rate increased with increasing concentrations of chymotrypsin. Degradation of ampicillin was complete with 50 μM chymotrypsin. As a control, albumin did not degrade ampicillin. There was no noticeable difference in residual ampicillin quantity with albumin or without albumin.

Table 2 The remaining quantity of ampicillin calculated by the linear regression equation

Sample (μM)	Concentration of residual ampicillin (μM)
Ampicillin (0.5)	0.48±0.03
Ampicillin (0.5)+chymotrypsin (0.05)	0.36±0.02
Ampicillin (0.5)+chymotrypsin (0.5)	0.32±0.03
Ampicillin (0.5)+chymotrypsin (5)	0.07±0.01
Ampicillin (0.5)+chymotrypsin (50)	0
Ampicillin (0.5)+albumin (0.05)	0.45±0.02
Ampicillin (0.5)+albumin (0.5)	0.46±0.02
Ampicillin (0.5)+albumin (5)	0.43±0.02
Ampicillin (0.5)+albumin (50)	0.43±0.01

Discussion

In this work, we documented and characterized the influence of chymotrypsin on bacterial growth in vitro. The effects of chymotrypsin on bacterial growth mostly depended on the strains of bacteria and the concentrations of chymotrypsin. Gram-positive and Gram-negative bacteria tested in this study responded differently to chymotrypsin. This may be due to their different structures. One principal difference between them is their cell wall structure (Hessle et al. 2005). Compared to Gram-positive bacteria, Gram-negative bacteria have an extra outer membrane (chemically different from the plasma membrane) external to the cell wall, and may also have a gelatinous sheath external to the outer membrane. In fact, the outer membrane acts as a selective permeation barrier (Lefevre et al. 2008). Indeed, while most abundant hydrophilic nutrients needed for Gram-negative bacterial growth diffuse passively into the periplasm through outer membrane channels, rare metals and large organometallic cofactors must be actively transported. This active transport through the outer membrane proceeds by coupling highly specific outer membrane transporters to an inner membrane (Lefevre et al. 2008). It is possible that the extra membrane in Gram-negative bacteria provides some protection against destructive forces of chymotrypsin. In contrast, the lack of an outer membrane renders Gram-positive bacteria more vulnerable to chymotrypsin. Another possibility is that Gram-negative bacteria have a higher capability to inhibit chymotrypsin activity. More studies are needed to answer this question.

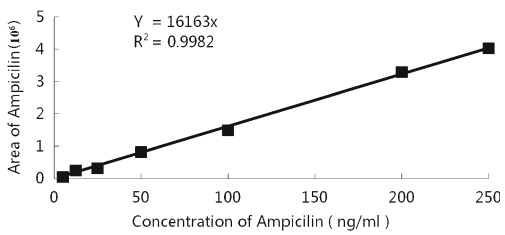

Fig. 6 Linear relationship of ampicillin concentration and peak areas

Interestingly, a weak stimulation of growth in three tested Gram-negative bacteria was observed with low concentrations of chymotrypsin. This is puzzling since the function of chymotrypsin is to break down amide bonds. It has been reported that serine proteases can induce proliferation in cancer cells through proteinase-activated receptors (PARs) (Ohta et al. 2003). PARs were activated enzymatically through proteolysis, which mediates transmembrane signal transduction and regulates a number of cellular functions (Kanke et al. 2005). Probably, there may be some similar receptors expressed in the membranes of Gram-negative bacteria, whose mechanism of activation is similar to that of PARs. As a common serine protease, a low concentration of chymotrypsin might accelerate the growth of Gram-negative bacteria through these activated receptors. Another trivial possibility is that chymotrypsin helps to digest extracellular proteins and facilitates these nutrients to be absorbed by the bacteria. The positive and negative (destructive) effects probably coexist. When the concentration of chymotrypsin rises, this positive effect could be offset by its potent destructive ability.

Chymotrypsin has also been found to have esterase activity. For example, fluorogenic synthetic ethers, amino acid derivatives, could also be substrates of chymotrypsin (Kraeva et al. 1991). This function reveals that chymotrypsin could degrade other materials besides peptides. Our study indicates that chymotrypsin might result in degradation of ampicillin. It has been reported that β-lactam derivatives are not only potent inhibitors of the chymotrypsin-like activity of 20 S proteasome but also very selective for this catalytic site (Imbach et al. 2007). So, we hypothesize that chymotrypsin plays this role possibly by reciprocal bonding between the catalytic site of chymotrypsin and the amide bond of β-lactam antibiotics. In fact, there are two amide bonds in the ampicillin molecule, with one of them being part of the β-lactam ring. Either or both of the two amide bonds may act as the target of chymotrypsin; it may be

partly responsible for the drop of ampicillin antibacterial activity. In addition, the binding of chymotrypsin per se may also make some ampicillin molecules inaccessible to the bacteria, which could contribute to the reduction in bactericidal activity. The exact mechanism of chymotrypsin-mediated ampicillin degradation remains subject to further investigations. Nevertheless, these results could be helpful in broadening current commercial usages of chymotrypsin and designing better strategies of clinical applications. For example, chymotrypsin may be used to clean possible contamination of ampicillin or other penicillin antibiotics (Jukes 1972). Such contamination is an issue in certain countries due to overuse of antibiotics in livestock. On the other hand, the undesired degradation might be avoided or minimized in patients receiving ampicillin treatment by including protein-rich diets. A high concentration of protein in the small intestine might protect the ampicillin by competitively binding to chymotrypsin, and therefore might potentially allow lower dosages to achieve the same antibiotic effect.

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