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A complex of trypsin and chymotrypsin effectively inhibited growth of pathogenic bacteria inducing cow mastitis and showed synergistic antibacterial activity with antibiotics

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ABSTRACT

The objective of this research was to assess the inhibitory effects of a complex of trypsin and chymotrypsin alone and in combination with antibiotics, on pathogenic bacteria involved in cow mastitis in vitro. We also conducted investigations of the antimicrobial mechanism and primary treatment trials on infected cattle. The presence of trypsin (0.16 mg/mL) alone only clearly hindered the growth of *Streptococcus agalactiae* (CVCC586) and *Streptococcus dysgalactiae* (ATCC12388). Chymotrypsin (0.16 mg/mL) alone, and the complex of trypsin and chymotrypsin at the dose C (0.16 mg/mL trypsin + 0.16 mg/mL chymotrypsin) or 2 C showed varying inhibitory effects on all the tested bacterial strains, including *Escherichia coli* (ATCC8739), *Pasteurella* (C51-3), *Staphylococcus aureus* (ATCC25923). The experiments also indicated that the complex of trypsin and chymotrypsin could hydrolyze bacterial outer-membrane proteins, damage the integrity of surface structures, and lead to leakage of intracellular material such as alkaline phosphatase, glucose and DNA. The complex of Trypsin and chymotrypsin showed well synergistic antibacterial effects when combined with specific *anti*biotics. In the field trial on 20 cows naturally suffering from clinical mastitis, the complex of trypsin and chymotrypsin reduced effectively *Streptococcus*, *E. coli*, *S. aureus*, and some other *Enterobacteriaceae* as well as the somatic cell counts in milk samples of treated cattle compared to controls. The complex of trypsin and chymotrypsin, and its combination with antibiotics, seems to have a potential in clinical veterinary medicine to treat mastitis.

1. Introduction

Antibiotics are widely applied to bacterial infections because of their high efficiency in controlling diseases. The popularity of antibiotics is seriously threatened by the emergence of bacterial drug-resistance (Güell et al., 2012). The situation is an increasing problem and especially serious in dairy farms (Aarestrup, 2000; Vaarst et al., 2002; McConnel et al., 2016). Cow mastitis is a common, worldwide disease in dairy herds. It is an inflammation of the mammary gland, mainly caused by various pathogenic microorganisms (Blosser, 1979; Viguier et al., 2009). Around 137 microorganisms can cause mastitis, including bacteria, fungi, yeast, mycoplasma, viruses and algae (Watts, 1988). Among the bacteria, Gram-positive bacteria such as *Staphylococcus aureus* usually cause subclinical mastitis, and Gram-negative bacteria such as *Escherichia coli* mainly induce acute, clinical mastitis (Shuster et al., 1991; Schukken et al., 2003). Cow mastitis is complex, and the costliest disease affecting production in dairy herds. Therefore, the search for new antimicrobial agents against pathogenic bacteria in cow mastitis is urgent.

Several new antimicrobial agents have been found. Ovochymase from the amphioxus *Branchiostoma belcheri*, a novel serine protease produced by *Sarcophaga peregrina*, and a serine protease homolog protein from human granulocytes, all show broad antibacterial activity (Gao and Zhang, 2009; Morgan et al., 1991). Trypsin and chymotrypsin, which exist widely in animals and plants, are considered the two main serine proteases, with extensive potential applications (Zhou et al., 2013). Chymotrypsin, a 25.8 kDa water soluble enzyme, has an ellipsoidal structure composed of about 50% β-sheet and 10% α-helices, with the remainder being relaxed and turn structures. The enzyme cleaves peptides to the carboxyl side of tyrosine, tryptophan, and phenylalanine by a

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hydrolysis reaction. Chymotrypsin has ampicillin degradation-mediated protective effects on bacteria (Zhou et al., 2013), and directly modulates bacterial growth. Trypsin, 2–30 kDa, is secreted from the pancreas as trypsinogen pattern, then activated by enterokinase in the small intestine into trypsin, and plays an important role in the digestive system (De Vecchi and Coppes, 1996; Chen et al., 2001; He et al., 2014). Trypsin consists of about 223 amino acid residues and has two size close tubbiness structural domains, each which has six antiparallel of β-foldings. Two domains were linked through six disulfide bonds, and His, Asp and Ser serving as the catalytic activity center of Try, the three of structures above produce a catalysis triplet (He et al., 2014). Trypsin cleaves peptide bonds on the carboxyl side of lysine, arginine and ornithine (Li et al., 2014). Trypsin and chymotrypsin are degraded during transit of the colon, principally by autolysis and gut microflora, to produce organic nitrogen-containing compounds that may contribute to the growth of enteric microorganisms (Macfarlane and Macfarlane, 1991).

Studies of simian rotavirus SA-11 demonstrated that trypsin and chymotrypsin functioned in viral polypeptide cleavage, but the specific cleavage products from the two treatments were different (Sato et al., 1987). Rajput and Gupta reported that the conjugate between trypsin and chymotrypsin was useful in probing complex biological structures (Rajput and Gupta, 1987). However, there are few reports on the effects of these two enzymes on bacteria. The urgent need for novel antibiotic substances in dairy farming prompted us to investigate the antimicrobial effect of the combination of trypsin and chymotrypsin on pathogenic bacteria in cow mastitis and the potential for reducing antibiotic use through the combination of antibiotics with trypsin and chymotrypsin.

2. Materials and methods

2.1. Drugs and media

We purchased chymotrypsin (Y-DCT-20130401, Geyuantianrun Bio-tech, China), Trypsin (Y-DT-20130401, Geyuantianrun Bio-tech, China), tilmicosin phosphate (20,110,401, Tairui Pharmaceutical, China), ceftiofur sodium (104,010-37-9, BODUN, China) and erythromycin thiocyanate (ETH-201306099, Tairui Pharmaceutical, China). Mueller-Hinton Agar (MHA, 130205) and Mueller-Hinton Broth (MHB, 130320) were purchased from Beijing Land Bridge Technology (China).

2.2. Bacteria

E. coli (ATCC8739), *S. aureus* (ATCC25923) and *S. dysgalactiae* (ATCC12388) originated from the American Type Culture Collection and were kindly provided by the College of Life Sciences, Tsinghua University, the College of Veterinary Medicine, Nanjing Agricultural University and by Prof. Lihua Xu, College of Agriculture, Ningxia University, respectively. *Pasteurella* (CVCC392) was purchased from the China Institute of Veterinary Drugs Control and *Streptococcus agalactiae* (CVCC586) was again kindly provided by Prof. Lihua Xu. All these bacteria are known as pathogens that cause cow mastitis.

2.3. Determination of the effects of the complex of trypsin and chymotrypsin on the growth of different bacteria

Trypsin or chymotrypsin were weighed and dissolved in PBS buffer (pH 7.4) to obtain 0.08 (0.5C), 0.16 (C) and 0.32 (2C) mg/mL trypsin and/or chymotrypsin solutions. C for the trypsin+chymotrypsin complex=0.16 mg/mL trypsin+0.16 mg/mL chymotrypsin. All enzyme solutions were filtered with Millipore Express Membrane filter (pore size 0.22 μm) before use.

50 mL of bacteria (diluted to 10^6 CFU/mL) was centrifuged, and the supernatant was discarded. 50 mL of different enzyme solutions were mixed with the bacteria above. PBS buffer and bacterial liquid were mixed for blank controls. Enzyme-bacteria mixtures and PBS-bacteria mixtures were incubated for 4 h at 37 °C with shaking at 180 rpm. 2 mL of each mixture were removed to another 50 mL centrifuge tube containing 28 mL MHB. Finally, all tubes were shaken at 37 °C, 180 rpm. 1.5 mL was sampled respectively to an aseptic Eppendorf tube at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, and 16 h. Samples were kept at 4 °C until detection. The optical absorbance (OD, 600 nm, the quantity of bacteria is proportional to the OD value within the range tested, Zhou et al., 2013) of all the samples was determined in 96-well plates using a microplate reader, and bacterial growth curves were drawn according to the OD values. Each treatment was carried out in triplicate.

2.4. Detecting the effects of the combination of antibiotics and the complex of trypsin + chymotrypsin on bacterial growth

2.4.1. Determination of Minimum Inhibitory Concentration (MIC) of antibiotics

Antibiotics were dissolved in PBS buffer and filtered to produce solutions of 2560 μg/mL. Microdilution was used to generate antibiotic solutions in 96 well plates with concentrations ranging from 1280 μg/mL to 0.000611 μg/mL, and then 20 μL microbial liquids were added to each well. Positive controls (bacteria added, no antibiotic) and negative controls (PBS, no bacteria, no antibiotic) were set. The plates were cultured at 37 °C for 18–24 h. Three parallel tests were performed in each antibiotic experiment. The MICs of antibiotics were used to select the optimal kinds of antibiotic for inhibiting bacterial growth.

2.4.2. Synergy test

Antibiotics were mixed respectively with trypsin and chymotrypsin in PBS. The final solutions were composed of antibiotics at the MIC and the complex of trypsin + chymotrypsin at the doses 2C, C and 0.5C, respectively. PBS was used in blank controls without any drug.

2.5. Detection of the impact of the complex of trypsin + chymotrypsin and antibiotics on bacterial ultrastructure

S. aureus and *E. coli*, as representatives of Gram-positive bacteria and Gram-negative bacteria respectively, were chosen to explore the effect of the complex of trypsin, chymotrypsin and *anti*biotics on bacterial cell ultrastructures. The optimal antibiotics against *S. aureus* and *E. coli*, respectively tilmicosin phosphate and ceftiofur sodium, were used. The drugs solutions were as described in the sections above. 9 mL drug solution for each treatment group was mixed with 3 mL bacterial liquid (10^6 CFU/mL). The control group was treated with PBS. All mixtures were cultured for 4 h at 37 °C with shaking at 180 rpm, and then were centrifuged at 6500 rpm for 20 min. After washing three times with PBS, the treated bacteria were immediately fixed with 2.5% glutaraldehyde solution. Samples were sent to the College of Life Science, Nanjing Agriculture University, for detection, and observed by scanning electron microscope (SEM) and transmission electron microscope (TEM).

2.6. Detection of bacterial intracellular leakage

S. aureus or *E. coli* culture in the logarithmic phase (3 mL) were centrifuged (6500 rpm, 20 min), washed three times with PBS, and