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Full Length Research Paper

Pathology of extracellular protease of *Stenotrophomonas maltophilia* isolated from channel catfish (*Ictalunes punctatus*) to mice

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A pthogenic bacterium *Stenotrophomonas maltophilia* was isolated from channel catfish (*Ictalunes punctatus*). We studied the pathogenicity of the extracellular protease (ECPase) of *S.maltophilia*. The extracellular protease was purified by 70% ammonium sulfate fractionation and DEAE-Sephadex A 50 and injected intraperitoneally to mice. The pathological changes of injected mice were observed. The injected mice showed different lethal rate and their typical symptoms include the demonstration of tachypnea and downcast, with mucous stool on their anuses. The tested animals showed visible lesions in livers, spleens, stomachs and intestines. They had serious gastroenteritis and flatulence. Mural stomach and intestines became thin with pallide-flavens mucus. The spleen was tumescent and hemorrhaging. The hepar was seriously tumid and had necrotic regions. The color of the kidney became light and the lung had no obvious change. The LD_{50} of the ECPase was 4.33 ug/g.

Key words: *Stenotrophomonas maltophilia,* extracellular protease, pathology, mice, channel catfish (*Ictalunes punctatus*)

INTRODUCTION

The gram-negative bacillus bacterium *Stenotrophomonas maltophilia,* formerly named *Xanthomonas maltophilia* or *Pseudomonas maltophilia* (Swings et al., 1983; Norberto, 1993), is found in a wide variety of environments and geographical regions as well as in ecological niches both inside and outside hospitals (Hugh and Leifson, 1963; Papapetropolou et al., 1994; Guerzoni et al, 1994; Nakatsu et al., 1995). It causes cystic fibrosis (Christopher et al., 2002; Marchac et al., 2004; Marcha et al., 2004; Samuel et al., 2010), urinary tract infection (Vartivarian et al., 1996; David, 2004; Andrews, 2008), respiratory tact infection (A'Court and Garrard, 1992; Pathmanathan and Waterer, 2005; Gasparetto et al., 2007), endocarditis (Baddour et al., 1991; Ijaz and Nirav, 2002) and bacteremia (Jang et al., 1992; Dong et al., 2004). Their resistance to many currently available broad-spectrum antimicrobial agents, including those of the carbapenem class, characterizes the majority of strains of *S. maltophilia* (Quinn, 1998), and it is increasingly recognized as an important cause of nosocomial infection. Beside the infection to human, it also infects goats, crocodile (Harris and Rogers, 2001), *Seriola quinqueradiata* (Furushita et al., 2005) and albacore tuna leading to death.

During April 2004 to 2006, there was an outbreak of an acutely epidemic disease of channel catfish (*Ictalunes punctatus*) infected by *S. maltophilia* in the Sichuan province, China. It made the breed aquatics of channel catfish a heavy hit. Little is known about the virulence factors and mechanism associated with the bacterium, and because of the broad antibiotics, it is difficult to control and huge economic losses are being incurred.

The study aimed to purify the extracellular protease and injected to mice. The results helped us to examine whether the extracellular protease was the virulence factor of S.maltophilia and how it caused death to mice.

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Figure 1. Haloes surrounded the colony of *S. maltophilia.*

We also described the pathology of extracellular protease of *S. Maltophilia* isolated from channel catfish to mice.

MATERIALS AND METHODS

Qualitative Detecting of Extracellular Protease

S.maltophilia was cultured in TSB 20℃ for 24h. 30μl culture was added to the punched skimmed milk agar plate and also cultured at 20℃ for 24h to observe if the haloes surrounded the colonies.

Purification of extracellular protease

The wild strain of *S. maltophilia* was isolated during naturally occurring outbreaks of *S. maltophilia* disease of channel catfish from a fish farm in china. The bacterial strain was routinely cultured in TSB at 20°C with shaking (150 rpm). Culture supernatant was collected by centrifugation (10000 × g, 30 min), concentrated by 70% ammonium sulfate overnight and centrifuged to collect the sedimentation, dissolved by 50 mM Tris/HCl pH 7.4. The diluted solution was applied to a column of DEAE-Sephadex A-50. Fifty fractions of 150 ml were collected and assayed for proteolytic activity. A single peak of activity was detected and all the active fractions were pooled and concentrated by polyethylene glycol. The extracellular protease proteolytic activity was measured referring to Allan and Stevenson, (1981).

Extracellular protease injected to mice

The purified protease was double diluted. At the same time, 35 mice were divided into six groups and injected intraperitoneally with diluted protease of 0.5 ml. The concentration of the injected protease of each team was: 0.860, 0.43, 0.215, 0.108, 0.054 and 0.027mg/ml, respectively. The control team was injected with equal amount of physiological saline. All animals were closely observed for 7 days, but most died within 6 - 52 h. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin. The fixed tissues were sectioned (6 μm) on a Lica and stained with haematoxylin and eosin.

Assay of proteolytic activity

Proteolytic activity was assayed by using azocasein (sigma) as a substrate. 50 ul of a suitable diluted enzyme solution was added to 50 ul of azocasein (1%, wt/vol) in a reaction buffer, and the mixture was incubated at 30°C for 30 min. The reaction was terminated by adding 1.25 ml of 5% (vol/wt) trichloroacetic acid and left for 30 min on ice, followed by centrifugation at 15,000×g, at 4℃ for 10 min. Adding 2 ml of 1.0 N NaOH neutralized 2 ml of the supernatant, and the absorbance at 440 nm (A_{440}) was measured by using a spectrophotometer. One unit of enzyme activity was defined as the amount which yielded an increased in A_{440} of 0.001 in 30 min at 30℃.

RESULTS

Qualitative Detecting of Extracellular Protease

After cultured 24h, haloes was observed surrounding the colonied of *S. maltophilia*. It demonstrated that *S. maltophilia* could secrete extracellular protease.

Purification of the enzyme

The fold purification of the enzyme is given in Table 1.

Lethal effect of extracellular protease on mice

The molecular weight of the purified protease is 45.7 KDa. When the purified protease is injected intraperitoneally into mice, the 50% lethal dose (LD_{50}) was found to be 4.33 ug/g of body weight. The first team entirely dies after being injected for 6 h and the lowest concentration of injected protease team were all alive. The death rate is different in each team (Table 2).

Clinical symptom of the tested mice

The tested mice demonstrated tachypnea and downcast and had mucous on their anuses. Tested animals show visible lesions in livers, spleens, stomachs and intestine. They exhibited serious gastroenteritis and flatulence. Mural stomach and intestine became thin with pallideflavens mucus in it. The spleen was tumescent and hemorrhaging. The hepar was seriously swollen and appeared in the necrotic regions. The color of kidney gets thin, but the lung has no obvious changes (Figures 2 and 3).

The fold purification of the enzyme (see table below:)

Table 1. Purification of extracellular protease of *S.maltophilia.*

Purification process	Volume (mL)	Total protein (mg)	Totalactivity (U)	Specific activity ($U \cdot mg^{-1}$)	Relative purification
Culture filter	500	113	7.4×10^{4}	6.55×10^{2}	0
Ammonium sulfate	20	35.04	1.84×10^{5}	5.24×10^{3}	8.0
DEAE Sephadex A-50 fast flow	10	11.21	0.92×10^{5}	8.22×10^{3}	12.5

Table 2. The death rate of mice injected with *S. maltophilia* extracellular protease intraperitoneally.

Team	No injected (mg/ml	Protein concentration (mg/ml)	Injected volume (ml)	No. of death	Death rate (%)
1	5	0.860	0.5	5	100
2	5	0.430	0.5	5	100
3	5	0.215	0.5	4	80
4	5	0.108	0.5	2	40
5	5	0.054	0.5	1	20
6	5	0.027	0.5	0	9
Control	5	0	0.5	0	0

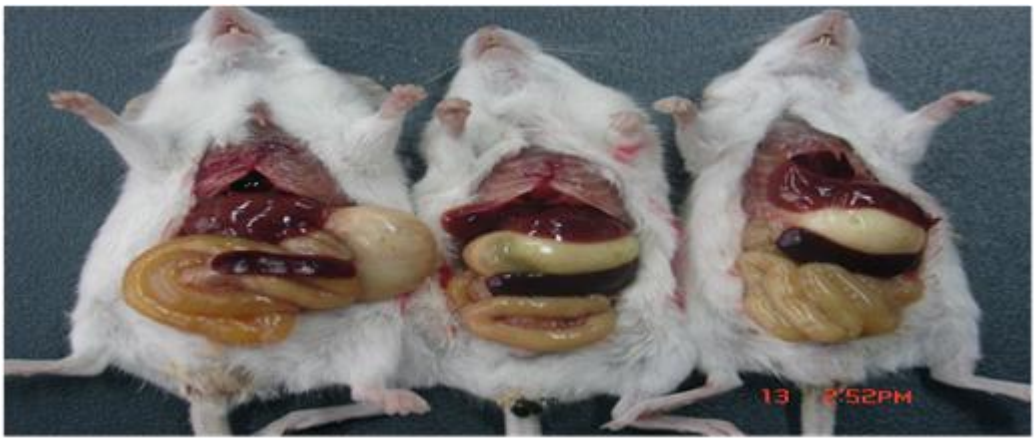

Figure 2. Dead rats had serious flatus, spleen and hepar swelling as well as splenorrhagia.

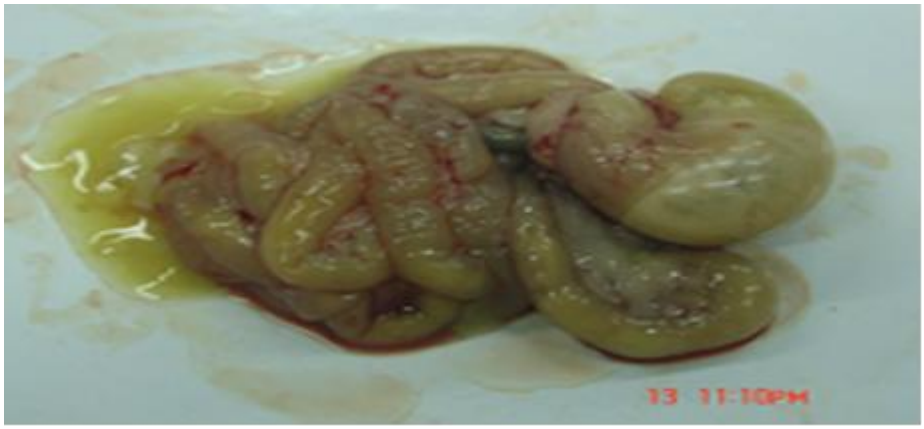

Figure 3. Gastrointestinal tract was filled with yellowy phlegm.

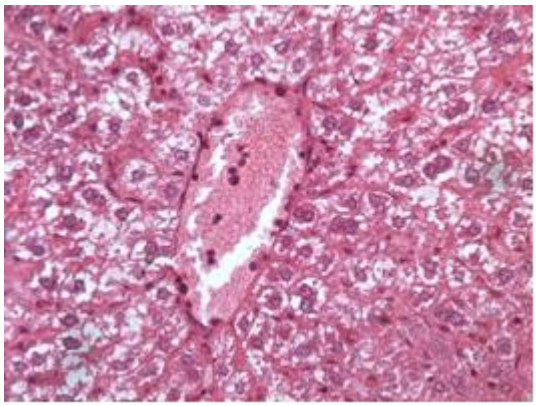

Figure 4. The hepatic cell had a serious vacuolar degeneration. Magnification: ×400.

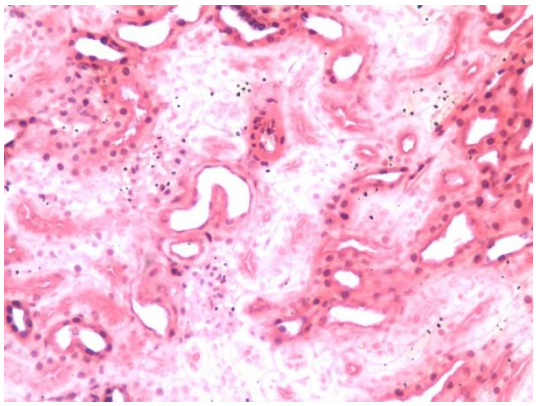

Figure 6. Nephric tubule had severe degeneration and necrosis. Magnification: ×400.

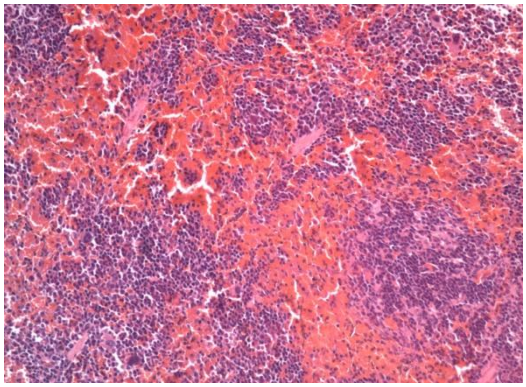

Figure 5. Splenorrhagia. Magnification: ×200.

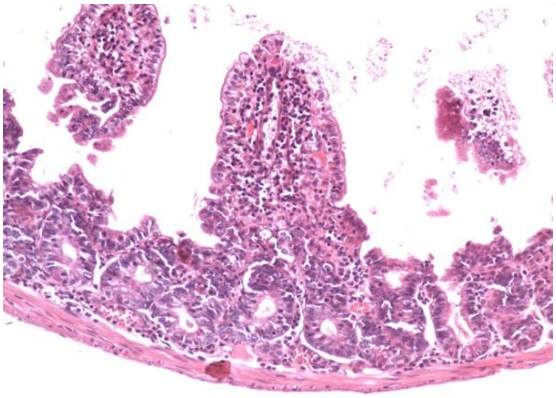

Figure 7. Intestinal mucosa had necrosis. Magnification: ×200.

Histopathologic Change

All the dead mice and the live mice that were killed later were selected for microscopic observations. They showed massive hepatic necrosis involving many lobules. Hepatic lobules vena showed dilatation and congestion. The hepatic cells showed swelling and granular degeneration. With the increase of protease concentration, vacuolar degeneration occurs. Some regions showed ballooning degeneration. Plasmolysis existed in many cells and liquifaction necrosis forms (Figure 4).

Splenorrhagis is seen in each tested team. Erythrocytes are filling with white pulp. The amount of lymphocyte reduces, and is replaced by erythrocytes (Figure 5).

The kidneys of mice in each tested team were affected. The lesions involved both cortical and medullary zones. The tubules were affected more, with damages consisting of denudation of the lining epithelium with red stained casts in the lumen. The epithelium showed granular degeneration when the concentration of the injected protein was low. The higher the concentration injected, the worse the lesion. Some came out as fatty degeneration. A lot of epithelium was lysed, forming different liquefacient necrosis zones. The normal shape of the tissue disappeared (Figure 6).

Protease injection is followed by marked tissue damage of gastrointestinal tract. There were many aneutrophil in the villi, the epithelial layer was severely damaged with superficial necrosis and sloughing of epithelium and some villi were completely absent. There was edema in the submucosa, with congested and dilated blood vessels. In animals treated with low concentrated protease, all the features of gastrointestinal damage were greatly reduced (Figure 7).

The cardiac cells showed granular degeneration in high concentration team and the tissues tended to be normal when the protease concentration was less than 0.108 mg/ml. Some part of the lungs hemorrhage and inflammatory cell was infiltrated (Figures 8 and 9).

DISCUSSION

Extracellular protease was an important virulence for

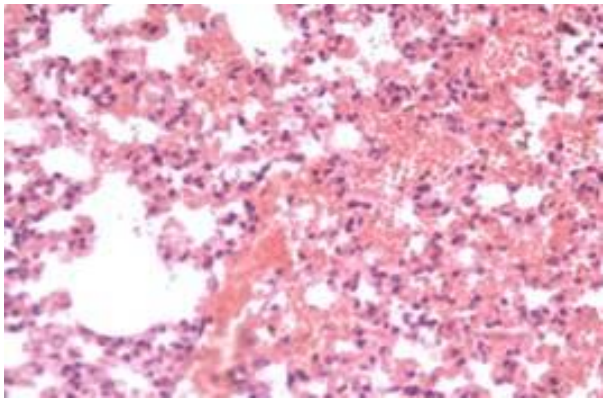

Figure 8. Pneumorrhagia. Magnification: ×400.

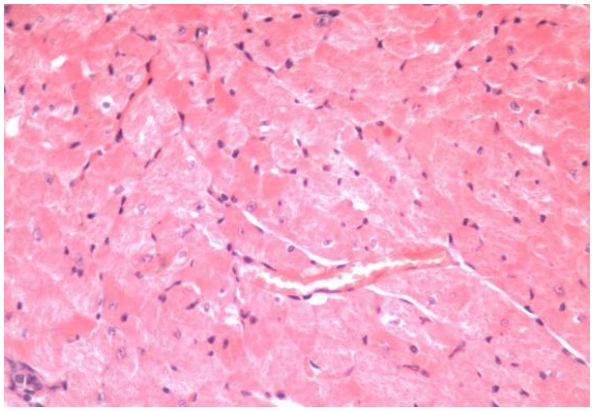

Figure 9. The cardiac muscle cell had granular degeneration. Magnification: ×400.

most bacteria. Reports showed that extracellular protease was the main virulence factor of many bacteria, such as *Aeromonas hydrophila* and *Pseudomonas aeruginosa***.** Sakai(1985) reported that the protease-deficient mutant (NTG-1) showed loss of virulence. Lee S (1998) demonstrated that protease IV was also a virulence factor of *Pseudomonas aeruginosa.* Fyle (1986) purified the extracellular protease, and intramuscularly injected it to Atlantic salmon, and the injected tissues was liquefied (Fyfe, 1986). The LD_{50} of extracellular protease of *S. maltophilia* to mice was 4.33 ug/g. However, the LD_{50} of the thermal instability serine protease P1 and the thermal medium-stability serine protease P3 are 3 and 8 ug/g respectively (Thune and Graham 1982), This is possible due to the variety of the bacteria. Little is known about the pathogenesis of *S. maltophilia*. Elaboration of a range of extracellular enzymes by *S. maltophili*a, including DNase, RNase, fibrinolysin, lipases, hyaluronidase, protease, and elastase has being carried out (Boethling, 1975; Arella and Sylvestre, 1979; O'Brien and Davis, 1981; Bottone et al., 1986), and Bottone et al. (1986) had proposed that these enzymes play a role in the pathogenesis of *S. maltophilia* associated infection.

Our research showed that the extrcellular protease of *S. maltophilia* isolated from channel catfish is an important virulence factor of the bacteria. It leads to the death of the tested mice and causes multi-tissue, organ hemorrhage and necrosis. The effect of the extracellular protease of *S. maltophilia* on channel catfish is to be studied in further research.

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