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SEPARATION, PURIFICATION AND MOLECULAR CHARACTERIZATION OF A NOVEL LUMBROKINASE FROM TUBIFICIDAE

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ABSTRACT

Using Tubificidae as the material was purified by historrhexis, salting out, ultra filtration, Sephadex G-50 gel filtration chromatography and DEAE Sepharose Fast Flow anion-exchange chromatography, acquired pure product of lumbrokinase. The activity of the lumbrokinase was detected by agarose-fibrin plate method. After purification by anion-exchange chromatography, the purity of the lumbrokinase with a specific activity of 5630.4 U/mg and increased 25.2-fold over the purity of the supernatant. By sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the molecular weight of the purified lumbrokinase distributed at the range of 15-29 kDa, namely, 15 kDa, 27 kDa, 29 kDa. The optimal temperature and pH for the killing activity of the purified killer toxin were 36 ℃ and 8.0, respectively, and the killing activity was stable over a temperature range of 0–50 ℃ and pH range of 7.0–10.0. Moreover, the ions increased the enzyme activity of K+, Na+, Mg2+, Ca2+ and Fe3+, while Pb2+ and Hg2+ have inhibitory effect. In addition, we used microorganism depressed method (cup method) to test the anti-microbial effects, we found that lumbrokinase can nhibit the growth of Staphylococcus aureus, Escherichia coli, klebsiella pneumoniae, Candida albicans and Bacillus subtilis. Especially for klebsiella pneumoniae and Candida albicans, the effects were extremely significant.

Keywords: Tubificidae; purification; molecular characterization; lumbrokinase; anti-microbial

Introduction

Earthworm is a kind of common medicinal materials, which contains rich protein and active substance. Earthworms are closely related to human beings, not only can be used as traditional Chinese medicine, but also can extract lumbrokinase, a variety of amino acids and other active ingredients. Lumbrokinase is a class of proteins with plasmin activity extracted from earthworms. It has the function of dissolving thrombus and is one of the most promising fibrinolytic drugs at present. In 1983, Mihara et al. isolated the crude extract with fibrinolytic

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activity from Lumbricus rubellus and named it Lumbrokinase (Mihara, et, al., 1983). In recent years, different components of lumbrokinase acquired from a variety of earthworms, and their fibrinolytic activity are not consistent (Rao et al. Mihara et 1986; al. 1991; Du 2007; Zhao 2012; Xie 2013). But hardly any studies have been done on the active constituents of Tubificidae, and no one has reported whether they have the activity of lumbrokinase. Lumbrokinase has the function of dissolving cellulose and thrombus, and the degree of difficulty of extraction, separation and purification are varies greatly with earthworm species.

Cheng (Cheng et al. 1990; Cheng 1996) acquired two fibrinolytic components with molecular weight of 29 kDa and 36 kDa from Lumbricidae Bimostos by extraction, ammonium sulfate salting out and column chromatography with 0.01 mol/L phosphate buffer (pH7.5), has a strong fibrinolytic effect, and has obvious dissolution effect on the experimental blood clot of rabbits. Zhang Wei (1997) purified a lumbrokinase as glycoprotein, the sugar content between 3%-15%, and the activity of the enzyme was lost after treated with three methane sulfonic acid; However, Nakajima reported that all the six plasmin they purified were non saccharide protains, and they were considered as serine protease with lumbrokinase and plasmin activity (Nakajima et al. 2002). A large number of in-vivo and in-vitro experiments showed that most of the lumbrokinase have anti-clotting, fibrinolytic, anti-thrombotic and thrombolytic effects (Li, and, Zhao, 2005; Gao, et, al., 2007; Lu, et, al., 2007; Zhang, and, Liu, 2004; Xie, 2013). Moreover, China has put its clinical application into the clinical practice, and has accumulated a wealth of research data on the prevention and treatment of CCVd (Dong et al. 2004; Zhou et al. 2006).

The Tubificidae also named as red filarial or red nematode, has high protein content, rich nutrition and good palatability, therefore, it is a natural food for many animals. In order to prove whether the Tubificidae has the activity of lumbrokinase, we studied the ingredients of Tubificidae for the first time. This study provides technological and data support for the development and utilization of Tubificidae resources, enriching the single use of bait and adding the additional value. We purified an lumbrokinase from Tubificidae and studied its characterization and antibacterial properties.

Materials and Methods

Materials

The Tubificidae purchased from aquaculture base in Jinan, Shandong province, China. All solvents and other chemicals were obtained from Sigma Aldrich. All aqueous solutions were prepared with deionized water unless otherwise stated.

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Bacterial strains were grown in luria-bertani (LB) medium containing 0.5% yeast extract, 1.0% peptone, 0.5% NaCl, and 2% agar. Yeast strains were grown in yeast extract peptone dextrose (YPD) medium containing 1.0% yeast extract, 2.0% glucose, 2.0% peptone, and 2% agar. Mould strains were grown in potato dextrose agar (PDA) medium containing 20 g glucose, 200 g potato, 15 g agar, amd 1000 mL distilled water.

Methods

Preparation of crude lumbrokinase

Take a certain amount of Tubificidae, washed several times, and then stimulate it to remove dirt from the body, and remove the excess water with the filter paper. After weighed, add buffer solution by volume of 1.5 times, homogenate l0 min in the homogenizer and put in the chromatography cabinet standing 4 h. After centrifugation (8,000 × g, 10 min, 4 °C), the supernatant was added solid ammonium sulfate to make it reach 30% saturation, then centrifugation (10,000 × g, 20 min, 4 °C), the supernatant was concentrated to a volume of 100 mL by ultrafiltration with a 10-kDa cutoff membrane in a Labscale TFF system (Millipore, Billerica, MA, USA). These concentrated supernatants were used as the crude lumbrokinase preparation, freeze-drying preservation (Peng 2010).

Determination of lumbrokinase activity

Agarose-fibrin plate method was conducted according to the method proposed by Dai (2008) to test the activity of the lumbrokinase. Taking 1 mL 40 U/mL thrombin solution and 5ml 0.5% agarose solution of temperature below 60 °C were quickly mixed with 5 ml 3 mg/ml fibrinogen solution, and poured into the culture plates. And 10 μL different concentrations of urokinase standard solution and sample solution were added in the same plate, respectively. The plates were placed at 28 °C for 18 h, after which the diameters of the dissolution zones were measured with a vernier caliper and recorded. Unit (U) of the standard urokinase was used as the abscissa, the product of two vertical diameters were used as the ordinate, and then acquired a regression equation. The unit (U) of sample titer was calculated through the regression equation (Xie 2013).

Purification of lumbrokinase

A concentrated crude lumbrokinase solution obtained by ultra filtration concentration was applied to a Sephadex G-50 gel chromatography column (Bestchrom), which was eluted with a gel filtration elution buffer (Phosphate Buffered Saline, pH7.5) at a flow rate of 1 mL/min. The eluent containing the lumbrokinase from the Sephadex G-50 column was fraction-collected by collection tubes and then pooled. The combined solution was eluted with phosphate buffered

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saline (pH7.5). After overnight equilibration of the resulting mixture, the mixture was applied to a anion-exchange chromatography column (DEAE-Sepharose Fast Flow; Bestchrom, Shanghai, China). After the entire sample entered the gel, the column was rinsed with an ion-exchange solution at a column pressure of 0.1 MPa and a flow rate of 1 mL/min for 1–2 h, followed by gradient elution with 1 M NaCl and the ion-exchange buffer under the same conditions. The eluent was automatically fraction-collected into several collection tubes, each collecting 4 mL. The collected samples were used to measure lumbrokinase activity and total protein content. The entire elution process was conducted at 4 °C in a protein purification system using ÄKTA™ prime with HiTrapTM (Amersham Biosciences, Uppsala, Sweden), and the light absorption of the sample was measured at a wavelength of 280 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method proposed by Laemmli (1970) to test the purity and molecular weight of the lumbrokinase. Electrophoresis was conducted according to the user manual of Mini-PROTEAN Tetra (Bio-Rad Biosciences, Orsay, France), and Coomassie brilliant blue R-250 was used for staining (George and Diwan, 1983). The protein marker was the Blue Plus Protein Marker (Transgen Biotech, Beijing, China), which comprises 7 pre-stained proteins with molecular weights in the range of 14–100 kDa, with the molecular weight of 50 kDa associated with an orange band; 25 kDa, 30 kDa, 40 kDa, 70 kDa, and 100 kDa associated with blue bands; and 14 kDa associated with a bright-yellow band. Electrophoresis was conducted on a two-dimensional electrophoresis system (Amersham Biosciences).

Effects of pH and temperature on lumbrokinase activity

The enzyme activity of the lumbrokinase was tested at a temperatures range of 16~44 °C. The purified lumbrokinase was placed in a thermostatic water bath for 1 h at 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C and 80 °C. The hydrolytic activity retained in the sample was tested, and the temperature stability of the lumbrokinase was determined.

The enzyme activity of the purified lumbrokinase was tested on test plates at range of 3.0~12.0. The purified lumbrokinase was mixed with the same volume of phosphate buffer of pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0. After incubation at 4 °C for 12 h, the hydrolytic activity retained was tested, and the pH stability of the lumbrokinase was determined.

Effect of metal-ion on lumbrokinase activity

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The effect of metal-ions on enzyme activity can usually be divided into inhibitors and activators. The activated effect of metal-ions mainly include K+, Na+, Ca2+, Fe3+ and Mg2+, while the inhibitory effect of metal-ions mainly are heavy metal ions. Therefore, we selected K+, Na+, Mg2+, Fe3+, Ca2+, Pb2+ and Hg2+ as the test ions. Meanwhile, the effect of EDTA on enzymes was determined in this study to determine whether the lumbrokinase is a metallo enzyme.

The concrete method is to add 0.1 mL 0.2 mol/L metal-ions to 1 mL samples of lumbrokinase (Zhao 2012). The added components were KCl, NaCl, MgSO4, CaCl2, FeCl3, EDTA, (CH3COO) 2Pb and HgCl2, with no metal ions as the blank.

Anti-microbial effects of lumbrokinase

The lumbrokinase was prepared with sterile distilled water into 4,000 U/mL, 6,000 U/mL, 8,000 U/mL and and 10,000 U/mL, respectively. Using the microorganism depressed method (cup method) and double-layer medium to test the anti-microbial effects of the lumbrokinase (Li et al. 2015). Before the bacterial layer has been solidified, 4 Oxford cups (6 mm × 10 mm) with a sterilization diameter of 6 mm were placed on the plates according to the experimental requirements, and 100-μL aliquots of the samples to be tested were added to the cups, with no lumbrokinase as blank. The plates were placed at 28 °C for 48 h, after which the diameters of the microbial inhibition zones were measured with a ruler and recorded

Results and Discussion

Purification of lumbrokinase

The prepared crude lumbrokinase was sequentially purified by ultra filtration concentration, gel filtration chromatography and anion-exchange chromatography (DEAE SepharoseTM Fast Flow). The elution curve of gel filtration chromatography showed 3 elution peaks (Fig. 1). The eluent at peak 4 had hydrolytic activity, and the eluent was collected in collection tubes numbered 17–31. All the collected eluent portions were applied to the anion-exchange chromatography column, which was subjected to gradient elution with 1 M NaCl and the ion exchange buffer under the same condition. The elution curve shown in Fig. 2 indicates that the eluent portion at the first elution peak had activity, with the peak vertex showing the highest activity. The finally lumbrokinase was purified 25.2-fold compared with the supernatant, with a specific activity of 5630.4 U/mg.

Gel electrophoresis

The final concentrated eluent showed multi-component band indicating a molecular weight range of 15-29 kDa (Fig. 1), very similar to the molecular weight of the lumbrokinase from Eisenia

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foetida range of 20-60 kDa (Xie et al. 2013). Through scientific literatures we find that the most purified lumbrokinases are oligomeric enzyme, the distribution of molecular weight from 15 kDa to 90 kDa, while Yang (1997) purified a dipolymer with lumbrokinase activity. The lumbrokinase from earthworm mainly have molecular weights of 15 kDa, 50 kDa, 70 kDa and 90 kDa (Zhao 2012). It is indicated that lumbrokinases from different earthworm are different.

Effects of temperature and pH on lumbrokinase activity

The results of lumbrokinase activity were tested at different temperatures are shown in Fig. 4(a). The samples showed the largest activity (320 U/mL) at a optimum temperature of 36℃. After incubation of the lumbrokinase at different temperatures for 1 h, the stability of the hydrolytic activity was tested, and the results indicated that the lumbrokinase had good stability in the temperature range of 0–50 ℃, rapidly declining at temperatures above 60 ℃ and almost totally disappearing at temperatures above 70 °C (Fig. 4(a)). While the results of the effect on pH are shown in Fig. 4(b), indicated that the activity was the largest at pH 8.0—with a activity of 318 U/mL, while no hydrolytic activity was formed at pH values lower than 3.0. After 12-h exposure of the lumbrokinase to different pH conditions, the hydrolytic activity of the purified lumbrokinase was tested to determine the pH stability. The results showed that the lumbrokinase had relatively good stability at pH 7.0–10.0 (Fig. 4(b)).

In the natural environment, most lumbrokinase had good stability. For example, the lumbrokinase still had relatively high activity after heated 30 min at 60 ℃, and activity began to decrease when the temperature above 60 ℃, while no hydrolytic activity was formed at temperature higher than 85 ℃ (Wang 2010); The lumbrokinase from Eisenia foetida in good stability at the range of 20-60 ℃ (Xie 2013). However, the lumbrokinase from Pheretima was only 33% of the remaining activity after heated in 30 ℃ for 1 h, and only had good stability in the range of pH4.0-8.0 (Zhang 2006).

Under normal conditions, purified lumbrokinase has no obvious change at the pH range of 3-11. For example, the lumbrokinase from Eisenia foetida is a polymers, the optimal pH range of 7.0–10.0 and stable in the pH range of 6-11 (Xie 2013); The earthworm fibrinolytic enzyme is stable in the pH range of 4-13 (Liu and Zhang 2002); And Wang et al. (2010) indicated that the enzyme activity decreased obviously when the pH was lower than 3 or higher than 11, very similar to our study of the lumbrokinase from Tubificidae, it may be the lumbrokinase from Tubificidae and Eisenia foetida have similar local structure.

Effect of metal-ion on lumbrokinase activity

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The results are shown in Fig. 5, indicated that the ions increased the enzyme activity of K+, Na+, Mg2+, Ca2+ and Fe3+, while Pb2+ and Hg2+ have inhibitory effect. And the EDTA has no effect on the enzyme activity, indicating that the enzyme is not a metalloenzyme. It is similar to the results that Hg2+ has a complete inhibitory effect on the lumbrokinase activity, and Mg2+ plays an active role in lumbrokinase activity (Xie, 2013). It's related to Mg2+ is an activator of many phosphorylation kinases, and Hg2+ can act with sulfhydryl, the active substance of enzyme related to cystine often exists at the center of enzyme activity (Han 2010).

Anti-microbial effects of lumbrokinase

The purified lumbrokinase has anti-microbial activity as the resuilts shown in Table 1. From the table, we can know that lumbrokinase can nhibit the growth of Staphylococcus aureus, Escherichia coli, klebsiella pneumoniae, Candida albicans and Bacillus subtilis. Especially for klebsiella pneumoniae and Candida albicans, the effects were extremely significant. Moreover, the diameter of inhibition zone increased with the increase of the concentration of lumbrokinase, and the effect on klebsiella pneumoniae reached the maximum inhibition zone at 10,000 U/mL (13.4mm).

Conclusions

The purified lumbrokinase from Tubificidae is a multimeric protein with molecular weight range of 15-29 kDa. The optimal temperature and pH for the purified lumbrokinase to act on the fibrinogen were found to be 36°C and 8.0, respectively. The lumbrokinase is stable over a temperature range of 0–50°C and pH range of 7.0–10.0. In addition, the ions increased the enzyme activity of K+, Na+, Mg2+, Ca2+ and Fe3+, but Pb2+ and Hg2+ have inhibitory effect. The anti-microbial effects experiment showed that lumbrokinase can nhibit the growth of Staphylococcus

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Conflict of Interest statement

We declare that we have no conflict of interest.

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