Sains Malaysiana 45(1)(2016): 115–118

Studies on Separation and Properties of Lumbrokinase in *Pheretima praepinguis*

(Kajian ke atas Pemisahan dan Sifat Lumbrokinase dalam *Pheretima praepinguis*)

TANG MEI, LIU CAO, LIANG ZI, GONG MINGFU* & HU DIE

ABSTRACT

In order to separate lumbrokinase in Pheretima praepinguis *and to study the enzymatic properties, with* Pheretima praepinguis *as material, lumbrokinase was separated with the salting out method. Lumbrokinase activity was measured with casein medium plate method. The effect of pH and temperature on lumbrokinase activity was studied. The activity of lumbrokinase separated from* Pheretima praepinguis *was relatively high. Lumbrokinase activity in neutral or slightly alkaline environment was higher. Lumbrokinase had tolerance ability to high temperature, with highly enzymatic activity under 60° and wide range of temperature adaptation.*

Keywords: Casein medium plate method; Lumbrokinase; Pheretima praepinguis; *salting out method; separation*

ABSTRAK

Pengasingan lumbrokinase dalam Pheretima praepinguis *dan untuk mengkaji sifat enzim, dengan* Pheretima praepinguis *sebagai bahan, lumbrokinase telah dipisahkan dengan kaedah penggaraman keluar. Aktiviti lumbrokinase diukur dengan kaedah kasein plat sederhana. Kesan pH dan suhu aktiviti lumbrokinase telah dikaji. Aktiviti lumbrokinase dipisahkan dari* Pheretima praepinguis *adalah agak tinggi. Aktiviti Lumbrokinase dalam persekitaran neutral atau sedikit alkali adalah lebih tinggi. Lumbrokinase mempunyai keupayaan toleransi kepada suhu tinggi, dengan aktiviti yang sangat enzim bawah 60° dan pelbagai penyesuaian suhu.*

Kata kunci: Kaedah kasein plat sederhana; kaedah penggaraman keluar; lumbrokinase; pengasingan; Pheretima praepinguis

INTRODUCTION

Earthworm fibrinolytic enzyme (EFE), also known as Lumbrokinase(LK) (Mihara et al. 1983) is a set of homoserine proteolysis enzyme with fibrinolytic activity derived from earthworm. With a strong antithrombotic and thrombolysis effect, Lumbrokinase can effectively degrade fibrinogen thereby reducing blood viscosity (Li 2009; Zhu et al. 2009). Applications of lumbrokinase in the field of heart and blood system diseases is very extensive because of good effect for the treatment of cerebral ischemic diseases (Qian et al. 2005; Yu et al. 2009), cardiovascular disease (Lukman et al. 2014; Wei 2010) and diabetes (Chu et al. 2008; Hang 2009). Lumbrokinase may have multiple single component (Chu et al. 2008; Wang et al. 2003), the enzyme activity is affected by many factors (Chi et al. 1999; Wang et al. 2003; Zhou et al. 2011).

Pheretima praepinguis, known as large earthworms of Mount Emei, a unique earthworm Emeishan, belongs to Pheretima, Megascolecinae, Megascolecidae. The general body length of *P. praepinguis* is 30 cm, the longest up to 80 cm (Li 1957).

In this experiment, crude products of lumbrokinase was extracted from *P. praepinguis* grew in Mount Emei. Plasmin activity of crude lumbrokinase was detected by using casein plate method (Naureen et al. 2014; Wu & Zhao 2004). In order to seek broader way of lumbrokinase biological preparation, the effects of pH and temperature on lumbrokinase activity was been further studied.

MATERIALS AND METHODS

P. PRAEPINGUIS

P. praepinguis were collected from cool, moist, humus-rich soil altitude 500-700 meters from Baoguo Temple to Qingyin pavilion in Mount Emei, Sichuan province, China.

PREPARATION OF CRUDE PRODUCTS OF LUMBROKINASE

Fresh *P. praepinguis* was cleaned with water several times, in order to make all the dirt excreted by earthworms surface moisture absorbed with filter paper, weighing, adding two volumes of pH7.8 phosphate buffer in tissue homogenates machine homogenized 5 min, at 4C° standing overnight, 4C° 5000 rpm centrifuge 10 min, discard the pellet. The supernatant was added with solid ammonium sulfate to a saturation then centrifugation (4C°, 4000 rpm, 10 m), then the supernatant was added with solid ammonium sulfate to a saturation and then allowed to stand overnight at 4 °C centrifugation conditions (4C°, 40000 rpm, 10 min). The precipitate collected was dissolved in a phosphate buffer solution, dialyzed with a dialysis bag and then desalted

using a freeze dryer and freeze-dried to obtain a powdery substance of a large number of light-brown, crude products is called lumbrokinase, 4C° constant temperature and stored at (Astrup & Mullertz 1952; Surhio et al. 2014).

Preliminary Determination Lumbrokinase Activity

The initial activity of lumbrokinase preform was determinatied by Casein plate method (Astrup & Mullertz 1952). The size of diameter of the transparent circle represents casein hydrolyzing activity of lumbrokinase.

Determination of The Activity of Lumbrokinase by Casein as Substrate

1 g of casein powder was added to 100 mL 0.05 mol • L^{-1} pH of 7.8 Tris-HCl buffer and lysed by heating, so that the substrate concentration was 1% and incubated at 37°C. Draw 2 mL lumbrokinase treated enzyme solution, then quickly add 2 mL of 37°C pre-insulated substrate solution, shake, continue incubation 30 min and finally add 2 mL 10% trichloroacetic acid solution to stop the reaction. The solution was kept at room temperature for 30 min and filtered. The absorbance of filtrate was determined at a wavelength of 280 nm and the retention of enzyme activity was calculated (Butt et al. 2015).

Determination of Optimum pH of Lumbrokinase Activity

The lumbrokinase freeze-dried powder was taken and put into the enzyme solution. After that, 1 mL of enzyme solution were added with 2 mL pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 phosphate buffer, mix. After 4 h at room temperature, the absorbance of filtrate was determined at a wavelength of 280 nm, retention of enzyme activity was calculated to determine the optimum pH of their lumbrokinase activity (Khaskheli et al. 2015).

Determination of Optimum Temperature and Thermal Stability of Lumbrokinase

2 mL of lumbrokinase enzyme solution and 2 mL of 1% concentration of casein solution, respectively, were incubated for 10 min at different temperatures of 20, 30, 40, 50, 60, 70, 80, 90°C. Then the enzyme solution was mixed with the casein solution at the same temperature. Mixed solution was incubated for 30 min at the original temperature. Finally, 2 mL of 10% trichloroacetic acid solution was added to the mixed solution to stop the reaction. The mixed solution was filtrated after standing at room temperature for 30 min. The absorbance of filtrate was determined at a wavelength of 280 nm, retention of enzyme activity was calculated, to determine the optimum temperature (Ashraf et al. 2013; Kiyani et al. 2014).

2 mL of enzyme solution incubated at the optimum temperature conditions for different time of 2, 4, 6, 8, 10, 12, 14, 16 and 24 h. Then the enzyme solution was mixed with 2 mL of 1% concentration of casein solution. Then the enzyme solution was mixed with the casein solution at the same temperature. The mixed solution was incubated for 30 min at the original temperature. Finally, 2 mL of 10% trichloroacetic acid solution was added to the mixed solution to stop the reaction. The mixed solution was filtrated after standing at room temperature for 30 min. The absorbance of filtrate was determined at a wavelength of 280 nm, retention of enzyme activity was calculated, to determine the holding time of lumbrokinase activity under optimum temperature (Batool et al. 2015).

Results and Discussion

Crude Extracts and Active Detection of Lumbrokinase

A total of 12.015 g freeze-drying crude product of lumbrokinase was obtained through the crude extract by ammonium sulfate salting precipitation method with 2053.125 g of clean fresh *P. praepinguis*. Lumbrokinase yield of 0.525% was relatively low.

Lumbrokinase activity was higher and the average diameter of the transparent circle was 15 mm with casein plate method (Figure 1).

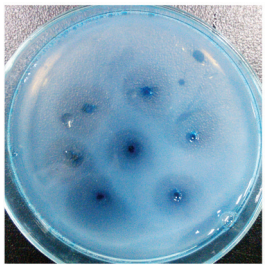

FIGURE 1. Lumbrokinase activity detection with Casein plate method

Determination of Optimum pH of Lumbrokinase Activity

Lumbrokinase activity was significantly affected by pH (Figure 2). Lumbrokinase activity was relatively stable and high activity in the alkaline environment (pH7-9), lumbrokinase activity retention rate maintain at 75% when pH increased to 11. Lumbrokinase activity gradually reduced in an acidic environment with pH decreased, lumbrokinase activity retention rate was less than 25% at pH4 and zero at pH3.

Effect of Temperature on Lumbrokinase Activity

Lumbrokinase activity was significantly affected by pH (Figure 3). Lumbrokinase activity retention rate was 100% below 60°C, rapid declined less than 10% when temperature rised to 70°C and 0% when temperature reached 80°C.

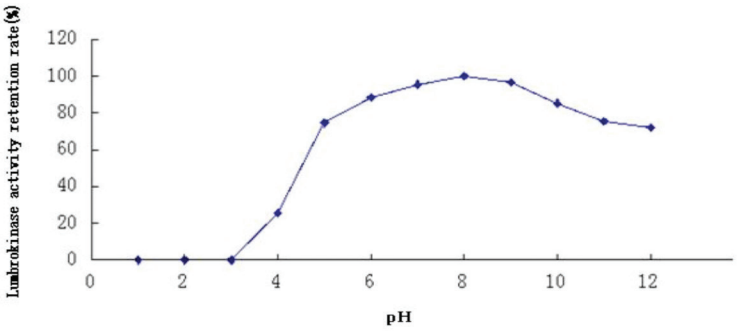

FIGURE 2. Effect of pH on lumbrokinase activity

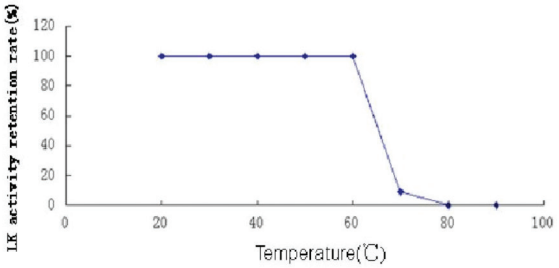

FIGURE 3. Effect of temperature on lumbrokinase activity

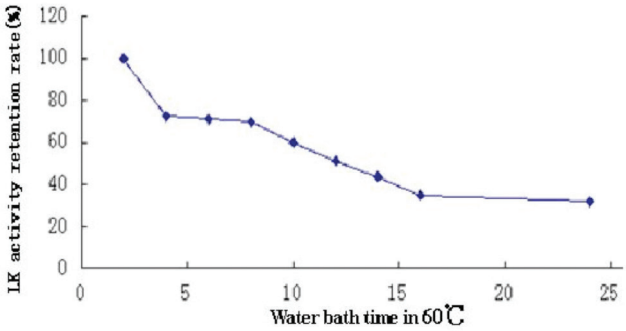

FIGURE 4. Effect of water bath time in 60°C on lumbrokinase activity

Lumbrokinase activity kept intact and Lumbrokinase activity retention rate maintained 100% in temperature range of 20 to 60°C, but the water bath time of enzyme solution at 60°C also affected the lumbrokinase activity (Figure 4). Lumbrokinase activity retention rate maintained at 100% with water bath 60°C for 2 h. With the water bath time in 60°C extended, lumbrokinase activity decreased gradually and stabilized with water bath 60°C for 16 h. Lumbrokinase activity retention rate stabled at around 33.0% after 16 h of water bath in 60°C.

CONCLUSION

The yield of lumbrokinase crude extract was only 0.525% which is lower than 2% from the previous studies. Low lumbrokinase yield in this experiment may be related with many factors such as using fresh wet earthworms as not all discharged dirt in earthworms, the homogenized earthworms were stored at 4°C for long time and other factors.

Lumbrokinase completely inactivated in pH conditions less than 3, but the stability of lumbrokinase in a neutral or alkaline environment was preferred (Lu et al. 2007; Yang & Ru 1997). This result maybe related with protein denaturation with strong acid environment. The results in this experiment was completely consistent with the results that the clinical drug of lumbrokinase and it can be well adapted to alkaline environment in the intestine and be inactivate in the acidic environment of gastric juice.

Lumbrokinase in this experiment has a good thermal stability, adaptation to a wide temperature range from 20 to 60°C, similar to the results described by Zhou et al. (2011). The properties of lumbrokinase extracted from *P. praepinguis* and *Eisenia fetida* was basically the same.

Therefore, the process of extraction and production of the lumbrokinase can be carried out at room temperature environment directly. Oral formulations and injections made from lumbrokinase can also be directly saved at room temperature, away from heat and exposure.

ACKNOWLEDGMENTS

The work was supported by Key Program of Department of Education of Sichuan Province (Grant NO: 09ZA041).

REFERENCES

Ashraf, M.A., Ullah, S., Ahmad, I., Qureshi, A.K., Balkhair, K.S. & Rehman, M.A. 2013. Green biocides, a promising technology: Current and future applications. *Journal of the Science of Food and Agriculture* 94(3): 388-403.

Astrup, T. & Mullertz, S. 1952. The fibrin plate method for estimating fibrinolytic activity. *Archives of Biochemistry and Biophysics* 40: 346-351.

Batool, S., Khalid, A., Chowdury, A.J.K., Sarfraz, M., Balkhair, K.S. & Ashraf, M.A. 2015. Impacts of azo dye on ammonium oxidation process and ammonia oxidizing soil bacteria. *RSC Advances* 5: 34812-34820.

Butt, M.A., Ahmad, M., Fatima, A., Sultana, S., Zafar, M., Yaseen, G., Ashraf, M.A., Shinwari, Z.K. & Kayani, S. 2015. Ethnomedicinal uses of plants for the treatment of snake and scorpion bite in Northern Pakistan. *Journal of Ethnopharmacology* 1: 1-14.

Chi, Y.J., Tian, B. & Zhang, Y.H. 1999. Studies on properties and thrombolytic activity of fibrinolytic enzyme from earthworm. *Journal of Northeast Agricultural University* 30(4): 267-270.

Chu, W.P., Fu, Q. & Zhang, M.H. 2008. The effect of lumbrokinase treatment on hemorheology, glucose and lipids in type 2 diabetes mellitus. *Chinese Journal of Diabetes* 16(8): 489-490.

Hang, R. 2009. Observation of 100 treatment cases of diabetes combined microcirculation with lumbrokinase. *China Practice Medicine* 20(4): 153-154.

Khaskheli, A.A., Talpur, F.N., Ashraf, M.A., Cebeci, A., Jawaid, S. & Afridi, H.I. 2015. Monitoring the *Rhizopus oryzae* lipase catalyzed hydrolysis of castor oil by ATR-FTIR spectroscopy. *Journal of Molecular Catalysis B: Enzymatic* 113: 56-61.

Kiyani, S., Ahmad, M., Zafar, M.A., Sultana, S., Khan, M.P.Z., Ashraf, M.A., Hussain, J. & Yaseen, G. 2014. Ethnobotanical uses of medicinal plants for respiratory disorders among the inhabitants of Gallies-Abbottabad, Northern Pakistan. *Journal of Ethnopharmacology* 156: 47-60.

Li, Z.Y. 2009. Efficacy of Ischemic stroke prevention with lumbrokinase capsules. *Capital Medicine* 16: 58.

Li, Y.L. 1957. Large earthworms in Mount Emei. *Bulletin of Biology* (5): 20-22.

Lu, L., Xu, Y.N., Liu, J., Qian, M. & Wang, J. 2007. Clinical efficacy of injectable lumbrokinase. *Acta Universitatis Medicinalis Nanjing (Natural Science)* 27(11): 1326-1327.

Lukman, A.L., Nordin, N.F.H. & Kamarudin, K.R. 2014. Microbial population in the coelomic fluid of Stichopus chloronotus and Holothuria (Mertensiothuria) leucospilota collected from Malaysian waters. *Sains Malaysiana* 43(7): 1013-1021.

Mihara, H., Sumi, H. & Akazawa, T. 1983. Fibrinolytic enzyme extracted from the earthworm. *Thromb Haemost* 50: 258-263.

Naureen, R., Tariq, M., Yusoff, I., Choudhury, A.J.K. & Ashraf, M.A. 2014. Synthesis, spectroscopic and chromatographic studies of sunflower oil biodiesel using optimized base catalyzed methanolysis. *Saudi Journal of Biological Sciences* 22: 322-339.

Qian, C.M., Dang, H.L. & Jing, X. 2005. Drug analysis of treatment of lacunar brain and cerebral arteriosclerosis. *Journal of Northwest Pharmaceutical* 20(5): 234-235.

Surhio, M.A., Talpur, F.N., Nizamani, S.M., Amin, F., Bong, C.W., Lee, C.W., Ashraf, M.A. & Shahid, M.R. 2014. Complete degradation of dimethyl phthalate by biochemical cooperation of the *Bacillus thuringiensis* strain isolated from cotton field soil. *RSC Advances* 4: 55960-55966.

Wang, X.Y., Wu, X.B. & Xu, F.C. 2003. Purification and some properities of fibrinolytic enzyme from Pheretima. *Pharmaceutical Biotechnology* 10(2): 88-91.

Wei, H.X. 2010. Compare treatment of angina pectoris with lumbrokinase and aspirin. *Capital Medicine* 2: 47-48.

Wu, J.X. & Zhao, X.Y. 2004. Casein plate method for determination of earthworm fibrinolytic enzyme. *Nature Magazine* 26(3): 184-185.

Yang, J.S. & Ru, B.G. 1997. Purification and characterization of an sds-activated fibbrinolytic enzyme from *Eisenia fetida*. *Comparative Biochemistry and Physiology* 183(3): 623-631.

Yu, N.W., Liu, J., Wu, J.F., Xu, D.X., Yang, Y.S., Guo, F.Q., Xie, Y. & Sun, H. 2009. Clinical research of improve carotid atherosclerotic plaques in patients ischemic cerebrovascular disease with Lumbrokinase capsule. *China Medicine* 4(3): 169-170.

Zhou, H., Wang, C.W., Zhang, Y. & Liu, Y. 2011. Purification and characterization of lumbrokinas. *Chinese Journal of Biochemical Pharmaceutics* 32(3): 212-216.

Zhu, X.J., Tan, J.F., Zhu, Y., Ding, X., Jin, X.Y., He, H., Dong, P. & Yang, Y. 2009. Clinical studies of treatment of acute cerebral infarctionc with lumbrokinase capsule. *Capital Medicine* (16): 46-47.

College of Life Science
Leshan Normal University, Leshan
Sichuan 614000
China

*Corresponding author; email: zumingli2@gmail.com

Received: 23 July 2014
Accepted: 21 November 2014