0273-2289/1559-0291 (Online)/07/136:251–264/$30.00

Biochemical and Enzymatic Properties of a Novel Marine Fibrinolytic Enzyme from *Urechis unicinctus*

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Received November 23, 2005; Revised May 9, 2006; Accepted May 25, 2006

Abstract

A novel potent protease, *Urechis unicinctus* fibrinolytic enzyme (UFE), was first discovered by our laboratory. In this study, we further investigated the enzymatic properties and dynamic parameters of UFE. As a low molecular weight protein, UFE appeared to be very stable to heat and pH. When the temperature was <50°C, the remnant enzyme activity remained almost unchanged, but when the temperature was raised to 60°C the remnant enzyme activity began to decrease rapidly. UFE was quite stable in a pH range of 3.0–12.0, especially at slightly alkaline pH values. Mn^{2+}, Cu^{2+}, and Fe^{2+} ions were activators of UFE, whereas Fe^{3+} and Ag^{+} ions were inhibitors. Fe^{2+} ion along with Fe^{3+} ion might regulate UFE activity in vivo. The optimum pH and temperature of UFE were about 8.0 and 50°C, respectively. When using casein as substrate and a substrate concentration <0.1% casein (w/v), the reaction velocity was increased with substrate concentration. Also when using casein as substrate, the determined K_m and V_{max} of UFE were 0.5298 mg/mL and 3.0845 mol of L-tyrosine equivalent, respectively. Our systematic research results are significant when UFE is applied for medical and industrial purposes.

Index Entries: Marine animal; fibrinolytic enzyme; protease; *Urechis unicinctus*; biochemical properties.

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Introduction

Urechis unicinctus looks like earthworm, whereas they are actually different species. *U. unicinctus* is classified in the phylum Echiura, while earthworm is a member of the Annelida. It is a marine animal mainly found in China, Korea, Russia, and Japan *(1)*.

In China, earthworm has been used as a traditional Chinese medicine for more than 2000 yr. In *Compendium of Materia Medica*, edited by a Chinese traditional medical doctor, Shizhen Li (1518–1593), earthworm is known as "earth-dragon," used in dried powder form to treat thrombi *(2)*. Lumbrokinase extracted from earthworm mainly directly degrades fibrin but could also activate plasminogen such as urokinase *(3–5)*. The capsules of lumbrokinase, referred to as Panford or Boluoke commercially, have already been used in Eastern Asian countries such as China, Japan, and Korea, as well as in North American countries such as Canada and the United States for the treatment of thrombi, especially for stroke *(2,6,7)*. Isolumbrokinases were secreted from the alimentary tract of earthworm *(8)* and then transported into blood to carry out their function. *U. unicinctus* is a more primitive animal compared to earthworm, because it has no blood vessels. The main body of *U. unicinctus* is full of coelomic fluid, acting as blood. Its body wall muscle is usually served as a well-known seafood and a condiment and is abundant in most flavorful amino acids including glutamine, aspartic acid, alanine, arginine, and glycine. By contrast, the coelomic fluid and gut are usually discarded as waste, from which we isolated *U. unicinctus* fibrinolytic enzyme (UFE), a novel fibrinolytic enzyme with a low molecular mass of 10.38 kDa.

Although novel fibrinolytic enzymes are so far continuously discovered, their molecular masses are usually above 20 kDa *(9–13)*. When they are used for medical purposes as drugs, there are usually side effects such as fever, drug resistance, and allergic reaction owing to their immunogenic characteristics *(14–18)*. Long-term administration of lumbrokinase usually causes side effects such as vomiting *(7)*. However, the molecular weight of UFE is only about 10.38 kDa. Our previous studies *(19)* revealed that UFE is a strong fibrinolytic enzyme, which not only can directly degrade fibrin but also can indirectly hydrolyze fibrin by transforming plasminogen into plasmin. Therefore, UFE is a possible potent thrombolytic drug applied in the clinical setting and a protease that can be applied in the food industry to process and handle proteins. For this reason, its enzymatic properties and dynamic parameters were of significance and we studied them further.

Materials and Methods

Chemicals and Equipment

UFE was isolated by our laboratory. D-Glucose was purchased from Shanghai Aibi Chemical (Shanghai, China). Sulfuric acid (oil of vitriol) was from Qingdao Chemical (Qingdao, China). Phenol, casein (chemical

reagent), Na_2HPO_3, trichloroacetic acid (TCA), l-tyrosine, citric acid, boric acid, barbital, Na_2CO_3, NaOH, KH_2PO_4, and NaH_2PO_3 were obtained from Shanghai Chemical (Shanghai, China). The TU-1800 spectrophotometer was from Beijing Puxitongyong (Beijing, China). The TGL-16C Centrifugal Machine was from Shanghai Anting (Shanghai, China). Other chemical reagents and instruments were preserved by our laboratory. All reagents were of analytical grade except for phenol and casein.

Determination of Sugar

Sugar in UFE protein was determined by spectrum colorimetric assay of a phenol-sulfuric acid method *(20)*. Four milligrams of UFE freeze-dried powder was dissolved in 8 mL of distilled water. In each of three test tubes was placed 2 mL of UFE solution, 1 mL of 6% phenol, and 5 mL of sulfuric acid. The tubes were shaken to mix the reaction components well and left to stand at room temperature for 20 min. Then the absorbance was measured at 490 nm. A blank was prepared in the same manner except that the reaction mixture contained 2 mL of distilled water. D-Glucose, as the standard, was placed at 105°C in order to dry it well prior to use. The absorbance of the reaction mixture was converted into the amount of D-glucose equivalent, and then the percentage of sugar in UFE protein was calculated.

Determination of Enzyme Activity

Enzyme activity was determined by the caseinolytic activity method of Mcdonald and Chen *(21)* and Zhao et al. *(22)* with minor modification. Briefly, the reaction mixture (pH 7.4) containing 1 mL of enzyme solution and 1 mL of 1% casein (w/v) was shaken slightly and incubated at 40°C for 30 min, and the reaction was stopped by adding 2 mL of 0.4 mol/L TCA. The reaction mixture was incubated at 30°C for 30 min. The mixture was centrifuged at 8000*g* for 10 min, and the chromogenic reaction mixture containing 1 mL of centrifuged solution and 5 mL of 0.4 mol/L Na_2CO_3 and 0.5 mL of Folin-phenol reagent B was shaken carefully and incubated at 40°C for 20 min, then cooled to room temperature. The absorbance was measured at 680 nm. A blank was prepared in the same manner except that the reaction mixture contained 1 mL of enzyme solution and 2 mL of 0.4 mol/L TCA without 1% casein (w/v). L-Tyrosine was used as the standard. The absorbance of the reaction mixture was converted to the amount of L-tyrosine equivalent. One unit of enzyme activity is defined as the amount of enzyme required to degrade casein to produce 1 μg of tyrosine equivalent at 40°C/min.

Effect of Temperature, pH, and Metal Ion on Stability

First, thermal stability was tested. UFE (0.1 mg/mL) in 0.01 *M* phosphate-buffered saline (PBS) (pH 7.4) was incubated at eight temperatures (4, 25, 37, 40, 50, 60, 70, and 80°C, respectively), and then their residual activities were determined at the end of each time interval (30 min). Second,

pH stability was examined. UFE (0.15 mg/mL) in different buffer solutions (pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0, respectively) was stored at 4°C for 24 h, and then their residual activities were determined as described above. Buffers of pH 3.0–12.0 were prepared by mixing different proportional volumes of solution A and B. One hundred milliliters of solution A contained 0.6008 g of citric acid, 0.3893 g of KH_2PO_4, 0.1769 g of boric acid, and 0.5266 g of barbital, and solution B was 0.2 *M* NaOH. Finally, metal ion stability was determined. UFE (0.1 mg/mL) in distilled water contained 0.1, 0.01, and 0.001 *M* different kinds of metal ions, respectively, and was stored at 4°C for 24 h. Then the pH was adjusted to 7.4 to determine the residual activities as described above. Metal ions were Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, Ag^{+}, Zn^{2+}, Li^{+}, Fe^{2+}, Ba^{2+}, K^{+}, Mn^{2+}, and Na^{+}, and were prepared by $MgSO_4 \cdot 7H_2O$, $CaCl_2$, $CuSO_4 \cdot 5H_2O$, $FeCl_3 \cdot 6H_2O$, $AgNO_3$, $ZnSO_4 \cdot 7H_2O$, $Li_2SO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, $BaCl_2 \cdot 2H_2O$, KCl, $Mn(CH_3COO)_2 \cdot 4H_2O$, and NaCl, respectively. After each metal ion is prepared, it should be used immediately, especially Fe^{2+} ion, because when exposed to air Fe^{2+} ion is easily changed into Fe^{3+} ion by oxidation. In addition, the reaction mixture should be well sealed during testing.

Effect of Temperature, pH, and Metal Ion on Activity

First, the dependence of temperature on enzyme activity was determined. One milliliter of UFE (0.162 mg/mL) in 0.01 *M* PBS (pH 7.4) was added to an equal volume of 1% casein (w/v), then well mixed. The mixture was incubated at each temperature (30, 40, 50, 60, 70, 80, and 90°C) in the range of 30–90°C for 30 min to determine enzyme activity as previously described. Second, optimum pH was measured. One milliliter of UFE (0.12 mg/mL) in different buffers was added to an equal volume of 1% casein (w/v) in distilled water as substrate, with a final pH in the range of 3.0–12.0, then well mixed and incubated at 40°C for 30 min to determine enzyme activity. Finally, the effect of metal ion was tested. One milliliter of UFE (0.1 mg/mL) containing metal ion was added to an equal volume of 1% casein (w/v) in deionized water as substrate at a metal ion concentration of 0.01 *M*, then well mixed and incubated at 40°C for 30 min to determine enzyme activity as previously described.

Calculation of Kinetic Parameters

Prior to measuring the kinetic parameters of UFE, the effect of substrate concentration on enzyme reaction velocity was tested using an enzyme concentration of 0.1 mg/mL and a substrate concentration of 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5% casein (w/v) at 40°C for 10 min. Then the Michaelis constant for hydrolysis with UFE was determined at 40°C. Substrate concentration was varied between 0.005 and 0.05% casein (w/v), and the enzyme concentration of 0.1 mg/mL was kept constant. Samples were incubated for 10 min and the enzyme was inactivated by adding 2 mL of TCA. A method of determining enzyme activity to measure the absorbance at 680 nm was

Table1
Measurement of Sugar in UFE

Test tube	D-Glucose equivalent (μg)	Sugar percentage (%)
1	6.182	0.6182
2	5.820	0.5820
3	5.410	0.5410
Average[a]	5.786 ± 0.723	0.5786 ± 0.0723

[a]Means ± SD; $n = 3$.

then used. The reaction velocity [V] was defined as the amount of increase in absorbance at 680 nm. The reciprocal of the substrate concentration was plotted against the reciprocal of [V]. The kinetic parameters K_m and V_{max} were calculated from the X and Y intercept of a Lineweaver-Burk plot. K_m is defined as the substrate concentration at which the reaction velocity is half of its maximum value.

Results and Discussion

Detrmination of Sugar in UFE

When using D-glucose as the standard, the percentage of sugar in UFE protein was 0.5786 ± 0.0723% (w/w) (Table 1). This result implied that UFE was not a glycoprotein, because it barely had sugar and the slight positive reaction was possibly the result of a protein reaction with phenol–sulfuric acid. Many proteins carry out their functions in a form of glycoprotein *(15,23,24)*, and without a sugar component, they are easily degraded by protease, losing their activities *(25)*, although when the polysaccharide chains in glycoproteins are intact they are usually stable. On the contrary, low molecular weight proteins existing in a form of monomer are very stable and active without sugar. It was reported *(26)* that six isozymes with fibrinolytic activity were isolated from *Lumbricus rubellus,* an earthworm. These enzymes are monomer proteins consisting of single polypeptide chains and are not glycoproteins, and they have molecular weights of 23–30 kDa. Hen egg-white lysozyme is also a nonglycolated protein with a molecular weight of 14.4 kDa, usually used as a molecular weight standard for polyacrylamide gel electrophoresis *(27)*. Compared to hen egg-white lysozyme and UFE, there are smaller proteins in nature such as aprotinin, with a molecular weight of only 6.5 kDa.

Effect of Temperature, pH, and Metal Ion on Stability

The thermal stability of UFE in 0.01 *M* PBS (pH 7.4) was examined at eight temperatures ranging from 4 to 80°C; Figure 1 presents the results. Interpretation of the individual temperature stability curves (Fig. 1A) is difficult, because some of them are overlapped for a two-dimensional plot.

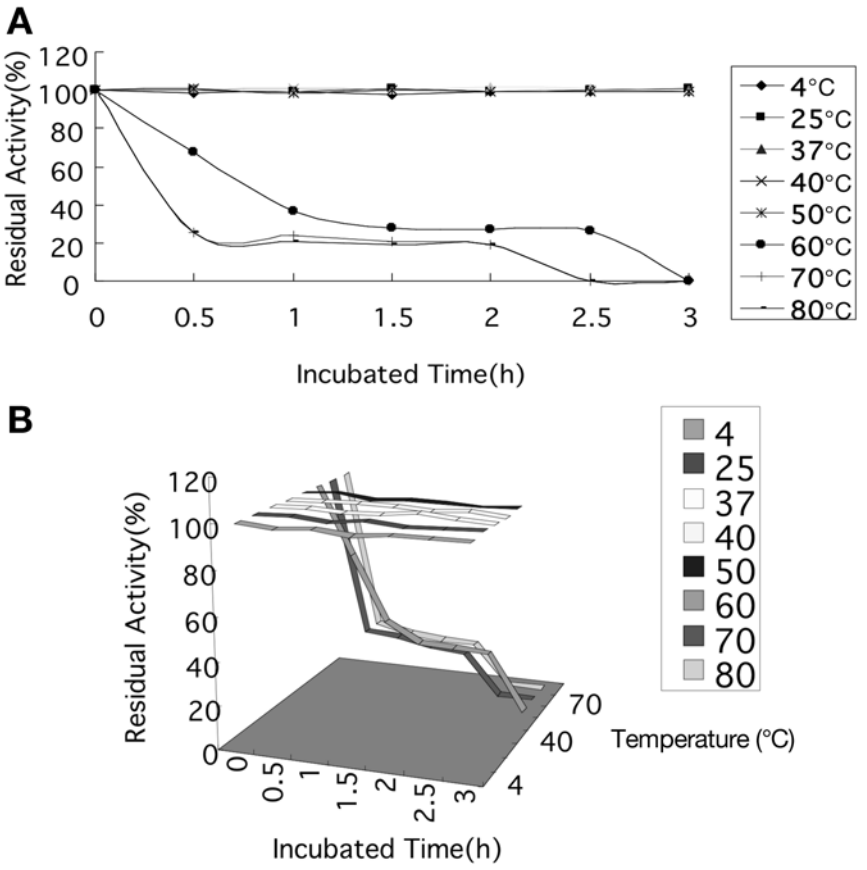

Fig. 1. Effect of temperature on stability of UFE: **(A)** two-dimensional plot; **(B)** three-dimensional plot. The enzyme samples (0.1 mg/mL) were incubated at eight temperatures ranging from 4 to 80°C, and at the end of each 30-min time interval, the residual activities were determined using casein (1% [w/v]) as substrate. The results are derived from absorbance values at 680 nm; each sample was measured three times and a mean of them with a reasonable standard deviation was used.

For this reason, a three-dimensional plot was prepared (see Fig. 1B). When the temperature was <50°C, enzyme activity remained almost unchanged. However, when the temperature was raised to 60°C, enzyme activity began to decrease rapidly, and at 60°C for 3 h, enzyme activity was not detected. UFE was stable at a relatively high temperature owing to its low molecular weight. In general, with continuous heating, high molecular weight proteins begin to gradually lose their activity and precipitate from solution owing to denaturation, which is often used in our laboratory to isolate peptide. UFE is expected to be of a higher thermal stability when used for medical purposes than in the present study, because the saline concentration (0.01 *M*) in UFE solution is remarkably lower than in vivo (0.9% physiologic saline, or 0.15 *M* NaCl), and higher saline concentration can improve

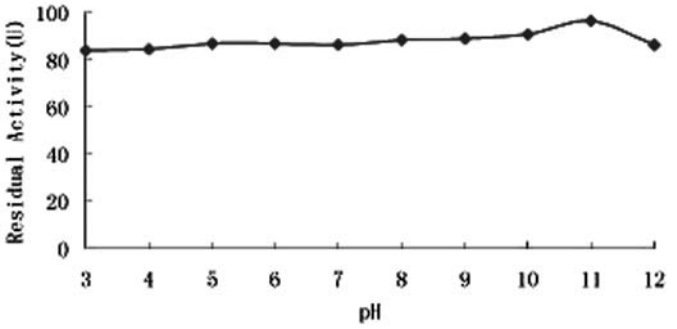

Fig. 2. Effect of pH on stability of UFE. The enzyme samples (0.15 mg/mL) in 10 buffers ranging from pH 3.0 to 12.0 were stored at 4°C for 24 h. Then the residual activity of each sample was determined using casein (1% [w/v]) as substrate. The results are derived from absorbance values at 680 nm; each sample was measured three times and a mean of them with a reasonable standard deviation was used.

the thermal stability of protein *(28)*. Additionally, thermal stability is of great importance for applying enzyme in industrial processes *(29)*.

The effect of pH on the stability of UFE was examined at 40°C using 10 pH values ranging from 3.0 to 12.0; Figure 2 presents the results. UFE was quite stable over the entire range of pH values examined, especially at slight alkaline pH values. When the pH was 11.0, the residual activity remained at 96.32%, whereas with an increasing pH residual activity began to drop. As a result of long-term evolution in nature, UFE from the marine animal *U. unicinctus* is well adapted to surviving in the environment of seawater. Therefore, it is more stable in alkaline than acidic pH, owing to the slight alkaline pH of seawater. The optimum pH at which *U. unicinctus* can survive in the seawater environment ranges from 7.5 to 8.5 *(30)*.

The effect of metal ion on UFE stability was examined for 12 metal ions (Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, Ag^{+}, Zn^{2+}, Li^{+}, Fe^{2+}, Ba^{2+}, K^{+}, Mn^{2+}, and Na^{+}) at three concentrations ranging from 0.1 to 0.001 *M*; Figure 3 presents the results. To make the measurement results more precise, none of the samples were dialyzed against metal ions prior to measurement, and because several metal ions are strong activators of the enzyme, their measurement results inevitably exceeded 100%. All of the tested metal ions were divided into three groups. The first group consisted of Na^{+}, K^{+}, Li^{+}, Mg^{2+}, Ba^{2+}, Ca^{2+}, Zn^{2+}, and Ag^{+}. UFE sample containing each of these metal ions was not stable; these metal ions might inhibit the activity of UFE, especially at the highest tested concentration except for Na^{+}, K^{+}, and Li^{+} ion. At different concentrations of Na^{+}, K^{+}, and Li^{+} ion, the residual activity was approximately half of the original, but at the highest tested concentration, the residual activity was slightly higher than that at the other concentrations, implying that the enzyme was more stable when at a higher Na^{+}, K^{+}, and Li^{+} ion concentration. The second group consisted of Mn^{2+} and Cu^{2+}. These two metal ions

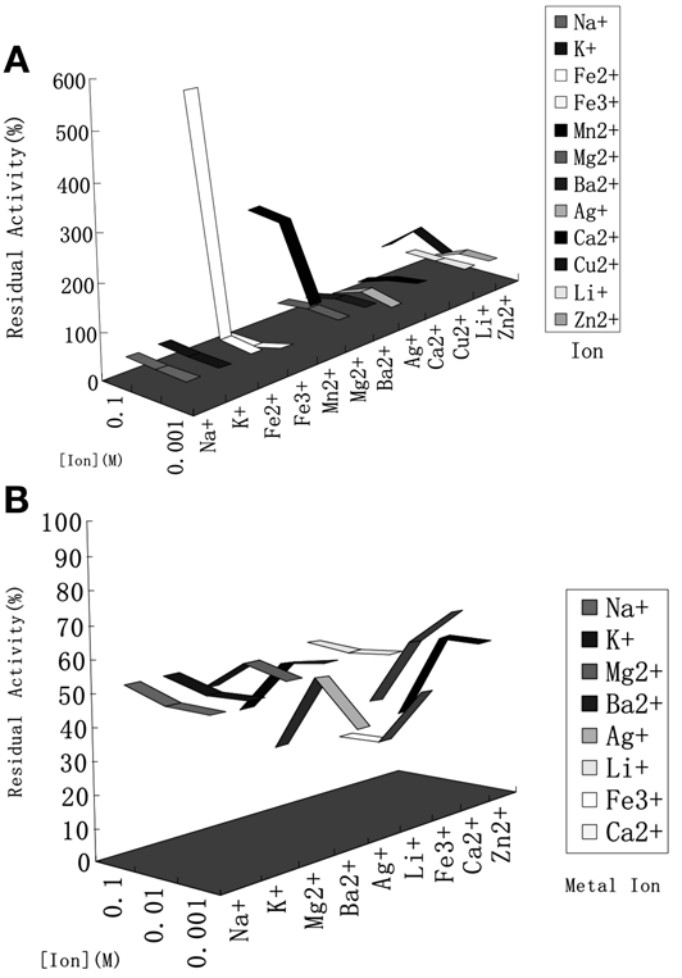

Fig. 3. Effect of metal ion on stability of UFE: **(A)** all results; **(B)** some results in detail. The enzyme samples (0.1 mg/mL) in distilled water contained 0.1, 0.01, and 0.001 *M*, respectively, of each kind of 12 metal ions (Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, Ag^{+}, Zn^{2+}, Li^{+}, Fe^{2+}, Ba^{2+}, K^{+}, Mn^{2+}, and Na^{+}) and were stored at 4°C for 24 h. Then their pH was adjusted to 7.4 to determine their residual activities. To ensure precise measurements, none of the samples were dialyzed against the metal ions, and the measurement results were therefore inevitably affected by the metal ions. Because several metal ions are strong activators of the enzyme, some of the results of these metal ions exceeded 100%. The results are derived from absorbance values at 680 nm; each sample was measured three times and a mean of them with a reasonable standard deviation was used.

were activators of UFE. At an Mn^{2+} ion concentration from 0.001 to 0.1 *M*, the residual activity was obviously increased over that of the original activity, indicating that Mn^{2+} ion was a strong activator of UFE. Cu^{2+} ion could also increase the activity of UFE at low concentrations, especially at 0.01 *M*. The third group consisted of Fe^{2+} and Fe^{3+}. When the ion concentration of Fe^{2+} ion increased from 0.001 to 0.01 *M*, the residual activity slightly in-

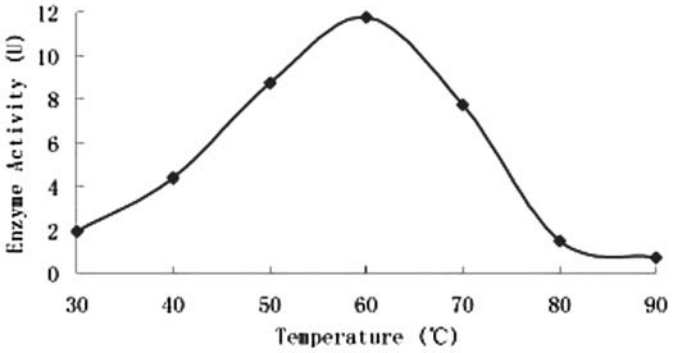

Fig. 4. Effect of temperature on enzyme activity of UFE. Activity was determined using 0.162 mg/mL of enzyme and 1% casein (w/v) as substrate at different temperatures ranging from 30 to 90°C for 30 min. The results are derived from absorbance values at 680 nm; each sample was measured three times and a mean of them with a reasonable standard deviation was used.

creased, and when the concentration continuously increased to 0.1 *M*, the residual activity sharply improved, to about 5.5-fold of the original. By contrast, at an Fe^{3+} ion concentration of 0.1 and 0.01 *M*, the residual activity remained unchanged, and when the ion concentration continuously decreased to 0.001 *M*, the residual activity began to increase. This is an interesting phenomenon and implies that Fe^{2+} and Fe^{3+} ion regulated UFE activity in vivo, because ferric ions abound in coelomic fluid of *U. unicinctus (1)* and UFE was mainly extracted from this fluid and Fe^{2+} and Fe^{3+} can be exchanged under certain circumstances. In our studies, coelomic fluid acting as blood was not clotted ex vivo for a long time; nevertheless, it was eventually completely clotted. The reason might be that UFE activity was gradually decreased as Fe^{2+} continuously changed into Fe^{3+} by the oxidation of air with the time elapsed.

Optimum Temperature and pH

Optimum temperature was determined using 0.162 mg/mL of enzyme in 0.01 *M* PBS (pH 7.4) and casein (1% [w/v]) as substrate at different temperatures ranging from 30 to 90°C for 30 min; Figure 4 presents the results. The optimum temperature was approximately 60°C. At above 70°C, activity was very low, because enzyme protein gradually lost its original bioactivity by thermal denaturation. However, at 30°C, the enzyme activity was also very low, a quite perplexing result, because *U. unicinctus*, as a marine animal, survives in the mud-sand cave of the coastal sea bed *(1)*, where the temperature is usually from 8 to 26°C *(30)*. This phenomenon might be explained by the fact that as previously studied Fe^{2+} and Fe^{3+} ion could regulate UFE activity with great ability (Fig. 3), and, hence, even under physiologic circumstances, UFE can carry out its function well. Nevertheless, this phenomenon warrants further study.

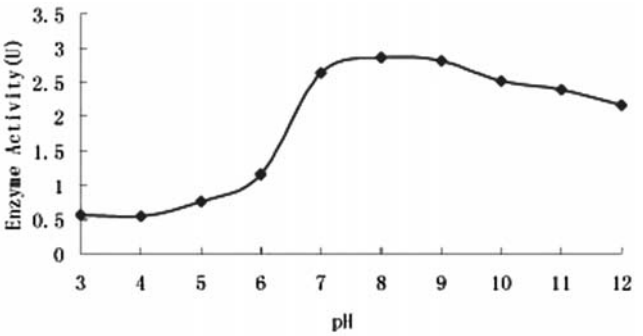

Fig. 5. Effect of pH on enzyme activity of UFE. Activity was determined using 0.12 mg/mL of enzyme in different buffers and 1% casein (w/v) in deionized water as substrate at a pH ranging from 3.0 to 12.0 and 40°C for 30 min. The results are derived from absorbance values at 680 nm; each sample was measured three times and a mean of them with a reasonable standard deviation was used.

Optimum pH was determined using 0.12 mg/mL of enzyme in different buffers and casein (1% [w/v]) in deionized water as substrate at pH values ranging from 3.0 to 12.0 and 40°C for 30 min; Figure 5 presents the results. The optimum pH was about 8.0. This can be explained by the fact that *U. unicinctus* survives in a seawater environment, where the most suitable pH is from 7.5 to 8.5 *(30)*, and as a result of long-term evolution, the optimum pH of UFE is about 8.0.

Effect of Metal Ions on Enzyme Activity

The effect of metal ions on enzyme activity was tested using 0.1 mg/mL of UFE and 1% casein (w/v) as substrate at a metal ion concentration of 0.01 *M* and 40°C for 30 min; Figure 6 presents the results. Compared with the control group, the enzyme activity of the tested groups of Mn^{2+} and Cu^{2+} ion was remarkably high. The results were indirectly verified by our previous studies described herein. Therefore, Mn^{2+} and Cu^{2+} ion are strong activators of UFE (Table 2), and Ag^{+} and Fe^{3+} ion are strong inhibitors (Table 2). The results were also indirectly verified by our previous studies. The effects of other ions (Mg^{2+}, Ca^{2+}, Zn^{2+}, Li^{+}, Ba^{2+}, K^{+}, Fe^{2+}, and Na^{+}) on the activity of UFE at 0.01 *M* were not obvious. However, at 0.1 *M*, Fe^{2+} ion is a strong activator of UFE, as demonstrated by our previous studies described herein (Fig. 3). Moreover, it is possible that other metal ions, such as organic and inorganic compounds, might also be activators or inhibitors of UFE, which warrants further study.

For enzyme application, it is important that activator or inhibitor is of great value in the field of enzyme industry. As a protease, besides application for medical purposes, UFE can be used in biochemical and food industrial areas *(31)* for processing protein. In industry, activator as additive is usually added to the reaction mixture to greatly improve the productivity, and inhibitor is used to prevent the unnecessary reaction from continuing. Therefore, our investigations of the effect of metal ions on UFE activity are of practical significance.

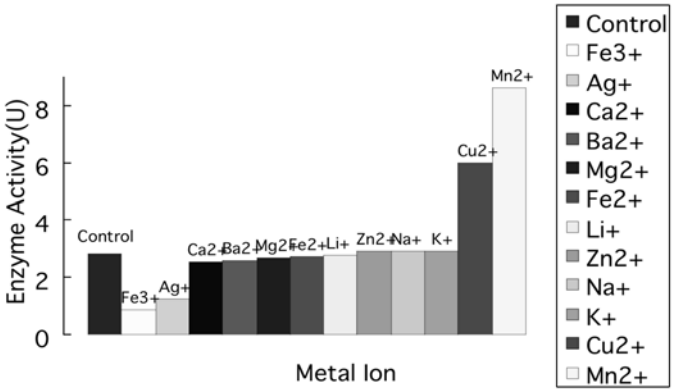

Fig. 6. Effect of metal ions on enzyme activity. Activity was tested using 0.1 mg/mL of UFE and 1% casein (w/v) as substrate at a metal ion concentration of 0.01 *M* and 40°C for 30 min. The results are derived from absorbance values at 680 nm; each sample was measured three times and a mean of them with a reasonable standard deviation was used.

Table 2
Activators and Inhibitors of UFE

Activator	Inhibitor
Cu^{2+}	Fe^{3+}
Mn^{2+}	Ag^{+}
Fe^{2+}	

Effect of Substrate Concentration on Enzyme Activity

The effect of substrate concentration on reaction velocity was examined using a UFE enzyme concentration of 0.1 mg/mL and substrate concentration of 0.05–0.5% casein (w/v) at 40°C for 10 min; Figure 7 presents the results. When substrate concentration was <0.1% casein (w/v), reaction velocity was at a direct ratio to substrate concentration, but with a continuous increase in substrate concentration, reaction velocity began to decrease. It is worthwhile to study this phenomenon further. The investigation of biochemical properties and dynamic parameters of UFE is of significance when UFE is used for industrial purposes, and by which the proportion of added enzyme and substrate in reactor can be regulated.

K_m *and* V_{max}

The Michaelis constant for casein hydrolysis with UFE was determined at 40°C and an enzyme concentration of 0.1 mg/mL; Figure 8 presents the results. K_m was 0.052981% casein (w/v), or 0.5298 mg/mL. V_{max} was 0.01903 absorbanceYalue/min, or 3.0845 mol of L-tyrosine equivalent.

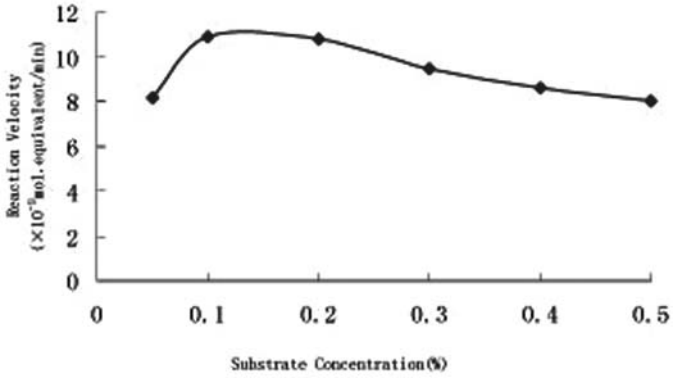

Fig. 7. Effect of substrate concentration on reaction velocity. The reaction was carried out at 40°C for 10 min with an enzyme concentration of 0.1 mg/mL and a substrate concentration of 0.05–0.5% casein (w/v). Th results are derived from absorbance values at 680 nm; each sample was measured three times and a mean of them with a reasonable standard deviation was used. Reaction velocity was the yield of L-tyrosine equivalent per minute.

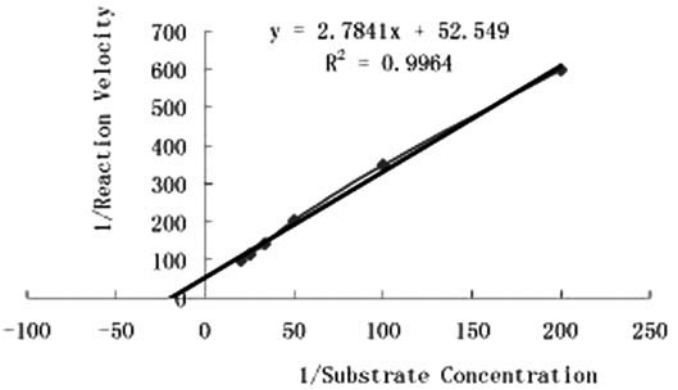

Fig. 8. Lineweaver-Burk plot for hydrolysis of casein with UFE. Determination of the kinetic parameters of the enzyme reaction was carried out at 40°C. Each sample was measured three times and a mean of them with a reasonable standard deviation was used. Reaction velocity was s the amount of increase in absorbance at 680 nm/min.

Conclusion

UFE is a small nonglycolated protein. Our previous studies *(19)* showed that its molecular weight is only 10.38 kDa. It is the smallest thrombolytic agent known to date. The molecular weight of currently used thrombolytic drugs, including lumbrokinase from earthworm, is usually >20 kDa. UFE is therefore expected to have fewer side effects than any other thrombolytic drug when applied in the clinical setting.

In the present study, we systematically investigated the biochemical and enzyme kinetic properties of UFE. It was discovered that UFE was

stable to heat and pH. When the temperature was <50°C, the residual enzyme activity remained almost unchanged, but when the temperature was raised to 60°C, the residual enzyme activity began to decrease rapidly. UFE was quite stable in a pH range of 3.0–12.0, especially when at slight alkaline pH values. The optimum pH was about 8.0, which is similar to that of the seawater in which *U. unicinctus* lives. The optimum temperature of the enzyme was approx 50°C. The effect of metal ion on UFE was also investigated. Mn^{2+}, Cu^{2+}, and Fe^{2+} ion were activators of UFE, whereas Fe^{3+} and Ag^{+} were inhibitors. The results of our investigations show that it is probable that Fe^{2+} ion along with Fe^{3+} ion may regulate UFE activity in vivo. Furthermore, some kinetic parameters of UFE enzyme were studied. It was found when using casein as the substrate and a substrate concentration <0.1% casein (w/v) that reaction velocity was at a direct ratio to substrate concentration. These investigations show that UFE is also of significance in industrial applications.

In general, as a strong potent thrombolytic agent and protease, UFE has many advantages. Thus, it can be applied in the medical field for treatment of thrombi and in the biochemical and food industries for processing proteins. For these reasons, further studies of UFE are important.

Acknowledgments

We are grateful to Dr. Changhong Wang for kindly providing technical assistance. This research was supported by grants from the China 863 High-technology Development Program (no. 2001AA625050) and the China Tenth 5-year Key Science & Technology Program (no. 2001BA708B04-07).

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