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Utilizing Enzymatic Method, Fibrin-Zymography and Liquid Chromatography Mass Spectrometry to Identify the Active Protein in Lumbrokinase Drug Substance

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Abstract

Lumbrokinase, extracted from the cultured earthworm of Eisenia fetida, is widely used to prevent or treat thrombosis as biochemical medicine in China. However, the molecular mechanism and protein components in the drug have not been clearly understood and the specification could not illustrate its characteristic comprehensively. In this study, we investigated the mechanism of lumbrokinase by the fibrin plate method. The results revealed that all the five manufacturers' products contained both fibrinolytic components and kinase components. We used the method of fibrin-zymography to show the existence and activity of lumbrokinase. The fibrin-zymography showed that lumbrokinase was a group of fibrinolytic proteins and there were slight differences between the products of different manufacturers. Besides, we proved the fibrin-zymogram gel could be used as the identification method to distinguish Eisenia fetida from other earthworm species. Then we identified the ingredients by mass spectrometry. The results suggested that fibrinolytic related components existed in the drug, including fifteen proteins. We further compared these proteins with other serine proteins. We observed the amino acid sequence and the catalytic triad of the identified proteins were similar with human trypsin and bovine trypsin. Besides, some also had similar characteristics with human tissue-type (tPA) plasminogen activators and urokinase (uPA) plasminogen activators. These results demonstrated lumbrokinase products contained two major groups of protein components, which suggested two different functions. One is the direct degradation of fibrin; the other is indirectly dissolving thrombus by activating the plasminogen into plasmin.

Introduction

Lumbrokinase (LK), which exists ubiquitously in earthworms, has been used as traditional medicines for thousands years in China. Due to its inconvenience for dosing and unpleasant smell, lumbrokinase has been manufactured into enteric capsules or enteric-coated tablets since 1990s. Lumbrokinase is safe, non-toxic, and has few side effects. The most common side effect is the hypersensitivity caused by heterologous proteins [1-9].

There are five manufacturers producing lumbrokinase drug substances and enteric coated products in China. The substances are extracted from the cultured earthworm (Eisenia fetida). Generally, process of lumbrokinase extraction includes three major procedures. First, the bodies of earthworm are milled and centrifuged to get the supernatant, then purified by DEAE(diethylaminoethyl) column and finally made into powder though the process of filtration, ultrafiltration and lyophilization. However, due to the rough manufacturing process, current products contain many different earthworm fibrinolytic enzymes and other contaminants which may cause side effects. Besides, although it has been used for a long time and believed to be effective, there is little knowledge about which kinds of proteins play an important role in the drug. So identification of protein components in lumbrokinase is urgently needed.

This research is intended to illustrate the mechanism of lumbrokinase in the related products to identify the proteins in lumbrokinase drug substances using ESI-MS (electrospray ionization mass spectrometry) and to provide data support for the improvement of its quality specification. In this study, we proved that lumbrokinase had two action mechanisms and identified the proteins existed in the lumbrokinase drug substances. We also recommended the fibrin-zymography technique as the identification method of earthworms.

Materials and Method

Materials: Sample loading buffer (5×, no DTT) and electrophoresis buffer (5×) were purchased from Beyotime biotechnology (China). Coomassie brilliant blue, dithiothreitol, formic acid, and PMSF were available from Sigma(USA);Chymotrypsin(Sequencer Grade V106A) was purchased from Promega (USA). Fibrinogen, lumbrokinase standard, thrombin and plasminogen were purchased from National Institute for Food and Drug Control (NIFDC, China). Other chemicals were analytical grade.

Enzymatic activity research

Sample preparation: For fibrin plate tests, the drugs from five manufacturers were dissolved in the 0.9% sodium chloride, and then diluted into a series of concentration (2000U/mL, 4000U/mL, 6000U/mL, 8000U/mL, 10000U/mL). The standard of lumbrokinase was diluted to obtain the same concentration.

Preparation of fibrin plate: Fibrin plates were prepared according to the standard method [10]. Added 39 mL of the fibrinogen solution (1.5 mg/mL, diluted with PBS buffer) into the agarose solution (55℃) and mixed toughly; added thrombin solution 3.0 mL, and transferred to the plastic culture plates after mixing evenly, waited for 1 hour at room temperature until the solution was solidified. The preparation of fibrin and kinase plates was the same except that 1mLplasminogen

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solution (3U/mL) was added into the plates.

For activity analysis, precisely measured 10 μL test solution of different concentrations into the pole, then incubated the plates at 37℃ for 18h. The diameter of the hydrolyzed clear zone was measured and the final values were an average of three replicates.

Fibrin-zymography

Lumbrokinase drug substances were dissolved into 3 mg/mL with the solution of 0.9% sodium chloride. SDS-fibrin gel was prepared as described by Kim and Choi [11,12]. The composition of the gel(10%) was described in the table 1. 10 μL of the supernatant from the drug substances that was diluted in the sample loading buffer were loaded into the fibrin gel. After running the gel (80V 30 min;120V 60 min), it was soaked in the 2.5% Triton-100 solution for 30min, then incubated in the reaction solution buffer (0.05M KH2PO4,0.04MNaOH; pH7.4) at 37℃ for 40 mins. The gel was stained with Coomassie Brilliant blue for 2h, then distained. The digested bands which reflected the enzymatic activities were visualized as clear bands of fibrinolysis against a dark-blue background of undigested fibrin substrate.

Liquid chromatography and mass spectrometry

Enzyme digestion: A solution containing about 1 mg of lumbrokinase per 1 ml was diluted with a solution containing 6 M urea, 50mMTris-HCl (pH 8.0) and 5 mm DTT, incubated for 20 mins at 95℃, then cooled to room temperature. A 6-fold volume of 50 mm ammonium bicarbonate solution (pH 7.8) was added. Chymotrypsin (chymotrypsin: sample = 1: 200) was added and digested at 37°C for 12h, then centrifugated (8000g) for 30mins before loading the sample to the mass spectrometry.

LC-ESI-MS/MS analysis an amount equivalent to 2μL of the digested peptide was then subjected to LC-MS/MS using a UPLC system and a Q-TOF mass spectrometry (Waters UPLC® G2S Q-TOF). Mobile phase A was 0.1% formic acid, while mobile phase B was acetonitrile. The flow rate was 0.3ml/min. The gradient elution program allowed for 5 min at 5%B,followed by a 40min step that raised eluent B to 40%, followed by a 10 min washing step at 40%, and then equilibrated at 5%B. The total analysis time was 60min.For mass spectrometry, the parameters were as follow: capillary voltage 3KV, first cone voltage 36V, sample cone voltage 60V, source offset voltage 80V, source temperature 100℃, desolvation temperature 400℃, cone hole gas 50 L / h, dissolvent gas flow rate 800 L/h, The acquisition range : 50 m/z-2000 m/z. The collected data were searched by ProteinLynxGlobal Server3.0 software, Database of Eisenia foetida was downloaded from the uniprot website (http://www.uniprot.org/). The protein identification was performed with the following searching parameters. Enzyme: trypsin; Protein mass range: 10-150KD; Tolerance: 50ppm; Missedcleavage: 1; Fixed modification: carbamidomethylation; Variable modification: methionine oxidation.

Table 1: Composition of SDS-fibrin polyacrylamide gel.

Components	10% (Separating gel)	5% (Concentrated gel)
Distilled water	2.0ml	1.6ml
1.5M Tris-HCl (pH8.8)	1.25ml	/
1.0M Tris-HCl (pH6.8)	/	1.0ml
30%acrylamide	1.7ml	0.38ml
Bovine fibrinogen (25mg/ml)	0.1ml	/
Bovine thrombin (42U/ml)	0.1ml	/
SDS (10%)	0.05ml	0.03ml
APS (10%)	0.05ml	0.03ml
TEMED	0.01ml	0.008ml

Results

Enzyme activity measurement

The fibrin plate was used to determine the direct fibrinolytic activity of lumbrokinase, as shown in Fig1a, and the fibrin-plasminogen plate was used to measure the fibrinolytic and kinase activity of lumbrokinase, as shown in Fig1b. The results showed that the diameter of the fibrin-plasminogen plate was significantly larger than that of the fibrin plate (Table 2), which indicated that lumbrokinase had not only direct degradation activity of fibrin, but also had the ability of activating plasminogen to produce indirect fibrinolysis which showed a kinase activity.

The current quality specification only measured its direct fibrinolytic activity by standard curve method and ignored the kinase activity. In our study, we observed that in the fibrin-plasminogen plate, the tendency of diameters of hydrolyzed clear zone between the sample and the standard was parallel, as shown in Figure 2. We further used the parallel line method to measure the total activity of lumbrokinase. As shown in table 3, the total potency calculation of all the five manufacturers 'products were complied with the method requirements and the precision of three independent tests was relatively well, which suggested that the parallel line method was feasible to measure total potency of lumbrokinase.

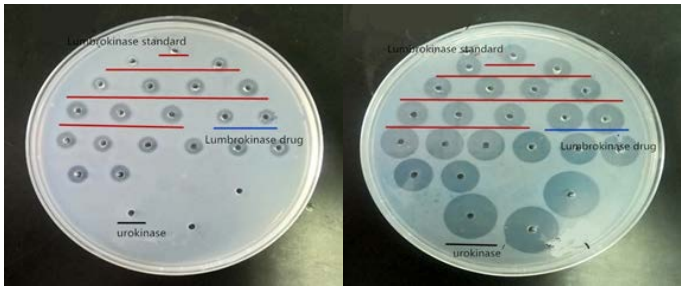
Fibrin-zymography

Then we used the zymography to show the existence of lumbrokinase, Gel based zymography which combined with separation principle of polyacrylamide gel electrophoresis and enzyme-substrate reaction mechanism could display the molecular weight of the active protein on the gel, and also could show the fibrinolytic band of lumbrokinase. The results of fibrin-zymography showed that there were six common active proteins in the products of the five manufacturers, as shown in Fig 3a.Besides, the products of one manufacturer had one more active protein band than others, and another one had two more bands. All the active bands were distributed in the range of 15~40KD.This suggested that lumbrokinase in the drugs was a group of fibrinolytic proteins. Then we further evaluated the consistency between different manufacturers, as shown in Fig 3b. Except that there was little difference between two companies, the active ingredients and manufacturing process of all products of the five manufacturers were highly similar.

According to the results, lumbrokinase is a mixture of fibrinolytic components and has different molecular masses in the polyacrylamide gel electrophoresis, so we next identified the species of earthworm based

Table 2: Diameter (mm) on the fibrin plate and the fibrin-plasminogen plate between sample and standard.

	Sample concentration (U/mL)	2000	4000	6000	8000	10000
Lumbrokinase sample	The fibrin plate	8.37±0.17	11.13±0.18	12.44±0.32	13.65±0.28	14.68±0.15
	The fibrin-plasminogen plate	16.32±0.10	18.60±0.07	19.04±0.05	19.94±0.13	20.29±0.28
Lumbrokinase standard	The fibrin plate	8.33±0.14	10.58±0.11	11.95±0.05	13.13±0.12	14.09±0.11
	The fibrin-plasminogen plate	13.51±0.03	15.10±0.17	15.97±0.08	16.79±0.15	17.50±0.23



1a 1b

Figure 1: Diameter on the fibrin plate(1a)and the fibrin-plasminogen plate(1b).

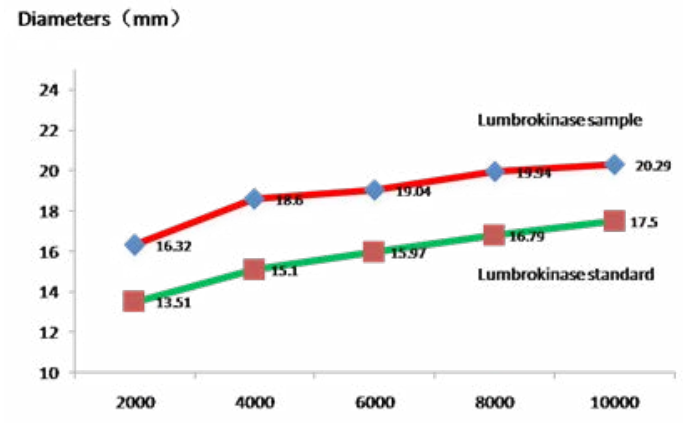


Figure 2: Diameter of fibrinolysis circle on the fibrin-plasminogen plate.

Table 3: Total potency of lumbrokinase (%), calculated by parallel line.

Total potency	1#	2#	3#	Mean±SD	Confidence limit (Mean)
Manufacturer A	80.0	78.8	80.9	79.9±1.1	5.8
ManufacturerB	75.2	76.3	81.6	77.7±3.4	5.2
ManufacturerC	73.5	73.0	74.1	73.5±0.6	5.0
ManufacturerD	74.7	76.1	83.0	77.9±4.4	7.0
ManufacturerE	99.3	97.0	95.8	97.3±1.8	5.5

on these results and further verified the method of fibrin-zymography.

In the specificity test, as shown in Figure 4a, inactive lumbrokinase and urokinase could not produce fibrinolytic lines. Defibrase from snake showed two fibrinolytic lines, but they were obviously different from the lumbrokinase. The fibrinolytic lines between the Eisenia foetida and Pheretima aspergillum were completely different, which indicated that fibrin-zymography could identify the active components which contained the fibrinolytic protein and distinguished Eisenia foetida from other earthworm species.

In the robustness test, as shown in Figure 4b and 4c, we explored the effects of fibrinogen concentration and incubation time on the clarity of fibrin-zymography. The results showed that when the fibrinogen concentration ranged from 0.25 to 1.0 mg/ml and the incubation time ranged from 10 to 60 mins, we could identify the fibrinolytic bands easily.

In the test of range of quantitation, as shown in Figure 4d, a clear figure of fibrin-zymography could be get when lumbrokinase concentration ranged from 0.5 to 37.5μg. Based on the validation results, the method of fibrin-zymography complies with the validation requirements and can be used as the identification method of Eisenia foetida.

Proteins identification in lumbrokinase drug substances by electrospray ionization mass spectrometry (ESI-MS)

Fibrin plate experiments revealed that lumbrokinase had two mechanisms of lysing fibrin, and fibrin-zymography confirmed the presence of fibrinolytic protein in lumbrokinase. So in the next step, we identified the protein in lumbrokinase drug substances using LC-MS. The drug substances were treated with chymotrypsin. As shown in table 4, fifteen fibrinolytic activity-related components were identified, and no other proteases were identified. 8-9 fibrinolytic proteins in each manufacturer were detected. Fibrinolytic protease 1, fibrinolytic protease P-III-1and protein ARSP1 existed in all products. The results of mass spectrometry showed that lumbrokinase drug substances contained fibrinolytic and kinase components, which was consistent with the results of lumbrokinase activity measurement.

Then the amino acid sequence of these proteins was compared with the known serine proteases using the sequence alignment tool. As shown in Fig 5, lumbrokinase shared the common features with the human trypsin, bovine trypsin, tPA(tissue-type plasminogen like activator) and uPA(urokinase-type plasminogen like activator)as their conserved catalytic triad, histidine(H), aspartate(D) and serine(S) were identical. The results indicated that these active sites were highly conserved in these serine proteins which played an important role in the activity of proteolysis [13]. Some lumbrokinases had a highly similar loop and specific pocket (Asn-Asp-iLe-Ala-Leu-Leu), similar to tPA and uPA [14-16]. Besides, the amino acids, asp507, ser532 and gly534 in tPA, which were important substrate recognition sites for tPA and uPA [17-19], also existed in some lumbrokinases. These proteins probably had the same activity with tPA or uPA [20-23]. The results suggested that some lumbrokinases might play a fibrinolytic activity and the others might have the ability of lysing fibrin indirectly by activating the plasminogen to plasmin in the lumbrokinase related products.

Discussion

The fibrin-zymography confirmed the presence of active proteins in the lumbrokinase. The results of fibrin plate showed that lumbrokinase had dual action mechanisms, which was further confirmed by mass spectrometry. These results were committed with the report that the protein property of these proteases both had the activity of degrading the fibrin directly and the activity of degrading the fibrin indirectly by activating the plasminogen into plasmin [14].However, the current fibrin plate method used in the potency determination of lumbrokinase only measured its direct fibrinolytic activity with its kinase activity excluding. Therefore, the current method should be improved to measure its total activity. Our study showed that it was theoretically

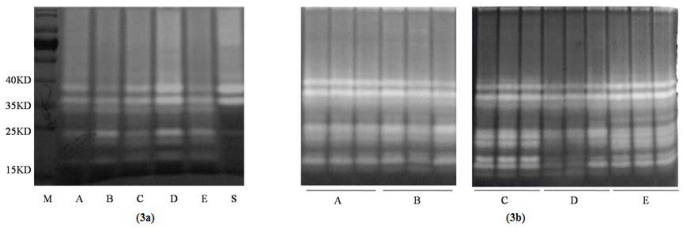


Figure 3: Fibrin-zymography reveals the active ingredients in the lumbrokinase bulk drugs. (3a) fibrin-zymography of lumbrokinase from five manufacturers and lumbrokinase standard. (3b) evaluation of consistency between different manufacturers, each manufacturer has three batches (M: protein marker; A~E: manufacturer A~E; S: lumbrokinase standard).

Table 4: Proteins identified by ESI-MS from five manufacturers.

Manufacturer A	Manufacturer B	Manufacturer C	Manufacturer D	Manufacturer E
Fibrinolyticprotease 1 (Q1ZZB7)	Fibrinolyticprotease 1 (Q1ZZB7)	Fibrinolyticprotease 1 (Q1ZZB7)	Fibrinolyticprotease 1 (Q1ZZB7)	Fibrinolyticprotease 1 (Q1ZZB7)
Fibrinolytic protease P-III-1(Q308Q8)	Fibrinolytic protease P-III-1(Q308Q8)	Fibrinolytic protease P-III-1(Q308Q8)	Fibrinolytic protease P-III-1(Q308Q8)	Fibrinolytic protease P-III-1(Q308Q8)
ARSP1(Q8I6N3)	ARSP1(Q8I6N3)	ARSP1(Q8I6N3)	ARSP1(Q8I6N3)	ARSP1(Q8I6N3)
Lumbrokinase-6 (Q6T373)	Fibrinolytic enzyme component A(Q8MX72)	Fibrinolytic enzyme large subunit(P81802)	Fibrinolytic enzyme component A(Q8MX72)	Fibrinolytic enzyme large subunit(P81802)
Fibrinolytic enzyme large subunt(P81802)	Lumbrokinase (A8ILP4)	lumbrokinase F238 (Q3HR18)	Fibrinolytic enzyme large subunit(P81802)	Lumbrokinase-6 (Q6T373)
Lumbrokinase F238 (Q3HR18)	Lumbrokinase (A8ILN1)	Lumbrokinase (A8ILP4)	Lumbrokinase-3 (Q6T376)	Lumbrokinase-5 (Q6T374)
Lumbrokinase (A8ILP4)	Lumbrokinase (A8ILP1)	Lumbrokinase (A8ILP1)	Lumbrokinase (A8ILP4)	Lumbrokinase-4 (Q6T375)
Lumbrokinase (A5HNU5)	Lumbrokinase (A5HNU5)	Lumbrokinase (A5HNU5)	Lumbrokinase (A8ILP1)	Lumbrokinase (A5HNU5)
Lumbrokinase (A8ILP1)		Lumbrokinase-6 (Q6T373)	Lumbrokinase (Q6DKQ2)	Lumbrokinase (A8ILN1)

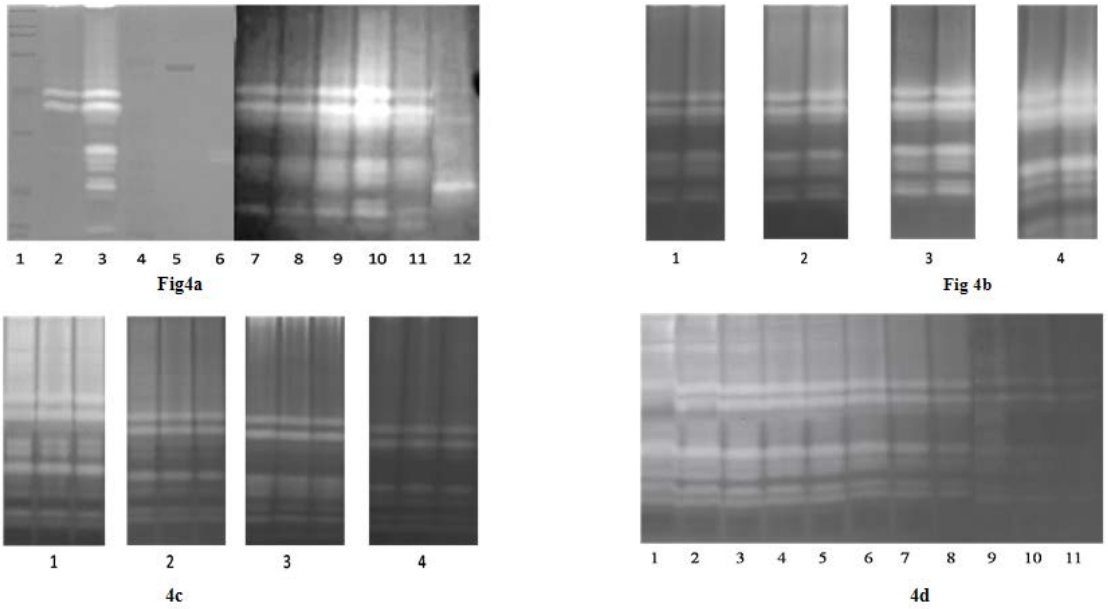


Figure 4: Verification of fibrin-zymography. **4a specificity test:** (1) marker; (2) lumbrokinase standard; (3) lumbrokinase sample (4) inactive lumbrokinase sample; (5) urokinase; (6) defibrase (7-11) manufacturer A/B/C/D/E; (12) Pheretimaaspergillum; **4b incubation time:** (1) 10mins; (2) 30mins; (3) 60mins; (4) 120mins; **4c fibrinogen concentration:** (1) 0.25mg/ml ; (2) 0.5mg/ml ; (3) 1.0mg/ml ; (4) 1.5mg/ml; **4d limit of quantitation:** (1-125μg;2-75μg;3-50μg;4-37.5μg;5 -25μg;6-20μg;7-10μg;8-5μg;9-2.5μg;10-1.5μg;11-0.5μg).

feasible to perform the potency improvement of lumbrokinase by using the parallel method.

It was reported that lumbrokinase (LK) was a group of fibrinolytic isozymes with molecular weights ranged from 25 to 32KD [2, 24-26]. These hydrolytic enzymes existed in different earthworm species, such as Lumbricus rubellus [27, 28], Lumbricus bimastus [9] and Eisenia fetida [29-31]. However, in the quality specification, identification of lumbrokinase based on the hemolysis test cannot differentiate the proteases from different species. Fibrin-zymography can provide dimensional separations based on the mechanisms of polyacrylamide gel electrophoresis and thus help to resolve the complex isozymes of lumbrokinase with relatively high sensitivity. In this study, fibrin-zymography was firstly introduced to identify the species of earthworm based on the hydrolytic bands on the gel, and used to show the existence of active protein in the drugs and to distinguish the earthworm according to the distribution of bright bands on the gel.

Lumbrokinase was a group of serine proteins with highly similar physical and chemical properties. The amino acid sequence of each protein was highly homologous [32]. The contents of lysine (K) and spermine (R) were low in the protein sequence, resulting in poor digestion of trypsin. Moreover, the isoelectric point of lumbrokinase was about 3-5, which was close to optimal pH of pepsin, so in the process of treating lumbrokinase drug substances with pepsin, we found the phenomena of protein precipitation. Therefore, chymotrypsin was selected as the treating enzyme because the restriction sites, phenylalanine, tyrosine, tryptophan were widely distributed in lumbrokinase.

It was worth mentioning that the database of Eisenia foetida contained very few types of proteins due to lacking of related research. Most of the proteins were fibrinolytic related. So the results of mass spectrometry showed the drugs contained fibrinolytic proteins and no other proteins were identified. Therefore, this study needs to be further

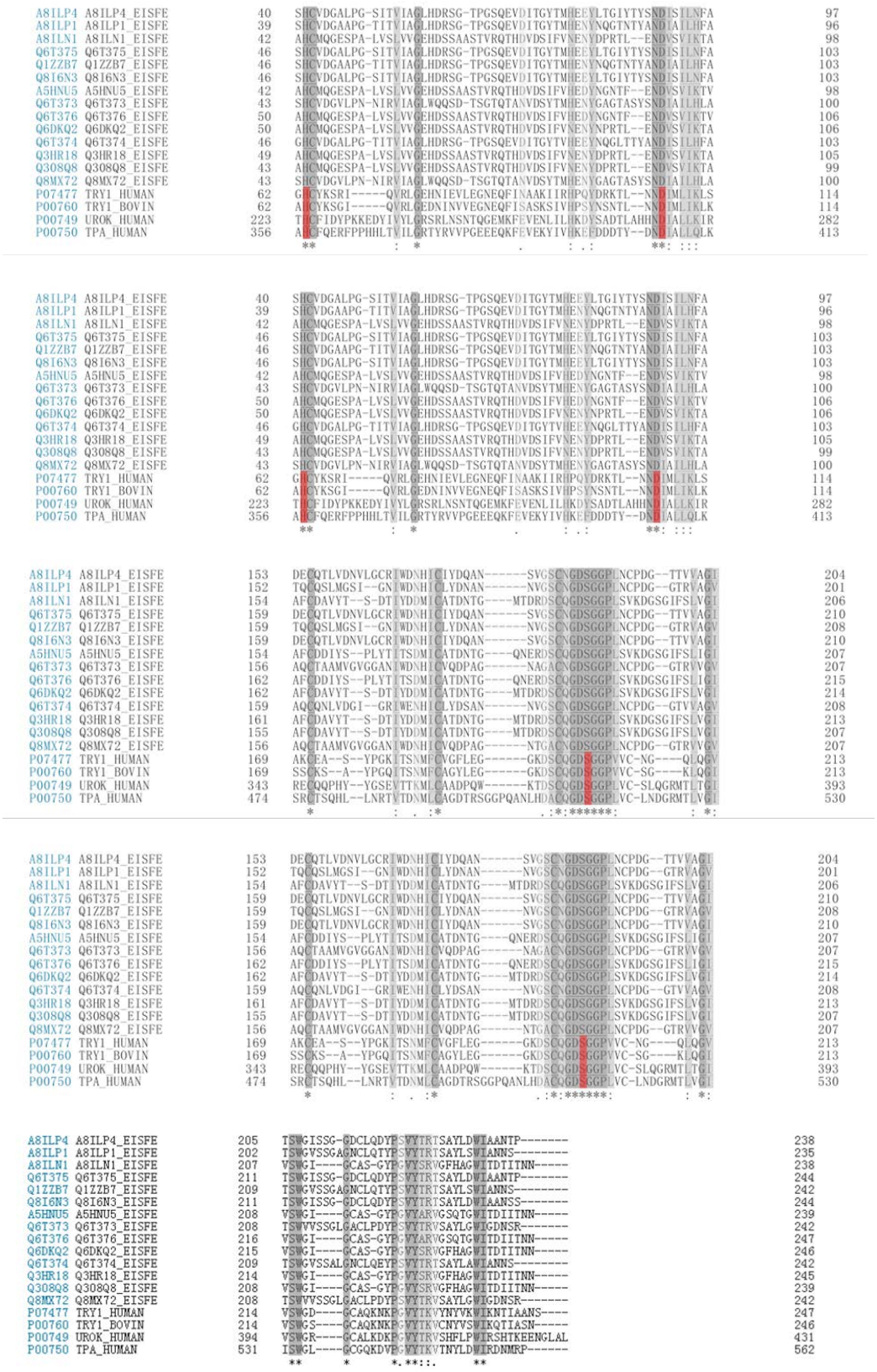


Figure 5: Sequence alignment of the identified proteins of E.fetida, human trypsin, bovine trypsin, human tPA and human uPA. The N terminal(or the C-terminal) part of the sequence was omitted. The numberings shown were based on those proteins, respectively. Gaps, indicated by a dash, were introduced in the sequences to maximize the homology. The amino acid residues of the catalytic triad were red, substrate recognition sites for tPA and uPA was indicated by an triangle (Δ). The loop and specific pocket was underlined.

performed with the gradual update of the database.

Since the lumbrokinase was not isolated and purified from the earthworm in our work, it could not be concluded that which proteins showed the fibrinolytic activity, which one showed the kinase activity and which one showed both. However, our work provided a foundation for the pharmacological study of lumbrokinase. To identify every component in the enteric-coated capsules is our future work.

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