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Research Article

Fractionation and Characterization of Proteins in *Lumbricus rubellus* Powders

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ABSTRACT
In order to find drugs from natural materials, earthworm powders *Lumbricus rubellus* can be used as a source of protein which can be developed into various types of drugs. The purpose of this study do fractionation and characterization of protein in *L rubellus* powders based on molecular weight. Protein in *L rubellus* powders extracted gradually to obtain a crude extract, precipitate and dialysate as final extract. Measurement of proteins levels in the crude extract, precipitate and dialysate is done by spectrophotometer, while electrophoresis was done to proteins characterization base on molecular weight. We conclude that 5 grams powder *L rubellus* in the dialysate fractions containing 2487 ug/mL proteins that show four dominant types of proteins with a molecular weight of 12.2, 13.3, 14.6 and 29.2 kiloDalton (kDa).

Keywords: *Lumbricus rubellus*, protein fractionation, electrophoresis, crude extract, precipitate, dialysate.

INTRODUCTION

Earthworms have been used as a traditional medicine in China, Japan and the other Far East countries for thousands of years.[1]Extraction and purification of the bioactive material contained in the protein earthworms have been conducted in various countries[2]. Protein earthworms widely studied because it has therapeutic effects, including anti–inflammatory, anti–oxidative, anti–tumor, anti–bacterial[3,4]and fibrinolytic activity[5]. Because that in order to find drugs from natural materials, earthworm powders of *L rubellus* can be used as a source of proteins for drugs development. Results of the research showed that *L rubellus* has a crude protein content 63.06%[6].Have observed that the protein content is derived from the earthworm has antibacterial capability. Earthworm protein contains bioactive components 'lumbricin' 0.1 ug/g,[7] and in vitro to inhibit the growth of bacteria *Escherichia coli*,[8]*Salmonella enteritidis*, *Staphylococcus aureus* and *Streptococcus aureus*[9]. Earthworms protein of *L rubellus* be expected as a source of new antibiotics. If the expectation is reached, it can replace bacitracin, tilosin, spiramicin, and virginiamicin which has been banned because it can lead to resistance of pathogenic bacteria[10]. Several studies of *L rubellus* focused on the activity of phenolic substance,[3,11]fibrinolytic enzymes,[5]glycoprotein,[12]and polysaccharides and proteins.[13] Study of *L rubellus* in Indonesia, has been focusing on DLBS1033.[14,15] Explorative study on the fractionation and characterization of proteins based on molecular weight contained in *L rubellus* very limited, therefore the purpose of this study do fractionation and characterization of proteins in *L rubellus* powders based on molecular weight. Expected each type of protein in *L rubellus* can be developed into a natural medicine.

MATERIALS AND METHODS

Preparation of L rubellus powder capsulation

Making the earthworms powder based on the method of Edwards[16]. Earthworm *L rubellus* obtained from fields in Bekasi, West Java, Indonesia. Earthworms are still fresh cleaned of growing medium and intestinal contents removed, then washed with running water. Earthworms are already clean stored in a refrigerator at –40ºC for 12 hours. The sample earthworms plus are 80% solution of formic acid as much as 3% of the weight of the sample, then grinded. The grinding process of the solution of earthworms do until it becomes smooth particle. The fine particles are then dried in an oven to 500ºC for 10–12 hours. Fine particles and dried then crushed and screened using a sieve with a particle size of +40 mesh. Earthworm powder obtained is then put into capsules.

Extraction of L rubellus powder

Five grams of earthworm powder plus 50 mL of 50 mM phosphate buffer, pH 7. The solution was centrifuged at 3000 rpm for 20 minutes, performed two times. Results

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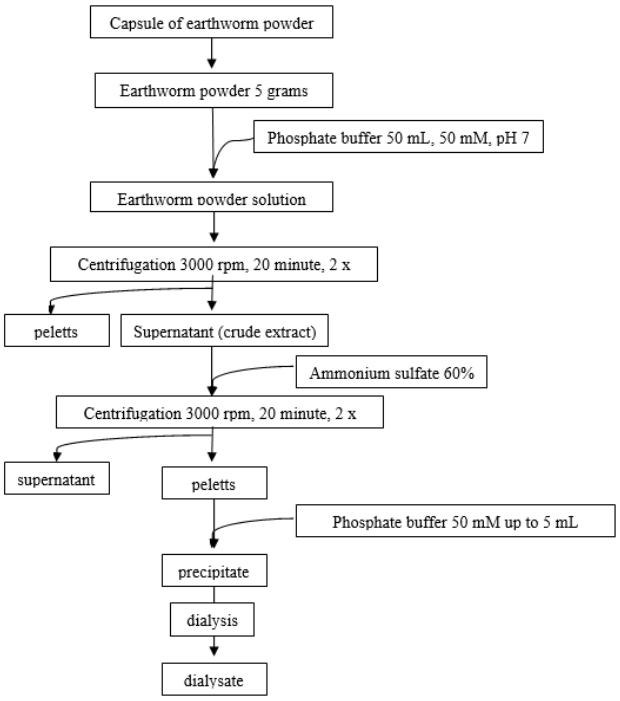

Figure 1 Extraction of *L rubellus* powder.
Abbreviations: mL, milliliters; mM, millimolar; rpm, revolutions per minute; %, percent.

of centrifugation namely peletts and supernatant (crude extract). Crude extract + 60% ammonium sulfate, and then centrifuged at 3000 rpm, 20 minutes, 2 x so that the resulting pellets and supernatant. Pellets + 50 mM phosphate buffer to a volume of 5 mL in order to obtain extracts of precipitation namely precipitate. Precipitate then dialyzed to produce extracts dialysis namely dialysate. The diagram of *L rubellus* powder extraction show in figure 1.

Measurement of protein levels in crude extract and precipitate

Bicinchoninic acid (BCA) method was done to measurement of protein levels in crude extract and precipitate. In this study was used BCA Protein Assay Reagent Kit from Pierce Biotechnology 3747 N. Meridian Road P.O. Box 117 Rockford, IL 61105. BCA method based on colorimetric detection and quantitation of total protein. Bovine serum albumin (BSA) solution which is used to make standard curve: 0, 25, 125, 250, 500, 750, 1000 and 1500 ug/mL. Absorbance is read using a spectrophotometer at a wavelength of 562 nm.

Measurement of proteins molecular weight in extract powder of L rubellus

Sodium dodecyl sulfat–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the earthworm powder of *L rubellus* use monogel 14%. Samples (crude extract, precipitates and dialysate) in a vial + sample buffer volume ratio of 1:1, then put in a 100°C water for 2 minutes. Buffer sample solution is then injected into the well in a chamber that contains a gel electrophoresis buffer. Running electrophoresis on a 150 Volt, 75 minutes. Gels were stained with dye solution (staining). Residual dye in the gel is washed with a destaining

Table 1 BSA absorbance at λ 562 nm with BCA method.

BSA concentrations (ug/mL)	Absorbance
0	0.000
25	0.025
125	0.044
250	0.068
500	0.150
750	0.202
1000	0.260
1500	0.326

Abbreviations: BSA, bovine serum albumin; λ, wave lenght; nm, nanometer; BCA, bicinchoninic acid; ug/mL, microgram per milliliter.

solution. Reagents used in electrophoresis is: 0.5 M Tris–HCl pH 6.8, 1.5 M Tris–HCl pH 8.8, sodium dodecyl sulfate (SDS) 10% weight/volume (w/v), ammonium persulfate (APS) 10% (w/v), the sample buffer (containing: distilled water, 0.5 M Tris–HCl pH 6.8, glycerol, SDS 10% w / v, blue bromfenol 0.5% w/v), β–mercaptoetanol, electrophoresis buffer (containing: Tris–base, glycine, SDS, distilled water), staining solution (containing coomassie brilliant blue R–250, absolute methanol, glacial acetic acid and distilled water), destaining solution (containing absolute methanol, glacial acetic acid and distilled water).

RESULTS AND DISCUSSION

Protein Levels of extract L rubellus powder

BSA absorbance at λ 562 nm show in table 1 and the correlation between BSA concentration and absorbance with BCA method show in figure 2. Protein levels of crude extract and precipitate of *L rubellus* powder show in table 2. Electrophoresis of proteins extracted from *L rubellus* powder. Protein electrophoresis of extract powder *L rubellus* show in figure 3.

Measurement of marker molecular weight

Measurement of marker molecular weight presented in table 3, while measurement of sample molecular weight presented in table 4. Curve of correlation between retention factor (Rf) and log of molecular weight (log of MW) presented in figure 4. The standard curve to determine the protein levels of extract powder *L rubellus* with $y = 0.0002 x + 0.0193$ ($R^2 = 0.9788$). Protein levels in crude extract in this study 1633.5 ug/mL and in precipitate 2003.5 ug/mL. The other research show that protein content of earthworm powder *Eudrillus euginae* were 05.21 ± 0.015 mg/gr. The analysis of earthworm powder *E euginae* showed that the percentage of protein reached 32.13% of the dry weight[17]accordance to analysis by Lourdumary and Uma (2012) that earthworm powder contained of protein 31.7%[18]. Result of SDS–PAGE in this study show that 8 protein fractions were A, B, C, D, E, F, G dan H (figure 3). Protein fractions A, B, C, D, F, G and H appear on electrophoresis fraction of dialysate 2487 ug / mL, while the dialysate fraction of 621.75 ug/mL showed protein fractions F, G and H. The fraction of protein E looks at the results of electrophoresis fraction

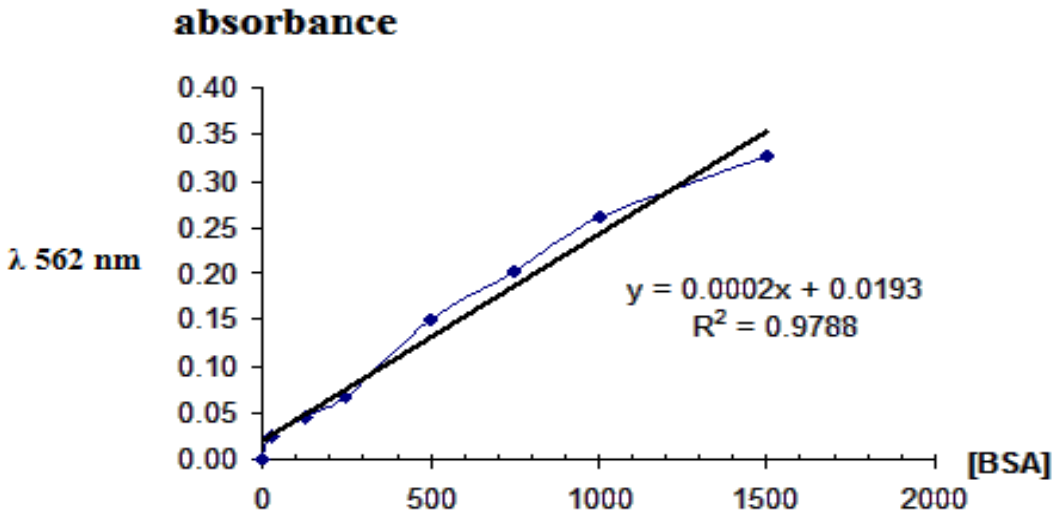

Figure 2 Correlation between BSA concentration and absorbance with BCA method.
Abbreviations: BSA, bovine serum albumin; BCA, bicinchoninic acid; ug/mL, microgram per milliliter; R2, correlation coefficient.

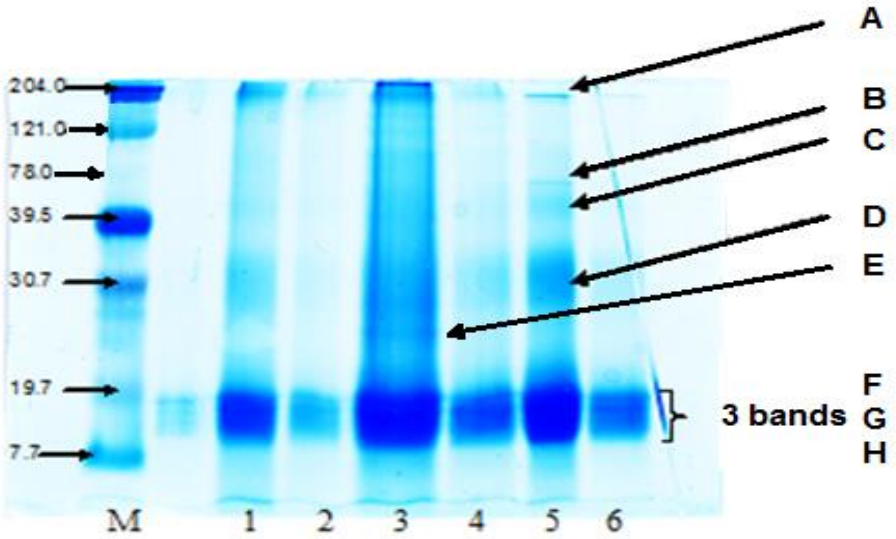

Figure 3 Protein electrophoresis of extract powder *L rubellus*.
Abbreviations: M, marker; 1, crude extract 1917 ug/mL; 2, crude extract 479.25 ug/mL; 3, precipitate 2487 ug/mL; 4, precipitate 621.75 ug/mL; 5, dialysate 2487 ug/mL; 6, dialysate 621.75 ug/mL; A, B, C, D, E, F, G and H, fractions of proteins sample.

of precipitate 2487 ug / mL. Based on the results of the electrophoresis, protein extract of *L rubellus* powder contains two types of protein fractions which is large protein fraction that include fractions C, D, F, G and H and a small protein fraction that include fractions A, B and E. Estimated molecular weight fractions of C, D, F, G and H below 100 kiloDalton (kDa) (Table 4), while the estimated molecular weight fractions of A, B and E each ranging from 204, 70 and 25 kDa. Result of this study show that 7 protein fractions (B, C, D, E, F, G and H) corespons to DLBS1033 extracted from *L rubellus.* DLBS1033 extracted from *L rubellus* has a specific group of proteins earthworm extract possesses 8 major proteins with molecular weight below 100 kDa. This specific protein pattern gives DLBS1033 its unique characteristic, named as Lumbricus low molecular–weight proteins[14]. This study shows that the fraction A is estimated to have a molecular weight of 204 kDa does not appear on DLBS 1033. Enzyme extraction of earthworms *L rubellus* has been carried out, and the enzyme was named Lumbrokinase (LK). There are 6 fractions of enzyme LK. Molecular weight of LK range between 23.5–34.2 kDa. Fraction I–0 has a molecular weight of 23.5 kDa, fraction I–1 27.4 kDa, fraction I–2 27 kDa, fraction II 28.5 kDa, fraction III–1 34 kDa and fraction III–2 34.2 kDa[19]. The other research show that the six LK fractions (F1 to F6) with fibrinolytic activities were purified from earthworm *L rubellus* lysates using the procedures of autolysis, ammonium sulfate fractionation, and column chromatography. A collective name for six fibrinolytic

Table 2 Protein levels of crude extract and precipitate of *L rubellus* powder.

	Dilution	Absorbance	Protein(ug/mL)	Total protein (ug)
Crude extract	1	0.346	1633.5	1633.5
	2	0.211	958.5	1917
	4	0.097	388.5	1554
	8	0.051	158.5	1268
	16	0.033	68.5	1096
	32	0.016	-16.5	-528
	64	0.059	198.5	12704
	128	0.017	-11.5	-1472
Precipitate	1	0.42	2003.5	2003.5
	2	0.268	1243.5	2487
	4	0.168	743.5	2974
	8	0.096	383.5	3068
	16	0.055	178.5	2856
	32	0.031	58.5	1872
	64	0.02	3.5	224
	128	0.022	13.5	1728

Abbreviations: ug/mL, microgram per milliliter; ug, microgram.

Tabel 3 Molecular weight of marker.

R	Rf	log of MW	MW
0.1	0.014493	2.30963	204
0.3	0.043478	2.082785	121
0.8	0.115942	1.892095	78
3.0	0.434783	1.596597	39.5
3.9	0.565217	1.487138	30.7
5.4	0.782609	1.294466	19.7
6.3	0.913043	0.886491	7.7

Abbreviations: R, ratio; Rf, retention factor = relative mobility of polypeptide bands; log of MW, log of molecular weight; MW,molecular weight; M, marker; 1, crude extract 1917 ug/mL; 2, crude extract 479.25 ug/mL; 3, precipitate 2487 ug/mL; 4, precipitate 621.75 ug/mL; 5, dialysate 2487 ug/mL; 6, dialysate 621.75 ug/mL.

isoenzyme proteins having molecular weights of 24.6 to 33 kDa. The molecular weights of each iso–enzyme, as estimated by SDS–PAGE, were 24.6 (F1), 26.8 (F2), 28.2 (F3), 25.4 (F4), 33.1 (F5), and 33.0 kDa. The proteolytic activities on the casein substrate of the six iso–enzymes ranged from 11.3 to 167.5 unit/mg with the rank activity orders of F4<F3<F6<F5<F1<F2. The fibrinolytic activities of the six fractions on the fibrin plates ranged from 20.8 to 207.2 unit/mg with rank orders of F4<F1<F3<F5< F2< F6[20].

The other research show that earthworm fibrinolytic enzymes are a group of serine proteases with strong fibrinolytic and thrombolytic activity and molecular masses of 24–30 kDa,[21]that corresponds to the molecular weight measurement of LK by Wang *et al*., (2003)[22]. Fan *et al.,* (2001) to show that earthworm fibrinolytic enzyme III–1 (EFE–III–1) isolated from *L rubellus* has molecular weight as 30 kDa.[23]LK a trypsin–like enzyme, was efficiently purified from a crude preparation of earthworm *L rubellus*. The purification procedure was as follows: dissolving crude powder in saline for 3 days; 30%–60% ammonium sulfate gradient fractionation of the soluble fraction; and column chromatography on DEAE–cellulose anion exchange and then *p*–aminobenzamidine sepharose 6B. Measurement result of LK molecular weight show around 34.2 kDa[24]. Some researchers have LK packed into capsules to treat clotting diseases such as acute myocardial infarction and cerebral embolism[25–27]. Futhermore has been tested in vitro that the extract powder of *L rubellus* able to inhibit the growth of bacteria *E coli*,[8]and *Salmonella pullorum*[28]. Results of research on extract powder of *L rubellus* with an added chitosan can improve the inhibition of *E coli.*[29]Molecular weight of *succinic semialdehyde dehydrogenase* from *L rubellus* was estimated to be 120 kDa. This enzyme suggested composed of 2 identical subunits. The subunit size of the enzyme was 56 kDa[30]. We suspect that *succinic semialdehyde dehydrogenase* only a fraction of protein extract *L rubellus*. This enzyme may include fraction of C in this study. The assumption is based on the results of SDS–PAGE showing that the molecular weight of the sample C around 43.29 kDa. SDS–PAGE analysis was performed to determine the molecular weight of proteins extracted from earthworm[21,31,32]. Has been made purification Fibronectinase of *Eisenia fetida* (EFNase) with affinity chromatography techniques. Enzyme precipitated using ammonium sulfate from the protein solution of earthworm *E fetida*. EFNase molecular weight approximately 34 kDa.[31] Result of the other research show that protein fractions of *E fetida* has molecular weight below 100 kDa[21]. Study result of protein purified

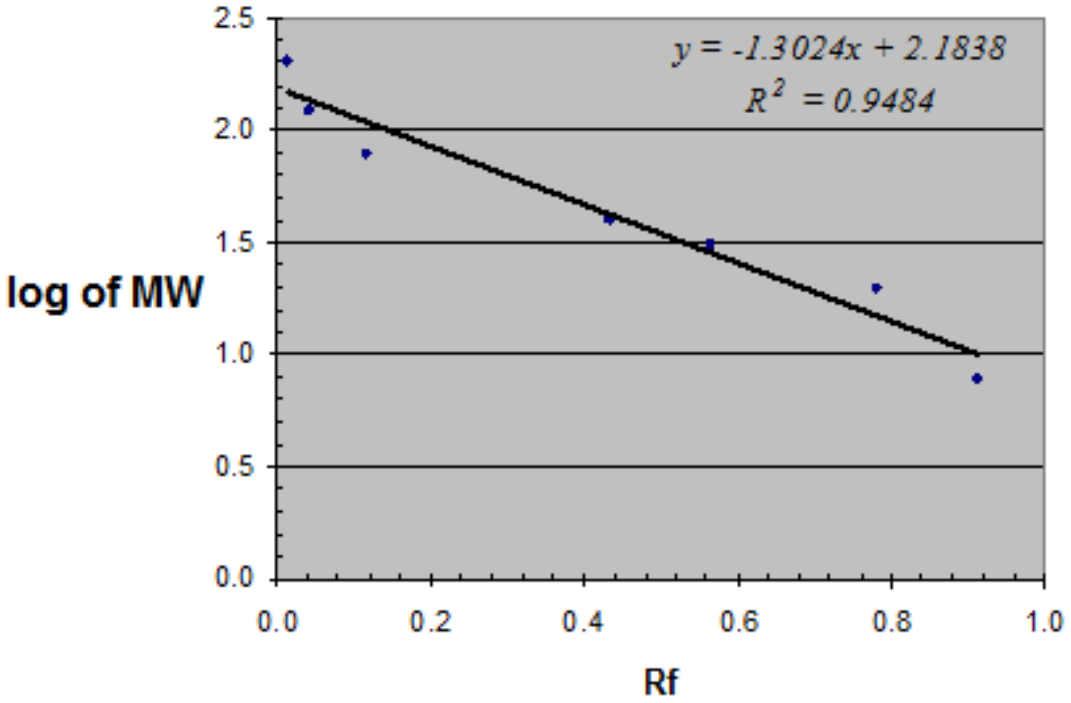

Figure 4 Curve of correlation between Rf and log of MW.
Abbreviations: log of MW, log of molecular weight; Rf, retention factor=relative mobility of polypeptide bands.

Table 4 Measurement of sample molecular weight

Sample fractions	R of sampel fractions	Rf of sample fractions (x)	log of MW (y)	MW
C	2.9	0.42029	1.636414	43.29268
D	3.8	0.550725	1.466536	29.27764
F	5.4	0.782609	1.16453	14.60597
G	5.6	0.811594	1.12678	13.38997
H	5.8	0.84058	1.089029	12.27521

Abbreviations: R, ratio; Rf, retention factor = relative mobility of polypeptide bands; log of MW, log of molecular weight; MW, molecular weight.

from *Lampito mauritii* show that 4 fractions were 100–110 kDa, 75–80 kDa, 45–50 kDa and 35–40 kDa. Protein fractions from the crude extract (F–11) has been found to possess strong protease function. This protein fraction has molecular weight around 45–50[32]. Results of protease profiling show that some fraction of earthworm *Perionyx excavatus* has molecular weight 25–35 kDa[33]. Anticoagulant protein that purified from *Scapharca broughtonii* was measured molecular weight about 26.0 kDa.[34] Earthworm fibrinolytic enzymes (EFE) are a group of serine proteases with strong fibrinolytic and thrombolytic activity with molecular weight of 23–30 kDa[35]. Has been done fractionation of the intestinal fluid *Pheretima posthuma*, then processed with a different chromatographic techniques. The results showed that the purified protein fraction having a molecular weight of 20 kDa[36]. Futher more, multiple SDS–PAGES were run to determine average molecular weight of candidate protease from Indian earthworm *P posthuma*. Based on standard low range protein ladder, average molecular weight of partially purified protease with presumed cytotoxic and anti–cancer activity was 15 kDa[37].

CONCLUSION

We was done 5 grams powders of *L rubellus* extracted gradually to obtain a crude extract, precipitate and dialysate as final extract. We conclude that in the dialysate fractions containing 2487 ug/mL proteins, and show four dominant types of proteins with a molecular weight of 12.2, 13.3, 14.6 and 29.2 kDa.

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