

Purification of Enzymes from Earthworms

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ABSTRACT

Earthworms are a very rich source of enzymes useful for bioremediation of contaminated soils, in animal feeds for improving the digestion of proteins, lipids and carbohydrates, in detergents, in the saccharification of starch, and in human and animal therapeutics. Powdered earthworms are very popular in the Orient due to their properties as antipyretic, diuretic and fibrinolytic. However, in order to improve the applicability of these enzymes, purification at different levels is necessary. Among them, proteins such as fibrinolytic enzymes are being used in therapeutic nowadays. This article reviews the methods used for purification of fibrinolytic enzymes (useful for clotting disorders treatment), ceramide glycanase (useful for structural elucidation of glycosphingolipids), xylanolytic enzyme (useful for waste treatment), alpha amylase (useful for starch saccharification), lipase (useful for detergents formulation), glutathione transferase (useful as a potential antioxidant agent) and succinic semialdehyde dehydrogenase (useful for pesticide detoxification), in addition to lombricine kinase. All these enzymes were obtained by earthworm homogenization and further subjecting the earthworms extract to several chromatographic steps. All types of chromatography were applied: size exclusion, ion exchange, hydrophobic interaction and affinity. Moreover, an integrative technique such as aqueous two-phase partition was utilized for obtaining a clear extract containing the earthworm enzymatic pool.

Keywords: aqueous two-phase systems, chromatography, precipitation, proteins, separation processes

Abbreviations: AC, affinity chromatography; APIs, active pharmaceutical ingredients; ATPS, aqueous two-phase system; CGase, ceramide glycanase; DEAE, diethylaminoethyl; EFEs, earthworm fibrinolytic enzymes; GSH, glutathione; GST, glutathione transferase; HIC, hydrophobic interaction chromatography; IEC, ion exchange chromatography; LK, lumbrokinase; SEC, size exclusion chromatography; SSD, succinic semialdehyde dehydrogenase; TLC, thin layer chromatography

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INTRODUCTION

Earthworms contain a number of enzymes that find utilization in animal feeds to enhance degradation and digestion of starch, proteins and lipids, in detergents, for bioremediation of contaminated soil and in human disease prevention and therapeutics. The therapeutic use of earthworm enzymes dates back to ancient Chinese acupuncture to increase blood and energy flow. Earthworms have a primitive digestive system rich in an enzyme mixture, not in vain Aristotle called them "the intestines of the soil". Earthworm has been used as a traditional medicine to treat clotting diseases in eastern countries for centuries. In Compendium of Materia Medica, edited by Doctor Shizhen Li (1518-1593), earthworm was described as a kind of drug prescribed for antipyretic and diuretic purposes in clinic. The dried earthworm prepared into powder with a medicinal name "Earth dragon"

is still used as a traditional folk remedy in various Asian countries.

Frédéricq (1878) pointed out that a fluid secreted by the alimentary tract of the earthworms could dissolve fibrin. He identified the pharyngeal region, crop gizzard and the anterior portion of the intestine as the secretory organs.

In 1881, Charles Darwin published his last book in which he described the important role of worms in soil fertilization, finishing with the contemporaneous farmers prejudice that considers worms as pests and nuisance. Regarding the kinds of food which worms consume, fats, raw meat and starch, Darwin agreed with the idea that the digestive fluid of worms was of the same nature as the pancreatic secretion of the higher animals. He also describes their ability to dissolve fibrin.

In 1920, Keilin isolated several earthworm proteases that could degrade casein, gelatin and albumin. Seventy years

later, Japanese scientists confirmed experimentally this observation when they isolated and purified six fibrinolytic enzymes from earthworms. They collectively named these enzymes lumbrokinase (LK) regarding the generic name of the earthworms used as the source of LK, *Lumbricus rubellus* (Mihara *et al.* 1991). Since then, many authors reported the extraction and purification of earthworm fibrinolytic enzymes (EFEs) from *L. rubellus* (Nakajima *et al.* 1993; Park *et al.* 1998; Cho *et al.* 2004), *Eisenia fetida* (Wang *et al.* 2003; Li *et al.* 2003; Iannucci *et al.* 2008), *Eisenia andrei* (Lee *et al.* 2007) and *Perionyx excavatus* (Thuy *et al.* 2006).

Purification methods of proteins such as enzymes constitute a relevant point which defines the price of the final product to commercialize. The downstream processing of a therapeutic protein will demand between 50 and 80% of the total production cost (Datar and Rosen 1990). Integrative procedures are an excellent alternative for reducing costs, increasing yield and productivity. These procedures integrate two or more recovery and purification methods in only one step. Commonly used recovery methods are precipitation, filtration and aqueous two-phase partition. Chromatography is the method of choice for protein purification. Several protein attributes could be exploited to separate it from a complex mixture, such as size, surface hydrophobic residues, surface charged residues and affinity for an immobilized ligand (antibodies, cofactors, substrates, synthetic analogs, ion metals, etc). The chromatographic methods based on the above characteristics are called: molecular exclusion (SEC), hydrophobic interaction (HIC), ion exchange (IEC) and affinity chromatography (AC), respectively.

Most of the methods reported start with a centrifugation or filtration clarification step of the earthworm homogenate to remove solid material. Then, some proteins of the supernatant – including the target enzyme – are precipitated by altering the solvation potential of the solvent by addition of ammonium sulfate, perchloric acid or polar organic solvents. Precipitated proteins are separated by centrifugation or filtration and dialyzed to remove the precipitating reagent (Mihara *et al.* 1986; Park *et al.* 1998; Wang *et al.* 2003; Cho *et al.* 2004). Finally, the target enzyme is further purified by chromatography. Integrative methods such as polymer-salt or polymer-polymer aqueous-two phase systems (ATPS) allow process integration on account of simultaneous clarification, separation and concentration of the target protein in one step (Kula 1990). They were also used to clarify and concentrate the earthworm extract (Iannucci *et al.* 2008).

Recently, Hu *et al.* (2005) reported the clonation, expression and purification of a novel fibrinolytic enzyme of *L. rubellus* in *Pichia pastoris*.

Purification of various earthworm enzymes was described in the literature, but most of them only for characterization purposes rather than for industrial implementation. Because of the high complexity of the earthworm extract as the raw material, only few enzymes were fully purified.

In this article, the direct extraction and purification of enzymes from different earthworms is reviewed.

EARTHWORM FIBRINOLYTIC ENZYMES (EFEs)

These enzymes have potential application as active pharmaceutical ingredients (APIs) in the development of novel therapeutic formulations for treatment of severe diseases such as heart or cerebral infarction and prevention of thrombus formation in surgery. Furthermore, EFEs have high stability, broad pH range and it was demonstrated that some of them could be absorbed intact into the blood stream through the intestinal epithelium (Fan *et al.* 2001). These facts make them attractive for the design of oral thrombolytic agents. EFEs could be extracted from various species of earthworms, while LK is the collectively name for the fibrinolytic isozymes extracted from *L. rubellus*.

EFE purification

Wang *et al.* (2003) purified to homogeneity seven fibrinolytic enzymes from *E. fetida* using ion exchange pre-packed columns. The enzymes were named EFE-a, EFE-b, EFE-c, EFE-d, EFE-e, EFE-f and EFE-g with isoelectric points of 3.46, 3.5, 3.50, 3.68, 3.62, 3.94, 3.46, respectively and molecular weights between 23 and 30 kDa. EFE-b, EFE-c and EFE-g are trypsin-like enzymes, EFE-d, EFE-e and EFE-f are chymotrypsin-like enzymes, and EFE-a is not a trypsin-like enzyme or a chymotrypsin-like enzyme, nor an elastase; its optimum substrate is still unknown.

Li *et al.* (2003) published a protocol for purification of glycosylated EFEs from *E. fetida*. They used AC with *m*-aminophenylboronic acid coupled to Sepharose as a selective ligand for binding to *cis*-pyranose on glycosylated proteins.

Zhao *et al.* (2007) reported the isolation of eight isozymes with fibrinolytic activity from *E. fetida*, following a stepwise purification procedure. It includes an ammonium sulfate precipitation followed by an AC step with soybean trypsin inhibitor coupled to Sepharose and IEC with a diethylaminoethyl (DEAE)-Sepharose matrix. Interestingly, all the proteases were glycosylated. They also found that one of the isozymes (EFE P-III-1) was active not only as a fibrinolytic agent but also as a fibrinogenic agent, thus suggesting that may play a role in the coagulation balance.

Thuy *et al.* (2006) isolated and purified a strong fibrinolytic enzyme from the earthworm of Vietnam *P. excavatus*. After homogenization of the earthworms in 0.9% NaCl, the supernatant was precipitated with a 35% and then 60% saturation of ammonium sulfate. The precipitate redissolved was subjected to SEC on Sephacryl S-200 and the active peak fractionated by IEC on DEAE cellulose and then by SEC on Superdex G-75. Two enzymes of molecular weights of 28 and 34 kDa and specific activities of 126 and 271 IU/mg were obtained.

Lee *et al.* (2007) analyzed the antithrombotic effects of the EFE fraction extracted from *E. andrei* by oral administration in rats. The EFE was partially purified by ethanol precipitation followed by filtration and ultrafiltration.

Iannucci *et al.* (2008) reported a simple and easy-to-scale-up method to purify EFEs from *E. fetida* in only two steps, using an aqueous two phase system (ATPS) composed by a polymer-salt mixture followed by IEC. ATPS allows the integration of simultaneous separation and concentration of the target protease in a clear extract being achieved. The low cost and easy scale-up of ATPS makes it potentially useful for large-scale downstream processing of proteins (Kula 1990). Moreover, the ATPS biocompatible environment promotes the preservation of the biological activity of enzymes extracts. A 97% pure enzyme with a yield of 80% was obtained with the above protocol.

LUMBROKINASE (LK)

LK is a group of enzymes that is widely distributed in the digestive cavity of *L. rubellus* earthworms. Possessing strong proteinase activity, LK not only has a direct effect on fibrin, but also can activate plasminogen (Mihara *et al.* 1991). LK is commercially available in capsules as a treatment of clot formation in order to prevent heart infarction and brain vascular accidents. There are two main mechanisms of action in LK: the first is the ability to activate the body's plasminogen into plasmin and the second is to dissolve the fibrin itself. Subsequent studies then demonstrated that LK is the main factor responsible for the earthworm extract anti-tumor activity due to its ability to dissolve fibrin clots. Chen *et al.* (2007) evaluated the anti-tumor activity of LK on the hepatoma cells both *in vitro* and *in vivo*, expanding its potential use in cancer treatment.

LK purification

Mihara *et al.* (1986, 1991) obtained six novel proteases with

fibrinolytic or thrombolytic activity from earthworms. These proteases differ from each other in their molecular mass, isoelectric point, substrate specificity and inhibition pattern against different trypsin inhibitors. The authors describe a thrombolytic medicament comprising at least one of the proteases derived from earthworms and one physiologically acceptable carrier for oral administration. Their work is protected by a US patent, in which they describe a series of extraction, recovery and purification methods for each of the six proteases. Extraction of earthworm tissues with an aqueous solution and precipitation of the proteases by adding a polar organic solvent are common procedures for all the proteases. The purification methods differ for each protease or group of them. Briefly, the described methods include a sequence of three IEC steps for one group of proteases, one IEC step followed by AC and a second IEC step for other group and an IEC step followed by a salting out precipitation followed by SEC and finally an ultrafiltration step for the last group.

Park *et al.* (1998) developed a rapid method for purification of LK from *L. rubellus* powder. After dissolution of the powder in buffer Tris-saline and centrifugation to remove particles, the supernatant was heated at 55°C. Following clarification of the supernatant, it was precipitated with ammonium sulfate. The pelleted proteins were dialyzed, loaded to a DEAE column and eluted with a linear gradient of sodium chloride. Pools of fibrinolytic activity obtained from the IEC were loaded onto a benzamidine-Sepharose column and eluted with 0.5 M arginine in equilibration buffer. Purified LK had a molecular weight of 34 kDa and accounted for 1.3% of the protein precipitated by ammonium sulfate.

Cho *et al.* (2004) purified six fibrinolytic enzymes from *L. rubellus*. After ammonium sulfate precipitation of an aqueous earthworm extract, an IEC allowed separation of three fractions: FI, FII and FIII. FI yielded two peaks after HIC: F1 y F2. FII yielded one peak after HIC: F3. FIII yielded three peaks after affinity chromatography in benzamidine agarose: F4, F5 and F6. The molecular weights of the six fractions, F1 to F6, were 24.6, 26.8, 28.2, 25.4, 33.1, and 33.0 kDa, respectively. By N-terminal microsequencing, it was demonstrated that F1 is the same as EFE-f, F2 is the same as EFE-d and EFE-e, F3 and F4 are the same as EFE-a, F5 and F6 are the same as EFE-b, EFE-c and EFE-g.

CERAMIDE GLYCANASE (CGase)

CGase cleaves the linkage between the ceramide and the glycan chain in various glycosphingolipids. This enzyme has been found in leeches, *Rhodococcus* sp. and earthworms from the *Lumbricus terrestris* species. In earthworms, the CGase was mainly found in muscle. Intestine was found to contain a very low level of this enzyme (Li *et al.* 1987). As the enzyme is very useful for the structural elucidation of glycosphingolipids, it is important to make it readily available (Carter *et al.* 1992).

CGase purification

Carter *et al.* (1992) describe a method where the enzyme is obtained from the muscle tissue of *L. terrestris* earthworms by homogenization in water followed by protamine sulfate and ammonium sulfate precipitation, gel permeation chromatography (Bio Gel A), and HIC with an Octyl-Sepharose column. Galactosidase was removed by affinity chromatography on a PAP-S-Gal-agarose column. This purification method is protected by a US Patent (Li and Li 1991). Two isozymes were obtained with yields of 10.3 and 26.0% and purification factors of 284 and 1123, respectively.

GLUTATHIONE TRANSFERASES (GST)

The cytosolic glutathione transferases (EC 2.5.1.18) play an important role in detoxication by catalyzing the conjugation of glutathione with electrophilic metabolites of natural or

manufactured hydrophobic chemicals. Glutathione transferases also play a role in preventing oxygen toxicity due to their activity towards organic hydroperoxides and hydroxyl alkenals (Borgeraas *et al.* 1996).

GST purification

Isoenzymes of GST were partially purified from the earthworm species *E. andrei* and *Eisenia veneta* by Borgeraas *et al.* (1996) by using AC followed by IEC. Briefly, worms were homogenized and the homogenate clarified by centrifugation and filtration. After buffer exchange by SEC, the eluate was applied to a glutathione (GSH)-agarose affinity column. Finally, isoenzymes of glutathione transferase were separated by IEC. At a laboratory scale, the yield was 43.3% with a purification factor of 193.

SUCCINIC SEMIALDEHYDE DEHYDROGENASE (SSD)

SSD catalyzes the conversion of succinic semialdehyde to succinic acid.

Earthworms play an important role in the ecology of soil. These animals may be exposed to pesticides and pollutants in the environment, and are, therefore, dependent on efficient detoxification systems, some of which may be based on succinic semialdehyde dehydrogenase.

SSD purification

Koh *et al.* (1994) purified SSD from *L. rubellus*.

A homogenate 25% (w/v) in a 15 mM sodium phosphate, pH 7.4, containing 1 mM EDTA, 0.1% 2-mercaptoethanol and 0.25 M sucrose was prepared. After centrifugation, the supernatant was treated with ammonium sulfate. The precipitate obtained at 30 to 65% ammonium sulfate saturation was dialyzed and applied to a column of DEAE-Sepharose. The enzyme was not retained by the anion exchange column, whereas contaminating proteins remained attached. Pooled active fractions were then applied to a column of Blue Sepharose CL-6B. After washing the column, proteins were eluted with an ion strength gradient. Active fractions were combined and applied to a column of AMP-Sepharose and SSD activity was eluted with equilibration buffer containing 0.2 mM NAD⁺. The procedure resulted in 1,500-fold purification with a yield of 22%.

LOMBRICINE KINASE

Phosphagen kinases (ATP: guanidine transferases) constitute a family of highly conserved enzymes which catalyze the reversible transfer of gamma phosphate of ATP to a guanidino acceptor thereby producing a phosphagen. A diverse array of phosphagens (and corresponding phosphagen kinases) are found in the animal kingdom including creatine phosphate (creatine kinase), arginine phosphate (arginine kinase), glycoyammine phosphate (glycoyammine kinase), tauroyammine phosphate (tauroyammine kinase), hypotauroyammine phosphate (hypotauroyammine kinase), opheline phosphate (opheline kinase) and lombricine phosphate (lombricine kinase).

Lombricine kinase purification

Ellington (1989) purified to homogeneity lombricine kinase from the body wall muscle of *E. fetida* with a modification of the method of Hoffmann (1981). Freshly thawed earthworms were homogenized in water and, after centrifugation, the supernatant was precipitated with perchloric acid and heated to 55°C. After treatment of the supernatant with solid K₂CO₃ to bring it to pH 5.5-6.0 and centrifugation, the supernatant was brought to pH 1.8 and loaded to a Dowex 50 column. The eluate was submitted to thin layer chromatography (TLC) and guanidine-containing compounds were pooled and applied to another Dowex 50 column. Fractions

containing lombricine were again submitted to TLC and lombricine eluted was further purified by three cycles of crystallization from ethanol.

XYLANOLYTIC ENZYMES

Many authors reported the absence of cellulase and mannanase activity in some species of earthworms, thus indicating that they rely on ingested microflora to degrade cellulose and mannan (Zhang *et al.* 1993; Lattaud *et al.* 1997).

Xylanolytic enzymes isolation

Merino-Trigo *et al.* (1999) demonstrate the occurrence of active xylanolytic enzymes in *E. andrei*; xylanase and xylan esterase being the most active. They displayed higher activity when the earthworms were fed with a xylan-containing medium. For the biochemical assays, empty gut earthworms were homogenized and, after centrifugation, the supernatant fractions were used. This isolation procedure was enough for enzymes destined to xylan cleavage for waste treatment.

ALPHA-AMYLASES

Earthworms of the phylum *Annelida* are known to hydrolyze carbohydrates, thus suggesting their ability to digest leaf litters, roots, yeast, brown algae and fungi on soil (Prat *et al.* 2002).

Ueda *et al.* (2008) described two raw-starch-digesting alpha-amylases from the body wall extract of *E. fetida* which are also active at 10°C. In the starch saccharification industry, the starch is gelatinized by heating up to 100°C, which increases the energy consumption and the production cost (Goyal *et al.* 2005). The enzymatic degradation of raw starch granules is important not only for industrial application, but also for elucidation of the structure of starch granules (Ueda *et al.* 2008). This was the first report of raw-starch-digesting alpha-amylases from *E. fetida*.

Purification of alpha-amylases

This is based on the protocol by Ueda *et al.* (2008). *E. fetida* earthworms were freeze-dried and powdered, and the resulting powder was suspended in 50 mM Tris-HCl buffer, pH 7.0. After centrifugation, the supernatant was precipitated with ammonium sulfate at 35% saturation. The precipitate was dissolved in the above buffer and, after dialysis against 20 mM Tris-HCl buffer, pH 7.0, loaded onto a DEAE-Toyopearl column. Two active peaks were obtained, and the protein contained in them was precipitated with ammonium sulfate at 80% saturation. Precipitated proteins were subjected firstly to SEC on a Sephacryl S-200 column and then to a HIC on a Butyl-Toyopearl column. Alpha-amylase I and alpha-amylase II thus obtained were purified to 85.2 and 263 folds with recoveries of 3.6 and 6.0%, respectively. Both enzymes resulted were purified at homogeneity and their molecular weights were estimated to be 60 kDa by SDS-PAGE.

LIPASE

Nakajima *et al.* (2005) described an enzyme from *L. rubellus* that catalyze the hydrolysis of triacylglycerol. The N-terminal amino acid sequence and the catalytic function of the purified enzyme were identical to those of the isozyme C of the earthworm serine proteases. No other lipases were found in earthworms. This enzyme might act on the hydrolysis of triacylglycerol as well as on protein decomposition.

Lipase purification

This is based on the protocol by Nakajima *et al.* (2005). The extract of *L. rubellus* was precipitated with ammonium sulfate at 35% saturation and the supernatant at 70% saturation. After dialysis, the redissolved precipitate was subjected se-

quentially to IEC on a DEAE column, IEC on a Mono Q column, HIC on a Phenyl-Superose column, IEC on a Mono Q PC column and finally to SEC on a Superdex 75 PC column. The overall yield was 4% with a purification factor of 202. The molecular weight of the purified enzyme was 28 kDa as judged by SDS-PAGE.

COMMENT

A lot of useful enzymes can be purified directly from earthworm homogenates, mainly by chromatographic techniques. Enzymes used in human therapeutics such as EFEs need to be extensively purified while those utilized for other applications such as soil bioremediation can be only isolated by precipitation.

In conclusion, earthworms are an endless source of enzymes useful for different applications, and the specific protocol for their purification will depend upon the requirements of the final product to be obtained.

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