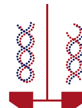

Journal of Bioscience and Bioengineering
VOL. xx No. xx, , 2017

www.elsevier.com/locate/jbiosc

High-pressure tolerance of earthworm fibrinolytic and digestive enzymes

Shin-ichi Akazawa,[1,*] Haruka Tokuyama,[1] Shunsuke Sato,[1] Toshinori Watanabe,[2] Yosuke Shida,[3] and Wataru Ogasawara[3]

Department of Materials Engineering, National Institute of Technology, Nagaoka College, 888 Nishikatakai, Nagaoka, Niigata 940-8532, Japan,[1] Waki Pharmaceutical, 898 Nango, Koryocho, Kitakaturagigun, Nara 635-0814, Japan,[2] and Department of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan[3]

Received 13 May 2017; accepted 22 August 2017
Available online xxx

Earthworms contain several digestive and therapeutic enzymes that are beneficial to our health and useful for biomass utilization. Specifically, earthworms contain potent fibrinolytic enzymes called lumbrokinases, which are highly stable even at room temperature and remain active in dried earthworm powder. However, the high-temperature sterilization method leads to the inactivation of enzymes. Therefore, we investigated the effect of high-pressure treatment (HPT) (from 0.1 MPa to 500 MPa at 25°C and 50°C) on the enzymatic activity of lumbrokinase (LK), α-amylase (AMY), endoglucanase (EG), β-glucosidase (BGL), and lipase (LP) of the earthworm *Eisenia fetida*, Waki strain, and its sterilization ability in producing dietary supplement. LK showed thermo- and high-pressure tolerance. In addition, HPT may have resulted in pressure-induced stabilization and activation of LK. Although AMY activity was maintained up to 400 MPa at 25°C, the apparent activity decreased slightly at 50°C with HPT. EG showed almost the same pattern as AMY. However, it is possible that the effects of temperature and pressure compensated each other under 100 MPa at 50°C. BGL was shown to be a pressure- and temperature-sensitive enzyme, and LP showed a thermo- and high-pressure tolerance. The slight decrease in apparent activity occurred under 200 MPa at both temperatures. Furthermore, the low-temperature and pressure treatment completely sterilized the samples. These results provide a basis for the development of a novel earthworm dietary supplement with fibrinolytic and digestive activity and of high-pressure-tolerant enzymes to be used for biomass pretreatment.

© 2017, The Society for Biotechnology, Japan. All rights reserved.

[**Key words:** Earthworm; *Eisenia fetida*; High pressure; Lumbrokinase; Fibrinolytic enzyme; Digestive enzyme]

Earthworms are well-known soil decomposers. Charles Darwin, one of the most acclaimed scientists, studied earthworms, leading to the compilation of the book “Worms” (1). Today, it is widely acknowledged that earthworms play a crucial role in the formation of nutrient-rich soil. Because earthworms are polyphagous animals and their cast contains a large amount of nutrients, the use of earthworms in composting has been extensively researched (2). Earthworms are also known to have many digestive enzymes such as amylase, cellulase, and lipase. In 1920, Keilin (3) found several earthworm proteases that could dissolve casein, gelatin, and albumin, and in 1951, Tracey (4) identified cellulase and chitinase from earthworms that could degrade biomass. In addition, we characterized highly active endoglucanases (*Ef*EG1 and *Ef*EG2), which belong to the family of cellulases, from the Waki strain of *Eisenia fetida* (5). These enzymes are useful for biomass utilization.

Earthworms have also been studied for their therapeutic effects on human diseases. In the 16th century, Shizhen Li, China’s greatest naturalist, used dried earthworm powder as an antipyretic and diuretic agent. The powder, called “Earth Dragon”, is still used as a traditional medicine for disease treatment (6,7). At the end of the 19th century, Frédéricq (8) discovered a protease from earthworm fluid that could dissolve fibrin, which may be the first report of a fibrinolytic enzyme from earthworms. Fibrinolytic enzymes may be used as active pharmaceutical agents to treat thrombotic diseases such as myocardial and cerebral infarction, which are difficult to treat. Thus, many researchers are studying the detailed function of fibrinolytic enzymes from earthworms. The importance of fibrinolytic enzymes from earthworms was recognized in 1991, when Mihara et al. (9) isolated and characterized a fibrinolytic enzyme called lumbrokinase (LK) from the earthworm *Lumbricus rubellus*. The potency and usefulness of earthworm fibrinolytic enzymes were subsequently widely recognized.

Sumi et al. (10) and Nakajima et al. (11) reported that fibrinolytic enzymes are very stable, which led to dried earthworm powder being sold as a dietary supplement in Asian countries, including Japan. However, the enzymes are inactivated by the high-temperature sterilization procedure used to produce dietary supplements. Because the sterilization method combining high-pressure and moderate temperature is useful in food processing, high-pressure treatment (HPT) has been employed in this field (12). In fact, some groups have reported that HPT can enhance enzymatic activity and increase the content of functional molecules such as γ-aminobutyric acid (GABA) (12,13). Furthermore, HPT can degrade allergenic proteins (14), and application of this technology can reduce the cost and duration of food processing. Use of HPT to produce earthworm powder would allow a provider to supply

* Corresponding author. Tel./fax: +81 258 34 9255.
E-mail address: s-akazaw@nagaoka-ct.ac.jp (S.-i. Akazawa).

1389-1723/$ – see front matter © 2017, The Society for Biotechnology, Japan. All rights reserved.
http://dx.doi.org/10.1016/j.jbiosc.2017.08.011

Please cite this article in press as: Akazawa, S., et al., High-pressure tolerance of earthworm fibrinolytic and digestive enzymes, J. Biosci. Bioeng., (2017), http://dx.doi.org/10.1016/j.jbiosc.2017.08.011

ARTICLE IN PRESS

novel products such as digestive supplements at low cost. However, no report on the high-pressure tolerance of earthworm enzymes was found in the literature. Therefore, we attempted to develop a novel earthworm dietary supplement having fibrinolytic activities and digestive enzymes using HPT with low-temperature.

Here, we investigated the high-pressure tolerance of the earthworm enzymes LK (fibrinolytic enzyme), α-amylase (AMY) (saccharification enzyme), endoglucanase (EG) (saccharification enzyme), β-glucosidase (BGL) (saccharification enzyme), and lipase (LP) (fatty acid degradation enzyme). The knowledge obtained will allow the development of an earthworm fibrinolytic and digestive dietary supplement, and also the use of earthworm enzymes for biomass utilization combined with HPT.

MATERIALS AND METHODS

Earthworms The earthworm *E. fetida*, Waki strain, was kindly provided by Waki Pharmaceutical, Nara, Japan. All experiments were conducted in accordance with the guidelines of the institutional Animal Care Committee.

Sample preparation and HPT Earthworms were starved for 24 h. Next, 40 g of earthworms were washed with Milli-Q-purified water and disrupted using a mixer in 100 mL of 50 mM sodium phosphate buffer at pH 7.0. The mixture was centrifuged at 10,000 ×*g* and 4°C for 15 min. A 5-mL aliquot of the supernatant was transferred to each plastic bag, and the HPT was performed under the following conditions: 0.1, 100, 200, 300, 400, and 500 MPa at 25°C or 50°C for 1 h using a Servo Pressure 500 (Sugino Machine, Toyama, Japan). A control sample was not subjected to heat or pressure treatment. The treated sample was centrifuged at 10,000 ×*g* and 4°C for 15 min. The supernatant was used for various assays. All experiments were performed in triplicate.

Measurement of enzymatic activity LK activity was measured at 37°C by determining the formation of *p*-nitroaniline (*p*NA) following the absorbance at 405 nm with a Corona Grating Microplate Reader SH-9000 (Corona Electric, Ibaraki, Japan). S-2251 (H-D-Val-Leu-Lys-pNA) and S-2288 (H-D-Ile-Pro-Arg-pNA) (Sekisui Medical, Tokyo, Japan) were used as a substrate for plasmin and tissue-plasminogen activator (t-PA), respectively. One unit of the enzyme was defined as plasmin or t-PA type LK activity that produces 1 μmol *p*NA/min. AMY activity was measured using the α-amylase measurement kit (Kikkoman Biochemifa, Chiba, Japan). EG activity was measured by determining the amount of reducing sugar produced from carboxymethyl cellulose (CMC) (Sigma−Aldrich Japan, Tokyo, Japan) using the Somogyi-Nelson method (15). One unit of the enzyme was defined as EG activity that produces 1 μmol glucose/min. BGL enzymatic activity was measured at 40°C by determining the amount of *p*-nitrophenol produced from *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) (Sigma−Aldrich) following the absorbance at 405 nm with a model 550 Microplate reader (Bio-Rad Laboratories, Tokyo, Japan). One unit of the enzyme was defined as BGL activity that produces 1 μmol *p*-nitrophenol/min. LP activity was measured using the lipase kit S (DS Pharma Biomedica, Osaka, Japan). Total activity was determined from a 5-mL aliquot of the supernatant.

Protein content determination The amount of protein was determined using the Bradford method (16) with a Bio-Rad protein assay kit (Bio-Rad Laboratories) and bovine serum albumin (BSA; Bio-Rad) as the standard.

Determination of viable cell counts The number of viable aerobic bacteria was determined using 3M Petrifilm Aerobic Count Plates (AC plate) (3M Japan, Tokyo, Japan). The number of viable coliforms was determined using 3M Petrifilm Rapid Coliform Count Plates (RCC plate) (3M). The number of viable yeast and mold cells was determined by 3M Petrifilm Yeast and Mold Count Plates (YM plate) (3M). All procedures followed the manufacturer's instructions.

RESULTS

High-pressure tolerance of enzymes from *E. fetida* Waki at 25°C Enzymatic activities of LK, AMY, EG, BGL, and LP were investigated under HPT at 25°C (room temperature). Fibrin (blood clot), which may cause thrombotic diseases such as myocardial and cerebral infarction, is formed by thrombin acting on fibrinogen (17,18). Plasmin, which can degrade fibrin, is activated by t-PA and urokinase-plasminogen activator (u-PA). Two mechanisms were proposed for earthworm LK; as a plasminogen activator and as a direct digestive action on fibrin (9,19,20). Therefore, we investigated both LK activities using chromogenic substrates S-2251 (substrate for plasmin) and S-2288 (substrate for t-PA). Fig. 1 shows that both LK activities remained almost unchanged up to 100 MPa, and were gradually enhanced above 200 MPa. The plasmin-like and t-PA-like total activities rose 1.6- and 1.4-fold, respectively (Fig. 1). These results revealed that LK has some pressure tolerance, and that the apparent enzymatic activity was enhanced by HPT.

AMY is a major amylase that catalyzes the hydrolysis of the α-1,4-glucosidic linkages of starch, with worldwide industrial applications (21,22). AMY showed thermotolerance and some small pressure-induced activation at 400 MPa (Fig. 2). The cellulolytic enzymes EG and BGL are useful not only for biomass utilization and bio-refining but also for human and animal health. Although EG activity was unchanged up to 400 MPa, it rapidly decreased to 0.7-fold of initial activity at 500 MPa (Fig. 3). In contrast, BGL activity decreased rapidly at 300 MPa and was almost eliminated at 500 MPa (Fig. 4). The enzyme did not show high-pressure tolerance. The thermotolerance of LP was similar to that of AMY, but its activity decreased to some extent above 200 MPa (Fig. 5).

High-pressure tolerance of enzymes from *E. fetida* Waki at 50°C Enzymatic activities were also investigated under HPT at 50°C. The total amount of protein was significantly reduced with only heat treatment, although LK showed thermotolerance and high-pressure tolerance (Fig. 6). The plasmin-like and t-PA-like total activities rose 1.7- and 1.9-fold, respectively, at 500 MPa (Fig. 6). The corresponding specific activities also increased 16- and 18-fold, respectively (Fig. 6). These results revealed that

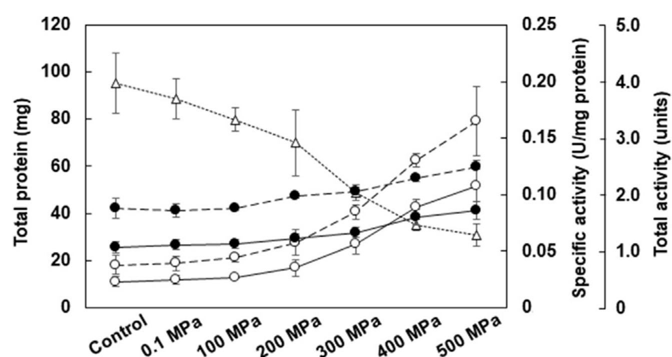

FIG. 1. Effects of pressure treatment on lumbrokinase at 25°C. S-2251 and S-2288 were used as chromogenic substrates for plasmin and t-PA. Data correspond to the mean of three replicates. Solid and dashed lines indicate S-2251 and S-2288, respectively. Open and closed circles represent specific and total activity, respectively. Open triangles represent total protein.

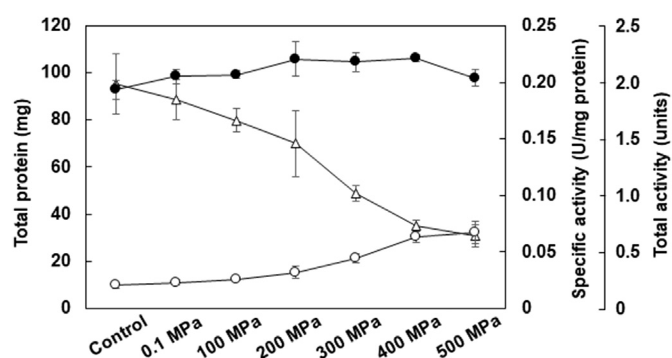

FIG. 2. Effects of pressure treatment on α-amylase at 25°C. Enzyme activity was measured using an α-amylase measurement kit. Data correspond to the mean of three replicates. Open and closed circles represent specific and total activity, respectively. Open triangles represent total protein.

Please cite this article in press as: Akazawa, S., et al., High-pressure tolerance of earthworm fibrinolytic and digestive enzymes, J. Biosci. Bioeng., (2017), http://dx.doi.org/10.1016/j.jbiosc.2017.08.011

Download English Version:

https://daneshyari.com/en/article/6489955

Download Persian Version:

https://daneshyari.com/article/6489955

Daneshyari.com