Collagenolytic activity of serine protease of *Perionyx excavatus*

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Abstract

A serine collagenolytic protease was purified from the earthworm *Perionyx excavatus* by DEAE-Sephadex A-50 and DEAE-Sephadex A-50 column. The molecular mass of the earthworm serine protease was estimated to be 35 kDa. The purified serine protease was optimally active at pH 6.8-7.8 and 55°C. In vitro studies on collagenolytic activity of this enzyme was also carried out using type I collagen. Hydrolysis of collagen using serine protease was characterized by the collagenolytic activity. The amount of hydroxyproline released (μg/ml) on untanned and tanned leather was monitored. Zymographic analysis was carried out using Collagen as substrate.

Keywords: Serine protease, collagen, *Perionyx excavatus*, earthworm, hydroxyproline.

Introduction

Proteolytic enzymes or proteases catalyze the cleavage of peptide bonds in proteins. Peptide bond cleavage is one of the most frequent enzymatic modifications of proteins; recent studies of proteolytic enzymes have focused on the regulatory roles in the variety of physiological processes. Proteases, proteinases or peptidases are described as the same group of enzymes that catalyze the hydrolysis of covalent peptidic bonds (In the case of serine protease the mechanism is based on the nucleophilic attack of the targeted peptidic bond by a serine). Earthworms secrete proteases which degrade casein, gelatin, collagen and fibrin and the lyophilized powder of earthworms have long been used for anti-pyretic and diuretic purpose in Chinese medicine under the name “Jiryu”. Collagenases are generally defined as enzymes that are capable of degrading the polypeptide backbone of native collagen under conditions that do not denature the protein. Two types of proteases with collagenolytic activity have been reported and are thought to play a vital role in different physiological functions. Metallo-collagenases, first discovered in tadpole tissue explants (Gross & Lapiere, 1962), are zinc-containing enzymes that also generally require calcium for their optimum activity and stability and cleave the collagen helix at a specific locus under physiological conditions (Stricklin et al., 1977; Cawston & Murphy, 1981; Sellers & Murphy, 1981; Harris & Vater, 1982). These enzymes have been widely studied from various mammalian tissues (Sellers & Murphy, 1981; Harris & Vater, 1982) as well as from bacteria, such as *Bacillus cereus* (Makinen & Makinen, 1987), *Clostridium histolyticum* (Peterkofsky, 1982; Bond & Van Wart, 1984a, b), *Achromobacter* (Nguyen et al., 1988), *Vibrio alginolyticus* (Takeuchi et al., 1992) and *Clostridium perfringens* (Matsuhita et al., 1994) and snake venom (Bjarnason & Fox, 1994). These enzymes are involved in the production of hormones, pharmacologically active peptides and in various cellular functions such as protein digestion, blood-clotting, fibrinolysis, complement activation and fertilization (Neurath, 1984; Bond & Butler, 1987). In addition, they are widely used in chemical, medical, food industries and molecular biology experiments. The objective of the present study is to purify a collagenolytic enzyme from *Perionyx excavatus* and to characterize the enzyme with respect to its responses to pH, temperature, solvents, collagenolytic activity and its molecular weight.

Materials and methods

The earthworms of species *Perionyx excavatus* were collected from the wet soil garden in the premises of Central Leather Research Institute, Adyar and also from Murugappa Chettiar Research Center, Chennai. After collection, all the earthworms were maintained in mud pot. Required nutrients and water were sprayed at alternative days. Growth was monitored by examining the length. Earthworms of length 10-12 cm were chosen for the extraction of enzyme for the present study.

**Enzyme assay:** Hydrolysis of native collagen was detected by hydroxyproline assay. The reaction mixture containing collagen (type I) in 200 mM Tris-HCl at pH 7 was incubated with an appropriate amount of enzyme for 2 h at 30°C. Samples (500 μl) were withdrawn and boiled in order to stop the reaction and then centrifuged. After hydrolysis of type I collagen by serine protease, concentration of hydroxyproline was measured in supernatant after 6 h of acid hydrolysis (HCl 6M, 105°C) as reported in International standard method (ISO 3496-1978 E).

**Extraction of enzyme:** The earthworms were narcotized on ice with 10% ethanol and cut in to pieces and homogenate was prepared by grinding it with the phosphate buffer of pH 6.4, with the help of mortar and pestle (Tillinghast et al., 2001). The earthworm autolysate was centrifuged at 10,000 rpm for 20 min at
4°C. The supernatant was collected and subsequently subjected for assessing the protease activity.  

**Purification of serine protease**

Purification of the extracted enzyme was carried out at three different stages. In the first stage, precipitation of enzyme was done using 80% ammonium sulphate followed by centrifugation at 10,000 rpm for 15 min. The precipitate thus obtained is subjected to dialysis using acetate buffer (0.2 M & pH 5.4). Dialysis was carried out for 3 days with intermittent changes in the acetate buffer solution. The dialyzed samples were again subjected for further purification using DEAE-Sephadex A-50 column with height and the bed volume. The influent was maintained at ml/min. The fractions thus collected were subjected for spectrophotometer analysis at 280 nm. Fractions exhibiting high optical density were pooled together and the molecular weight of that particular fraction was assessed electrophoretically using SDS-PAGE gel electrophoresis using 12% acryl amide.

**Protein determination**

The amount of protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

**Effects of pH & temperature**

Stability of the enzyme towards the pH changes was monitored by incubating the reaction mixture containing enzyme and substrate and the required buffer at various pH starting from acidic to alkaline range for 30 min. The thermosability of the enzyme was assessed by incubating the enzyme at different temperatures and almost every enzyme has characteristic temperature at which their activity is maximum. Above or below the optimum temperature the activity is minimized or lost. Hence it is very much necessary to determine the optimum temperature of the enzyme.

**Effect of solvents**

The stability of the enzymes to the organic solvents was tested at room temperature in 1 ml of 100 mM Tris-HCl buffer (pH 8) containing 25% (v/v) of solvents. Acetone, 2-propanol, toluene, n-hexane were used for the present study. The experimental procedure was followed as per the method reported by Nakajima et al. (2000).

**Molecular mass determination**

The molecular weight of the fraction was determined by 12% SDS-PAGE and its proteolytic activity was determined by using collagen as a substrate. SDS-PAGE is a method of separating proteins within a sample for the analysis and molecular weight determination.  

**Effect of serine protease on hydrolysis of collagen**

Proteases are found to play an important role in hydrolysis of proteins. However, hydrolysis of skin protein, especially collagen using serine protease was not reported. So the present study has been extended to evaluate the potential of the extracted serine proteases on collagen hydrolysis. Moreover the hydrolyzed product of collagen whether by chemical or biological method is hydroxyproline, the measurements of quantity of release of hydroxyproline by the test sample will in turn provide the extent of hydrolytic effect of test proteases on collagen.

**Zymography**

Zymography is an analytical method (Neely et al. 1991) to evaluate both latent and active forms of MMPs based on the denaturation of the enzyme followed by renaturation before incubation with enzyme buffer for activity determination steps, which activates the various proenzyme forms (Matsubara et al., 1991).

**Results**

**Purification & substrate specificity**

Throughout our experimental study, all the earthworm species shown good health and was maintained under natural environment. For extraction, only live organism was used. The collagenolytic enzyme from earthworm, *Perionyx excavatus* was purified by ammonium sulfate (80%) and consecutive column chromatography using DEAE-sephadex A-50 column. Table 1 summarizes the serine protease activity with respect to different substrates namely casein, bovine serum albumin and collagen. There was an increase in activity in the order of collagen followed by casein and BSA. The proteolytic activity of any enzyme source is important role in hydrolysis of proteins. However, hydrolysis of skin protein, especially collagen using serine protease was not reported. So the present study has been extended to evaluate the potential of the extracted serine proteases on collagen hydrolysis. Moreover the hydrolyzed product of collagen whether by chemical or biological method is hydroxyproline, the measurements of quantity of release of hydroxyproline by the test sample will in turn provide the extent of hydrolytic effect of test proteases on collagen.

**Table 1. Substrate specificity of the enzyme.**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Protease activity (μg/min)</th>
<th>Specific activity (μg/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>271.2</td>
<td>71.37</td>
</tr>
<tr>
<td>Casein</td>
<td>64.4</td>
<td>16.7</td>
</tr>
<tr>
<td>Collagen</td>
<td>130.48</td>
<td>30.34</td>
</tr>
</tbody>
</table>

**Table 2. Collagenolytic activity of earthworm serine protease on untanned & tanned skin/hide.**

<table>
<thead>
<tr>
<th>Enzyme activity (μg/ml)</th>
<th>Amount of hydroxyproline released (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untanned skin/hide</td>
<td>Tanned skin/hide</td>
</tr>
<tr>
<td>130</td>
<td>0.465</td>
</tr>
<tr>
<td>200</td>
<td>0.625</td>
</tr>
<tr>
<td>400</td>
<td>0.712</td>
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</table>
maximum activity was observed between the pH of 6-7.8 (Fig. 1). The activity of the enzyme with respect to temperature revealed that most of the proteases are highly stable towards higher temperature. In the present study, the maximum activity was observed between 55-57°C. Further increase in temperature reduces the activity vigorously (Fig. 2). Similar observations were made by Nakajima et al. (1993) and Nakajima et al. (2002).

**Effect of organic solvents**

Regarding the effect of organic solvents on the protease activity, it has been observed that irrespective of the solvents chosen, there was an increase in activity with an increase in incubation period. Fig. 3 illustrated the activity pattern with respect to solvent at the concentration of 1:5 (enzyme: solvent). This implies that the isolated proteases exhibited stability towards wide range of solvents and also with an increase in incubation period. The maximum activity of protease was observed in 4th day (Fig. 3). Similar observations were made by Nakajima et al. (2000).

**Molecular weight determination**

Molecular weight of the isolated serine protease was carried out using 12% SDS-PAGE and the molecular weight was assessed as a range between 22-35 KDa and was in unison with earlier report (Nakajima et al., 2003).

**Collagenolytic activity**

The isolated enzyme exhibited a highest activity when collagen was used as substrate, further collagen hydrolysis of the enzyme was performed using pure collagen and also for the tanned and untanned collagen. Table 2 summarizes the quantity of release of hydrolyzed product namely hydroxy proline after hydrolysis. About 40-60 μg/ml of collagen was released after 24 h of incubation at 37°C. Similar reports were reported on the hydrolytic activity of serine proteases by Kim et al. (2003).

**Zymogram**

Further confirmation using native gel technique (Fig. 4) clearly indicated that serine protease hydrolyzes the proteins completely.

**Discussion**

Enzyme technology is one of the best technologies, applied in the food production and drug formulation, etc. Enzymes of various classes find their way in different usage and currently a number of enzymes are being extracted from various sources based on its requirement and application. In the overwhelming population and the quantity of solid waste generated from major industries, a special attention has to be given to degrade/mineralize the solid materials via eco-friendly techniques. Earthworms, as an eco-friendly organism, serve much more to keep the nature clean. Its beneficial effects are immense. Recently it has been observed that proteases are of high need to hydrolyze the solid...
protein substrates. Much importance has been given to serine proteases for its wide application in medical, pharmaceutical, chemical, detergent formulation, dairy backing, food processing, etc. In addition to all, further research has to be focused on hydrolysis of leather wastes generated in leather manufacturing industries. In the present study, extraction of serine proteases from earthworms (Indian variety) was carried out and the physical and chemical characterization of this enzyme was completely analyzed. In addition, stability of the enzyme with respect to various environmental conditions, pH, temperature, solvents, and also the inhibitory effect of other compounds was also assessed. The molecular weight determination was done according to the procedures and it was found that the molecular mass is in the range of 23 - 35 kDa. Further confirmation by zymogram procedures using various protein substrates substantiate the purity of the enzyme extracted. Regarding the application of the isolated serine protease on leather waste, it has been found that, the enzyme hydrolyzes the untanned leather up to 50 - 60% and only up to 25% in tanned leather.

**Fig. 4. Zymographic analysis of the enzyme using collagen substrate.**

**References**