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Purification and characterization of collagenase from Bacillus altitudinis

Aditi Mohindra[1], Aditi Chauhan[2] and Vijay Prabha[3]

Author Affiliation: [1]Student, [2]Research Scholar, [3]Professor, Department of Microbiology, Panjab University, Chandigarh-160014, India.

Corresponding Author: **Vijay Prabha,** Professor Department of Microbiology Panjab University Chandigarh -160014. India

E-mail: satishvijay11@gmail.com

Abstract

Microbial collagenases are secreted by anaerobic as well as aerobic pathogenic and non-pathogenic microorganisms to utilize collagen as a source of nutrition. The collagenase production from aerobic non-pathogenic strains can increase its application by decreasing the production time and avoiding pathogenicity. Keeping this in view, previously, collagenase had been isolated and partially purified by gel permeation chromatography (Sephadex G-200) from an aerobic non-pathogenetic microorganism, Bacillus altitudinis, in our laboratory. Thus, the present study was undertaken to purify further and characterize the collagenase. For this, collagenase was purified by anion exchange chromatography using a DEAE cellulose column. SDS-PAGE was carried out, where a single band of apparent homogeneity with the molecular mass of ~23 kDa was observed. Finally, the purified collagenase was characterized in terms of its stability at different temperature and pH ranges. It showed stability at temperature ranging from 4 to 45°C and pH ranging from 4 to 10 up to 60 minutes.

Keywords: Purification and Characterization; Bacillus altitudinis.

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Introduction

The use of chemicals in different industries has increased enormously, which is affecting life tremendously. So, present-day research is going on to replace these toxic chemicals with environmentally friendly products. Therefore, the focus is shifting to determine different enzymatic processes instead of chemical processes. Proteases are successfully considered an alternative to chemicals and an eco-friendly indicator for nature. They are one of the three largest industrial enzymes, and their global market is drastically increasing annually (Razzaq et al., 2019). Out of the various protease sources, microbial proteases have shown importance as they can be produced in large amounts rapidly and cost-effectively, thus extensively utilized in various fields (Nisha and Divakaran, 2014). Among these proteolytic enzymes' collagenases play an important role as they are the only enzymes that can hydrolyse the insoluble fibrous collagen, a major fibrous element of skin, bones, tendons, cartilage, and blood vessels. This enzymatic degradation of collagen results various peptides which have diverse industrial applications e.g., an immunotherapeutic agent, a moisturizer for cosmetics, a preservative, dietary material and significant applications in medical industries (Pal and Suresh, 2016).

The collagenases are mainly found in various animals, microorganisms, and plants but differ in substrate specificities (Bhagwat et al., 2018). Microbial collagenases have broad specificities. They can degrade both native and denatured collagens and attack various sites of the collagen chain in contrast to other collagenases that only cleave the native collagen at a specific site (Baehaki et al., 2012). These collagenases have been isolated from various organisms, most of them being pathogenic. This pathogenic nature of the microorganisms poses the possibility of an outbreak of microorganisms, leading to an increase

in the cost of enzyme production and limiting the applications (Bhagwat et al., 2015). Hence, studies on microbial collagenase production and purification from non-pathogenic sources and further exploring their applications would be beneficial to improve the growth in various industrial sectors. Previously in our laboratory, collagenase was isolated and partially purified from an aerobic, non-pathogenic Bacillus altitudinis. Considering this, the present work was carried out to further purify and characterize the collagenase from Bacillus altitudinis.

Materials and Methods

Microorganism

Bacillus altitudinis, capable of producing collagenase, isolated previously in our laboratory was used in the present study (Chauhan and Prabha, 2017).

Isolation and partial purification of collagenase from Bacillus altitudinis

Collagenase was isolated and partially purified by gel permeation chromatography (Sephadex G-200) from 72 hours old cell culture of Bacillus altitudinis by the method earlier standardized in the laboratory (Chauhan and Prabha, 2017).

Purification of collagenase

The purification of collagenase was carried out by anion exchange chromatography. The pooled and concentrated fractions of collagenase after gel permeation chromatography (Sephadex G-200) were passedthrough DEAE cellulose column. 80 mL of elution buffer PBS (50 mM, pH 7.2) was allowed to run down the column. Elution of protein was carried out with PBS containing 0.05, 0.1, 0.2, 0.4 and 0.6 M NaCl. Fractions of 4 mL each were collected, and the absorbance was read at 280 nm on UV spectrophotometer. The fractions showing collagenase activity were pooled, concentrated and stored at -20 °C.

Molecular mass estimation of purified collagenase

The molecular mass of the purified collagenase was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli (1970).A 10% gel was prepared and SDS was added accordingly. Purified collagenase was denatured and loaded onto the gel along with the standard molecular weight markers. After the gel was run, Coomassie blue staining was done and molecular weight was estimated.

Characterization of collagenase

Effect of temperature

The purified collagenase was incubated for 5, 15, 30, 45 and 60 minutes at various temperatures i.e., 4, 28, 37, 40, 45, 50 and 55 °C with 50 mM Tris–HCl buffer (50 mM CaCl2, pH 7.5). The activity of collagenase was examined by gelatin plate method (Suphatharaprateep et al., 2011)in which wells were cut in gelatin agar plates with a sterile cork borer and loaded with the protein sample. The plates were flooded with 35% (w/v) Trichloroacetic acid and observed for clear zones around the wells after 24 hours of incubation at 37 °C.

Effect of pH

For studying the effect of pH, the purified collagenase was incubated for 5, 15, 30, 45 and 60 minutes at different pH i.e., 3, 4, 5, 6, 7, 8, 9 and 10 with 50 mM Tris–HCl buffer (50 mM CaCl2, pH 7.5). and collagenase activity was examined by gelatin plate method(Suphatharaprateep et al., 2011).

Results

Purification of collagenase

For purification, the pooled and active fractions from Sephadex G-200 column (Figure 1) were applied on DEAE cellulose anion exchange column. It was found that collagenase bound in the column could be eluted with PBS. The fraction showing collagenase activity were 4-6 with peak value in fraction 5 (Figure2).

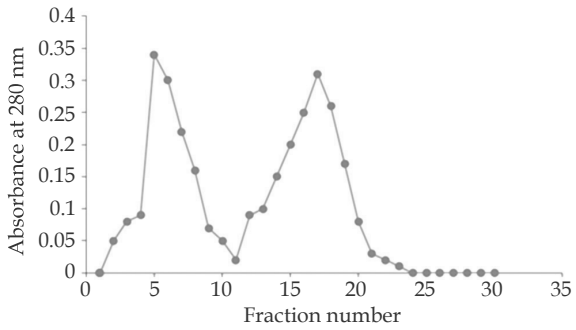

Fig. 1: Elution pattern of collagenase from B. altitudinis after gel permeation through sephadex G-200 column is showing the presence of collagenase in fractions 5-8 with peak value in fraction 5

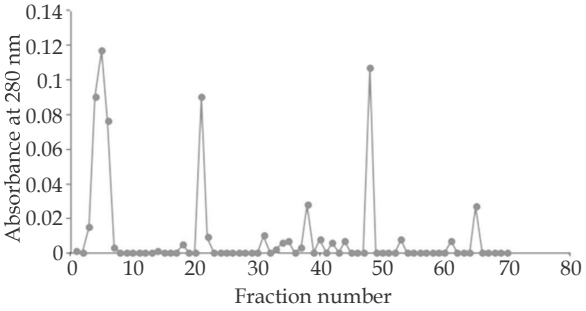

Fig. 2: Elution pattern of collagenase by DEAE cellulose ion exchange chromatography showing collagenase in fractions 4-6 with peak value in fraction 5

Estimation of molecular mass

SDS-PAGE was carried out to estimate the molecular mass of purified collagenase. The Coomassie blue stained gel showed that the purified collagenase was a ~23 kDa protein (Figure3).

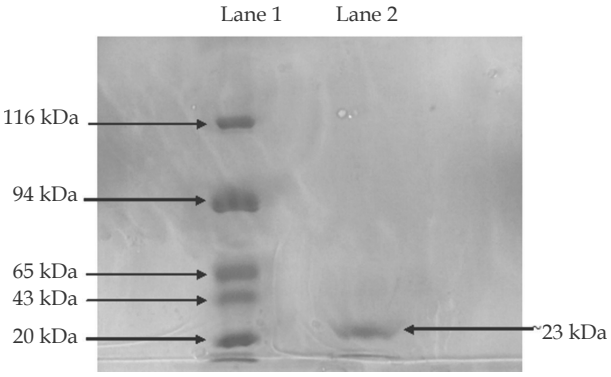

Fig. 3: SDS-PAGE of collagenase; Lane 1: Marker; Lane 2: DEAE-cellulose pooled and concentrated fraction

Characterization of collagenase

Characterization of enzymes is important to determine its applicatory use with respect to dierent sectors in which it is going to be exploited. Therefore, the purified collagenase has been characterized by their stability profiles corresponding to temperature and pH.

Effect of temperature

The purified collagenase was incubated for 5, 15, 30, 45, 60 minutes at various temperatures ranging between 4-55 °C with 50 mM Tris–HCl buffer (50 mM CaCl2, pH 7.5). It was observed that collagenase was stable at temperatures 4 to 45 °C till 60 minutes (Figure 4).

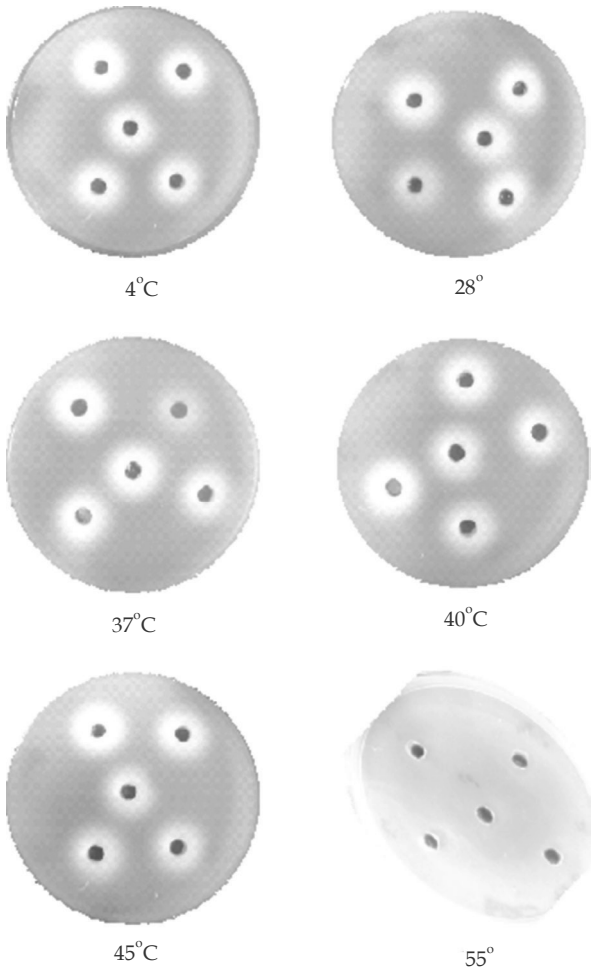

Fig. 4: Clear zones on gelatin agar medium after addition of TCA around wells loaded with purified protein after incubation for 5, 15. 30, 45 and 60 minutes at 4, 28, 37, 40, 45 °C and no clear zones at 55 °C

Effect of pH

The purified collagenase was incubated for 5, 15, 30, 45, 60 minutes at different pH ranging from 3-10 with 50 mM Tris–HCl buffer (50 mM CaCl2, pH 7.5). It showed collagenase was stable from pH 4 to pH 10 till 60 minutes (Figure 5).

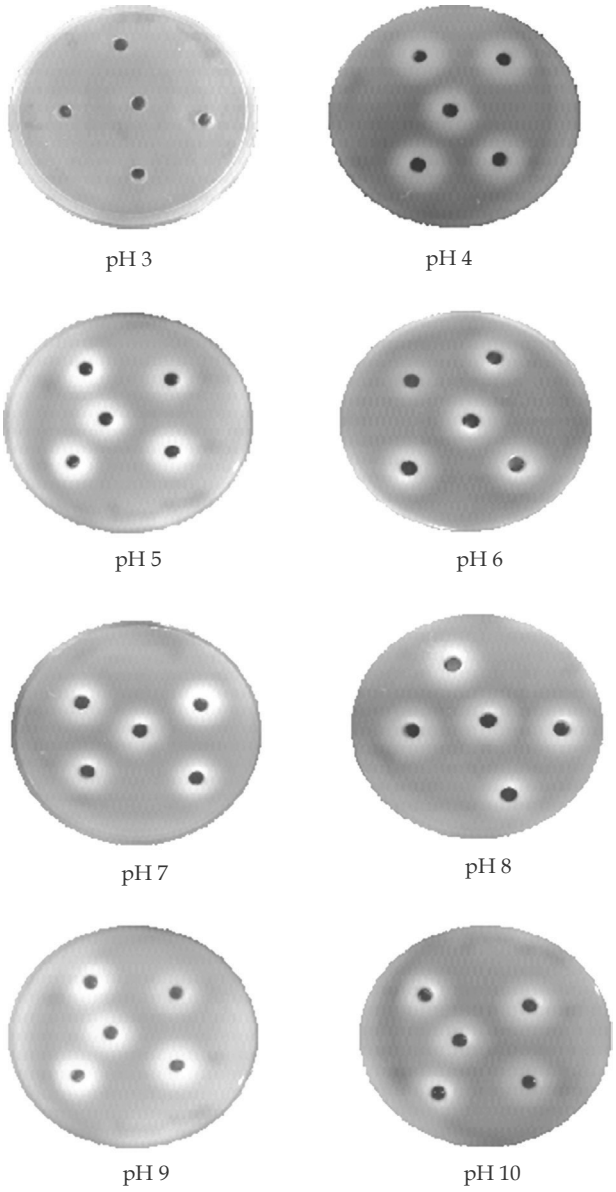

Fig. 5: Clear zones after addition of TCA on gelatin agar medium around wells loaded with purified protein after incubation for 5, 15, 30, 45 and 60 minutes at pH 4, 5, 6, 7, 8, 9, 10 and no clear zones at pH 3

Discussion

Collagens are the major proteins in the skin, tendons, teeth, blood vessels, bone, dentin, and other body structures. They determine the organ shape, tissue integrity, and cell attachment. Collagen molecules have a very rigid structure; therefore, only certain types of proteases, i.e., collagenases, can degrade them. Valuable collagen peptides, produced by the action of collagenases, have several biological applications in medicinal and industrial fields. Therefore, research is going on microbial collagenase purification where collagenases from Bacillus licheniformis, Pseudomonas sp. SUK, Pseudomonasmarinoglutinosa, Porphyromonasgingivali s, Rathayibacter sp., Bacillus cereus MBL13, Clostridium histolyticum, Vibrio vulnificus, Thermoactinomyces sp. E21, Alicyclobacillus sendaiensis, Bacillus pumilus Col-J are being purified and characterized (Baehaki et al., 2012; Bhagwat et al., 2016; Hamdy, 2008; Kato et al., 1992; Labadie and Hebraud, 1997; Liu et al., 2010; Matsushita et al., 1999; Miyoshi et al., 1998; Petrova et al., 2001; Tsuruoka et al., 2003; Wu et al., 2010). In this light, collagenase was isolated and partially purified in our laboratory. Hence, as an addendum to previous work, the present study was carried to further purify and characterize collagenase.

Numerous procedures have been used for the purification of microbial collagenases, such as gel permeation chromatography, ion-exchange chromatography, immobilized metal anity chromatography, amylose anity chromatography, and removal of N-terminal tag (Bhagwat et al., 2016; Ducka et al., 2009). Therefore, the fractions of gel permeation chromatography showing collagenase activity were pooled and subjected to anion exchange chromatography (DEAE cellulose). It is by far the most common ion-exchange technique that has been used by several researchers to purify collagenase and is based on diethylaminoethyl (DEAE) cellulose or agarose (Daboor et al., 2010).

The molecular mass of collagenases generally ranges from 20 to 120 kDa. Different molecular masses for different collagenases have been reported which include 125 kDa for B. subtilis FS-2 collagenase (Nagano and To, 2000); 120 and 29 kDa for B. licheniformis N22 (Asdornnithee et al., 1994); 42.8 kDa for Bacillus cereus (Sela et al., 1998); 58.64 kD for B. pumilus Col-J (Wu et al., 2010); 50 kDa for Thermoactinomyces sp. 21E (Petrova et al., 2006b); and 33 and 19.8 kDa for Pseudomonas sp. (Hisano et al., 1989). After ion-exchange chromatography, the fractions showing collagenase activity were pooled, and SDS-PAGE was carried out to determine the molecular mass of purified collagenase. A single band of apparent homogeneity with a molecular mass of ~23 kDa was observed.

A handful of purified microbial collagenases have been characterized by their activity and stability profiles corresponding to temperature and pH. These are considered to be the most important factors in retaining collagenolytic activity. Therefore, the purified collagenase was characterized in terms of its stability at different

temperatures and pH. The collagenase purified from Bacillus altitudinis showed stability up to 60 minutes at a temperature ranging from 4 to 45°C and pH ranging from 4 to 10. Earlier researchers have reported different optimum temperatures and pH for collagenases from various organisms, i.e., optimum temperature of 30–42°C for C. perfringens (Matsushita et al., 1994), 50°C for B. subtilis FS-2 (Nagano and To, 2000), 70–75°C for both Bacillus sp. MO-1 (Okamoto et al., 2001) and Thermoactinomyces sp. 21E (Petrova et al., 2006) and optimum pH of 8.5 for P. logei (Xu et al., 2004), 9.0 for B. subtilis FS2 (Nagano and To, 2000) and Bacillus sp. MO-1 (Okamoto et al., 2001), and 9.0–9.5 for Thermoactinomyces sp. 21E (Petrova et al., 2006),respectively.

Conclusion

From the observations, it can be concluded that collagenase from Bacillus altitudinis could be purified by gel permeation chromatography followed by ion-exchange chromatography, and the purified collagenase is stable at a temperature range of 4 to 45°C and a pH range of 4 to 10 up to 60 minutes.

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