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Purification and characterization of protease from *Emericella nidulans*

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Abstract: In order to purify protease(s) produced by *Emericella nidulans* AUMC 9198, several steps were performed including ammonium sulphate precipitation at 80% and chromatography on Sephadex G-100 and on DEAE-cellulose columns. The optimum pH, temperature, pH and thermal stability were pH 8, 55°C, pH 7-10 and up to 55°C respectively. Protease activity was completely inhibited with PMSF and Hg^{2+} while not affected with EDTA. The purified enzyme had a V_{max} value of 595 U/ml and K_m value of 1.092 mg/ml. Anticoagulant potency of the purified serine protease recorded prolonging action on the extrinsic coagulation pathways using prothrombin time (PT) bioassay.

Key words: *Emericella nidulans*, proteases, purification, anticoagulant potency.

Introduction

There is an increased demand for newer and novel medicinal products with improved characters and less undesirable side effects to heal various important diseases. Among enzymes, proteases have a renewed interest mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Gupta *et al*. 2002). Proteases (EC 3.4.21-24 and 99), a large and complex group of enzymes that catalyses the hydrolysis of protein molecules into peptides and amino acids (Özkan and Ertan 2012). Proteases constitute one of the most important groups of enzymes in industrial field, and account for at least 60% of the total world-wide enzyme sales (Singh *et al*. 2001) and constitute two thirds of the total enzymes used in various industries (Gupta *et al*. 2002). Majority of these enzymes have application in textile and detergent industries (Charles *et al*. 2008), leather, food and pharmaceutical industries (Coral *et al*. 2003), and waste industry (Pastor *et al*. 2001). Their involvement in the life cycle of disease-causing organisms had led them to become a potential target for developing therapeutic agents against fatal disease such as cancer and AIDS (Rao *et al*. 1998). Proteolytic enzymes are found in all living organisms and they are important for the cell growth and differentiation. The inability of the plant and animal proteases to meet current world demands (due to extensive use in food, pharmaceutical and detergent industry) has led to an increased interest in microbial proteases (Gupta *et al*. 2002). Several fungi have the ability to produce proteolytic enzymes including genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Alternaria*, *Mucor*, *Trichoderma*. Although the extensive works on enzymes production from various sources, there is still searching for new sources of enzymes to result in novel process applications. So, the present investigation has been carried out to study the purification and characterization of protease enzyme by the soil isolate *Emericella nidulans* (AUMC 9198).

Materials and Methods

Fungal isolate

Emericella nidulans AUMC 9198 was isolated from soil in the area of Faculty of Science, Helwan University. Taxonomic identification of this isolate was carried out in our Mycology lab and confirmed in Assuit University Mycological Center (AUMC).

Production medium

The culture medium used for *Emericella nidulans* had the following composition (Sandhya *et al*. 2005): dipotassium hydrogen phosphate, 0.1 g; magnesium sulphate, 0.5 g; sodium chloride, 0.5 g; ferrous sulphate, 0.004 g; wheat bran, 2 g and 100 ml distilled water.

Protease assay

Protease activity was determined using modification of Anson's method (Keay and Wildi 1970), using casein as a substrate.

Protein determination

Protein concentration was estimated by the method of Bradford (1976).

Enzyme purification

One liter of the optimized medium (Sandhya *et al*. 2005) was prepared, inoculated and incubated at 28°C. At the end of incubation

period, the supernatant was obtained by filtration using Whatman filter paper followed by centrifugation at 10,000 rpm for 20 min. at 4°C and was used as the source of the enzyme. To the cell free broth was added ammonium sulfate to the saturation of 80% as mentioned by Dixon and Webb (1964). The precipitated protein was collected by centrifugation for 20 min. at 10,000 rpm at 4°C. The resulting pellets were suspended in 0.05 M phosphate buffer pH 7, dialyzed against the same buffer, then were concentrated by dialysis against sucrose. Three ml of the dialyzed preparation was applied to Sephadex G-100 column (2×70cm) equilibrated with the same phosphate buffer. Sixty fractions, each of 5 ml, were collected at a flow rate of 40 ml /h and tested for its proteolytic activity and protein content. The protease peak fractions were pooled together and tested for both its proteolytic activity and protein content, then concentrated for further purification step. Three ml of the concentrated pooled fractions of sephadex G-100 were applied on the top of Diethylaminoethyl cellulose (DEAE-C) (30×1 cm) pre-equilibrated with 0.05 M phosphate buffer pH 7. The exchanged material were eluted stepwise with NaCl ranging from 0.0 to 0.5 M at a flow rate of 30 ml /h and collected in 5 ml fractions. Fractions exhibiting protease activity were collected and dialyzed against sucrose for concentration.

Effect of different factors on the purified protease activity

Effect of temperature

To estimate the best temperature for purified enzyme activity, the reaction mixture (enzyme and substrate) was incubated at different temperatures (15°, 25°, 35°, 45°, 55°, 65° and 75°C) and the assay completed under standard conditions. On the other hand, thermal stability of the enzyme was determined by exposing only the purified enzyme solution to different temperatures (15° to 75°C) for 20 min. then immediately cooled to 4°C then the activity was determined under standard assay conditions.

Effect of pH

The reaction mixture was incubated at different pH values (6, 7, 8, 9, 10 and 11) with pH buffer solutions (0.2 M phosphate buffer 6-8, 0.2 M glycine-NaOH 9-11) and the assay was carried out under standard assay conditions. Furthermore, stability of the purified enzyme was investigated by exposing the purified enzyme solution to various pH values (5 to 11) using different pH buffer solutions (0.2 M sodium acetate buffer 5, 0.2 M phosphate buffer 6-8, and 0.2 M glycine-NaOH 9-11). The activity was determined under standard assay conditions.

Effect of different inhibitors

The purified enzyme was incubated with various inhibitors **(**ethylene diamine tetraacetic acid (EDTA), phenyl methyl sulfonyl fluoride (PMSF) and Hg^{2+}) in concentration of 10mM for 20 minutes. The enzyme activity was determined as mentioned before.

Determination of kinetic parameters K_m and V_{max}

The kinetic constants K_m and V_{max} were estimated by double reciprocal plots of the data according to the method of Lineweaver and Burk (1934).

Anticoagulant properties

The prothrombin time (PT) studies the total extrinsic clotting system. It measures the clotting time of plasma at 37 ºC in the presence of excess tissue calcium-thromboplastin (Becker *et al.* 1984). Different concentrations of the purified enzyme were incubated with 100 µl of platelet poor plasma (PPP) [fresh human blood was mixed with 0.11 M sodium citrate in the ratio of 4:1 and the mixture was centrifuged for 15 min. at 7000 rpm and incubated for 3 min. at 37 ºC. 100 µl of calcium-thromboplastin reagent (pre-warmed at 37 ºC), was added and the clotting time recorded. For determination of the control time, the experiment was performed by using 0.05 M Tris-HCl buffer, pH 8.5 (100 µl) instead of crude extract or purified enzyme.

Results and Discussion

Purification of proteases is an important step for the perspective of developing a better understanding of the functioning of the enzyme (Takagi 1993). In the current study, in order to purify protease produced from *Emericella nidulans* several steps were performed including preparation of cell-free filtrate, ammonium sulphate precipitation and application on column chromatography. All purification steps were performed at or below 4°C to prevent microbial contamination as well as to maintain enzyme activity and stability. In the present work, when the cell-free filtrate was subjected to different concentrations of ammonium sulphate, it was found that the enzyme was best precipitated at 80% saturation (Fig. 1). Similar results were reported for proteases from *Vibrio fluvialis* (Wang *et al.* 2007) and *Aspergillus flavus* strain K-03 (Kim 2007).

Precipitates obtained were dissolved and dialyzed against 0.05M phosphate buffer then against sucrose to concentrate the sample. The sample was then loaded on Sephadex G-100 column gel filtration chromatography which resulted in one enzyme peak (Fig. 2) that was

collected, concentrated and further applied for purification on DEAE-cellulose column resulting in one enzyme peak (Fig. 3). A summary of purification steps was presented in Table (1).

The optimum temperature for purified enzyme activity was 55°C (Fig. 4), with the thermal stability up to 55°C for *Emericella nidulans* (Fig. 5). Almost similar optimum temperature was reported for other fungi, at nearly 50°C like *Rhizopus oligosporus* (Devi *et al*. 2011), *A. fumigatus* (da Silva *et al*. 2013), and *Aspergillus flavus* (Muthulakshmi *et al*., 2011). Differently, Charles *et al*. (2008) reported the maximum enzyme activity from *A. nidulans* HA-10 at 35°C with thermal stability up to 50°C. Pena-Montes *et al*. (2008) reported 40°C for *Aspergillus nidulans* PW1.

The optimum pH value of the purified protease was found to be 8 with stability range of pH 7-10 (Figs. 6 &7). Likewise, Charles *et al*. (2008) reported the maximum enzyme activity from *A. nidulans* HA-10 at pH 8. These reports are in accordance with previous findings that the optimum pH of 8.0 was reported for *Rhizopus oligosporus* (Devi *et al*. 2011), *Nomuraea rileyi* (Ramachandran and Arutselvi 2013) and the medicinal mushroom *Pleurotus sajor-caju* (Ravikumar *et al*. 2012). In addition, Pena-Montes *et al*. (2008) reported pH 8.5 for *Aspergillus nidulans* PW1 with stability range 8-11.

Concerning the effect of various inhibitors on the purified enzyme activity, the results demonstrated that the enzyme activity was not affected by using EDTA compared to control, while the activity was sharply inhibited by PMSF and Hg^{2+} ion indicating that the enzyme is serine protease type (Fig. 8). Sulfonation of the essential serine residue in the active site by PMSF results in the complete loss of activity (Kumar and Takagi 1999). Protease activity of *A. nidulans* HA-10 was completely inhibited by PMSF (Charles *et al*. 2008). Other investigators supported this finding as Sharma and De (2011) for *Aspergillus tamarii*, da Silva *et al*. (2013) for *Aspergillus fumigatus* and Murthy and Naidu (2010) for *A. oryzae* CFR305.

The effect of substrate concentration on the rate of enzyme activity was tested. The substrate concentration of 1-40 mg/ml casein was taken for this experiment. The kinetic constants K_m and V_{max} were estimated by double reciprocal plots of the data according to the method of Lineweaver and Burk (1934). The result showed that V_{max} for protease from *E. nidulans* was 595U/ml, while K_m was 1.092 mg/ml **(**Fig. 9**).** El-Shora and Metwally (2008) reported that V_{max} for alkaline protease from *A. terreus* was 29 U ml^{-1}, while alkaline proteases from *A. niger* was 166 U ml^{-1}. In addition, Chakrabarti *et al*. (2000) reported the K_m of the purified protease from *Aspergillus terreus* (IJIRA 6.2) to be 0.055 mM.

Blood clotting is a very complex process, involving many factors found in the plasma and tissues. It involves both the intrinsic and extrinsic pathways (Jandl 1996). Blood coagulation was affected by many inhibitors and activators. The prothrombin time (PT) test is used to show the effects of test agents on the extrinsic pathway (Brown 1988). Substances that affect the PT are thought to act on the extrinsic pathway factors: factors V, VII, X, prothrombin and fibrinogen, (Osoniyi and Onajobi 2003) The anticoagulant potency of the purified serine protease from *Emericella nidulans* was determined by studying its action on the extrinsic coagulation pathways using prothrombin time (PT) bioassay. The purified serine protease had anticoagulant actions by all concentrations used (Table 2). In addition, increasing the concentration of the purified enzymes up to 15 µg protein, prolonged the PT time up to >900 seconds.

In conclusion: The purified enzyme was active at 55°C, pH 8, stable till 55°C and at pH range 7-10 and was inhibited with PMSF indicating that it is serine protease. The purified enzyme had a V_{max} value of 588 U/ml and K_m value of 0.112 mg/ml. Studying the anticoagulant potency of the purified serine protease showed its prolonging action on the extrinsic coagulation pathways using prothrombin time (PT) bioassay.

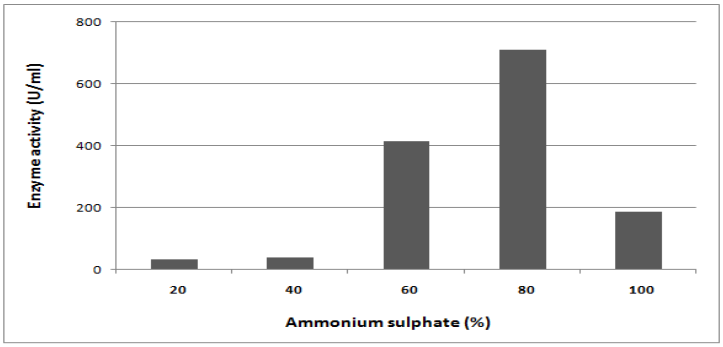

Figure 1: Effect of ammonium sulphate concentrations on protease production

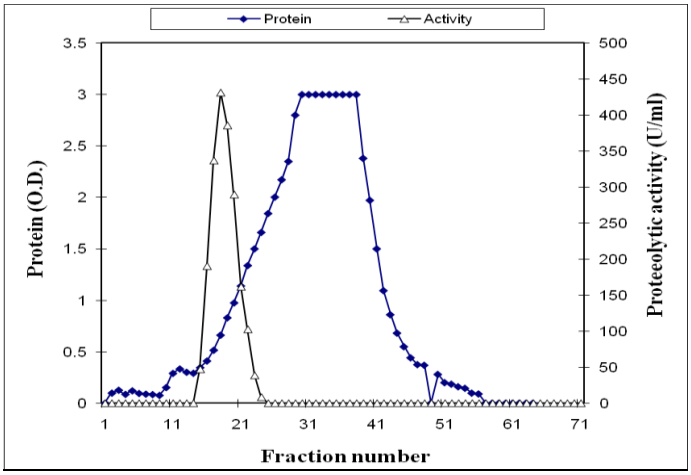

Figure 2: Purification of protease produced by *Emericella nidulans* using Sephadex G-100 column.

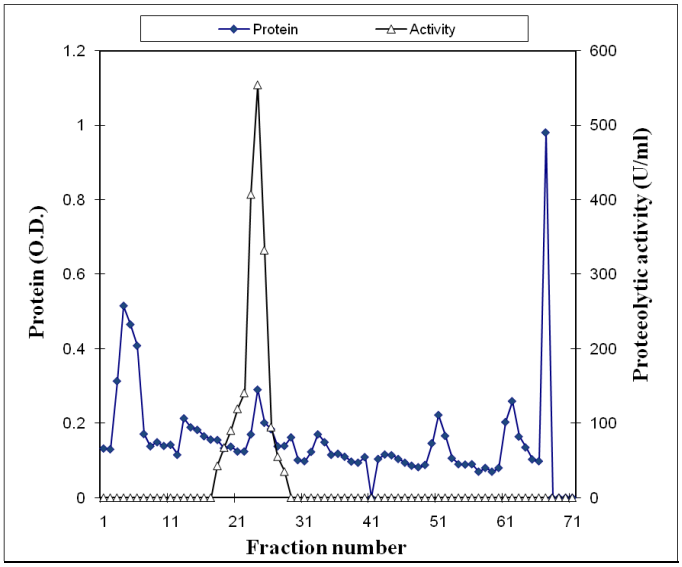

Figure 3: Purification of protease produced by *Emericella nidulans* using DEAE- cellulose column.

Table 1: A summary of the purification steps of protease enzyme produced by *Emericella nidulans*.

Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold
Crude extract	336960	720	468	1
Ammonium sulph.	57570	70	822.4	1.8
Dialysis	53855	34	1605	3.4
Sephadex G-100	8420	2.4	3508	7.5
DEAE cellulose	5052	0.9	5613	11.9

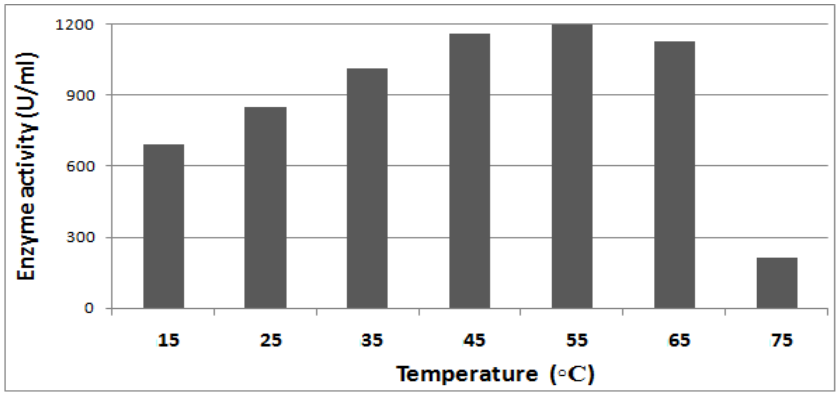

Figure 4: Effect of temperature on the purified enzyme activity produced by *Emericella nidulans.*

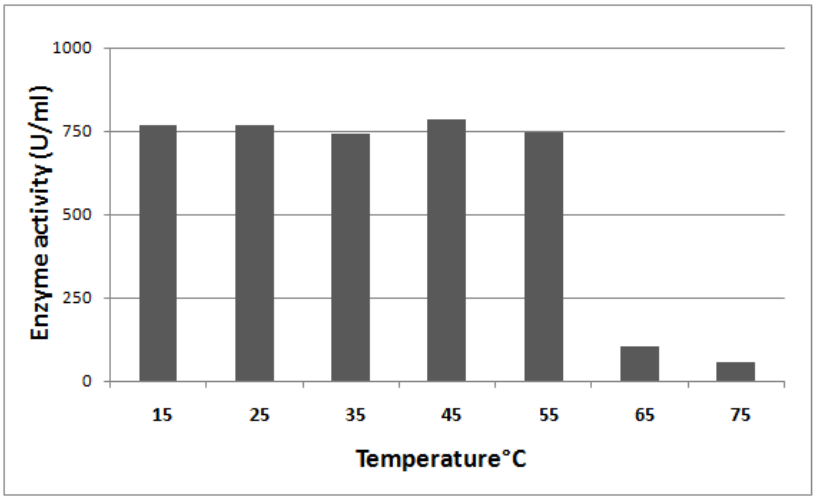

Figure 5: Effect of temperature on the stability of the purified enzyme produced by *Emericella nidulans*.

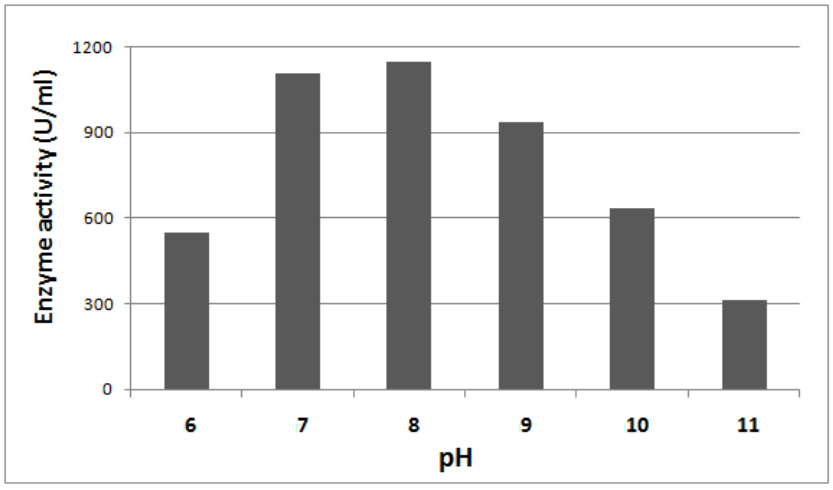

Figure 6: Effect of pH on the purified enzyme activity produced by *Emericella nidulans.*

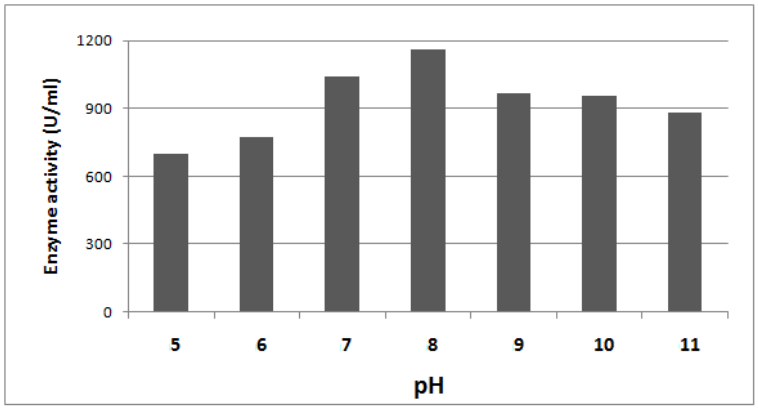

Figure 7: Effect of pH on the stability of the purified enzyme produced by *Emericella nidulans*.

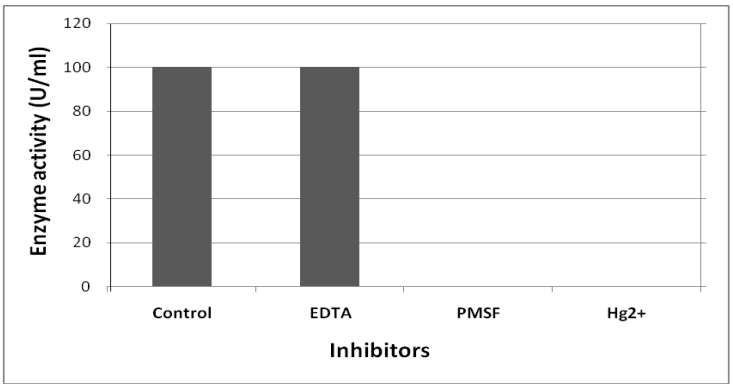

Figure 8: Effect of different inhibitors on the activity of the purified enzyme produced by *Emericella nidulans.*

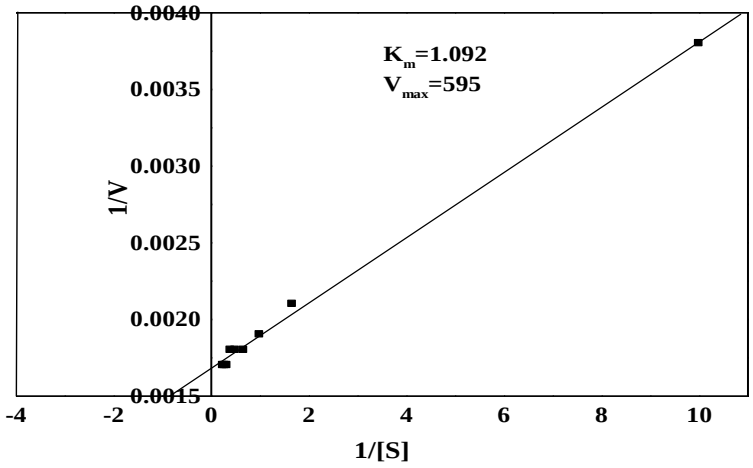

Figure 9: Lineweaver-Burk plot of experimental data for serine protease from *Emericella nidulans*: K_m =1.092 mg/ml; V_{max}=595U/ml.

Table 2: Bioassay for the inhibition of the extrinsic blood coagulation pathway

Proteins (µg)	Prothrombin time (seconds)
3	40
6	50
15	>900
Control	16

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