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# Isolation of a putative virulence agent, cytotoxic serine-elastase, from a newly isolated *Pseudomonas aeruginosa* ZuhP13

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A 48 kDa ZuhP13 elastase from *P. aeruginosa* isolated from a urine sample was successfully purified to 8.8-fold and 39% recovery by DEAE-Sepharose CL-6B and Sephadex G-100 chromatography. Its ideal reaction values were pH 7.5 and 40°C. It showed stability at pH 6–9 for 1 h and up to 60°C for 30 min with midpoint temperature ($T_m$) at 61.3°C and isoelectric value (p*I*) at 5.6±0.2. Its $K_m$ and catalytic efficiency ($K_{cat}/K_m$) for the substrate azocasein were 1.3 mg/mL and $4.62\times10^7$ $M^{-1}s^{-1}$, respectively. On contrary to most *P. aeruginosa* proteases, $Zn^{2+}$, EDTA, 2,2′-bipyridine and *o*-phenanthroline showed slight inhibition upon its activity, while, the elastase inhibitors (elastatinal and elastase inhibitor II) and the serine protease inhibitors (TLCK, PMSF, SBTI, and aprotinin) markedly decreased the enzymatic activity. Taken together, we suggest that ZuhP13 is a serine elastase-type. Interestingly, the tested enzyme showed both hemolytic and hemorrhagic activities *in vivo*. Furthermore, it induced nuclear lysis yielding hyperchromatism within leaky and malformed hepatocytes, suggesting ZuhP13 elastase as a high molecular weight potential pathological agent.

**Keywords.** Elastase; *Pseudomonas aeruginosa*; purification; virulence factor

**Abbreviations:** BoNT, botulinum neurotoxin; EDTA, ethylene diamine tetraacetic acid; i.p, intraperitoneal; LB broth, Luria-Bertani broth; LB plot, Linweaver-Bulk plot; p*I*, isoelectric point; PMSF, phenylmethyl sulfonyl fluoride; SBTI, soybean trypsin inhibitor; TEM, transmission electron microscope; TLCK, tosyl lysine chloromethyl ketone; $T_m$, midpoint temperature

## 1. Introduction

Proteases are the leading group of enzymes in worldwide markets with many applications in food industry. In this regard, caution should be done concerning the producing bacterium as proteases for some are part of their virulence towards their hosts. During infection, these pathogenic bacteria secrete tremendous amounts of heterogeneous proteases to provide them with peptidic nutrients. These foreign proteases alter the function of the endogenous proteases secreted by the human or animal body. Furthermore, they affect the integrity of body's structural proteins causing tissue damage and several diseases, like atherosclerosis (Frees *et al*. 2013).

Interestingly, the lethal fraction of anthrax toxin produced by *B. anthracis* is a metallo-type protease which specifically cleaves MAP kinase protein of human body causing the disease (Young and Colliers 2007). The BoNT neurotoxin of *Clostridium botulinium* is also a metallo-type protease that cleaves SNAP-25 protein responsible for the storage of the neurotransmitter, acetylcholine. Other pathological proteases that were also reported are the serralysin of *Serratia marcescens* and the hemagglutinin of *V. cholera* (Kotb *et al.* 2013).

Recently, Esp, EcpA, and SepA proteases of *Staphylococcus epidermidis* were found to facilitate the instrumental associated nosocomial infections by proteolysis and inactivation of the adhesion molecules thus facilitate the formation of biofilms (Martínez-García *et al.* 2018). Other proteases are produced by the pathogens to protect them from glycoproteins of the immune system. For example, ZapA protease secreted by *Proteus mirabilis* protects the bacterium from the rising immunoglobulins by their proteolytic action. A second example is the metalloprotease produced by *B. thuringiensis* to degrade the antimicrobial glycproteins secreted by the host pest (Aneas *et al.* 2001). For this, many researchers consider them as the main virulence factor among all microbial metabolites. The exact mechanism through which microbial proteases trigger diseases is still lacking but their major role in the maturation *of Listeria monocytogenes*

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toxins and activation of diphtheria, pertussis and tetanus toxins is very clear (Stehling *et al*. 2008; Frees *et al.* 2013).

Thus, inactivation of these pathological proteases is of special interest among researchers. It was found that, inactivation of ClpP protease system in *Staph. aureus*, *Listeria monocytogenes*, *Streptococcus pneumoniae* D39, *Salmonella typhimurium* rendered them avirulent with rapid elimination from their hosts and they become more susceptible to environmental and nutritional stresses (Frees *et al.* 2013).

*P. aeruginosa* may be acquired from the environment thus, causing otitis externa, keratitis, skin or soft tissue infections. In addition, it may be acquired from hospitals thus, causing infections to the respiratory tract, urinary tract, blood, surgical wounds or skin burns. Protease-deficient strains of *P. aeruginosa* are generally less virulent than protease producing strains. There are three well known proteases enhancing the virulence of *P. aeruginosa* viz. lasA elastase, lasB elastase and alkaline protease-type (Stehling *et al*. 2008). However, detailed characterization of microbial proteases from pathogenic bacteria such as *P. aeruginosa* is not fully done, herein, we primarily aimed to screen the proteolytic action of *P. aeruginosa* isolates associated with medical samples. The objective of the study was also extended to demonstrate the subcellular changes triggered by injection of *P. aeruginosa* ZuhP13 protease in mice.

## 2. Materials and methods

### 2.1 *Materials*

Cetrimide agar, azocasein, elastatinal, elastase inhibitor II, aprotinin, EDTA, *o*-Phenanthroline, 2,2′-bipyridine, PMSF, SBTI and TLCK were procured from Sigma-Aldrich (USA). DEAE-Sepharose CL-6B, Sephadex G-100 FF and the chemicals of SDS-PAGE electrophoresis were obtained from Pharmacia Biotech (Sweden). While, chemicals of analytical grade were obtained from local suppliers

### 2.2 *Strain isolation and characterization*

During this survey a total of 120 clinical specimens were collected from the hospitals of Zagazig University (Zagazig, Egypt) in the period from October 2016 to March 2017. The clinical data were obtained from the respective units and wards of the patients. For the specific isolation of *P. aeruginosa,* specimens were cultured on cetrimide agar consisting of (g/L) gelatin (20.0), agar (13.6) dipotassium sulphate (10.0), magnesium chloride (1.4) and cetrimide (0.3) with pH adjusted at 7.2, then transferred to 37°C incubators for 24 h. Growing isolates were assayed for protease production on 25% (v/v) skimmed-milk agar involving (g/l) agar (12), peptone (4), NaCl (3), yeast extract (2), and beef extract (1) with pH adjusted at 7.2. Incubation was done at 37°C for 24 h. Appearance of clear halos around proteoltyic isolates were observed. Pure cultures obtained from quadrate streaking were maintained on 20% glycerol at −20°C for further studies.

Cetrimide agar is both differential and selective for the isolation and identification of *P. aeruginosa* in which cetrimide acts as a selective inhibitor against other bacteria and as an indicator by enhancing the production of pyocyanin and pyoverdine pigments by *P. aeruginosa* only. The biochemical characterization of the most potent isolates was done till the species level as defined in *Bergey's Manual of Systematic Bacteriology*. Sequencing of *16srDNA* gene revealed sequence similarity with *P. aeruginosa*.

### 2.3 *Enzyme production*

Crude ZuhP13 protease was produced in a basal medium constituting (%, w/v) casein (1.0), glycerol (0.7 mL), fructose (0.5), NaCl (0.3), $KH_2PO_4$ (0.08), $MgSO_4{\cdot}7H_2O$ (0.05), yeast extract (0.04), $K_2HPO_4$ (0.02), $CaCl_2$ (0.01), $MnSO_4$ (0.01), $FeSO_4$ (0.002) and $ZnSO_4$ (0.002). The final pH was adjusted at 9.0 and the fermentation process was operated at 40°C for 48 hr. Only twenty percent (v/v) of 250 ml Erlenmeyer flasks volume were used that were inoculated with 2% (v/v) ($\sim 3\times10^8$ cells/mL) of 12 h *P. aeruginosa* previously cultured in LB broth composed of (%, w/v) tryptone (1.0), yeast extract (0.5) and NaCl (0.5), with final pH of 7.0. Incubation was done for 48 h at 35°C with constant shaking at 180 $rev^{-1}$ min. The bacterial cells were harvested at 7,000*g* for 10 min. Supernatants were used for assessment of total soluble proteins and enzyme activity.

### 2.4 *Enzyme assay and protein measurement*

The assay mixture comprised of 1 mL of 1% (w/v) casein solution at pH 7.2 in 0.2 M borate buffer and 1 mL of the enzyme solution. Enzyme catalysis operated at 37°C and terminated after 30 min by 2 mL of 10% (w/v) of stop solution, trichloroacetic acid then left in crushed ice bath for 1 h. The unit of protease activity (**U**) was equilibrated as the sum of enzyme necessary to release 1 μg of the amino acid L-tyrosine per min under the usual assay settings. The concentration of solubilized proteins (C) was quantified (mg/mL) according the following equation; C (mg/mL) = 1.55 $OD_{280}$ − 0.76 $OD_{260}$.

### 2.5 *Enzyme purification*

The bacterium *P. aeruginosa* ZuhP13 was grown under optimized conditions then bacterial biomass was harvested after 10 min centrifugation at 7,000*g*. Supernatant was fractionated by 30–60% $(NH_4)_2SO_4$ and resulting proteins were collected at 10,000*g* for 20 min and resuspended in 20 mM borate buffer (pH 8.5). The crude enzyme initially

passed DEAE-Sepharose CL-6B column (2.5×30 $cm^2$) equilibrated with typical buffer. Unadsorbed proteins were eluted with same buffer with linear increase in ionic strength 20–800 mM at 1 mL/min elution rate. Concentrated active fractions then passed at an 0.5 ml/min elution rate through Sephadex G-100 FF column (2×70 $cm^2$) using 20 mM borate buffer (pH 7.4). Finally, potent fractions lyophilized and analyzed for purity by SDS-PAGE that was done following the method of Andrews (1986) with 10% resolving gel and 4% stacking gel with constant volt of 60V.

### 2.6 *Effect of temperature on protease activity and stability*

This was done at 20–50°C in 0.2M borate buffer, pH 7.4 using the substrate, milk casein. The heat stability was measured by standing the enzyme at 50–80°C for 60 min with interval measurements of remaining activity each 15 min. At the end of incubations, replicates were cooled to 40°C to assess remaining activity.

### 2.7 *Effect of pH on protease activity and stability*

Mixtures of purified protease and the substrate casein were adjusted to a wide pH range at 40°C by three buffers at 100 mM strength; dimethylglutarate (pH 5.0–7.2), borate (pH 7.4–9.0) and glycine-NaOH (pH 9.2–11.0). For pH stability experiment, preincubation of buffered enzyme fractions without substrate at 40°C for 2 h in range of pH 5–11 was done, then remaining activity was determined at pH 7.5 and 40°C.

### 2.8 *Determination of enzyme isoelectric point*

This was done by modification of Kantardjieff and Rupp (2004) method. Protease fractions were incubated for 12 h at pH range of 3.0–10.8 at 4°C using the aforementioned buffer systems. In each separate test, precipitated proteins after centrifugation at 10,000*g* for 15 min were quantified by resuspending in 1 mL borate buffer (pH 7.5) then, measuring the absorbances $A_{280}$ and $A_{260}$ and applying the following equation; soluble protein (mg/mL) = 1.55 $OD_{280}$–0.76 $OD_{260}$. The p*I* is defined as, the pH value at which maximum protein was measured.

### 2.9 *Kinetic parameters*

The kinetic values, $K_m$ and $V_{max}$ for proteolysis of the dyed-substrate azocasein by ZuhP13 were determined following Michaelis–Menten kinetics using Lineweaver–Burk plot. Herein, several concentrations (17.39–4350 µM) of azocasein in 100 mM borate buffer (pH 7.5) were used for catalysis of the purified enzyme at 40°C.

### 2.10 *Effects of metallic ions and protease inhibitors*

Several cations in addition to EDTA, aprotinin, 2,2′-bipyridine, TLCK, *o*-Phenanthroline, PMSF, SBTI, elastatinal and elastase inhibitor II on enzymatic activity was tested. Protease activity was assessed by measuring substrate hydrolysis activity after preliminary incubation with metal ions and/or inhibitors at 40°C for 60 min at the standardized values.

### 2.11 *In vitro cell damaging activity*

Assay of cytotoxicity was done by 24 h incubation of 15 µg ZuhP13/mL with HT29-cell line with concentration adjusted to $1\times10^5$ cells/mL. The percentage of enzyme cytotoxicity was expressed as the percentage of HT29-cell death by the end of incubation. For negative control, boiled enzyme was used, while, for blank, HT29-cells were omitted.

Assay of hemolytic activity was done at 37°C by quantifying the amount of released hemoglobin after 90 min of mixing equal volumes of 15 µg ZuhP13/mL and 4% (v/v) washed human RBCs suspended in 100 mM borate buffer at pH 7.5. Absolute (100%) hemolysis was attained by addition of triton X-100 to RBCs suspension at concentration of 1% (v/v). For assay of *in vitro* tissue damaging activity, mice organs were removed and incubated separately with ZuhP13 preparation at concentration of 15 µg enzyme /ml at 37°C for 5 h. The percent of released hemoglobin was quantified and was taken as a measure of tissue damage.

### 2.12 *In vivo damaging activity*

BALB/c mice weighting 17–20 g housed in two groups of six per cage and allowed to acclimatize laboratory conditions for one week before experimentation. Animals were fed and kept at constant environmental and nutritional conditions through the experimental period. Briefly 100 U of purified protease in 1 mL was intraperitoneally injected in each mouse. Controls were received an equivalent amount of heat-inactivated enzyme preparation. Mice were then witnessed at constant intervals over 2 days. Sacrificed and control animals were autopsied immediately to determine the histological effects. Livers were picked and fixed in 5% glutaraldhyde then 1% osmium tetraoxide. Seventy nm ultrathin slices were cut using RMC ultramicrotome. They were carried on copper grids and examined under JEOL 1010 TEM.

### 2.13 *Statistical analysis*

Unless otherwise stated, all treatments and experimentation were done in triplicates. Final data were in form of averages ± standard deviations.

## 3. Results

### 3.1 *Strain isolation and characterization*

From 120 medical samples, we obtained fifty-four isolates of *P. aeruginosa* that were positive for protease productivity. Exactly, 31.5% of isolates were obtained from pus, 20.4% from urine, 16.7% from wounds, 12.9% from sputum, 7.4% from eyes, 7.4% from blood and 3.7% from ears. Sex wise prevalence of clinical isolates showed that, *P. aeruginosa* are more common in males (66.7%) compared with females (33.3%). While, age wise prevalence of clinical isolates showed that, most patients (61.1%) were aged between 26 and 50 years.

The most potent isolates (ZuhP43, ZuhP6, ZuhP13, ZuhP39, ZuhP50 and ZuhP21) in respect with protease production (table 1) were characterized using the biochemical tests outlined in *Bergey's Manual of Systematic Bacteriology.* For further characterization, *16S rDNA* gene fingerprint was applied. The PCR gave DNA pieces at the expected length (supplementary figure 1).

### 3.2 *Fermentation conditions*

The fermentation period required to achieve the highest enzyme productivity is mainly influenced by culture characteristics and the bacterial growth rate. The maximum growth monitored at $A_{600}$ was 1.34 and the maximum level of enzyme production reached 108.8 U/mL after 48 h of incubation thereafter, both parameters decreased gradually.

As shown in figure 1a, maximum protease productivity was obtained with 1% fructose (115.0 U/mL) while, maximum bacterial growth was obtained with 1% glycerol. Optimization of carbon source concentration (data not shown) resulted in higher levels of protease production at 0.5 % of fructose. Maximum protease productivity of strain ZuhP13 was obtained at 0.5% casein (129.6 U/mL) followed by 0.5% peptone (111.4 U/mL), while ammonium sulphate drastically repressed enzyme production (figure 1b). Casein also induced maximal bacterial growth (0.974) as judged by $A_{600}$. Optimization of N-source concentration (data not shown) resulted in maximal productivity at 1 % of casein.

### 3.3 *Enzyme purification*

Exactly, 3 L of fermentation medium was produced, thereafter ZuhP13 protease separated as briefed in table 2. Last eluted protein showed a specific activity of 39.2-fold with 8.8% recovery and a major band at 48 kDa (figure 2).

### 3.4 *Reaction temperature and thermal stability of the purified enzyme*

Temperature achieved maximal protease activity was 40°C (132 U/mL). At lower and higher temperatures lesser activities obtained (figure 3a). Results represented in figure 3b indicated that, purified protease was heat stable for 30 min up to 60°C. The calculated half-life times ($t_{1:2}$) were 297.7 min, 97.3 min, 26.5 min and 0 min at temperatures 50, 60, 70 and 80°C, respectively and the deduced $T_m$ was 61.35°C (figure 3c).

### 3.5 *Reaction pH, pH stability and pI of the purified enzyme*

Maximum protease activity was obtained at pH 7.5 (146 U/mL) and enzyme was stable for 1 h at pH 6–9. Above or below this value activity decreased gradually (figure 4a). On basis of protein precipitation pattern (figure 4b), the isoelectric point of tested enzyme was 5.6±0.2.

### 3.6 *Kinetic parameters*

Kinetic parameters $V_{max}$ and $K_m$ of ZuhP13 were determined from the double reciprocal plot of initial rates by using the

**Table 1.** A comparative data of protease production by the most potent isolates

| | | Maximal yields | | |
|---|---|---|---|---|
| Strain | Source of isolation | Protease productivity (U/mL) | Biomass ($A_{600}$) | Fermentation time (h) |
| ZuhP6 | Pus cells | 95.6 ± 5.8 | 1.066 ± 0.08 | 60 |
| ZuhP13 | Urinary tract infection | 108.8 ± 7.2 | 1.345 ± 0.14 | 48 |
| ZuhP21 | Complication of corneal ulcer | 100.6 ± 6.4 | 0.923 ± 0.11 | 48 |
| ZuhP39 | Septicemia | 92.8 ± 4.8 | 1.411 ± 0.15 | 60 |
| ZuhP43 | Burn surgery | 95.8 ± 7.5 | 0.866 ± 0.07 | 54 |
| ZuhP50 | Urinary tract infection | 82.7 ± 5.9 | 0.945 ± 0.09 | 48 |

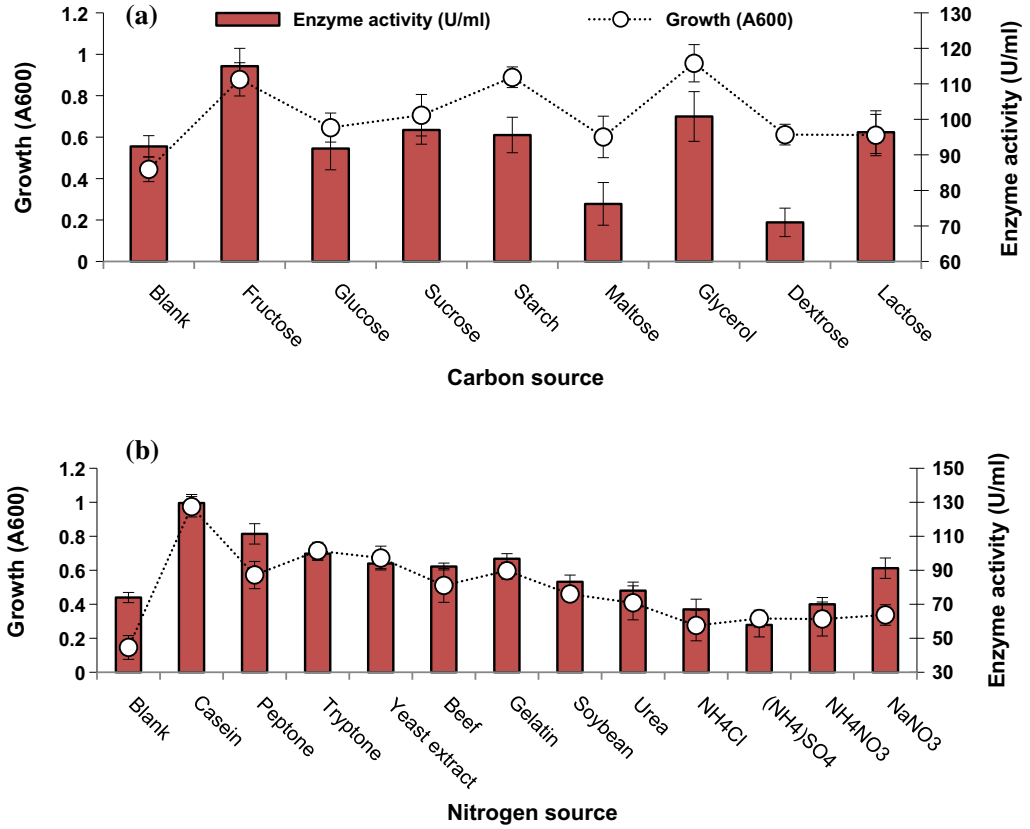


**Figure 1.** Relation of carbon (**a**) and nitrogen sources (**b**) to protease productivity by *P. aeruginosa* ZuhP13.

**Table 2.** A brief of ZuhP13 protease purification steps

| Purification step | Total activity (U) | Protein content (mg) | Specific activity (U/mg) | Purification folds | Recovery (%) |
|---|---|---|---|---|---|
| Crude supernatant | 113168.5 | 5.87 | 19279.13 | 1.0 | 100.0 |
| Ammonium sulfate fractionation | 67912.0 | 1.98 | 34298.99 | 1.8 | 60.0 |
| DEAE-Sepharose CL-6B | 52121.0 | 1.17 | 44547.86 | 2.3 | 46.1 |
| Sephadex G-100 | 44328.2 | 0.26 | 170493.08 | 8.8 | 39.2 |

substrate, azocasein (supplementary figure 3). The apparent $K_m$ was 1.32 mg/mL and $V_{max}$ was equivalent to 126.6 U/mL. The $K_{cat}$ was 1.27 $s^{-1}$ and the catalytic efficiency was $4.62 \times 10^7$ $M^{-1}$ $s^{-1}$.

### 3.7 *Inhibition study*

Concerning effect of metallic ions and protease inhibitors upon activity of ZuhP13 (table 3), it was noticed that, $Zn^{2+}$ did not affect enzymatic activity whereas, $Hg^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Ca^{2+}$, $Mn^{2+}$, $Mg^{2+}$, $Ba^{2+}$, $Cd^{2+}$ and $Fe^{3+}$ decreased activity. Furthermore, enzymatic activity was not affected by metalloproteinase inhibitors, *o*-phenanthroline, 2,2′-bipyridine and EDTA but inhibited by elastatinal and elastase inhibitor II and drastically ceased by serine inhibitors, PMSF, TLCK, aprotinin and SBTI.

### 3.8 *In vitro cell damaging activity*

As shown in table 4, ZuhP13 at dose 15 µg/mL demonstrated 79.2% cell death of HT29 cell line and triggered 1.71-fold increase in hemolysis of washed RBCs. Furthermore, *in vitro* studies showed, detrimental tenderizing effects towards mice organs especially lung and liver (supplementary figure 4). This was judged by amount of released hemoglobin from tissues (table 4).

### 3.9 *In vivo study*

In this experiment, intraperitoneal injection route was employed at concentration of 100 U enzyme/ml. Active and heat-inactivated ZuhP13 were used to assess relationship between enzymatic activity and toxicity. Deaths noticed for

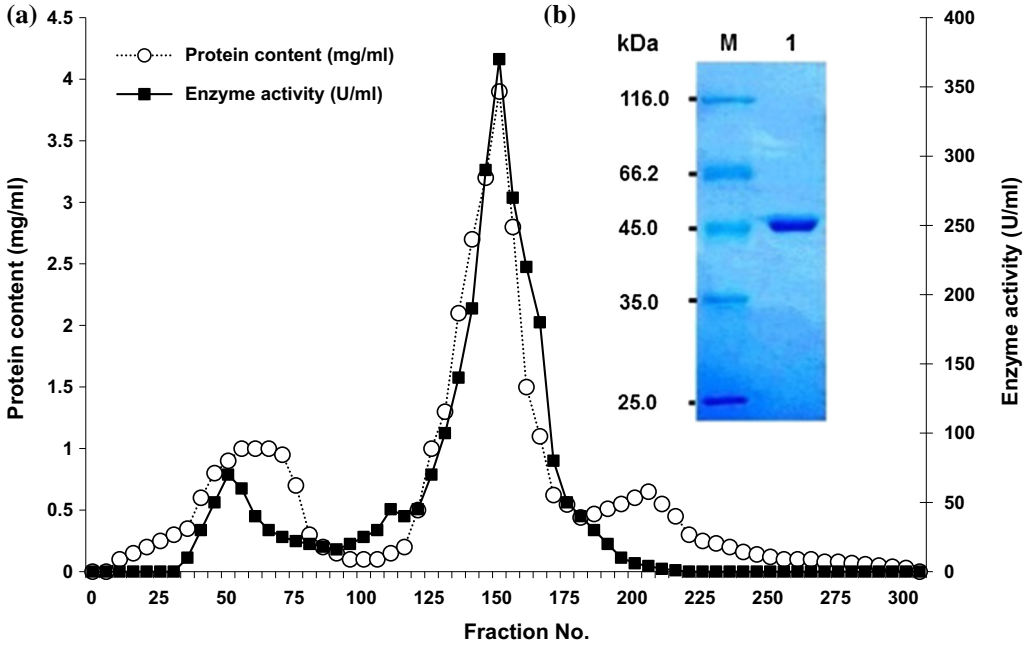


**Figure 2.** Elution pattern of protease through DEAE-Sepharose CL-6B (panel **a**) and SDS-PAGE (panel b).

most mice within 2 h of initial injection, while no lethality was observed with heat-inactivated preparation. Dying hepatocytes showed programmed necrosis with extensive intracellular vesiculation. Heterochromosomes were accumulated intensively yielding marginal hyperchromatism (figure 5b) in contrary with blank cells (figure 5a) where, chromatin is uncondensed giving mottled appearance.

## 4. Discussion

In a previous study concerning protease KB76, it became obvious that, it is a pathologic factor involved in many infections of the rare bacterial pathogen, *Brevibacterium otitidis* (Kotb *et al.* 2013). In this study, we tested the virulence of proteases from one of the most widespread bacterial pathogens, *P. aeruginosa.*

Growth and protease productivity (109 U/mL) by the most potent strain, ZuhP13 was synchronous at maximal levels at 48 h of fermentation. Thus, ZuhP13 enzyme seems essential for bacterial growth unlike protease produced by a fish pathogen, *Yersinia ruckeri* that showed maximal levels at 12 h (Secades and Guijarro 1999) and the antimicrobial protease from *Pseudomonas* sp. CL 1457 at 18 h of fermentation (Shastry and Prasad 2002). Values of 0.5% fructose and 1% casein as C/N-source enhanced maximal ZuhP13 production. In general, organic nitrogens were better than inorganic types for both *Pseudomonas* and *Bacillus* species for both growth and enzyme productivity (Puri *et al.* 2002). Interestingly, ZuhP13 production was under ammonium control where, addition of ammonium salts ceased enzyme productivity, due to failure of *P. aeruginosa* ZuhP13 to consume ammonia. This outcome matches protease productivity by *Yersinia ruckeri* (Secades and Guijarro 1999).

Size of purified ZuhP13 as estimated by SDS-PAGE to be equivalent to 48 kDa, with band profile similarity to *Yersinia ruckeri* virulent protease (47 kDa, Secades and Guijarro 1999). In general, proteases of *P. aeruginosa* are between 20 and 50 kDa (Karadžić *et al.* 2004). This variation may due to presence of bounded processing protein from which mature enzyme formed (Karadžić *et al.* 2004). Where, value of 36 kDa was reported by Yadav *et al.* (2010) for a strain of *P. aeruginosa*, 35 kDa by Patil and Chaudhari (2011) for alkalophilic *P. aeruginosa* MTCC 7926 and 30 kDa by Palpperumal *et al.* (2016) for another strain of *P. aeruginosa*. On the other hand, few reported proteases of higher molecular weights like that of *P. aeruginosa* MNI (65 kDa, Bayoudh *et al.* 2000).

Best reaction pH for ZuhP13 was found at 7.5, which is closely similar to the pathological proteases of *Br. otitidis* KB76 (Kotb et al. 2013) and *P. aeruginosa* ME4 (Cheng *et al.* 2009) but lower than those of the fish pathogen *Yersinia ruckeri* (pH 8.0, Secades and Guijarro 1999), *B. cereus* BG1 (pH 8.0, Ghorbel-Frikha *et al.* 2005) and aeruginolysin protease-type (pH 9.0, Karadžić *et al.* 2004). The drastic decline in ZuhP13 activity at low pH values may be explained on basis of its lower isoelectric point (p*I*, 5.6) where, enzyme precipitation occurs. Similar p*I* was reported for KB76 of *Br. otitidis* (Kotb et al. 2013). pH stability of ZuhP13 was between 6 and 9 for 1 hr which is related to *P. aeruginosa* ME4 (Cheng *et al.* 2009) and *Ps aeruginosa* san-ai strain (Karadžić *et al.* 2004). While, proteases from

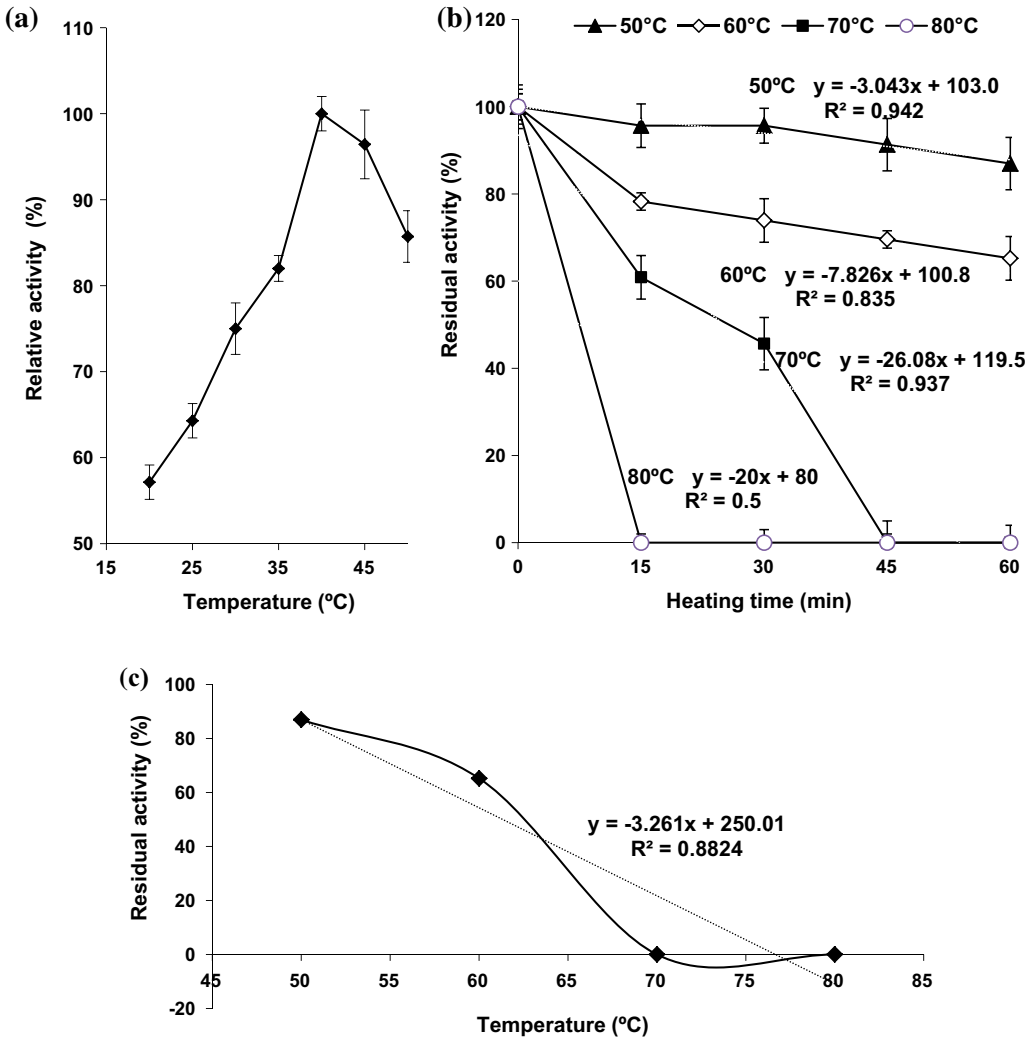


**Figure 3.** Relation of reaction temperatures to protease activity (**a**) and stability (**b**). (**c**) Calculated $T_m$.

*Aeromonas veronii* PG01 (Divakar *et al.* 2010) showed stability over a wide basic pH range (8 to 11).

Best temperature for purified-ZuhP13 was found at 40°C, as exhibited by virulent proteases of *Br. otitidis* KB76 (Kotb et al. 2013) and *Yersinia ruckeri* (37°C, Secades and Guijarro 1999). On the other hand, 50°C was optimal for protease from *P. aeruginosa* ME4 (Cheng *et al.* 2009) and 60°C was optimal for proteases of various strains of *P. aeruginosa* (Bayoudh *et al.* 2000; Karadžić *et al.* 2004) and *B. cereus* BG1 (Ghorbel *et al.* 2003). Our results indicated that, purified ZuhP13 was thermally stable for 30 min up to 60°C (figure 3b) with resemblance with aeruginolysin, while it was more heat sensitive when compared with san-ai protease (90 min at 60°C) (Karadžić *et al.* 2004). While, it was more stable than protease of *Yersinia ruckeri* (100% inhibition at 55°C, Secades and Guijarro 1999) and Br. otitidis KB76 (50% inhibition at 60°C for 2 hr, Kotb et al. 2013).

Concluded data from LB plot for azocasein revealed that, protease from *P. aeruginosa* ZuhP13 with catalytic efficiency of $4.62\times10^7$ $M^{-1}$ $s^{-1}$, $K_m$ of 1.32 mg/mL and $K_{cat}$ of 1.27 $s^{-1}$. In view of other investigators, catalytic efficiency of ZapA from *Proteus mirabilis* N17-12 against Phe-Ser was 291 mM/s, $K_m$ was equivalent to 13.6 µM and $K_{cat}$ was equivalent to 3.96 $s^{-1}$ while, its catalytic efficiency against Phe-Leu was 13 mM/s, $K_m$ was 2.3 µM and $K_{cat}$ was 0.031 $s^{-1}$ (Aneas *et al.* 2001).

Surprisingly, on confliction to former *P. aeruginosa* proteases including elastase (Fukuchi and Nishikawa 2001), san-ai (Karadžić *et al.* 2004) and ME4 protease (Cheng *et al.* 2009) $Zn^{2+}$ ions not affected protease activity of ZuhP13. In addition, *o*-phenanthroline, 2,2′- bipyridine and the metal chelating agent, EDTA, showed slight inhibition. While, elastase inhibitors (elastatinal and elastase inhibitor II) and serine protease inhibitors (TLCK, PMSF, SBTI, and aprotinin) markedly decreased enzymatic activity. Taken together, we suggest that, ZuhP13 is a serine protease-type like espP virulence protease of *E. coli* (Khan *et al.* 2009). Virulent serine-proteases are less common than virulent

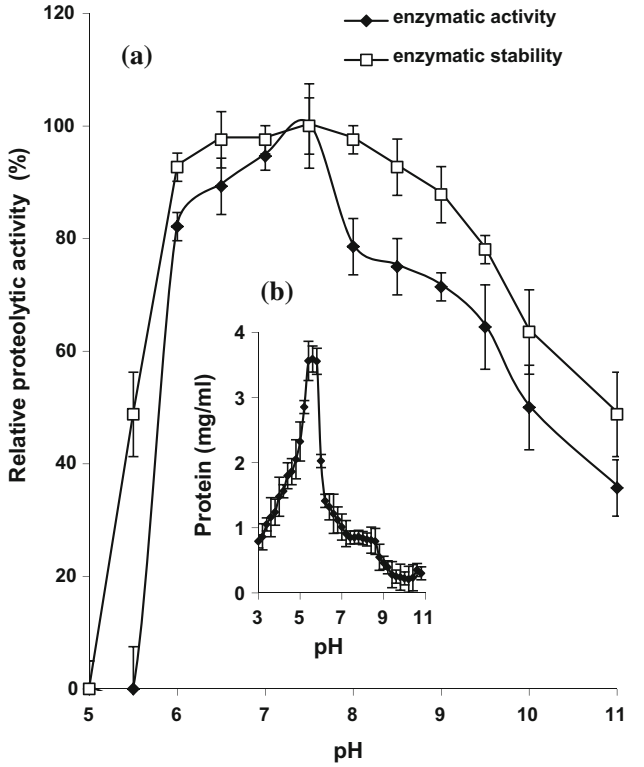


**Figure 4.** Relation of pH values to protease activity and stability (panel **a**). Isoelectric point based on pH precipitation profile is shown in panel **b**.

**Table 3.** Effect of metallic ions and protease inhibitors upon the enzymatic activity of ZuhP13

| Chemical | Concentration (mM) | Enzyme activity (U/mL) |
|---|---|---|
| Blank | – | 484 ± 32 |
| $HgSO_4$ | 5 | 419 ± 25 |
| $CuSO_4$ | 5 | 426 ± 18 |
| $CoCl_2$ | 5 | 431 ± 23 |
| $CaCl_2$ | 5 | 441 ± 15 |
| $ZnSO_4$ | 5 | 483 ± 22 |
| $MnSO_4$ | 5 | 438 ± 27 |
| $MgSO_4$ | 5 | 449 ± 28 |
| $BaCl_2$ | 5 | 452 ± 31 |
| $CdCl_2$ | 5 | 462 ± 12 |
| $FeCl_3$ | 5 | 431 ± 38 |
| EDTA | 5 | 481 ± 17 |
| 2,2′-Bipyridine | 0.1 | 489 ± 21 |
| TLCK | 0.1 | 56 ± 4 |
| Aprotinin | 0.1 | 72 ± 6 |
| *o*-Phenanthroline | 0.1 | 459 ± 32 |
| PMSF | 10 | 2 ± 1 |
| SBTI | 0.1 | 21 ± 3 |
| Elastatinal | 200 | 67 ± 5 |
| Elastase inhibitor II | 200 | 92 ± 5 |

Blank, enzyme incubated with borate buffer; PMSF, phenyl methyl sulfonyl fluoride; EDTA, ethylene diamine tetraacetic acid; SBTI, soybean trypsin inhibitor; TLCK, tosyl lysine chloromethyl ketone

**Table 4.** *In vitro* damaging activity of ZuhP13

| *In vitro* damage | Treated | Control |
|---|---|---|
| Cytotoxicity | 79.2 ± 4.1 | 1.9 ± 0.2 |
| Hemolysis | 1.247 ± 0.3 | 0.729 ± 0.1 |
| *In vitro* tissue damaging activity ($A_{520}$) | | |
| Liver | 0.456 ± 0.03 | 0.104 ± 0.01 |
| Kidney | 0.292 ± 0.02 | 0.077 ± 0.04 |
| Lung | 0.381 ± 0.05 | 0.102 ± 0.03 |
| Pancreas | 0.268 ± 0.06 | 0.065 ± 0.01 |

metalloproteases found in *Proteus mirabilis* N17-12 (Aneas *et al.* 2001), *Yersinia ruckeri* fish pathogen (Secades and Guijarro 1999) and almost all *P. aeruginosa* proteases (Karadžić *et al.* 2004; Cheng *et al.* 2009). The presence of sulfhydryl groups in primary structure of serine protease ZuhP13 lead to formation of disulfide bonds that explain the thermal stability of tested enzyme (Karadžić *et al.* 2004).

Hemolytic activity and significant cytotoxicity of ZuhP13 towards HT29 mammalian cells may be attributed to proteolysis of cell's membrane proteins. Furthermore, *in vitro* detrimental macerating effects on mice organs especially liver and lungs may be attributed to its elastase activity upon elastin fibers in tissues and blood capillaries thus, releasing hemoglobin. This was confirmed during the next *in vivo* experiment, while dissecting mice. Where, a hemorrhage was noticed inside abdominal cavity. These hemorrhagic and hemolytic activities agree with protease A of *V. parahemolyticus* no. 93 (Lee *et al.* 2002) and KB76 protease of *Br. otitidis* (Kotb *et al.* 2013).

TEM study revealed that, i.p injection of ZuhP13 in mice caused cellular and nuclear lysis by necrosis rather than apoptosis because there is no damage to mitochondrial structures (Kotb *et al.* 2013). Furthermore, heterochromosomes were accumulated intensively especially at inner lining of nuclear membrane, giving hyperchromatism appearance. Finally, cells became leaky and malformed. In this regard, proteases from *Vibrio vulnificus* and *Pseudoalteromonas* sp. N10 showed extensive wound necrosis and triggered damage of muscle proteins, respectively (Ridgway *et al.* 2008). Furthermore, *Yersinia ruckeri* triggered massive cell damage of host fish (Secades and Guijarro 1999). While, streptococcal proteases triggered cellular apoptosis and were involved in necrotizing fasciitis (Kagawa *et al.* 2000).

As a conclusion, there are many instances of how proteolytic enzymes support microbial infections, however, the exact machinery is infrequently known. In this study, we have isolated a 48-kDa serine-elastase from *P. aeruginosa* as a putative virulence factor with cytotoxicity. It is a potential candidate for the membrane degradation, penetration and digestion of internal tissues. Use of chimeric proteins of such

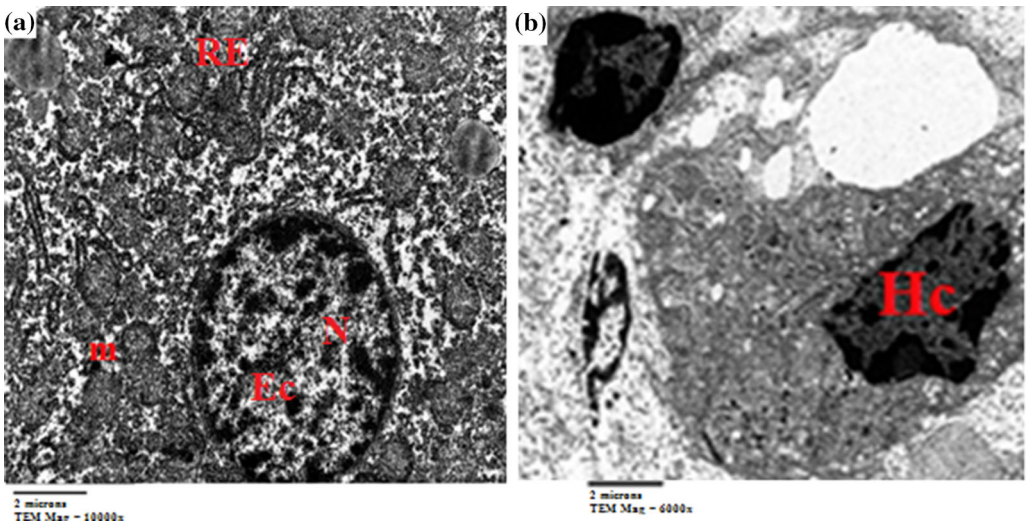


**Figure 5.** TEM micrographs of mice hepatocytes. (**a**) Normal hepatocyte with euchromatin (Ec) inside nucleus (N), rough endoplasmic reticulum (RE) and mitochondria (m). (**b**) Active protease-treated hepatocyte with heterochromatin (Hc) and vesiculation.

protease to target undesirable cells should be an aim in future research.

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