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Extraction and Purification of Anticancer Enzyme: Protease from *Pseudomonas aeruginosa*

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Abstract : This study investigated the anticancer and wound-healing potential of protease enzymes derived from Pseudomonas aeruginosa against cancer and normal cell lines. Out of 110 bacterial isolates obtained from clinical samples like urine, burns, and wounds, 67 were identified as Pseudomonas aeruginosa using biochemical tests and the VITEK-2 Compact system. Qualitative and quantitative screening revealed that 56 isolates produced protease. Notably, clinical isolate 9A from burn and wound samples demonstrated high protease activity, with specific activities of 280U/mg, indicating potential cytotoxic and wound healing properties. Optimal conditions for protease production were found to be at 37°C and pH 8 in Trypticase soy broth. The extracted enzymes underwent purification via gel filtration using Sephadex G-150 chromatography, resulting in significant purification folds for the protease. Cytotoxicity assays using MTT revealed that the protease showed increased cell viability, indicating possible growth-promoting effects. However, excessive protease activity led to cytotoxicity in normal cell lines, highlighting the need for controlled enzyme activity. Moreover, in vitro scratch tests demonstrated that protease showed a further pronounced decrease in wound area, revealing its potential role in processes of wound-healing.

Keywords: Pseudomonas aeruginosa, protease, purification anticancer enzymes, Wound healing

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Introduction

Pseudomonas aeruginosa, a ubiquitous Gram-negative bacterium, exhibits remarkable adaptability, thriving in diverse environments ranging from soil and water to various living organisms (1)(2). *Pseudomonas aeruginosa* has become an increasing threat to immunocompromised patients and, in most circumstances, within health care settings. Despite being perceived as harmless, it is a formidable opportunistic pathogen because it has sophisticated virulent factors, including enzymes, and shows intrinsic resistance mechanisms, which increases the challenges of treating mesothelioma effectively (3).

Considering the rapidly escalating rate of cancer cases worldwide, it comes as no surprise that the detection and treatment of the disease have become a critical priority for researchers and scientists worldwide (4)(5). Microbial-derived enzymes are prime contenders for the development of new treatment methods due to their target specificity (6)(7). Among these, *Pseudomonas aeruginosa* enzymes, such as proteases, exhibit specific breast cancer cell targeting.

Various protease types are an important set of enzymes that facilitate distinct processes in human diseases, including cancer cell metastasis and tumor progression (8). They destroy the structural portion of cells, which inhibits cancerous cell activity. They also interfere with cell signaling pathways and attempt to control or minimize cell migration and growth (9)(10)(11)(12).

There has been an upsurge in interest in using enzymes as potential anticancer agents lately. However, to use them as therapeutic agents, effective extraction and purification methods are required. Several techniques, including chromatography, ultrafiltration, or precipitation, have been designed to recover and purify enzymes from microbial sources while maintaining their structural stability, biological activity, and specificity (13(14). Besides their anticancer activity, the *Pseudomonas aeruginosa* enzymes, proteases, for example, also help in the healing of wounds. This enzyme degrades extracellular matrix components that impede cell migration, and thus, these enzymes can help regulate the host’s immune response, aid tissue remodeling, and enable wounds to heal more rapidly (15)(16).

This study leverages a new potential treatment for cancer and wound healing by utilizing *P. aeruginosa* enzymes for therapeutic intervention. This opportunistic pathogen possesses a variety of virulence factors, including protease, that play important roles in pathogenicity, making treatment a challenging problem. From 2000 to 2019, 72,022 cases of breast cancer were diagnosed among women in Iraq. The age-standardized incidence rate (ASIR) was 37.883 per 100,000 women. A significant increase in breast cancer cases was observed during this period, with an average annual percentage increase of 3.192%. This upward trend was consistent across various age groups, with notable increases in women aged 40-49, 50-59, 60-69, and 70 and older. Compared to global rates, Iraq's breast cancer ASIR falls within a moderate range (17(18)). Breast cancer is a major global health concern, with a significant impact on women's health and mortality. Current treatment options, such as chemotherapy and radiotherapy, often have severe side effects and can lead to the development of drug resistance. Therefore, there is an urgent need to develop novel and targeted therapies with improved efficacy and reduced toxicity. P. aeruginosa harbors several proteases with great anti-tumor potential in breast cancer. These include inhibition of tumor cell proliferation and migration via disruption of growth-promoting signaling pathways.

In addition, several proteases could trigger cancer cell apoptosis and thus promote their destruction.

Further research is beyond their anticancer activity. P. aeruginosa protease possesses wound-healing activity. The enzyme could adjust the host immune response, encourage tissue remodeling, and enable wound closure by destroying extracellular matrix components and promoting cell migration (15).

This study aims to investigate the therapeutic potential of P. aeruginosa-derived proteases in cancer treatment and wound healing.

Materials and Methods

Collection of samples

Samples (110) were collected between October 2022 and January 2023. Samples were collected from clinical sources (urine, wounds, and burns) from different hospitals (AL Yarmouk Teaching Hospital, AL Karma

Hospital, Teaching Hospital of Baghdad Medical City, and their laboratories). All samples were collected from females, and males' ages were between (5-50). The samples were gathered using transport media and then placed in a cool ice box before being taken to the laboratory. The samples were cultured for 24 hr. at 37°C in the nutrient broth.

Bacterial identification

All isolates were classified based on their form and biochemical reaction promoted by (19). The bacteria have been examined after their morphological properties defined by Cetrimide agar rising (Selective media for P. aeruginosa) improving Pseudomonas development such pigments as pyocyanin and pyoverdine. (19).

Qualitative screening for protease production

For qualitative screening, *pseudomonas aeruginosa* was examined for protease production. *Pseudomonas aeruginosa* isolates were streaked on skim milk agar as suggested by (20), finding whether bacteria might generate the alkaline protease enzyme. It was carried out by centering aseptically loopful of culture in the middle of the plate and then circularly distributed to cover an area about (5 to 18mm in diameter) then incubate the plate inverted at 37°C for 24 to 48 hours. Clearing the zone of proteolysis, the cloudiness of the agar, shows success.

Quantitative screening of local protease-producing isolates

Growth conditions for enzymes

The growth of *pseudomonas* aeruginosa was estimated as OD. per unit time. 1 mL of culture was taken to prepare an appropriate dilution with normal saline using a reader at 620nm, and the inoculum was prepared with an initial absorbance 0.5. Then, after 50 mL of the culture was prepared in 250 mL flask and inoculated with a prepared inoculum. The cultures grew at 37°C for 24 hr. in a shaker incubator at 150 rpm. (21).

Enzymes production

The efficiency of the selected isolates in producing protease was tested; these isolates were grown

in soya broth as detailed by (22). This medium weighed 46.7 g, melted in two 1000 mL Erlenmeyer flasks. After autoclaving, sterilized. Each fresh (24hr) isolated fresh (1 ml) was seeded into each flask, then cultured in a shaker incubator (150 rpm. 37°C for 24 hr). Using casein as a substrate working solution at pH 8, the synthesis of alkaline protease was ascertained; thus, the protease activity and protein concentration for the isolates will be discussed.

Determination of Protease Enzyme Activity

The activity of alkaline protease enzymes was determined using a method initially described by (23) and later modified by (24). In this procedure, 0.8 ml of 0.5% casein solution (pH 8) was preincubated at 37°C for 10 minutes in a water bath. Subsequently, 0.2 ml of crude enzyme was added to the substrate solution and incubated for 20 minutes. The reaction was terminated by adding 3 ml of 5% TCA, with a blank prepared identically except TCA was added before the enzyme. The solutions were then centrifuged at 5000 rpm for 15 minutes using a cooling centrifuge. The optical absorbance of the supernatants was measured at 280 nm using a UV spectrophotometer, and enzymatic activity was calculated based on protein hydrolysis into peptides and amino acids. Enzymatic activity (units/ml) was determined using the formula: (Absorbance at 280 nm) / (0.01 × 20 ×

0.2), where 0.01 represents the enzyme unit definition, 20 is the reaction time in minutes, and 0.2 is the volume of enzyme added. The unit of enzyme activity was defined as the amount of enzyme required to increase absorbance by 0.01 per minute under standard conditions. Specific activity (U/mg) was calculated by Activity(U/ml)/ Protein conc. (mg/ml).

Optimization of Protease Enzyme Production Conditions

To determine the optimal production conditions for protease, Pseudomonas aeruginosa isolate 9A were selected out of 15 isolates. 9A was cultivated in trypticase soy broth medium (25) for protease production. The media studied under various pH, temperature, and ventilation to find the most favorable parameters for successful production of the enzyme.

Effect of pH

To determine the optimum pH, production medium for protease was cultured. The media consisted of pH 6, 7, and 8. Flasks containing the local isolate were inoculated in the media and incubated in the same conditions. Following incubation, enzyme activity and protein concentration were measured to evaluate the effect of pH on production.

Effect of temperature

To determine the optimal temperature for protease production from the local isolate, inoculated flasks containing the production medium (optimized in prior experiments) were incubated at different temperatures (35, 37, and 40°C) for 18 hours at 150 rpm. After incubation, enzyme activity and protein concentration were measured to assess the impact of temperature on production efficiency.

Extraction and Concentration of Crude Protease Enzymes Using Sucrose

Protease was extracted from the selected local isolate after cultivation under optimal production conditions. Cells were separated by centrifugation at 6000 rpm for 20 minutes, and the filtrate was prepared as a crude extract for purification. For enzyme concentration, 500 ml of the production medium was used, and extracellular enzymes were extracted by centrifugation at 8000 rpm for 15 minutes. The supernatants for protease were concentrated using sucrose in dialysis bags, reducing volumes to 36 ml. Enzyme activity and protein concentration were measured according to (26) and the concentrated enzyme was used as crude extracts for further purification.

Enzyme purification by Sephadex G-150

Sephadex G-150 was prepared by suspending 20g in 500ml of distilled water at 90°C for 3 hours with gentle agitation to allow gel bead swelling, followed by overnight storage at 4°C. The resin was washed twice with 0.2M potassium hydrogen phosphate buffer (pH 8.0), resuspended in the same buffer, degassed using a vacuum pump, and packed into a glass column (22.6 × 1.7 cm). The column was equilibrated with the buffer before use. For enzyme separation, concentrated protease (5ml) was separately purified by passing through the Sephadex G-150 column. Elution was performed using 0.2M potassium hydrogen phosphate buffer at a flow rate of 20 mL/hr., collecting 3ml fractions. Absorbance at 280 nm was measured for each fraction until it reached zero. Fractions with protease activity were pooled, and enzyme activity and protein concentration were measured. Post-purification, the resin was stored at 4°C in 0.2M potassium hydrogen phosphate buffer with 0.1% sodium azide.

Protein concentration measurement

Protein concentration was determined using the Bradford method.

The cytotoxic effect of purified enzymes

This in vitro method was used to analyze the cytotoxic effect of protease. The purified protease was prepared in different concentrations, and its cytotoxic effect was tested on tumor (MCF7) and normal cell lines (HFF).

Maintenance of cell lines

The maintenance of cell lines (27) involved conducting experiments when cells formed confluent monolayers in a vessel. The growth medium (complete RPMI medium with 10% FBS) was aspirated, and the cells were washed with PBS. The monolayer was treated with 2-3 ml of trypsin/versine solution, gently rocked to detach the cells, and incubated at 37°C for 1-2 minutes until the cells detached. Fresh complete RPMI medium (15-20 mL) was added, and the cells were dispersed by pipetting. The cells were then redistributed into culture vessels (plates or flasks) at the required concentration and incubated at 37°C in a 5% CO2 incubator. Cell concentration was determined using a hemocytometer, and live cell concentration (cells/mL) was calculated using the formula: (viable cells / number of squares) × dilution factor × 10^4.

Cytotoxic effect of purified enzymes

The cytotoxic effect of purified enzymes from *P. aeruginosa* was evaluated using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) with a ready-to-use kit containing MTT solution and solubilization solution. MCF7 and HFF cells ($1x10^4$–$1x10^6$ cells/mL) were seeded in 96-well plates with 200µL of culture medium per well, covered with parafilm, and incubated at 37°C with 5% CO_2 for 24 hours. After incubation, the medium was replaced with serial dilutions (2.5, 5, 7.5, 10 mg/mL) of the enzymes, with triplicates for each concentration and controls (serum-free medium). Plates were incubated for another 24 hours, followed by the addition of 10µL MTT solution to each well and further incubation for 4 hours. The medium was then removed, and 100µL solubilization solution was added to each well for 5 minutes. Absorbance was measured at 575 nm using an ELISA plate reader, and optical density data were analyzed to determine the concentration causing 50% reduction in cell viability (IC_{50}) for each cell line.

Wound healing (Scratch Assay)

The wound healing (scratch assay) was performed following the method described by (28). HFF cells were seeded in 24-well plates at a density of 2 x 10^5 cells/mL and incubated for 24 hours at 37°C with 5% CO_2 until a confluent monolayer formed. A scratch was created in the monolayer using a sterile pipette tip, and cellular debris was removed by washing with fresh RPMI medium (without FBS). The cells were then treated with 5 mg/mL of protease, while untreated cells served as the control. Bright-field microscopy (Axio Observer 5, Zeiss, Germany) was used to capture images at 0, 12, 24, and 48 hr. post-treatment. Untreated scratched cells were used as the negative control, and all treatments and controls were maintained under the same conditions.

Results and Discussion

Sample Collection, Isolation, and Identification of *Pseudomonas aeruginosa*

From October 2022 to January 2023, 110 clinical samples (urine, wounds, burns) were collected from Iraqi health institutions. Eighty-seven samples showed characteristics of *Pseudomonas* spp., with 67 identified as *P. aeruginosa* and 20 as other *Pseudomonas* species. Cetrimide agar, along with biochemical tests, was used for *P. aeruginosa* identification. *P. aeruginosa* cultural characteristics included yellow-green colonies on Cetrimide agar, colorless colonies on MacConkey agar, and large, fluorescent colonies on Nutrient agar. Simmons Citrate agar showed a green to blue color change.

Morphological Identification

The assessment of colonial shape revealed colorless colonies (1-3 mm diameter) on MacConkey Agar, indicating non-lactose fermentation in Gram-negative species (29). P. aeruginosa displayed mucoid, colorless characteristics on Nutrient Agar, forming smooth, elevated colonies. Positive identification of P. aeruginosa was confirmed by green pigmentation on Cetrimide agar, indicating pyocyanin production. The results from culturing P. aeruginosa on Nutrient Agar, MacConkey Agar, and Cetrimide agar.

Biochemical Test

Bacterial isolates were analyzed through biochemical tests after morphological and microscopic identification, as detailed in Table 1. The tests included the oxidase test, catalase test, indole test, urease test, and Simmons citrate test to determine specific characteristics of the isolates (30)(31)(32). Notably, these tests helped differentiate Pseudomonas aeruginosa from other Pseudomonas species based on enzymatic activities and growth patterns, as outlined in the results presented in Table 2.

Table (1): Biochemical test results are used to identification *p. aeruginosa*.

Test	Result
Oxidase test	+
Catalase test	+
Indole test	_
Simmons citrate test	+
Grow at 42 ˚C and 4 ˚C	+
Grow on Cetrimide agar	+
Urease	_

Qualitative Screening for Protease Enzymes in *Pseudomonas aeruginosa*

A total of 67 *P. aeruginosa* isolates were screened to produce protease enzymes. 56 out of the 67 isolates (83.6%) tested positive, whereas 11 isolates (16.4%) were negative when cultured on skim milk agar (Figure 1.).

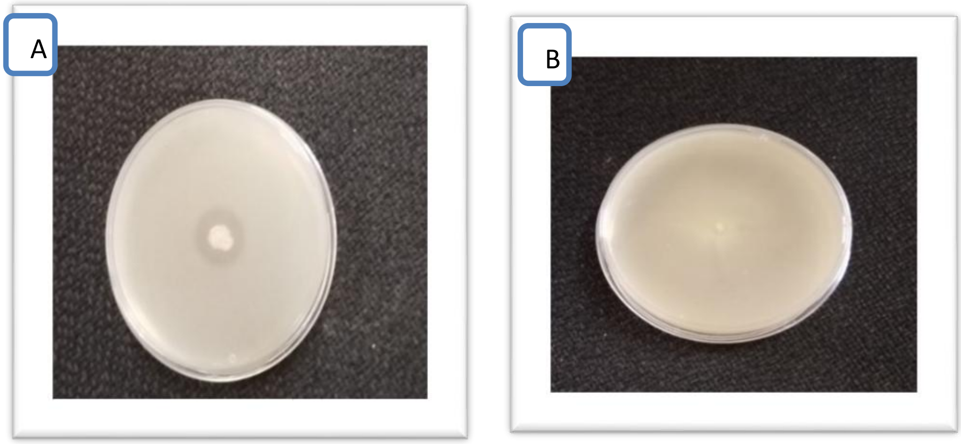


Figure 1: Enzyme Production by *Pseudomonas aeruginosa* (9A) on Skim Milk Agar after 24 Hours at 37°C: A- clear zone showed positive protease enzyme. B- without clear zones showed the negative producing protease enzyme.

Quantitative Screening for Protease Enzymes

Fifteen *P. aeruginosa* isolates, pre-selected based on clear zone formation in qualitative screening, were further assessed for protease production, 15 isolates with clear zones of 1-3 cm on skim milk agar were grown in Trypticase soy broth. Isolate 9A (from a bacteremia patient) showed the highest specific activity (280 units/mg protein) compared to 200-275 units/mg protein for other isolates. Isolate 9A was selected for further study. These variations in enzyme production may be attributed to genetic differences, isolation source, infection type, and environmental factors like media composition, temperature, pH, aeration, and other growth conditions (22)(33)(34).

Optimization of Protease Production

Optimal conditions for protease production by *P. aeruginosa* isolates 9A were investigated. This included determining the optimal pH and temperature for enzyme production.

Effect of pH on Enzyme Production

Protease production by *P. aeruginosa* 9A was highest at pH 8, with a specific activity of 281 U/mg (Figure 2.). This result agrees with studies on protease production in other bacteria like *Serratia marcescens* (35)(36), although other studies have reported a pH of 7.2 for this species. pH influences enzyme production by affecting nutrient solubility, transport, and ionization, ultimately impacting microbial growth and enzyme synthesis (37).

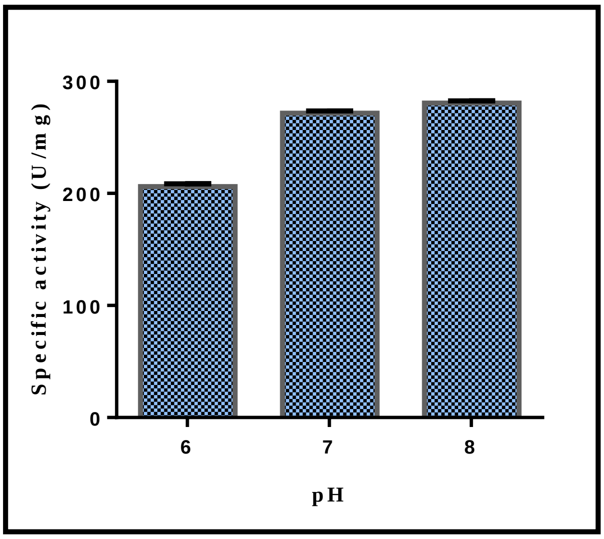


Figure (2): Effect of pH on Protease Production by *P. aeruginosa* 9A after 24 Hours at 37°C.

Effect of Temperature on Enzyme Production

Protease production by *P. aeruginosa* 9A was also optimal at 37°C (282 U/mg), decreasing at other temperatures (Figure 3.). This aligns with some studies on *Serratia marcescens* (38), though other optimal temperatures (28°C, 30°C) have been reported (39). Similar variations are seen in *Enterobacter* spp. (40). Temperature significantly affects enzyme production; excessive heat can cause cell death, halt production, alter enzyme structure, and inhibit function (41).

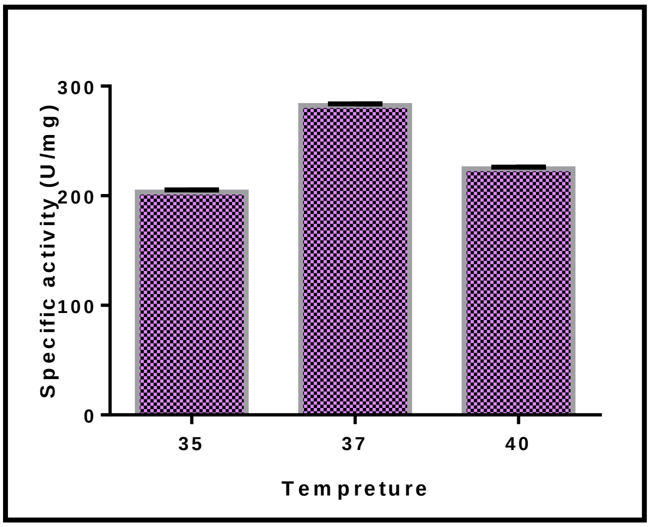


Figure (3): The effect of Temperature on protease production from *P. aeruginosa* 9A for 24hr.

Purification of Protease: Sucrose Concentration and Gel Filtration Chromatography

Protease from *P. aeruginosa* 9A was purified using sucrose concentration and gel filtration chromatography (Sephadex G-150). Sucrose concentration was used due to enzyme deactivation by ammonium sulfate precipitation. Gel filtration (Sephadex G-150) was then employed, separating proteins based on size (5,000-300,000 Da) (42).

Protease was eluted using 0.2 M Tris-HCl buffer. Three protein and three protease activity peaks were observed (Figure 4.). Peak 1 had a purification time of 3.9 and a recovery of 69%, peak 2 had 0.788 and 39.9%, respectively, and peak 3 had 2.65 and 21.27%, respectively (Table 2.). This contrasts with (43), who observed one major and three minor protein peaks, with only one enzyme activity peak, during protease purification from *E. coli*. The degree of enzyme purification depends on its intended use (44).

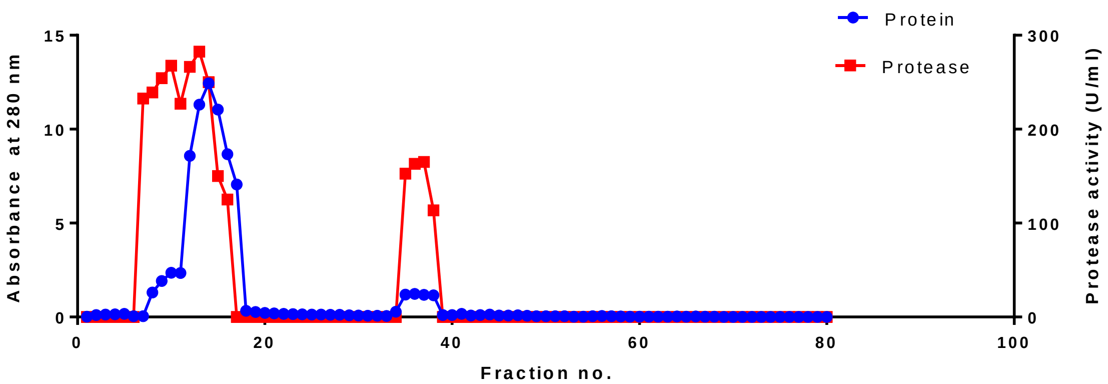


Figure (4): Chromatography gel filtration for partial purification of Protease produced from *P. aeruginosa* 9A through used (Sephadex G-150) column (22.6 × 1.7cm) at a flow rate of 20 ml/hr.

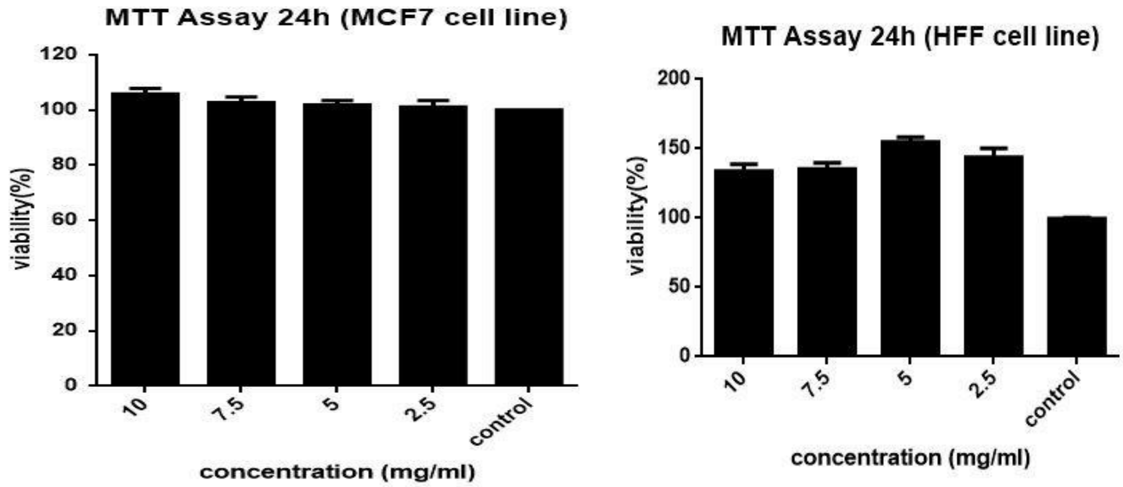
Table (2) : Partial purification of Protease produced from *P. aeruginosa* 9A.

Purification steps	Volume (ml)	Enzyme activity (U/ml)	Total activity	Protein conc. (mg/ml)	Total protein	Specific activity (U/mg)	Yield %	Purification fold
sucrose conc.	10	880	8800	2.17	21.7	405.5	100	1
Gel filtration								
first peak	15	405	6075	0.254	3.81	1594	69.03	3.932
second peak	15	234	3510	0.657	9.855	356.2	39.89	0.878
third peak	12	156	1872	0.145	1.74	1076	21.27	2.653

Cytotoxic Effects of partially Purified Protease on Tumor and Normal Cell Lines

The MTT assay was used to assess the cytotoxic activity of purified protease on MCF-7 tumor cells and HFF normal cells. Cell viability was measured after 24 hours of incubation with varying concentrations (2.5, 5, 7.5, and 10 mg/mL) of each enzyme.

Protease, at concentrations of 2.5, 5, 7.5, and 10 mg/mL, was tested for its effect on MCF-7 and HFF cell viability over 24 hours (Figures 5.A and 5.B). At 10 mg/mL, cell viability was 106% for MCF-7 cells and 137% for HFF cells, suggesting increased viability in both cell lines. While proteases break down proteins and excessive activity can be detrimental, the observed increase in viability suggests a different mechanism of action in these cell lines. However, it's important to note that even though viability has increased, proteases can still target essential cellular proteins (45).



Figure(5): Cytotoxicity of protease on: A- MCF-7 cell line and B- HFF cell line after 24hr treatment

Wound Healing (Scratch Assay) and Enzymatic Activity:

The management of burns and complex wounds often requires agents with antimicrobial and regenerative properties. The in *vitro* scratch assay serves as a valuable tool to evaluate the efficacy of therapeutic compounds, such as nanoparticles or enzymes, in promoting wound repair by modeling cell migration and proliferation during healing (46). This assay determines whether a test substance accelerates or inhibits cellular migration and tissue closure.

Figure 6. depicts wound healing progression in human foreskin fibroblast (HFF) cells treated with protease, monitored at 0, 12, 24, and 48 hours. Each row compares untreated (control) and protease-treated samples at corresponding time points. At 12

hours, treated cells exhibit a modest reduction in wound area compared to the control, indicating early-stage closure. By 24 hours, protease-treated samples demonstrate a marked acceleration in wound closure, with significantly smaller wound areas relative to both the control and earlier time points. After 48 hours, treated cells show near-complete wound resolution, contrasting with the slower closure observed in untreated samples.

Proteases, enzymes that catalyze protein degradation, play a dual role in wound repair. While excessive proteolytic activity can damage healthy tissue, controlled protease function is indispensable for effective healing (47)(48). These proteases promote debridement by removing necrotic debris, break down components of the extracellular matrix (ECM) to enable cell migration, and activate growth factors necessary for tissue regeneration (49)(50). The noted acceleration of wound closure in protease-treated cells is consistent with these mechanisms, implicating effective proteolysis as beneficial for ECM remodeling and proliferation.

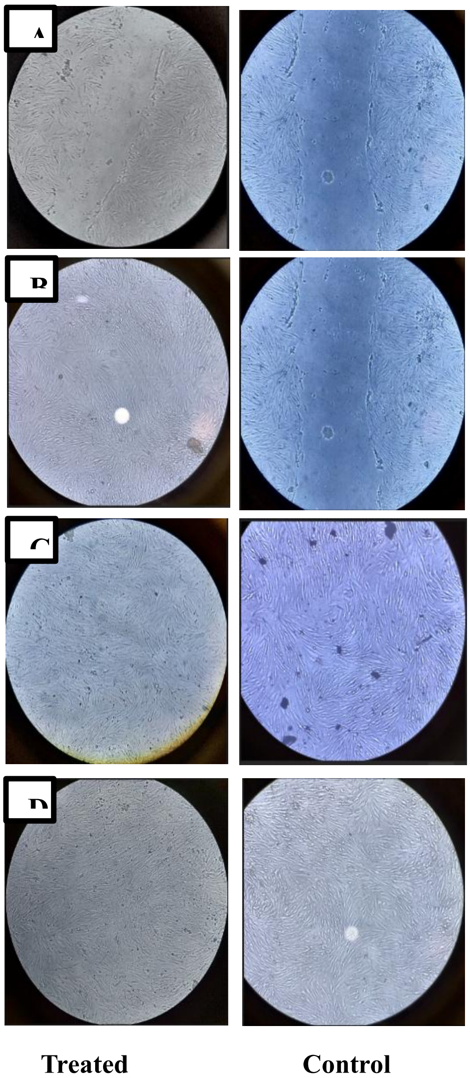


Figure (6): wound healing of normal cell line (HFF) using protease for 0,12,24,48 hrs. A- The control and treated for (0 hrs.) B- The control and treated for (12 hrs.) C- The control and treated for (24 hrs.) D- The control and treated for (48 hrs.)

Conclusion

This study sheds light on the potential wound healing and anticancer activity of protease enzymes from *Pseudomonas aeruginosa*. The study reveals that the enzyme, particularly from clinical isolates 9A, exhibits significant activity under optimal conditions, with protease enhancing wound healing but requiring strict regulation due to its cytotoxic effects. Characterization and purification of these enzymes are beneficial in their clinical use. Nevertheless, further research has to be undertaken in order to maximize their activity and utilize them for therapeutic purposes safely.

References

1. Mehrad, B.; Clark, N. M.; Zhanel, G. G. and Lynch, J. P. (2015). Antimicrobial resistance in Pseudomonas aeruginosa: Current understanding and future perspectives. Clinical Infectious Diseases, 60(Suppl 2), S76–S80.
2. AL-Shamaa, N. F.; Abu-Risha, R. A. and AL-Faham, M. A. (2016). Virulence genes profile of Pseudomonas aeruginosa local isolates from burns and wounds. Iraqi journal of biotechnology, 15(3).
3. Silby, M. W.; Winstanley, C.; Godfrey, S. A.; Levy, S. B. and Jackson, R. W. (2011). Pseudomonas aeruginosa: Environmental and genetic adaptation. Nature Reviews Microbiology, 9(7), 514-525.
4. Al-Ani, A. and Alsahlanee, R. (2024). Designing of human serum albumin nanoparticles for drug delivery: a potential use of anticancer treatment. Karbala Int. J. Mod. Sci, 10, 13.
5. Aseel, S. M.; S. Omar Sh, and S. S. Mohamed. (2019). Uranium concentration variation dependency on gender correlated with age of bladder cancer patients. International Journal of Research in Pharmaceutical Sciences. 10(3), pp. 1730–1734.
6. Stover, C. K.; Pham, X. Q.; Erwin, A. L.; Mizoguchi, S. D.; Warrener, P.; Hickey, M. J.*, et al.* (2007). Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature, 406(6799), 959-964.
7. Rasheed, H. A.; Alsahlanee, R. and Khalfa, H. M. (2022). Anticancer Mechanisms of Zoledronic Acid-Based Graphene Oxide Nanoparticles for Prostate Cancer Bone Metastases Treatment. Iraqi Journal of Science, 4241-4253.
8. Zahraa, H. H. and Rasheed, H. A. (2023). Cytotoxic Effect of Asparaginase Produce from E. coli and Conjugated with ZnO Nanoparticles on Cancer Cell Lines. Iraqi Journal of Agricultural Sciences, 54(6), 1664-1678.
9. Al-Azzawi, R. M.; Raheem, A. and Ghizar, R. (2012). Extraction and partial purification of Protease from local Serratia marcescens isolate. Iraqi Journal of biotechnology, 10(2), 261-272.
10. Li, H.; Qiu, Z.; Li, F. and Wang, C. (2019). The role of matrix metalloproteinases in tumor invasion and metastasis. International Journal of Molecular Sciences, 20(17), 4203.
11. Manaf, N. A.; Wahab, A. A.; Rasheed, H. A.; Aziz, M. N. C.; Salim, M. I. M.; Sahalan, M.*, et al*. (2023). Investigation of single beam ultrasound sensitivity as a monitoring tool for local hyperthermia treatment in breast cancer. Multimedia Tools and Applications, 82(4), 5011-5030.
12. Mahmood, A. S. and Issa, Y. W. (2024). Impacts of microRNA-155 on the Expression of Interleukin-1β and Tumor Suppressor Gene JADE-1 in Iraqi Women with Cervical Cancer. Al-Rafidain Journal of Medical Sciences (ISSN 2789-3219), 7(1 (Special)), S24-28.
13. Mohammed, S. A. (2013). Advances in enzyme purification and stabilization techniques. Biotechnology Advances, 31(8), 1543-1551.
14. Jawad, L. Q. and Rasheed, H. A. R. R. (2022). Isolation and Purification of anticancer protein Exotoxin A from Pseudomonas aeruginosa. Iraqi Journal of Agricultural Sciences, 53(1), 48-56.
15. Sarwar, S. (2018). Microbial enzymes in wound healing: Mechanisms and applications. Applied Microbiology and Biotechnology, 102(18), 7725-7733.
16. Shawkat, A. A. K. M. S. (2022). Primary and Secondary Screening of Pseudomonas aeruginosa for Protease Production. Iraqi journal of biotechnology, 21(2).
17. Al-Hashimi, M. M. (2021). Trends in breast cancer incidence in Iraq during the period 2000-2019. Asian Pacific journal of cancer prevention: APJCP, 22(12), 3889.

18. Hamd, A. H. and Al-Lami, N. (2023). Anti-breast cancer activity of some synthesized pyrazole derivatives bearing imidazo [1, 2a] pyridine moiety. Iraqi Journal of Science, 3205-3217.
19. Atlas, R. M.; Brown, A. E. and Parks, L. C.; 1995. Laboratory manual of experimental microbiology. Mosby.
20. Du, B.; Meng, L.; Liu, H.; Zheng, N.; Zhang, Y.; Zhao, S.*, et al*. (2022). Diversity and proteolytic activity of Pseudomonas species isolated from raw cow milk samples across China. Science of the Total Environment, 838, 156382.
21. DeBell, R. M. (1979). Production of exotoxin A by Pseudomonas aeruginosa in a chemically defined medium. Infection and Immunity, 24(1), 132-138.
22. Hussein,S.S.(1996).Production, Purification and characterization of alkaline protease from Aspergillusoryzae by solid material fermentation method, PHd.Thesis,College of Science,Baghdad university.(In Arabic).
23. Murachi, T. (1970). Bromelainenzymes. In: Methods in Enzymology (eds. Perlman, G.E. and Lorand, L.) Vol. 19: 273-284. Academic Press, New York.
24. Senior, B. W. (1999). Investigation of the types and characteristics of the proteolytic enzymes formed by diverse strains of Proteus spp. bacterial pathogencity. J. Med. Microbiol. 48: 623-628.
25. Liu, P. V.; Yoshii, S. and Hsieh, H.; 1973. Exotoxins of Pseudomonas aeruginosa.II. Concentration, purification, and characterization of exotoxin A. Journal ofInfectious Diseases, 128(4), 514-519.
26. Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem.; 72 : 248-254.
27. Freshney, R. I.; 2010. Culture of animal cells. A manual of basic technique and specialized applications. 6th (ed.), John Wiley and Sons, Inc. USA.
28. Martinotti, S. and Ranzato, E. (2020). Scratch wound healing assay. Epidermal cells: methods and protocols, 225-229.
29. Collec, G. J.; Fraser, G. A.; Marmion, P. B. and Sinmons, A. (1996). Edinburgh, Practical Medical Microbiology. Churchill Livingstone, 11, 163.
30. Alexander, S. K. and Strete, D. (2001). Microbiology: a photographic atlas for the laboratory.
31. Bartelt, P. E. and Peterson, C. R. (2000). A description and evaluation of a plastic belt for attaching radio transmitters to western toads (Bufo boreas). Northwestern Naturalist, 122-128.
32. Leboffe, M.J. and Pierce, B.E. (2011) A Photographic Atlas for the Microbiology Laboratory: Catalase Test. 4th Edition, Morton Publishing Company, Englewood, 75.
33. Rüchel, R.; Tegeler, R. and Trost, M. (1982). A comparison of secretory proteinases from different strains of Candida albicans. Sabouraudia: Journal of Medical and Veterinary Mycology, 20(3), 233-244.
34. Cassone, A.; Bernardis, F. D.; Mondello, F.; Ceddia, T. and Agatensi, L. (1987). Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. Journal of Infectious Diseases, 156(5), 777-783.
35. Lyerly, D. and Kreger, A. (1979). Purification and characterization of a Serratia marcescens metalloprotease. Infection and Immunity, 24(2), 411-421.
36. Morita, Y.; Kondoh, K.; Hasan, Q.; Sakaguchi, T.; Murakami, Y.; Yokoyama, K. and Tamiya, E. (1997). Purification and characterization of a cold-active protease from psychrotrophic Serratia marcescens AP3801. Journal of the American Oil Chemists' Society, 74(11), 1377-1383.
37. Jobling S. (2004) Improving Starch for Food and Industrial Applications. Current Opinion in Plant Biology, 7, 210-218.
38. Liu, P. V. and Hsieh, H. C. (1969). Inhibition of protease production of various bacteria by ammonium salts: its effect on toxin production and virulence. Journal of Bacteriology, 99(2), 406-413.
39. Matsumoto, D. (1984). People: Psychology from a Cultural Perspective. Belmont, CL: Brooks/Cole Publishing Company.
40. Green, A. (2005) Production of chitilytic enzyme by Serratia marcescens using various protase. J.Chem. Technol.Biotechnol. 80 (7):28-34.
41. Vial C.; Tobin A. B. and Evans R. J. (2004) G-protein-coupled receptor regulation of P2X(1) receptors does not involve direct channel phosphorylation. Biochem. J. 382, 101–110.
42. Hagel, L. (1998). Gel-filtration chromatography. Current protocols in molecular biology, 44(1), 10-9.
43. Alsa'ady, A. J.; Ibrahim, A. H.; Hassan, H. A. and Abdulameer, Z. W. (2023). Biodisintegration of human mucin protein by protease produced from Escherichia coli

AJ55 isolated from Urinary Tract Infection of Iraqi pa-tients. Iraqi Journal of Science, 5640-5653.

44. Voet, D.; Voet, J. G. and Pratt, C. W. (2013). Fundamentals of biochemistry: life at the molecular level (pp. 581-620). Hoboken, NJ: Wiley.
45. Turk, A.; Nakai, S. and Sugahara, M. (2006). Acoustic segment durations in prosodic research: A practical guide. In S. Sudhoff, D. Lenertova, R. Meyer, S. Pappert, P. Augurzky, I. Mleinek, N. Richter, & J. Schliesser (Eds.), Methods in Empirical Prosody Research (pp. 1-27). Berlin: Walter de Gruyter. doi:10.1515/9783110914641.1
46. Felder, M.; Trueeb, B.; Stucki, A. O.; Borcard, S.; Stucki, J. D.; Schnyder, B.*, et al*. (2019). Impaired wound healing of alveolar lung epithelial cells in a breathing lung-on-a-chip. Frontiers in bioengineering and biotechnology, 7, 3.
47. McCarty, S. M. and Percival, S. L. (2013). Proteases and delayed wound healing. Advances in wound care, 2(8), 438-447
48. López-Otín, C. and Bond, J. S. (2008). Proteases: multifunctional enzymes in life and disease. Journal of Biological Chemistry, 283(45), 30433-30437.
49. Lu, P.; Takai, K.; Weaver, V. M. and Werb, Z. (2011). Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harbor perspectives in biology, 3(12), a005058.
50. Moali, C. and Hulmes, D. J. (2009). Extracellular and cell surface proteases in wound healing: new players are still emerging. European Journal of Dermatology, 19(6), 552-5.