Antibacterial and Anticancer Activities of (Free and Immobilized) Elastase Produced by Klebsiella Pneumoniae

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Abstract
Elastase is a type of protease that specifically degrades elastin. The elastase produced by Klebsiella pneumoniae isolates and purified by three steps ammonium sulfate precipitation, ion-exchange chromatography, and Sephadex G-150 chromatography. The optimal condition for elastase production was showed high specific activity with starch (3.8 U/mg protein), casein as nitrogen source with a specific activity reached to (3.3 U/mg protein). The maximum elastase production was obtained when the pH value was (7.5) with specific activity (4.4 U/mg protein). Elastase (free and immobilized on TiO2- NPs) used in application as antibacterial and anti-cancer, and results showed high antibacterial activity against pathogenic isolates especially Lactobacillus acidophilus and Pseudomonas aeruginosa were affected by immobilized elastase. Free and immobilized elastase have anti-cancer activity against lung cancer using A549 cell line, and immobilize elastase was potent cytotoxic effect on A549 cells with IC50 142.8 µg/ ml compared with IC50 of normal cell line HdFn on 655.0 µg /ml.
Keywords: Klebsiella pneumoniae, Elastase, Immobilization, TiO2-Nps- Antibacterial, Anticancer.

1. Introduction

Klebsiella pneumoniae is a Gram-negative that belongs to Enterobacteriaceae family. It normally occurs in healthy humans including gastrointestinal tracts (Martin & Bachman, 2018).
Elastase is an enzyme from the class of proteases that break down, and degrade elastin. An elastic fiber that, together with collagen, determines the mechanical features of connective tissue (Everett & Davies, 2021). Elastase is one of proteolytic enzymes (also termed peptidases, proteases and proteinases), its capable of hydrolyzing peptide bonds in proteins. Elastases belong to the families of aspartic, cysteine, serine, and metalloproteases. There are many distinct forms of elastases in mammals (Fujii et al., 2020). Some microbes, such as Clostridium, Vibrio, Pseudomonas, Aeromonas, Bacillus, Streptomyces, and Aspergillus, have elastases with proteolytic activity (He et al., 2003).
Novel antibacterial drugs should be added to the arsenal to combat multidrug-resistant (MDR) microorganisms, especially before they evolve new resistance mechanisms against already sensitive antibiotics, in order to save public health (Culp et al., 2020). Proteolytic enzymes have great medical and pharmaceutical importance due to their key role in biological processes and in the life-cycle of many pathogens (Mótyán et al., 2013). Because of their improved antimicrobial action and longer longevity, nanoscale antibacterial materials have received a lot of attention in recent years as additional or alternative agents (Loh et al., 2010). This study aimed to evaluate the effect of elastase enzyme (free and immobilized) - purified from isolates of Klebsiella pneumoniae- as antibacterial against pathogens and as anticancer in vitro.

2. Material and Methods

Isolation and identification of K. pneumoniae isolates

Clinical samples (163) were collected from 15th of April to the 10th of July 2021 from Hospitals in the center of Wasit Governorate- Iraq. These samples included: (burns and wounds, Urine, and sputum). Isolation and identification of K. pneumoniae isolates was conducted by culturing on McConkey agar plates in a septic procedure, and incubated at 37°C for 24 hours (Patel et al., 2017).Then Gram stain used to study morphological features (Atlas et al., 1995). After that 16S rRNA analysis used to diagnosis the target genes (Abbas et al., 2020).

Screening of elastase production by Quantitative Assay

Forty -eight of K. pneumoniae isolates were cultured in brain heart infusion broth medium at 37 °C for 24hrs at shaking incubator (200 rpm). Cells were removed by centrifugation at 10000 rpm for 10 min and then sterilized with Millipore filter (0.22µm), 1 ml sterile supernatant was incubated with 20 µg elastin Congo red (ECR) for 30 minutes at shaking incubator (200 rpm). Then insoluble ECR was removed by centrifugation at 10000 rpm for 10 min

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at room temperature, and the absorbance was measured at 495 nm. Fresh BHI medium was used as the blank control (Li et al., 2019).

Optimization of Elastase Production

Optimization of parameters for elastase production by using composition of minimal medium and 1% elastin substrate**,** then added nitrogen sources (casein, peptone, ammonium sulfate, yeast extract and meat extract), Various carbon sources (glucose, maltose, sucrose, glycerol and starch**),** at concentration of 1% for the production of elastase, the best nitrogen and carbon sources was adjusted with different pH values (6, 6.5, 7, 7.5, 8, 8.5) and incubated at different temperature (25, 30, 35, 37, 40)°C**.** Culture medium was inoculated with 1% of isolate and incubated at 37°C for different time periods (24, 48, 72) hrs , and this culture was used to measuring elastase production (Kumar *et al.*, 2016).

Extraction and Purification of Elastase

Crude extracellular elastase was extracted from a maximum producter isolate of K. pneumoniae (NJ22) after growing in optimum conditions (24 hours, centrifugation at (10000 rpm) for 10 minutes), and the supernatant was used. At 4 C°, solid ammonium sulfate was gradually added to 75 ml of crude enzyme at saturation ratios (20- 90 percent), and the enzyme activity content was measured (Lei et al., 2018).

Purification by Ion exchange chromatography

DEAE-cellulose column (2 x20 cm) was used to purify elastase, and the flow rate was 30ml/hr throughout the column, and the absorbance of each fraction was measured using a spectrophotometer at 280 nm (Shinji et al., 2019)

Purification by Gel filtration chromatography

The a Sephadex G-150 (3 x 25 cm) column was used. Phosphate buffer pH8 was used for equilibration and elution at a flow rate of 30 ml/hr, and the absorbance of each fraction was measured using a spectrophotometer at 280 nm.

Elastase activity assay

One ml sterile supernatant was incubated with 1 ml of elastin Congo red buffer (20mg ECR in 6 ml Tris- HCL buffer of 200 mM, pH 8) for 1 h in water bath at 37°C. To stop the reaction, 0.1Mof NaOH was added. Then insoluble ECR was removed by centrifugation at 10000 rpm for 10 min at room temperature, and the absorbance was measured at 495 nm. Tris- HCL buffer (200 Mm, pH8) was used as control group. One unit of elastase activity was defined as the amount of enzyme required to hydrolyze 1 mg elastin per minute at 37 C (Lei et al., 2018).

Elastase immobilization on TiO2 nanoparticles

Adsorption of elastase on TiO2 nanoparticles was done by incubating 1 ml enzyme (containing 86 U/mg, dissolved in 50 mM sodium acetate buffer, pH 4.8) with 10 mg TiO2 nanoparticles suspended in 1 ml of the aforementioned buffer. The mixture was incubated at 25°C for 3 hours with constant shaking, after being centrifuged at 2000 rpm for 5 minutes at 4°C. The adsorbed enzyme-coated TiO2 nanoparticles were washed in a 50 mM sodium acetate buffer (pH 4.8) containing 1 M NaCl and 50% ethylene glycol. The enzyme activity was determined in the supernatant and washings (Ahmad & Sardar, 2014). Characterization of immobilized elastase was determined by using UV-Vis spectroscopy and FTIR analysis.

Antibacterial activity of free and immobilized elastase on TiO2NPS

The agar well diffusion method was used to detect antibacterial activity of free and immobilize elastase produced by Klebsiella pneumoniae (NJ22) as mentioned in (Indarmawan et al., 2016) Pathogenic bacteria used in this assay was gram negative, (klebsiella pneumoniae, E.coli and pseudomonas aeruginosa), and gram positive, (Streptococcus pneumonia and lactobacillus acidophilus).

Anticancer effect of free and immobilized elastase with TiO2- NPS

MTT-assay was done for cytotoxicity analysis on normal cell line (HdFn) and lung cancer cells line (A549). Medium of RPMI 1640 was used with different concentrations of free and immobilize elastase (12.5,25,50,100, 200 and 400, µg/mL). Cell viability could be evaluated as follows: Total Cell Count mL-1 = Cell count x Dilution Factor (Sample Volume) x 104

3. Results and discussion

Characterization of K pneumoniae

Initial identification of 48 isolates (29.44 %) of *K.pneumoniae* based on morphological characteristics on MacConkey agar which they appeared mucoid, large, and pink on MacConkey agar due to lactose fermenting. The microscopic examination of *K.pneumoniae* isolates as gram negative. The 16S rRNA gene was revealed in all isolate of *K. pneumoniae* (100%) as in Figure (1).

Figure 1: Identification of Klebsiella pneumoniae. A-16SrRNA, B-on MacConkey agar medium

Screening the ability of *K. pneumoniae* isolates in elastase production

The cell free extracts of *K. pneumoniae* isolates were tested spectrophotometrically using a method reported earlier by (Li *et al.*, 2019), to detect the effective bacterial isolates in elastase production. Figure (2) demonstrates that all isolates could generate the enzyme with specific activities ranging from (0.003-1.613) U/mg protein, but the isolate symbolled (NJ22) having the highest specific activity of 1.613 U/mg protein and so being chosen for future study.

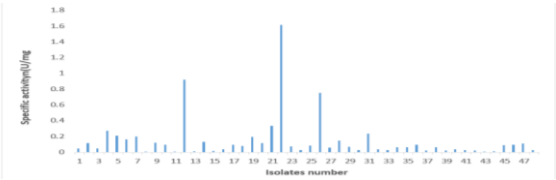

Figure 2: Specific activity of elastase produced by isolates of K. pneumoniae after 24hrs incubation at 37°C. on elastin nutrient broth medium

Optimal nitrogen source for elastase production

Casein was found to be effective in the production of enzyme from K. pneumoniae (NJ22) with a specific activity reached 3.3 U/mg protein (Fig. 3). Peptone was the second nitrogen source suitable for elastase production, which gave a specific activity of 2.7 U/mg protein. Yeast extract represented the low nitrogen source for enzyme production 0.55 U/mg protein.

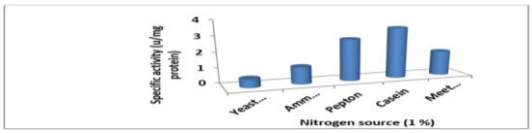

Figure (3): Optimal nitrogen source for elastase production by K. pneumoniae (NJ22) incubated at 37°C for 24 hrs

Because nitrogen provides the microorganism with the building blocks of organic molecules such as proteins, the availability of a nitrogen supply is essential for enzyme production (Pant et al., 2015). In P. aeruginosa requires that to induce elastase and enable growth on casein when casein is the sole nitrogen as well as carbon source (Yan et al., 2018).

Optimal carbon source for elastase production

Elastase enzyme showed high specific activity with starch of 3.8 U/mg protein, then began to decrease with other carbon sources until reached the low specific activity of 0.8 U/mg protein with glucose (Fig. 4). Therefore, starch was used as the main carbon source in later experiments.

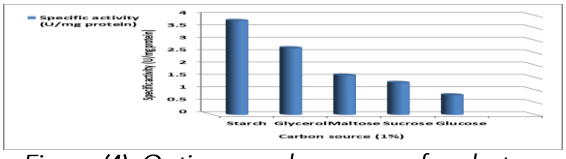

Figure (4): Optimum carbon source for elastase production by K. pneumoniae (NJ22)

The use of several carbon sources by Klebsiella sp. for the synthesis of an extracellular biosurfactant was investigated. The largest generation of crude biosurfactant was discovered with starch among the several carbon substrates examined *(Jain et al., 2013).* The elastase enzymes production by microorganisms has been reported to be enhance by carbon source and other operating parameter (He *et al.*, 2003).

Optimal pH of medium elastase production

The maximum elastase production was obtained when the pH value of the production medium was adjusted to 7.5. At this value, the enzyme specific activity recorded 4.4 U/mg protein (Figure 5).

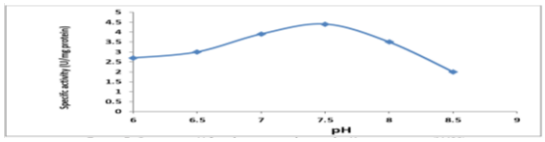

Figure 5: Optimum pH for elastase production by K. pneumoniae (NJ22)

This results agree with (Ghorbel-Bellaaj *et al.*, 2012) in which most elastase from *P. aeruginosae* exhibited optimum pH values ranging from 7 to 9. The pH of culture media changes when the concentration of hydrogen ions (H+) lowers or rises. Because H+ and/or OH compete with hydrogen and ionic bonds in an enzyme, this can result in denaturation of the enzyme(Bhunia *et al.*, 2013). On the other hand, the ionization states of the substrate or amino acid side chain are altered by pH, which is a determining factor in the expression of enzyme activity(Turner & Romero, 2010).

Optimal incubation temperature for elastase production

The specific activity was 3.8 U/mg protein at 37° C. At this temperature maximum production of elastase was observed (Fig. 6). This result similar to the result recorded by Ghorbel-Bellaaj *et al.*, (2012) exhibited that highest production of elastase from *P. aeruginosa* was at 37°C temperature. But (He et al., 2004) showed the best temperature for the production of Elastase from *Bacillus sp.* was 30°C. This variance may be due to the difference in bacterial isolate or to geographical factors.

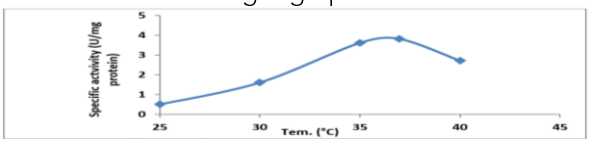

Figure (6): Effect of incubation temperature on elastase production by K. pneumoniae

Optimal incubation period for elastase production

Results in Figure (7) indicated that the specific activity of enzyme production reached to the maximum at 4.5 U/mg protein at 24 hr.

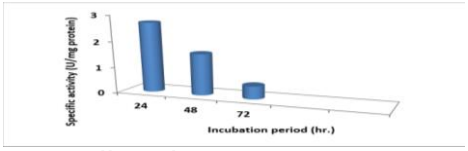

Figure (7): Effect of incubation period on elastase production by K. pneumoniae (NJ22)

This result is consistent with (Shinji *et al.*, 2019), who found that *P. aeruginosa* produces the most elastase after 24 hours of shaking incubation. The decrease in elastase production after 24 hours could be attributed to a lack of nutrients in the medium, the accumulation of excess acid in the media as a result of sugar utilization, or the development of oxygen tension. It was also related to the development of acetate, which inhibits cell growth and causes the production of toxic chemicals, resulting in lower protein production (El-Naggar & El-Ewasy, 2017).

Purification of elastase

The best precipitation of enzyme when ammonium sulfate saturation was (60%) when applied with the crude enzyme (supernatant). The result of DEAE-cellulose ion exchange chromatography column showed in Figure (8), and appeared one protein peak in the wash step, and another protein peak appeared after elution in (Fractions 47 to 53) which contained most of the elastase activity, and the enzyme specific activity was measured to be 36 U\mg protein.

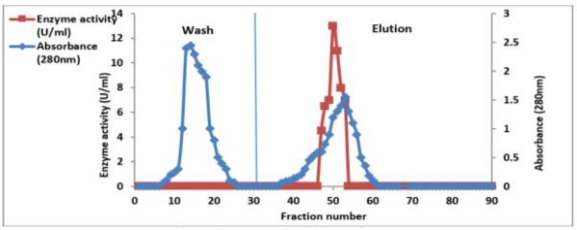

Figure (8): Ion exchange chromatography of elastase from K. pneumoniae (NJ22)

The results showed that protein with 76.6 % overall yield and 8.2 purification fold after purification by DEAE cellulose. On the basis of their affinity for the ion exchanger, ion chromatography separates ions and polar compounds. It operates on large proteins, tiny nucleotides, and amino acids, among other charged molecules. However ion chromatography must be done in conditions that are one unit away from the isoelectric point of a protein (Din et al., 2021).

After using Sephadex G-150 for further purification of elastase, and the results in Figure (9), showed that the high specific activity of the purified enzyme reached to 96 U/mg protein.

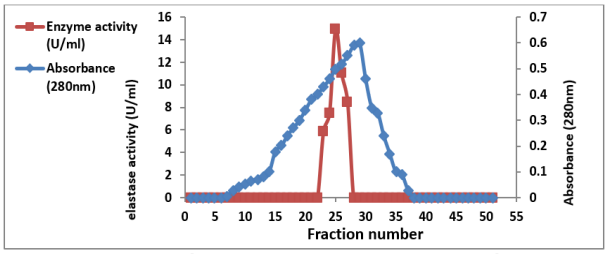

Figure (9): Gel filtration chromatography of elastase from K. pneumoniae (NJ22)

Lei *et al.*, (2018) recorded the purification of elastase from *Chryseobacterium indologenes* use different procedure of ammonium sulfate, gel filtration on Sephadex G-75 column, which gave a final purification fold of 8.3 and 5.8% yield. The specific activity was170 U/mg with purification folds 39.2 U/mg and(8.8%) overall yield time of elastase purified from *P. aeruginosa* recorded *by* (Kotb *et al.*, 2019).

Characterization of immobilized elastase

Figure (10, A) showed UV-visible spectroscopy analysis for immobilized elastase comparing with free enzyme and nanoparticles. An absorption peak at 362 nm was appeared which indicates the presence of TiO2 NPs, and absorption peak of enzyme at 281nm (figure 10, B). While two absorbance peaks exhibited for elastase immobilized on TiO2-NPs at 280 and 360 nm (figure10, C).

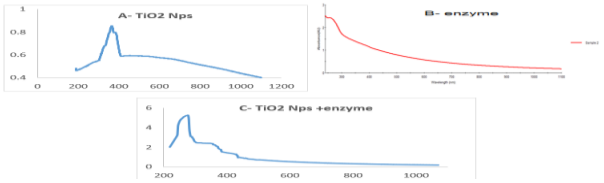

Figure (10) : UV–visible absorption spectrum (A)TiO2NPs (B)elastase (C) enzyme +TiO2 NPs

Figure 11 a, b and c) showed FTIR spectra of a TiO2 showed that TiO2 Nanoparticles have peak in 575 cm-1 were characteristic of O-Ti-O bond. The broad band is observed at 1171.15 cm-1 is corresponding to C=O stretch region showed in figure (11, a). While the peak for elastase at 3435 cm-1 reflected an OH functional group along with an H-bounded vibration. In addition, a detected peak at 2930.40 cm-1 was belong to NH primary and secondary amines and amides that have higher strength. The peak at 2327.79 and 2359.32 cm-1 corresponded to carbonyl groups, and peak at 599.62 for NH2 bonds showed in figure (11, b).

Spectra of TiO2NPs with immobilized elastase showed common characteristic peaks, while a number of differences were existed as a result of slight chemical interaction of elastase within the TiO2NPs conjugate. As shown in Figure (11 c), new peaks were generated and appeared in FTIR spectra of immobilize enzyme, which were not TiO2NPs spectra; the strong NH group with a peak at 3435.06 cm-1 indicated the presence of elastase on the TiO2NPs, the carbonyl group at 2955.23 and 2881.65 cm-1. These new peaks in the elastase conjugate TiO2NPs confirmed the successful synthesis of the enzyme immobilize with nanoparticles.

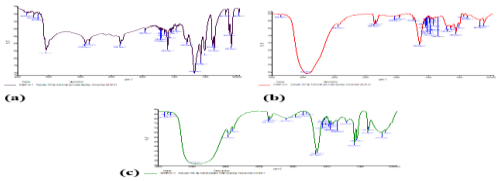

Figure 11: FT-IR analysis of (a) TiO2NPs, (b) elastase and (c) immobilized elastase

Antibacterial activity of purified free and immobilized elastase

The results indicated that free and immobilized enzyme possess significant antibacterial activity against all pathogenic isolates contrast with control. The antibacterial activity of immobilized elastase was significantly higher than free enzyme and TiO2 NPs against all isolates, as shown in figure (12). Lactobacillus acidophilus and Pseudomonas aeruginosa were the most bacterial isolates affected by immobilized elastase followed by E coli and Klebsiella pneumoniae with inhibition zones of 26, 11 and 9 mm respectively at highest concentration.

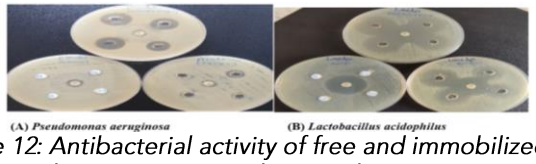

Figure 12: Antibacterial activity of free and immobilized elastase against pathogenic bacteria

Nanoparticles have also been shown to detect bacteria that cause infections in samples in a short period of time with greater accuracy than traditional methods (Sundaramoorthy & Nagarajan, 2022).The major types of antimicrobial enzymes are proteolytic enzymes and polysaccharide-degrading enzyme amongst the proteolysis enzymes, which belong to the serine protease (Cao et al., 2020).

Anticancer activity of purified free and immobilize elastase

Anticancer effect of free elastase was evaluated against A549 cell line as well as the normal HdFn cells using MTT assay for 24 hrs., with reduction rate of 69.98 ± 2.18 to 94.79± 2.81 from the concentrations 400 to 12.50 µg ml, and showed that elastase enzyme exhibited significantly the most potent cytotoxic activity with IC50 value of 243.8 µg/ml comparison with IC50 of 384.5 µg/ml on HdFn normal cell line figure (13).

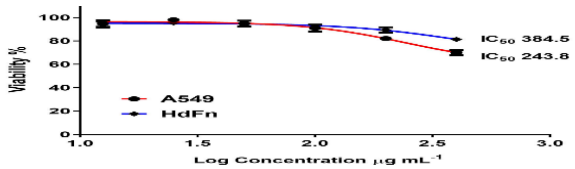

Figure (13): Cytotoxic effect of elastase on A549 and HdFn cells

The cell viability is reduced by increasing the concentration of immobilize elastase enzyme. The decreasing in A549 cell viability was noted by 400µg/ml (55.71 ± 3.60) while the highest A549 cell viability at 12.50 µg/ml reached to (94.87 ± 0.29). Figure (14) showed immobilize elastase was potent cytotoxic effect on A549 cells and the IC50 values of immobilize enzyme was 142.8 µg/ ml, while IC50 of normal cell line HdFn was 655.0 µg /ml.

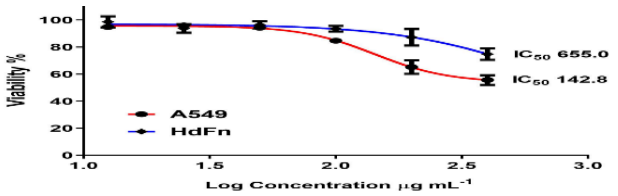

Figure (14): Cytotoxic effect of immobilize elastase on A549 and HdFn cells after 24 hrs incubation at 37 °C.

Cancer is a mutation-driven disease with significant geographical and temporal genetic variability (Vogelstein *et al.*, 2013). Finding medicines that combine wide efficacy across cancer types with selectivity to limit host damage has been difficult. The fundamental idea behind enzyme therapy methods is to use enzymes capable of depleting cells of a certain type of amino acid that is required for malignant cell growth but not for normal cell survival. In fact, a single amino acid deficiency has been identified as a potentially effective cancer treatment method (Zolfaghar *et al.*, 2019).

4. Conclusion

The results of the present investigation revealed that the klebsiella pneumoniae bacteria are a potential source of elastase enzyme. High specific activity for elastase purified from klebsiella pneumoniae (NJ22) is obtained after three steps of purification by ammonium sulfate precipitation (60%), DEAE-Cellulose, and Sephadex G150. It is concluded from the present study that the elastase enzyme isolated from bacterial strains possess significant antibacterial activity against gram negative and gram- positive bacteria. Free and immobilize elastase had cytotoxic activity against A549 cancer cell line.

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