The Egyptian Journal of Hospital Medicine (January 2023) Vol. 90, Page 1838-1843

Cleavage of Mucin by Partial Purified Protease Produced from Gastrointestinal Escherichia Coli A29 Isolated from Iraqi Patients

Ataa R. Khayoon*, Ali J.R. AL-Sa'ady
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq
***Corresponding author:** Ataa R. Khayoon**, Mobile: +9647739988271,**
ORCID: 0000-0002-6699-4103**, E-mail:** ataa.raghad1206a@sc.uobaghdad.edu.iq

hABSTRACT

Background: Escherichia coli is one of the significant bacteria that belongs to Enterobacteriaceae bacteria family, which found in human intestinal tracts. Several Escherichia coli clone have been known to be extremely virulent and multidrug resistant. Escherichia coli poses a significant public health challenge for Iraq.

Objectives: The current study aimed to cleavage the mucin protein by partial purified protease enzyme produced by pathogenic Escherichia coli bacteria. **Materials and methods:** This study was conducted on isolates of Escherichia coli bacteria that isolated from stool of people with gastroenteritis and diarrhoea. These isolates were examined on skim milk agar medium in order to screening of protease enzyme. Escherichia coli A29 was efficient isolate for protease production. The protease enzyme was purified by ion exchange (CM-Cellulose column) and gel filtration chromatography (Sephacryl S-300) after precipitated by ammonium sulphite saturation of 80%. Characterization for protease enzyme was done for effect of pH and temperature on activity and stability. The next step was treatment of protease (19250 U/mg) with mucin (0.11 mg/ml) and passed across the Sephacryl S-300 column.

Results: The results showed that the purification of protease by these chromatography techniques was given specific activity of 19250 U/mg, purification fold 4.94 and yield 49. The maximum activity for purified protease was at pH 6.0 (77.527 U/ml) and pH stability for enzyme activity was between 5.5 and 9, while the optimum temperature was 37˚C (77.7 U/ml) and the stability for activity was kept between 15 and 50°C.

Conclusion: The protease was cleavage of mucin for 3 peaks which represent fragments of mucin.

Keywords: Purification, Characterization, Temperature, Stability, Biodegradation, Experimental Study, University of Baghdad.

INTRODUCTION

Escherichia coli is One of the significant bacteria that belongs to Enterobacteriaceae bacteria family [(1)], which found in human intestinal tracts. It was first known as "Bacterium coli commune" because Theodor Escherichia found it in the colon of an infant's feces in 1885. Depend on their shape and motility; these bacteria were categorized as prokaryotes in the beginnings under the name "Monera" [(2)].

In addition to E. coli strains that are harmless commensals found in human gastrointestinal system, there are other strains which are pathogenic E. coli of humans and animals [(30)], which classified as those that cause disease inside the gastrointestinal tract and those that can infect outside the gastrointestinal tract [(3)]. The path type of E. coli has unique pathogenic mechanisms and unique virulence factors that are encoded by particular gene clusters [(4)], one of the most important virulence factors is protease activity [(5)]. Proteases are degradative enzymes that act as a catalyst for protein hydrolysis, have a molecular weight ranging from 18 to 90 KDA. These enzymes can be created by animals, plants, and microorganisms; however, they are typically formed by bacteria and fungi [(6)]. Mucins are proteins have high-molecular weight which synthesized and secreted in many organs [(7)]. They considered a highly glycosylated proteins family, that make up the majority of the organic parts of the mucus layer, which protects the epithelial cells in various organs of both humans and animals, including the gastrointestinal tract [(8)].

The current study aimed to cleavage the mucin protein by partial purified protease enzyme produced by pathogenic Escherichia coli bacteria.

MATERIALS AND METHODS

Some of material that used in this research was Skim milk agar (Hi-media_ India), Trichloroacetic acid (TCA) (Alpha- chemika-India), Coomassie Brilliant Blue G-250 (BDH-England), Carboxyme-thyl cellulose (CMC), Sephacryl S-300.

Protease production: A 147 bacterial isolates were obtained from hospitals in the city of Maysan which were cultured on MacConkey agar plates and nutrient agar [(9)]. These isolates were tested for their ability to the protease production, and these experiments were performed via primary and secondary screening on skim milk agar and brain heart infusion broth, respectively. The isolate of E. coli A29 which produced the largest protease, were cultured (75 ml) in Brain Heart Infusion Broth (flask of 250 ml) and incubated for 24 hours at 37 °C. After that, the culture was filtered through filter paper and centrifuged at 10000 rpm for 30 minutes. The supernatant was used to measure protease activity and protein concentration [(10)].

Protease activity assay: Protease activity was estimated according to method as describes in [(11)] by using (1%) casein as substrate.

Protein concentration assay: Protein concentration was calculated according to Bradford (1976) [(12)].

Protease precipitation by ammonium sulfate: The supernatant of crude enzyme was fractionated at 4°C with ammonium sulfate (0-80% saturation), ammonium sulfate was gradually mixed with the enzyme solution for 2 hours on an ice bath with gentle stirring. After that the enzyme solution was centrifuged for 20 minutes at

Received: 03/12/2022
Accepted: 03/02/2023

10,000 rpm. The supernatant was eliminated while the precipitate was dissolved in 10 ml of 0.1 M phosphate buffer solution [13]. Protease activity and protein concentration were determined. The protease product from the bacterial culture was preliminary concentrated by salt precipitation and then purified by ion exchange chromatography [14].

Purification of protease by Ion exchange chromatography: After precipitation of enzyme by ammonium sulfate step, the resulted concentrated enzyme was applied to column of exchange chromatography loaded with CM-Cellulose (28-1.7 cm) column by using clean Pasteur pipette. The column was equilibrated with Tris-HCl (0.005 M, pH 8.0), and eluted with NaCl gradient (0.1-1 M) in flow rate 30 ml/ hourr, 3 ml for each fraction. The absorbance of all fractions from the washing and elution steps was measured at wavelength 280 nm by spectropho-tometer. Thereafter, the activity was measured for the fractions proteins and these parts were then collected.

Gel Filtration Chromatography: This step involved using gel filtration chromatography as a second technique for protease purification. Proteins and peptides can be separated according to their size using this technique. Sephacryl S-300 column (85×1.5 cm) was prepared and packed based on manufacturing company (Pharmacia-Sweden). The activation parts that gave the enzyme activity (14 ml) from ion exchange chromatog-raphy was add to the Sephacryl S-300 column with flow rate of 30 ml/hour and equilibrated with Tris-HCl (0.005 M, pH 8.0), 3 ml for each fraction. After fractions collection, the absorbance for all the tubes was measured at 280 nm.

Characterization of partial purified protease enzyme

Effect of pH on the activity and stability of enzyme: Casein was used as substrate to determine the optimum pH for partial purified protease, casein was prepared in acetate buffer (pH 3, 3.5, 4, 4.5, 5, 5.5, and 6), phosphate buffer (pH 6.5, 7, and 7.5), and Tris-HCl buffer (pH 8, 8.5, and 9). Then the enzyme activity was determined. In order to determine the pH stability of a protease, equivalent volume of partial purified enzyme was combined with various buffers at a ratio of 1:1, and the mixture was then incubated at 40 °C for 30 minutes. The samples were immediately transferred to an ice bath; thereafter calculate the remaining activity (%) for each sample.

Effect of temperature on the activity and stability of enzyme: A 0.2 ml of the partial purified protease add to prepared casein 1% and incubated at various temperatures (15-70) °C which used to determine the optimal temperature for protease activity, incubated for 20min then the enzyme activity was measured. The partial purified enzyme was incubated at various temperatures (15-70°C), and then the samples were immediately transferred to an ice bath, and then calculate the remaining activity (%).

Gel Filtration Chromatography for mucin: This step involved using gel filtration chromatography for determination of mucin location. Sephacryl S-300 column (85×1.5 cm) was prepared and packed based on manufacturing company (Pharmacia-Sweden). A 2 ml of mucin (0.11 mg/ml) was add to the column, eluted with Tris-HCl (0.005 M, pH 8.0) and 3 ml for each fraction was collected. After collection of fractions, the absorbance for all the fractions was measured at 280 nm.

Cleavage of mucin by protease enzyme

Sample preparation: A 2 ml of mucin protein (0.11 mg/ml) was mixed and incubated with 0.2 ml partial purified protease with specific activity 19250 U/mg and 0.004 mg/ml of protein concentration for 37ºC at 24 hours.

Determination of mucin Cleavage: After treatment of mucin with partial purified protease, the mixture was loaded on Sephacryl S-300 column (85×1.5 cm) carefully by pasture pipette. Then eluted with 0.005M Tris-HCl (pH 8), three ml for each fraction was accumulated. The absorbance for each fraction was calculated at 280 nm and the results were record.

Ammonium sulfate precipitation: The resulting crude extract was first precipitated with ammonium sulfate salts. In the current study, protease precipitation was obtained by using an ammonium sulfate saturation of 0-80%. As shown in the table (1), the results showed that 73% of the protease was gained with a purification fold of 1.95 and specific activity 7606U/mg. In the study by Winarti et.al., [15] how showed that ammonium sulfate saturation 70% for precipitation of protease from Bacillus cereus was gave the highest specific enzyme activity (78.296 U/mg). **Lakshmi *et al.*** [16], found that the precipitation of protease from Bacillus cereus by ammonium sulfate saturation 50%, was gave the highest specific activity (29.47 U/mg), yield 53.8% and 1.67 purification fold [16]**.** Historically, ammonium sulfate precipitation was used extensively to concentrate proteins and was almost considered a cheap method of precipitating a protein extract. Salting out was performed to separate the contaminated proteins from solution of protein since that the solubility of protein in various salt solution concentrations is known [17]. Ammonium sulfate has special properties make it important manner for enzymes precipitation such as, availability, high solubility, susceptibility to stabilize proteins, and low cost [18].

Purification of protease by Ion exchange chromatography: The next step for protease purification was ion exchange chromatography which is a technique for separating organic molecules based on size, shape, charge, and solubility [19]. There are mobile and stationary phases in this procedure, CM- Cellulose is a type of weak cation exchanger; it can bind to the reverse charge of the target protein [20]. In this experiment, the washing step with 0.005 M Tris-HCl (pH 8.0), allows of the occurrence of one peak which

represented by fractions 14-41, and also three peaks in elution step which represented by fraction 61-71, 70-77, and 79-85 as seen in figure (1).

Protease activity and protein concentration were determined for each fraction. Protease activity was showed by the fractions in washing step 13-29. The result indicates that protease produced from E. coli A29 carried positive charge which binds to CM-cellulose of negative charge.

As found in table (1) protein concentration of 0.005 mg/ml, activity of 90.56U/ml, and specific activity of 18112 U/mg, with a purification fold of 4.6 and yield of 62 were gained, in contract with other study by Chung and Goldberg [29] that used CM-cellulose exchanger for purification of protease from E. coli, protein concentration was 3.8, activity 285U/ml. Another study by Sharma and De involved purification of protease that produced from Aspergillus tamari showed that fold enzyme purification was 26 with a specific activity of 11078 U/mg, protein 0.4mg/ml and yield 50% [21].

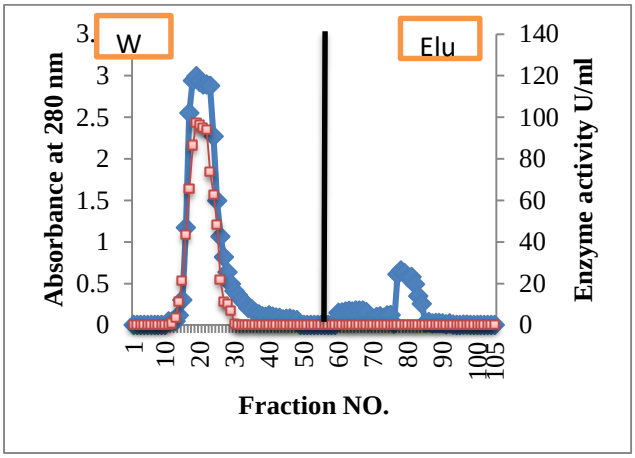

Figure (1): Ion exchange chromatography for purification of protease from E. coli by using CMC-column (28-1.7 cm) equilibrated with Tris-HCl (0.005 M, pH 8.0), eluted with Tris-HCl with NaCl gradient (0.1-1 M) in flow rate 30 ml/ hour , 3 ml for each fraction.

Gel Filtration Chromatography: Gel Filtration Chromatography used for protease purification, Sephacryl S-300 column (85×1.5 cm) was prepared and used for this purpose.

The results as seen in figure (2) two peaks was appeared which represented by fractions 29-41 and 63-67, one of them (29-41) referred to protease enzyme, with specific activity 19250 U/mg, purification fold 4.94 and yield 49 %, in contract with other study for purification of protease enzyme produced from Bacillus sp, the results were specific activity of 916.76 U/mg, purification fold 4.31and yield 1.68 % [22].

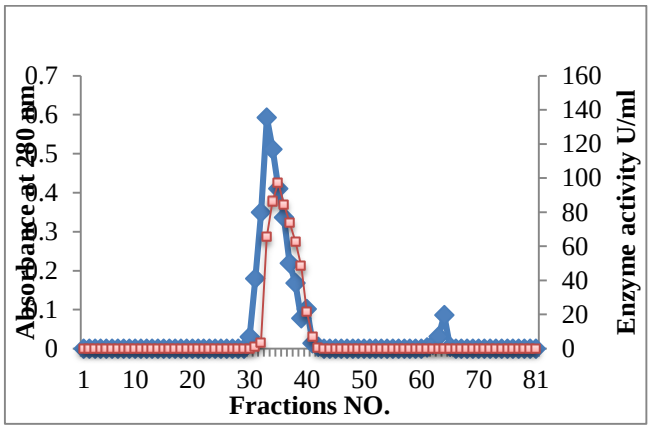

Figure (2): Purification of protease by using Sephacryl S-300 column (100×1.5 cm) with flow rate of 30 ml/hour, eluted with Tris-HCl (0.005 M, pH 8.0) and 3 ml for each fraction.

RESULTS AND DISCUSSION

Table 1. Purification steps of protease from E. coli.

Step	Volume ml	Activity U/ml	Protein concen. mg/ml	Specific activity U/mg	Total activity	Fold	Yield %
Crude enzyme	75	27.27	0.007	3896	2045	1	100
Precipitation by ammonium sulphate (0-80 % saturated)	15	98.88	0.013	7606	1483	1.95	73
Purification by Ion-exchange chromatography	14	90.56	0.005	18112	1267.9	4.6	62
Purification by Gel-filtration chromatography	13	77	0.004	19250	1003	4.94	49

Characterization of partial purified protease

Effect of pH on the activity and stability of enzyme: Protease activity was evaluated at various pH levels, and it was concluded that this enzyme is most active at pH range of 6 to 7, and the maximum activity occurring at pH 6.0, where it was 77.527 U/ml as exhibited in **Figure 3**. At pH 5.5 and below and at pH 7.5 and above 9, the activity was decreased. In contract with other research by **Cheng and Zipser** [23], the optimal pH for purified protease from E. coli was 7.4 while in another study by **Kramer et al.** [24] on purified protease that produced by E. coli was demonstrated that the best pH for higher protease activity was 6.5.

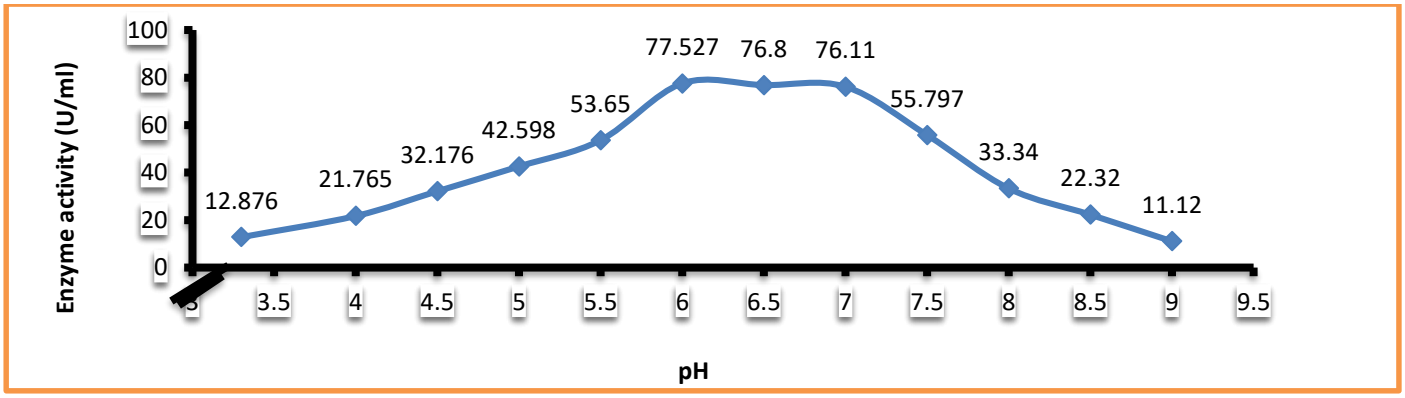

Figure (3): Optimum pH for partial purified protease activity from E. coli.

Stability of pH for protease activity was measured and it was observed that pH ranged between (5.5-9) were the optimal pH for protease stability, at pH 5.5, the enzyme activity was 96% while retained 100% of its activity in pH 7.0, and 7.5, also about 97% for pH 9. The remaining activities was 87% for pH 5.0, the activity was very decline to reach of 55% at pH 4.5 (figure 4). These findings were referred that the protease produced from E. coli A29 is steadier and it has large range of pH stability. Previous study by **Ghorbel *et al.*** [25], how found that the pH stability of protease from Bacillus cereus bacteria was 6.0 and 9.0.

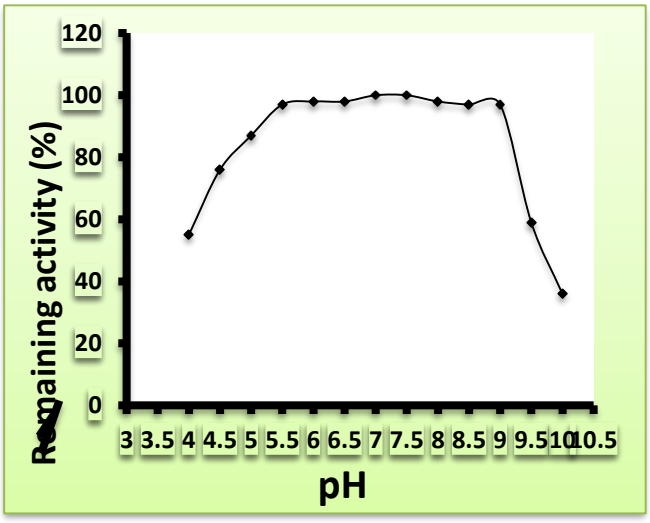

Figure (4): pH Stability for partial purified protease from E. coli

Effect of temperature on the activity and stability of enzyme: The best temperature of protease activity was determined by measuring the activity at different temperature ranges (15- 70°C). The outcomes in **Figure 5** indicated that the better temperature for partial purified protease activity was at 37°C with 77.7 U/ml. Then the enzymatic activity was decreased in temperatures above 45ºC which may be as a result of protein denaturation and change in the active sites of enzyme, which leads to loss the activity as described in **Figure 6**. In contract with other study by **Biver *et al.*** on purified protease from E. coli, the optimum temperature was 50ºC [26]. In other study by **Kothary *et al.*** on production of protease from Enterobacter sakazakii, the optimal temperature was 37ºC [27].

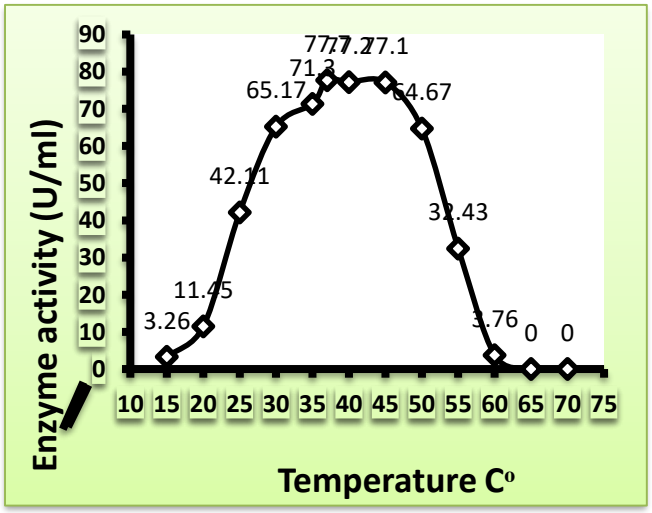

Figure (5) Optimum temperature for partial purified protease activity from E. coli.

The thermal stability for the partial purified enzyme involved incubated at several temperatures ranges (15-70) °C, the results as shown in figure (6), demonstrated that the protease enzyme was kept its activity between 15-50°C temperatures and the activity was decreased above 50 degree, which become 38% at 60°C, while at 65°C, there was no remaining activity (loss of enzyme activity).

In contract with other study by Kumar for protease from Bacillus pumilus, the enzyme activity was maintained between 55-60 °C. The majority of enzymes are kept at low temperatures because they are steadier there, and the sensitivity of protease to high temperatures, which is reflected in the effect of temperature on the protein's three-dimensional structure by damaging R-groups of amino acids, causes the enzyme's activity to decrease at temperatures higher than 0°C [20].

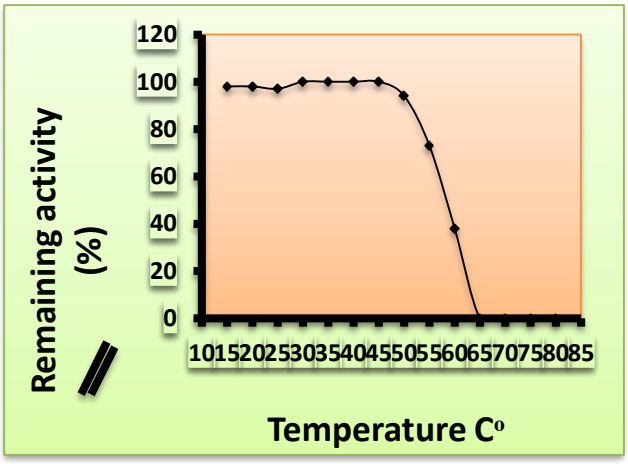

Figure (6): Thermal stability of partial purified protease from E. Coli.

Determination of mucin location: The next step of using Sephacryl S-300 column (85×1.5 cm) involved added 2ml of mucin protein which eluted with Tris-HCl (0.005 M, pH 8.0) and flow rate of 30 ml/hour. A 3 ml for each fraction was collected and the absorbance was estimated at 280mnm. The result in figure (7) that between fractions 11-24, two peaks was appeared which referred to existing of mucin protein depend on the absorbance reading of fractions.

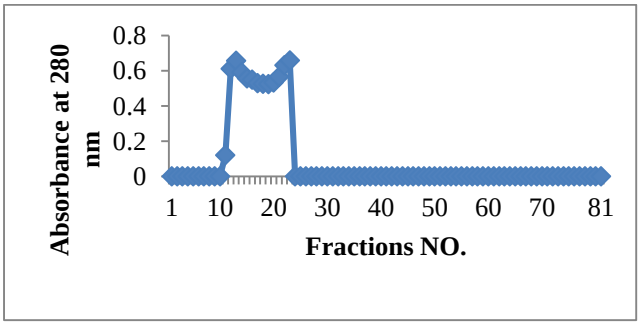

Figure (7): Elution of Mucin protein using Sephacryl S-300 column (85×1.5 cm) with flow rate of 30 ml/hour, eluted with Tris-HCl (0.005 M, pH 8.0) and 3 ml for each fraction.

Cleavage of mucin by protease enzyme: A mixture solution consists of 200 µl partial purified protease and 2 ml mucin (0.11 mg/ml) was prepared and incubated for 24 hours at 37 °C, after that the mixture was added to the Sephacryl S-300 column (85×1.5 cm) by clean pipette carefully. After collection of fractions and measured the absorbance at 280nm for each fractions. Results as in figure (8) displayed occurrence of 4 peaks, the first represented by fractions (30-40), the second peak represented (51-63), the third represented by fractions (71-74) and the forth represented by fractions (75-78). The first peak referred to protease based on it activity against the casein. The second, third and fourth peaks belonged to the fragments of mucin protein, which mean that the protease cleavage the mucin protein to these fragments. In previous study by **Abdalah *et al.*** on cleavage of mucin by protease produced from Native Strain of Streptococcus, in which the protease cleaved the mucin in three peaks [7].

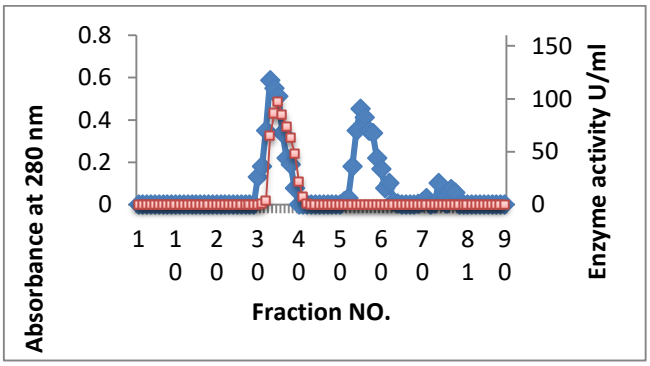

Figure (8): Gel filteration chromatography for mucin fractions treated with protease using Sephacryl S-300 column (85×1.5 cm) with flow rate of 30 ml/hour, eluted with Tris-HCl (0.005 M, pH 8.0) and 3 ml for each fraction.

Financial Disclosure: No financial disclosure.
Conflict of Interest: None to declare.
Ethical Clearance: "All trial protocols were approved under the University of Baghdad, College of Science, Department of Biotechnology were carried out in accordance with approved guidelines".

REFERENCES

1. **Dutta P, Cappello R, Navarro-García F, Nataro J (2002):** Functional comparison of serine protease autotransporters of Enterobacteriaceae. Infection and Immunity, 70(12):7105-13.
2. **Clark C, Kruczkiewicz P, Guan C *et al.* (2013):** Evaluation of MALDI-TOF mass spectroscopy methods for determination of Escherichia coli pathotypes. Journal of Microbiological Methods, 94(3):180-91.
3. **Kaper J, Nataro J, Mobley H (2004):** Pathogenic escherichia coli. Nature Reviews Microbiology, 2(2):123-40.

4. **Pakbin B, Brück W, Rossen J (2021):** Virulence factors of enteric pathogenic Escherichia coli: A review. International Journal of Molecular Sciences, 22(18):9922.
5. **Nanda A, Nayak S (2020).** Virulence factors and drug resistance of gastrointestinal Escherichia coli isolated from different animals. Indian J Anim Hlth., 59(2):159-68.
6. **Ikram-Ul-haq H, Umber H (2006):** Production of protease by Penicillium chrysogenum through optimization of environmental conditions. Journal of Agriculture & Social Sciences, 2(1):23-5.
7. **Abdalah M, Al-Saady A, ALBahrani M *et al.* (2018):** Cleavage of mucin and salivary agglutinin by partial purified protease from a native strain of Streptococcus mutans AN67. Journal of Global Pharma Technology, 10(06):404-10.
8. **Derrien M, van Passel M, van de Bovenkamp J *et al.* (2010):** Mucin-bacterial interactions in the human oral cavity and digestive tract. Gut Microbes, 1(4):254-68.
9. **Das G, Prasad M (2010):** Isolation, purification & mass production of protease enzyme from Bacillus subtilis. Int Res J Microbiol., 1(2):26-31.
10. **Maitig A, Alhoot M, Tiwari K (2018):** Isolation and screening of extracellular protease enzyme from fungal isolates of soil. Journal of Pure and Applied Microbiology, 12(4):2059-67.
11. **Afsharnezhad M, Shahangian S, Sariri R (2019)**: A novel milk-clotting cysteine protease from Ficus johannis: Purification and characterization. International Journal of Biological Macromolecules, 121:173-82.
12. **Bradford M (1976):** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1-2):248-54.
13. **Abdullah S, Flayyih M (2015)**:Production, purification and characterization of uricase produced by Pseudomonas aeruginosa. Iraqi Journal of Science, 56(3B):2253-63.
14. **Bradford M (1976)**: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1-2):248-54.
15. **Winarti A, Fitriyanto NA, Pertiwiningrum A, Bachruddin Z, Pranoto Y, Erwanto Y (2018)**: Optimizing of protease purification from Bacillus cereus TD5B by ammonium sulfate precipitation. Chemical Engineering Transactions, 63:709-14.
16. **Lakshmi B, Kumar D, Hemalatha K (2018):** Purification and characterization of alkaline protease with novel properties from Bacillus cereus strain S8. Journal of Genetic Engineering and Biotechnology, 16(2):295-304.
17. **Novák P, Havlíček V (2016): Protein extraction and precipitation. https://doi.org/10.1016/B978-0-444-63688-1.00004-5**
18. **Pulicherla K, Gadupudi G, Rekha V *et al.* (2011)**: Isolation, cloning and expression of mature staphylokinase from lysogenic Staphylococcus aureus collected from a local wound sample in a salt inducible E. coli expression host. International Journal of Advanced Science and Technology, 30: 35-42.
19. **Ebere E, Obinna I, Wirnkor V (2019)**: Applications of column, paper, thin layer and ion exchange chromatography in purifying samples: Mini review. SF Journal of Pharmaceutical and Analytical Chemistry, 2(2):1018.
20. **Noori H, Aziz G (2020):** Purification, Characterization, and Evaluation of Fibrinolytic Activity of Staphylokinase from Locally Isolated Staphylococcus aureus GH38. The Iraqi Journal of Agricultural Science, 51(4):1195-203.
21. **Sharma N, De K (2011)**: Production, purification and crystallization of an alkaline protease from Aspergillus tamarii. Agriculture and Biology Journal of North America, 2(7):1135-42.
22. **Margino S, Jumi'ati J, Ngadiman N (2013):** Purification and Characterization of Protease from Bacillus sp. TBRSN-1. Indonesian Journal of Biotechnology, 18(2):151-60.
23. **Cheng Y, Zipser D (1979)**: Purification and characterization of protease III from Escherichia coli. Journal of biological chemistry, 254(11):4698-706.
24. **Kramer R, Zandwijken D, Egmond M *et al.* (2000):** In vitro folding, purification and characterization of Escherichia coli outer membrane protease OmpT. European Journal of Biochemistry, 267(3):885-93.
25. **Ghorbel B, Sellami A, Nasri M (2003):** Stability studies of protease from Bacillus cereus BG1. Enzyme and Microbial Technology, 32(5):513-8.
26. **Biver S, Portetelle D, Vandenbol M (2013):** Characterization of a new oxidant-stable serine protease isolated by functional metagenomics. Springerplus, 2(1):1-10.
27. **Kothary M, McCardell B, Frazar C *et al.* (2007):** Characterization of the zinc-containing metalloprotease encoded by zpx and development of a species-specific detection method for Enterobacter sakazakii. Applied and Environmental Microbiology, 73(13):4142-51.
28. **Kumar C (2002):** Purification and characterization of a thermostable alkaline protease from alkalophilic Bacillus pumilus. Letters in Applied Microbiology, 34(1):13-7.
29. **Chung C, Goldberg A (1983):** Purification and characterization of protease So, a cytoplasmic serine protease in Escherichia coli. J Bacteriol., 154(1):231-8.
30. **Zahid A, AL-Mossawei M, Mahmood A (2016):** In vitro and in vivo pathogenicity tests of local isolates APEC from naturally infected broiler in Baghdad. Int J Adv Res Biol Sci., 3(3):89-100.