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Isolation and Purification of Chickpea (*Cicer arietinum*) Seeds Protein, Testing Their Antibacterial Activity, and Using to Extend the Shelf Life of Beef Patties

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Abstract: Antimicrobial proteins (AMP) from chickpea (*Cicer arietinum* L.) seeds were isolated and purified using the saturation of $(\text{NH}_4)_2\text{SO}_4$ by 80% and gel filtration through Sephacryl S-200, the inhibition zone of the separated peak was 24, and 22 mm for growth of *Escherichia coli* and *Salmonella typhimurium* respectively. The molecular mass was 28385 Da estimated through Sephacryl S-200. The optimum pH for activity was 5.5. It was stable at 4.5-7.5, while it lost 21.07 and 55.65% from its activity at pH 3 and 8 respectively, the optimum temperature for activity was 35°C and it was stable at 35°C for 60 min, while it lost all inhibitory activity at 65°C for the same time. The use of AMP at 100 mg resulted in an inhibition zone of 37 ± 2.92 , 35 ± 1.68 , 32 ± 2.33 , and 33 ± 2.09 mm, with a significant difference at ($P \leq 0.05$) against *E. coli* and *S. Typhimurium*, *Staphylococcus aureus*, and *Bacillus cereus*, respectively. The use of AMP to extend the shelf life of beef patties at 100 mg.kg^{-1} resulted in a decrease in the total count of bacteria, as it reached $7.4 \times 10^2 \pm 0.18$, $4.6 \times 10^2 \pm 0.22$ and $2.8 \times 10^2 \pm 0.19$ CFU.g⁻¹, while it was $7.4 \times 10^2 \pm 0.23$, $8.2 \times 10^2 \pm 0.31$, and $9.5 \times 10^2 \pm 0.27$ CFU.g⁻¹ in the control sample during a storage period of 0, 3, and 6 d at 4°C. It was observed that there was no significant difference in the control treatment and AMP added on 0 d, while a significant difference was observed at ($P \leq 0.05$) for treatments at a storage period of 3 and 6 d at 4°C.

Keywords: Antimicrobial proteins (AMPs), Beef patties, Chickpea seeds, Extending shelf life of food, Inhibition of microorganisms.

Introduction

Plants are considered a valuable and important source of many active biological compounds which have the ability to treat various diseases, infection of humans, animals, and plants caused by pathogenic microorganisms (Aljazy *et al.*, 2019; Alsoufi & Aziz, 2019). Proteins that have low molecular weight represented these compounds which were isolated and purified from different plant sources, such as,

Datura stramonium seed (Muhammad *et al.*, 2019), *Cucumis sativus* L. seeds (Al Akeel *et al.*, 2018), *Gastrodia elata* Blume tubers (Chen *et al.*, 2018), seedlings of *Bauhinia purpurea* L. (Sakthivel & Palani, 2016) and others. The molecular weight range of these AMP/peptides AMPs is 2 to 50 kDa, the helical structure shows that it is high in

hydrophobic amino acids (Thapliyal *et al.*, 2016).

There are many proteins with AMPs including enzymes such as chitinases and β -1-3-glucanases which can collaborate with membrane elements such as lipid transfer proteins (LTPs); cyclophilin-like proteins and thaumatin-related protein; glycine/histidine-rich proteins, defensins, all of them act as potent defensive mechanisms for these cells against microbial infections through the action of peptides on the bacterial membrane by forming pores which lead changes in the membrane permeabilization of bacteria (Thapliyal *et al.*, 2016; Muhammad *et al.*, 2019). The nature and charge of AMPs such as hydrophilic and hydrophobic ion groups that interact with phospholipid groups and lipid elements of the cell membrane, respectively, are important for destroying the cell membrane through the attraction of AMPs to phospholipids and lipopolysaccharides in the cells of G^- bacteria, and teichoic acids in G^+ bacteria. (Epanand *et al.*, 2010; Sakthivel & Palani, 2016).

The AMP/peptides have large potential for the improvement of new biological agents having an effect against some diseases that are as of now difficult to treat due to the spread of microbial resistance to antibiotics, therefore, it is imperative to increase the research in this field to find more biologically safe agents from plants and herbs against pathogenic microorganisms (Al Akeel *et al.*, 2018). So, this study aims to isolation and purification of AMP from chickpea seeds and testing their antibacterial activity and use it in extending shelf life of beef patties.

Materials & Methods

The raw material

Chickpea seeds were obtained from the local markets of Baghdad.

Microorganism's strains

E. coli, *S. typhimurium*, *S. aureus* and *B. cereus* that use in this study were obtained from the laboratories of the Department of Science, College of Basic Education, University of Al-Mustansiriah, Baghdad, Iraq.

Estimation of protein

Total protein mg.ml^{-1} of the *C. arietinum* through purification steps and applications was estimated by the method of Bradford (1976) using BSA stock solution at 595 nm.

Antimicrobial activity

The antimicrobial activity of *C. arietinum* seeds extract through purification steps and applications was evaluated using agar well diffusion method according to the method of Gupta *et al.* (2016). The Mueller-Hinton medium was allowed to cool at 45 to 50°C immediately after autoclaving. Then was poured into Petri dishes (90 mm in diameter) and placed on a horizontal surface to give a depth of almost 4 mm. The agar media was allowed to cool and at room temperature and the plates were incubated at 35°C for 24h before used to confirm sterility. Then spreader 100 μl of the bacterial suspension (1.5×10^8) on the surface of agar by using sterile spreader. Wells of 5 mm in diameter and 3 mm in depth were made on the agar plate and were filled by 100 μl of protein solution and incubated aerobically at 37°C for 24h, the inhibition zone was measured in millimeters.

Isolation and purification of AMP

Antimicrobial protein was isolation and purification according to the method of Kumar *et al.* (2014) with some modifications from author (saturation percentage and type of gel filtration). Chickpea seeds (100 g) were soaked in deionized distilled water for 24h at 4°C, then, homogenized in an electrical blender by 0.01 M Tris-HCl buffer solution pH 7.2 for 5

min, subsequently centrifuged at 10000 rpm for 30 min at 4°C, the supernatant was treated with (NH₄)₂SO₄ by 80% saturation for 6h with stirring at 4°C, then centrifuged at 10000 rpm for 30 min at 4°C, the precipitate was dialyzed through (Dialysis membrane Spectra/Por® 7 MWCO 10000 Da) against 0.01 M Tris-HCl buffer solution pH 7.2 for 24h at 4°C and concentrated by freeze dryer, subsequently 10 ml (10 mg.ml⁻¹) of crude extract was loaded onto Sephacryl S-200 column (1.5×60 cm) which was equilibrated with 0.01 M Tris-HCl buffer solution pH 7.2 containing 0.15 M NaCl, the proteins were eluted using the same buffer solution at a flow rate of 18 ml.h⁻¹. All fractions were monitored at 280 nm and tested for antimicrobial activity, then the active fractions were collected, dialyzed using deionized distilled water for 12h at 4°C, and freeze dried.

Characterization of AMP

The gel filtration Sephacryl S-200 was used for estimating the molecular mass of the proteins according to the method of Al-Soufi *et al.* (2016) using a column 1.5×60 cm with standard proteins Lysozyme, Trypsin, Ovalbumin and Lactoferrin with molecular mass of 14400, 23000, 43000 and 81000 Da, respectively.

The effect of pH on AMP activity was estimated by dissolving 10 µg of AMP in 3 mL of the 0.05 M citrate phosphate, sodium acetate, sodium phosphate, and Tris-HCl buffer pH 3.5-4.5, 5.0-5.5, 6.0-7.0 and 7.5-9, respectively at 25°C for 1h. Then estimated of antimicrobial activities as a method of Sakthivel & Palani (2016).

The effect of temperature on AMP activity was estimated by dissolving 10 µg of AMP in 3 ml of the 0.05 M Tris-HCl buffer at pH 7.5 and incubating the mixture at 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80°C for 60 min.

then the antimicrobial activities were estimated by the method of Sakthivel & Palani (2016)

Application

The total bacterial count was estimated by pour plate method preparing dilutions 10⁻¹, 10⁻², and 10⁻³ for 5 g of beef patties meat using peptone water (0.1%). The number of bacteria (CFU.g⁻¹) was calculated after incubation of the samples at 37°C for 24-48h according to the method of Alsoufi & Aziz (2022).

The optimum concentration of purified AMP was estimated using wells diffusion method by adding 0.1 ml (25, 50, 75 and 100 mg) of purified AMP to the wells and incubating them at 37°C for 24h, the inhibition zone was measured in millimeters for microorganism's strains (Gupta *et al.*, 2016).

Extending shelf life

The beef patties were preparation under aseptic conditions to prevent contaminations. The extended shelf life was estimated by adding 0.01 g of purified AMP to 100 g of meat [0.01% (w/w)] and the all was mixed by blending well, then the patties were wrapped with polyethylene film and storing for 10 d at 4°C (Przybylski, *et al.*, 2016).

Statistical analysis

The Statistical Analysis System-SAS (SAS, 2018) program was used to detect the effects of different factors in the study parameters. Least significant difference-LSD test (Analysis of Variation-ANOVA) was used to significantly compare between means in this study.

Results & Discussion

Purification of AMP

The precipitation of protein from chickpea (*C. arietinum*) seeds was achieved using 80% saturation of ammonium sulphate to isolate the

AMP and get rid of other non-antimicrobial proteins. The purification by Sephacryl S-200 (Fig. 1) gave many proteins peaks at 280 nm, while the test of antibacterial activity showed that fourth peak had the ability to inhibit the growth of *E. coli* and *S. typhimurium* with inhibition zones 24 and 22 mm, respectively.

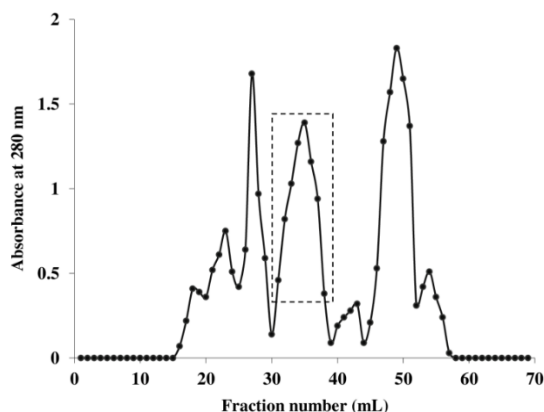


Fig. (1): Purification of antimicrobial protein (AMP) from chickpea (*Cicer arietinum* L.) seeds using Sephacryl S-200 column (1.5×60cm) with 0.01 M Tris-HCl buffer solution pH 7.2 containing 0.15 M NaCl as a buffer solution for equilibration and elution at flow rate of 18 ml.h⁻¹.

More than one method was used for the purification of AMPs from the plant sources, as in Muhammad *et al.* (2019) the purification of AMP from *D. stramonium* seed using ammonium sulphate 20-80% saturation and CM Sephadex cation exchange gel column observed higher activity of basic proteins compared with acidic proteins against *E. coli* and *Klebsiella pneumoniae*, or Chen *et al.* (2018) which used ammonium sulphate 40-80% saturation, DEAE-cellulose anion-exchange column, and gel-filtration chromatography using Sephadex G-50 to purify AMPs from *G. elata* Blume tubers. While, Sakthivel & Palani (2016) showed that the inhibition activity was 19 and 18 mm against *B. cereus* and *E. coli*, respectively for purified protein from *B. purpurea* by using

ammonium sulphate (60-80%) and CM-Cellulose ion exchange chromatography. Also, Thapliyal *et al.* (2016) noticed that the extracted AMPs from *Ficus glomerata* leaves had a strong inhibition activity against some types of bacteria, the inhibition zones in growth agars of *Pseudomonas aeruginosa*, *E. coli*, *Salmonella enterica*, and *Bacillus subtilis* was 19.6, 18.3, 18 and 17.3 mm, respectively. Also, Kumar *et al.* (2014) found that the purified AMPs from *C. arietinum* using 30% saturation ammonium sulphate and Sephadex G-100 gel filtration column had antifungal activity against human pathogenic fungi.

Molecular mass

The molecular mass of AMP was 28385 Da as estimated by Sephacryl S- 200 (Fig. 2).

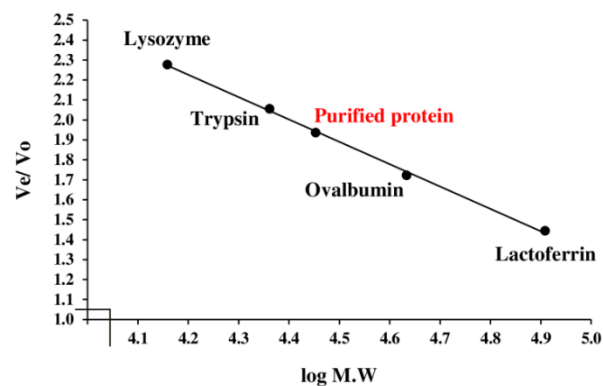


Fig. (2): Molecular mass of purified antimicrobial protein (AMP) from chickpea (*Cicer arietinum* L.) seeds using Sephacryl S-200.

The antimicrobial activity of some proteins in plants depends on many characteristics such as the molecular weight, structures, net charge, sequence and nature of amino acids (Sakthivel & Palani, 2016). In general, the molecular masses of proteins were estimated by gel filtration chromatography and SDS-PAGE method, therefore it may give various values depending on the source of protein and the method of estimation (Al-Soufi *et al.*, 2016). The molecular mass was 20900, 35000, 25000, 27000, 30000 and 35000 Da of purified protein

from *B. purpurea* (Sakthivel & Palani, 2016), *F. glomerata* leaf (Thapliyal *et al.*, 2016), *C. arietinum* (Kumar *et al.*, 2014), *Bauhinia forficata* (Silva *et al.*, 2012), *Bauhinia unguulate* (Silva *et al.*, 2014) and *Murraya koenigii* (Ningappa *et al.*, 2010), respectively by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as a method for estimation.

Effect of pH

The optimum pH for the activity of purified AMP was 5.5. It was stable at 4.5-7.5, while it lost 21.07 and 55.65% from its activity at pH 3 and 8 respectively (Fig. 3).

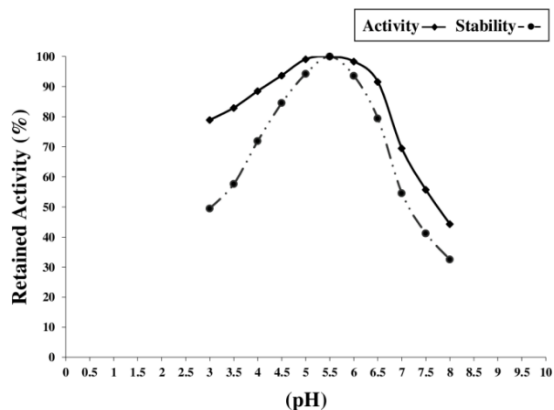


Fig. (3): Effect of pH on purified antimicrobial protein (AMP) from chickpea (*Cicer arietinum* L.) seeds.

The differences in optimum pH among proteins are attributed to the variance in physiological functions in the cells (Al-Soufi *et al.*, 2016), as shown in the results of many research in this field, as Chen *et al.* (2018) indicated that the optimum pH of purified AMPs from *G. elata* BI tubers at pH 6 against *S. aureus* and *Candida albicans*. Also, Sakthivel & Palani (2016) showed that antimicrobial activity of purified proteins from *B. purpurea* was observed at pH below 3.0 and above 9.0, and the optimum pH was 5.5. Also, Zheng *et al.* (2010) observed that proteins from *Clitocybe sinopica* showed active inhibition at

pH 5.0-8.0, while not showing any activity at pH below 3.0 and above 9.0.

Effect of temperature

The optimum temperature for the activity of purified AMP was 35 and it was stable at 35°C for 60 min, while it lost all inhibitory activity at 65°C for the same time (Fig. 4).

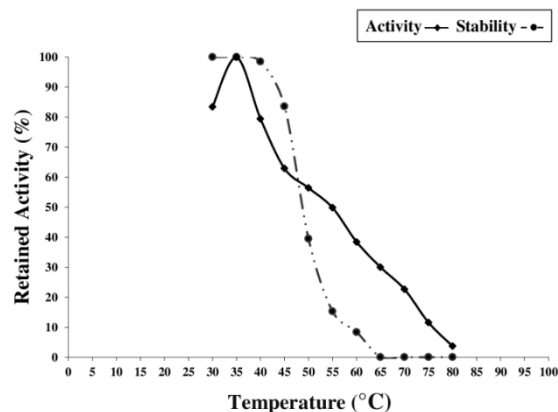


Fig. (4): Effect of temperature on purified antimicrobial protein (AMP) from chickpea (*Cicer arietinum* L.) seeds.

The optimum temperature of AMP activity can differ depending on the source of purification, the differences are attributed to the variance in the number and type of amino acids, components (metal and carbohydrate), and the type of bonds in the protein structure (Al-Soufi *et al.*, 2016). On this basis, Chen *et al.* (2018) observed that the purified protein from *G. elata* BI tubers showed good thermal stability up to 60°C. While, Sakthivel & Palani (2016) showed that the purified AMPs from seedlings of *B. purpurea* exhibited optimum antimicrobial activity at 35°C. It was stable for 60min at the same temperature, while, it lost inhibition activity at 65°C. Also, Zheng *et al.* (2010) observed that the purified protein from *C. sinopica* was 100% inactivated after incubation for 30 min at 80°C.

Extending shelf life

The results of the optimum concentration of purified AMPs are shown in (Table 1) which indicates that the use of 100 mg of AMP gives a higher inhibition compared to others concentrations (25, 50 and 75 mg) used in this study, the results showed that inhibition zone of *E. coli*, *S. typhimurium*, *S. aureus* and *B. cereus* were 22±2.37, 20±1.08, 18±0.77 and 16±0.81 mm respectively, at 25 mg; 27±2.44, 26±1.93, 21±1.17 and 22±1.52 mm respectively, at 50 mg; 30±2.41, 29±2.52, 27±1.38 and 29±2.20 mm respectively, at 75 mg; and 37±2.92, 35±1.68, 32±2.33 and 33±2.09 mm respectively. At 100 mg of purified AMP, the LSD values were 5.49, 4.91,

5.06 and 5.17 at (P≤0.05) for *E. coli*, *S. typhimurium*, *S. aureus* and *B. cereus* respectively, therefore, this concentration was used for extending shelf life of minced beef, it was noted that the total bacterial count for the control sample was 7.4×10²±0.23, 8.2×10²±0.31 and 9.5×10²±0.27 CFU.g⁻¹, while being 7.4×10²±0.18, 4.6×10²±0.22 and 2.8×10²±0.19 CFU.g⁻¹ for the study sample treated with 100 mg of purified AMPs stored for 0, 3 and 6 d respectively, at 4°C, the LSD value was 2.35 NS and 2.96 at (P≤0.05) for control and (AMP) respectively, while, the LSD value for the treated meat (control and AMP) was 2.37 NS, 3.07 and 3.66 at (P≤0.05) stored for 0, 3 and 6 d respectively, at 4°C (Table 2).

Table (1): Effect of concentration for purified antimicrobial protein (AMP) from chickpea (*Cicer arietinum* L.) seeds on inhibition of growth of types of bacteria.

Microorganism's strains	Inhibition zone (mm)				LSD value
	Antimicrobial protein (AMP) from chickpea seeds				
	25 (mg)	50 (mg)	75 (mg)	100 (mg)	
<i>E. coli</i>	22±2.37c	27±2.44bc	30±2.41b	37±2.92a	5.49*
<i>S. typhimurium</i>	20±1.08c	26±1.93b	29±2.52b	35±1.68a	4.91*
<i>S. aureus</i>	18±0.77b	21±1.17b	27±1.38a	32±2.33a	5.06*
<i>B. cereus</i>	16±0.81c	22±1.52b	29±2.20a	33±2.09a	5.17*
Average	19±1.03c	24±2.08b	28.75±2.61b	34.25±2.52a	4.88*

Means having with the different letters in same row differed significantly.

*(P≤0.05): Significant

Table (2): Total bacterial count (CFU.g⁻¹) of treated 100 g of beef patties by 0.01 g of purified antimicrobial protein (AMP) from chickpea (*Cicer arietinum* L.) seeds that storage for 0, 3 and 6 d at 4°C.

Treatment	Storage (d)			LSD value
	(CFU.g ⁻¹)			
	0	3	6	
Control	7.4×10 ² ±0.23a	8.2×10 ² ±0.31a	9.5×10 ² ±0.27a	2.35 NS
Antimicrobial protein from chickpea seeds (AMP)	7.4×10 ² ±0.18a	4.6×10 ² ±0.22b	2.8×10 ² ±0.19b	2.96*
LSD value	2.37 NS**	3.07*	3.66*	---

Means having with the different letters in same row differed significantly.

*(P≤0.05): Significant

**NS: Non significant

The use of herbs, plant extracts, and biological products from safe microorganisms in food has increased recently due to its many benefits such as improving flavor, and taste and extending shelf life because they include biological compounds with antimicrobial activity against pathogenic microorganisms that cause food spoilage (Al-Soufi, 2015; Al-Sahlany, 2017; Alsoufi & Aziz, 2017; Alsoufi & Aziz, 2021). Antimicrobial peptides/proteins (AMPs) are a natural compounds that found in plants, animals, and microorganisms that are responsible for defense of host from pathogenic infections. Therefore, they use be alone or in combination with other antimicrobial in food industries an alternative to the chemical preservatives to extending shelf life of foods (Rai *et al.*, 2016).

In this context. Alsoufi & Aziz (2022) found that treated minced beef with Welsh onion extract and killer toxin from bakery yeast led to a decrease in the total bacterial and peroxide values during storage for 6 d at 4°C. Also, the use of edible films that made from a mix of whey proteins and chitosan on fresh cut turkey pieces inoculated with *S. typhimurium* and *E. coli* stopped the growth of these bacteria during storage for 6 d at 5°C (Brink *et al.*, 2019). While, Al Akeel *et al.* (2018) showed that crude and purified proteins from *C. sativus* seeds could be used as antimicrobial agents when added to food products to extend the shelf life due its effectiveness on both G⁺ and G⁻ bacteria such as *S. aureus*, *E. coli*, *P. aeruginosa*, and *Proteus vulgaris*. Also; the use of antimicrobial peptide derived from slaughterhouse byproduct inhibited the microbial growths for beef patty during storage for 14 d at 4°C (Przybylski, *et al.*, 2016). Many antimicrobial peptides/proteins as a preservative for extending shelf life was widely used in meat production, such as Nisin

(Rai *et al.*, 2016), bacteriocin (Galvez, *et al.*, 2014).

The mechanism of bacterial inhibition is its antimicrobial effect on the bacterial surface layers which leads to damage to the cell membrane (Sakthivel & Palani, 2016). Also, the antimicrobial activity is attributed to targeting some intracellular biological activities such as DNA synthesis (Al Akeel *et al.*, 2018).

Conclusion

This study showed the ability of purified antimicrobial protein (AMP) from chickpea (*C. arietinum*) seeds in extending the shelf life of beef patties during storage.

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Contributions of authors

M. A. A.: Research proposal, purification of antimicrobial proteins, application, explaining of results, and manuscript writing.

R. A. A.: Providing and dealing with microorganism strains, characterization of antimicrobial proteins, application, explaining of results, and manuscript writing.

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Conflict of interest

The authors declare that the present study was performed in the absence of any conflict of interest.

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عزل وتنقية البروتينات المضادة للأحياء المجهرية من بذور الحمص *Cicer arietinum* واستعمالها في إطالة العمر الخزني للحم البقر المفروم

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المستخلص: تم عزل وتنقية البروتين المضاد المضادة للأحياء المجهرية من بذور الحمص (*Cicer arietinum* L.) باستعمال 80% تشبع من كبريتات الامونيوم والترشيع الهلامي باستعمال Sephacryl S-200 وظهرت القمة المفصولة قطر تثبيطي مقداره 24 و 22 ملم تجاه كل من بكتريا *Escherichia coli* و *Salmonella typhimurium* على التوالي، وكان الوزن الجزيئي 28385 دالتون عند تقديره باستعمال الترشيح الهلامي بعمود Sephacryl S-200، وبلغ الاس الهيدروجيني الأمثل للفعالية 5.5 وكان البروتين ثابتا عند 4.5-7.5، بينما فقد 21.07 و 55.65% من فعاليته الاصلية عند الأس الهيدروجيني 3 و 8 على التوالي، ولوحظ ان درجة الحرارة المثلى لفعالية البروتين المنقى 35م، وكان ثابتا عند درجة حرارة 35م لمدة 60 دقيقة، بينما فقد كامل فعاليته التثبيطي عند درجة حرارة 65م لنفس الوقت، وادى استعمال البروتين المنقى بتركيز 100 ملغم الى الحصول على قطر تثبيطي مقداره 37±2.92 و 35±1.68 و 32±2.33 و 33±2.09 ملم، بوجود فرق معنوي عند (P≤0.05) تجاه بكتريا *S. typhimurium* و *Staphylococcus aureus* و *Bacillus cereus* على التوالي، كما ادى استعمال البروتين المنقى لإطالة العمر الخزني للحم البقر المفروم بتركيز 100 ملغم.كغم⁻¹ الى انخفاض العدد الكلي للبكتريا، اذ بلغ $7.4 \times 10^2 \pm 0.18$ و $4.6 \times 10^2 \pm 0.22$ و $2.8 \times 10^2 \pm 0.19$ وحدة تكوين مستعمرة.غم⁻¹، بينما كان $7.4 \times 10^2 \pm 0.23$ و $8.2 \times 10^2 \pm 0.31$ و $9.5 \times 10^2 \pm 0.27$ وحدة تكوين مستعمرة.غم⁻¹ في عينة السيطرة خلال مدة خزن 0 و 3 و 6 يوم بدرجة حرارة 4م، ولوحظ عدم وجود فرق معنوي في معاملة السيطرة والبروتين المضاف عند وقت الصفر، بينما لوحظ حدوث فرق معنوي عند (P≤0.05) للمعاملات المذكورة عند مدة خزن 3 و 6 يوم بدرجة حرارة 4م.

الكلمات المفتاحية: البروتينات المضادة للأحياء المجهرية، اللحم المفروم، بذور الحمص، اطالة العمر الخزني للغذاء، تثبيط الاحياء المجهرية.