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Modern methods for extraction and purification of colicin produced from Escherichia coli

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ABSTRACT:

The current study aimed to extract and purify colicin extracted from *Escherichia coli (E,coli)*. One isolate was selected from 10 bacterial isolates obtained from Hilla Teaching Hospital in Hilla Governorate / Babylon, and all diagnostic and biochemical tests were carried out to ensure the purity of the bacterial isolates and to investigate their production of bacteriocin. Bacteriocin was extracted by centrifuging the bacterial suspension with a cooling centrifuge and concentrated by ammonium sulfate at a saturation rate of 70% and then by dialysis and ultrafiltration to get clear of the largest possible percentage of salts and impurities present in the solution. The protein extract was purified by SDS-PAGE polyacrylamide technique and its purity was confirmed by the appearance of a single band when electrophoresis was performed on polyacrylamide gel in the presence of SDS denaturants. The results of protein characterization showed that the molecular weight by electrophoresis on polyacrylamide gel was estimated to be 66 kDa. It was shown by matching the molecular weight of the protein with the molecular weights of standard proteins that the protein is colicin.

Keywords: *Escherichia coli*, colicin, ammonium sulfate, dialysis, ultrafiltration, SDS Page polyacrylamide.

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Introduction

Bacteriocins are proteinaceous or peptidictoxins produced by bacteria to inhibit the growth of similar or closely related bacterial strains. They are similar to yeast and paramecium killing factors, and are structurally, functionally, and ecologically diverse. Applications of bacteriocins are being tested to assess their application as narrow-spectrum antibiotics (Cotter, Ross *et al*. 2013).

Bacteriocins were first discovered by André Gratia in 1925 (Gratia 1925). He was involved in the process of searching for ways to kill bacteria, which also resulted in the development of antibiotics and the discovery of bacteriophage, all within a span of a few years. He called his first discovery a colicin because it was made by *E.coli*.

The bacteriocins from *E.coli* are called colicins called 'colicines', meaning 'coli killers'). They are the longest studied bacteriocins. They are a diverse group of bacteriocins and do not include all the bacteriocins produced by *E.coli*. In fact, one of the oldest known so-called colicins was called colicin V and is now known as microcin V. It is much smaller and produced and secreted in a different manner than the classic colicins.

The *E.coli* is a gram-negative, facultative anaerobe, nonsporulating coliform bacterium (Hada, Chaturvedi et al. 2023). Cells are typically rod-shaped, and are about 2.0 μm long and 0.25–1.0 μm in diameter, with a cell volume of 0.6–0.7 μm3 (Yu, Loo et al. 2014).

Colicins are plasmid-encoded protein antibiotics produced by some strains of Escherichia coli under conditions of nutritional or environmental stress to eliminate other closely related strains, thereby enhancing the chances of survival of the colicin-producing strain (Cascales, Buchanan *et al*. 2007). Colicins have been shown to promote microbial diversity within structured environments such as the mammalian colon (Kirkup and Riley 2004). Once released from the producing cell, the colicin gains entry into its target bacterium through the parasitization of proteins within the outer membrane and periplasm (Lazdunski, Bouveret *et al*. 1998).

Colicins are grouped according to the periplasmic proteins with which they interact to facilitate delivery of their cytotoxic domains into the target cell. Group A colicins bind components of the Tol system, while group B colicins interact with members of the Ton system. Both the Tol and Ton systems access the inner membrane proton motive force; therefore, interacting with these systems may provide energy for driving the colicin into the cell. The protein–protein interactions that occur between colicins and proteins within their target bacteria are critical to the processes that bring about cell killing. Here, we examine these interactions for two group A colicins, the pore-forming colicin N (Bourdineaud, Boulanger *et al*. 1990) that brings about cell killing through destruction of the proton motive force, and the nonspecific DNase colicin E9 (Housden and Kleanthous 2011). The receptor-binding and translocation domains of eight enzymatic E colicins (E2–E9) are highly conserved, indicating a common mechanism for the delivery of varied toxic enzymes to the bacterial cytoplasm. The cytotoxic nuclease domain of enzymatic E colicins takes the form of a ribosomal RNase, a transfer RNA-specific RNase, or a nonspecific DNase which target the bacterial genomic DNA. Each of the enzymatic E colicins is produced as a heterodimer with its own specific immunity protein to prevent the colicin-producing cell from committing suicide (Kleanthous and Walker 2001).

2. Methods

2.1. Bacteriocin extraction and Purification

Precipitation with Ammonium Sulfate

Ammonium sulfate is used in precipitation because it has high solubility, availability, reasonable cost, and does not harm enzymes or proteins (Ryan, Kinsella et al. 2023).

Ammonium sulfate is used in precipitation because it increases the ionic strength of the solution and reduces the solubility of the protein, thus leading to the precipitation of the protein from the solution (Burgess 2009).

Certain weights of ammonium sulfate crystals were gradually added to the crude extract with continuous stirring to obtain a saturation rate of 70%. The solution was discarded after each addition at a speed of 3000 rpm at a

temperature of (0-4)°C for 15 minutes. The clear solution was separated and another weight of ammonium sulfate was added to it to obtain The final percentage of saturation, As in the procedure detailed below in the book of protein methods according to (Fisher, Cabelli *et al*. 1994).

The procedure:

1) The baker containing the protein solution was placed in a another cold baker on top of the magnetic stir plate.

2) The 56.8g of ammonium sulfate was added slowly, While stirring gently with on a magnetic stirrer. The salt was added more slowly when reaching final saturation. This step was completed in 5-10 minutes.

3) The stirring was performed continuously for 10-30 minutes after adding all the salt.

4) The mixture was centrifuged at 10,000 x g for ten minutes.

5) The supernatant was collected using a centrifuge.

6) Ammonium sulfate was removed by the following dialysis or ultrafiltration.

Dialysis

Dialysis is usually used to remove salts from extracted protein, i.e. it is used to change the buffer solution of the protein, but it is also used as a method to concentrate protein solutions if carried out in a hygroscopic environment.

- **Preparing the dialysis tubing**

The dialysis tubing or sack contains chemical contaminants from manufacturing processes. To remove them, the tube is supposed to be boiled for 30 min in 10 μM sodium bicarbonate (NaHCO3)/1 μM EDTA (Richmond, Denis *et al*. 1985). After boiling, the tubing should be washed with distilled water and kept at 4°C to prevent microbial contamination. Prepare dialysis tubes every six months (Schleif and Wensink 2012).

Procedure:

1) Two tight knots were formed at one end of the tube.

2) The protein solution was added to the dialysis tubing.

3) A knot was tightly tied at the other end of the tubing. Then the tube was putted in more than 10 sizes of dialysis buffer (phosphate buffered saline). The beaker was moved gently with a magnetic stirrer at a cooling temperature, as equilibrium happen after several hours of dialysis. The dialysis beaker was changed several times until some of the buffer components are sufficiently diluted.

Ultrafiltration

This method is less likely to denature the protein than other precipitation methods, which are carried out according to the following protocol, according to (Thermo Scientific company).

1. The molecular weight cut-off (MWCO) that best suits the sample was chosen. For maximum recovery, a MWCO 10,000 Da was selected, According to the molecular weight of the protein.
2. The maximum of 10 ml of the sample was added to the filtration tube according to the instructions of the tube and device.
3. If the sample is thick or smaller than required tube size, it was diluted with distilled water to reach the appropriation size, This helps increase the dissolution of the salt and precipitation the sample faster .
4. The sample was centrifuged at an appropriate spin speed 4,500 x g for 30 min in cooling centrifuge.
5. The sediment was discard in the filtrate container.
6. The filtration tube was refilled with an appropriate sample.
7. The centrifuge was performed one more time as before.
8. The sediment was empty in the filtrate container.
9. The filtration tube was recovered and the sample was drawn from the bottom of the tube pocket with a pipette.

10. **preparation the SDS PAGE Polyacrylamide**

The discontinuous vertical gel system was used in polyacrylamide. This means that the gel consists of two different layers. The lower gel layer was poured that prepared with the additives in table (3-9) at a medium speed to ensure that bubbles do not occur that affect the protein separation process, the gel polymerization process will take place in 60 min. After the casting process, the upper gel was prepared with the same components as the lower gel with different additives as in Table (1), which is approximately 1.5 cm high. The comb was placed and the cassette is closed before the polymerization process, which takes place in 30 min. Acrylamide was used at a low concentration of 12.5%, according to the molecular weight for protein, remove the comb after pouring the Running buffer, then connect the device to the electric current to carry out the electrophoresis process at a voltage of 60 V for 45 minutes. After separating the protein, the gel was removed and cover it completely with Coomassie Blue dye for 24 hours to identify the bands indicating the protein.

Table (1) : Calculation for X% Separation gel and Stacking gel

Gel name	Reagents					
	Acrylamide solution	Separating gel buffer	Stacking gel buffer	H2O	10% ammonium persulfate	TEMED
Separating gel	2.7 ml	2.5 ml	0 ml	4.8 ml	50 ml	5 µL
Stacking gel	0.67 ml	0 ml	1.0 ml	2.3 ml	30 ml	5 µL

3. Results

3.1. Bacteriocin extraction and purification

Precipitation with ammonium sulfate

When ammonium sulfate is added, the solubility layer will be compressed and protein interactions will increase. Which leads to an increase in the concentration of the salt buffer solution, so that the water mass becomes more bound with the ions, and thus the amounts of water available to participate in the protein solubilization layer will decrease. The protein may exhibit hydrophobic interactions and the solution precipitates completely.

According to studies that proved that a saturation level of 80% of ammonium sulfate is used to precipitate bacteriocins (Lü, Yi *et al*. 2014), bacteriocin produced from *E.coli* was precipitated with 70% ammonium sulfate at 0 ºC, since the low temperature of thawing avoids the risk of protein denaturation that can occur when the sample temperature rises . This is because the increasing of salt concentration has a negative effect on the production of bacteriocin, which leads to an inhibitory effect and an effective decrease in bacteriocin (Leroy and De Vuyst 1999).

Other studies have pointed out that although ammonium sulfate precipitation is popular, there are still limitations to this method. For example, there is no clear convention for ammonium sulfate saturation in published protocols (Burgess 2009). However, some bacteriocins do not precipitate even at high ammonium sulfate concentration of 75–80% because they are low molecular weight (Borzenkov, Surovtsev *et al*. 2014). I agree with (Leroy and De Vuyst 1999; Burgess 2009; Borzenkov, Surovtsev *et al*. 2014).

Dialysis and Ultrafiltration

The colicin produced from *E.coli* was concentrated by dialysis to remove salts, water, and impurities to obtain a degree of purity. The some compounds were removed with low molecular weights, as well as reduce the ionic strength of the extract and prepare it for the next steps of purification. This step achieved molecular purification of colicin by balancing the concentration inside and outside the membrane. This was achieved by changing the dialysis buffer (phosphate buffered saline) several times at a temperature of 0 ºC, as shown in figure (4-8).

Dialysis was used as a first step in many studies to purify proteins and enzymes. This study is consistent with (Harman, Hayes *et al*. 1993), Which indicated its use in purifying proteins and enzymes extracted from fungi and bacteria.

The protein solution was filtered by ultrafiltration using a 10,000 Da permeation membrane. This membrane allows water and small molecules to pass through it and retain the protein through centrifugation.

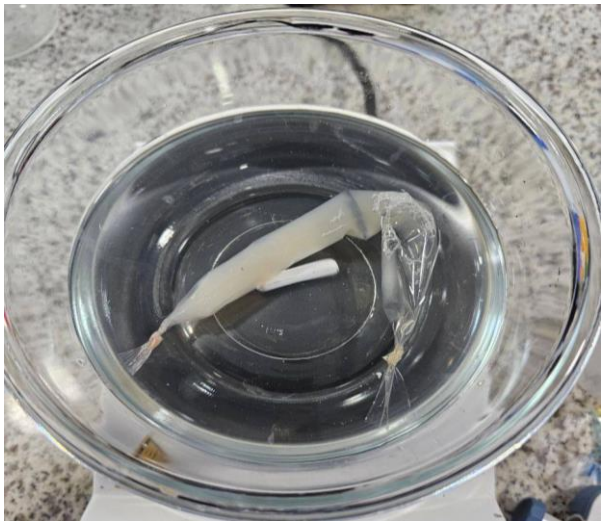

Figure (4-8): Purification of colicin extracted from *E.coli* from salts, water, and impurities by dialysis.

Colicin Electrophoresis via SDS PAGE Polyacrylamide

The colicin was purified by SDS-PAGE polyacrylamide that run in (12.5% acrylamide according to the molecular weight of colicin, low voltage of 60 V to avoid the band appearing as a smear and time 45 min), where the purification of colicin was confirmed by the appearance of a single band in polyacrylamide gel after staining with Coomassie Blue, which is the preferred stain for protein determination, as there are two types of Coomassie Blue: R-250 (R for reddish) and G-250 (G foe greenish) (Simpson 2010). Coomassie Blue type G-250 was used because it requires only water for rinsing and removing the stain, it provides better sensitivity than traditional R-250 formulations and has a simpler and faster protocol (Neuhoff, Arold *et al*. 1988). Then the molecular weight of 66,000 kDa was estimated based on the molecular weight of the standard colicin according for (CUSABIO enterprise). This study is consistent with (Herschman and Helinski 1967, Dankert, Hammond *et al*. 1980) showed that colicins after purification appear to have a high molecular mass ranging from 40,000 kDa to 80,000 kDa.

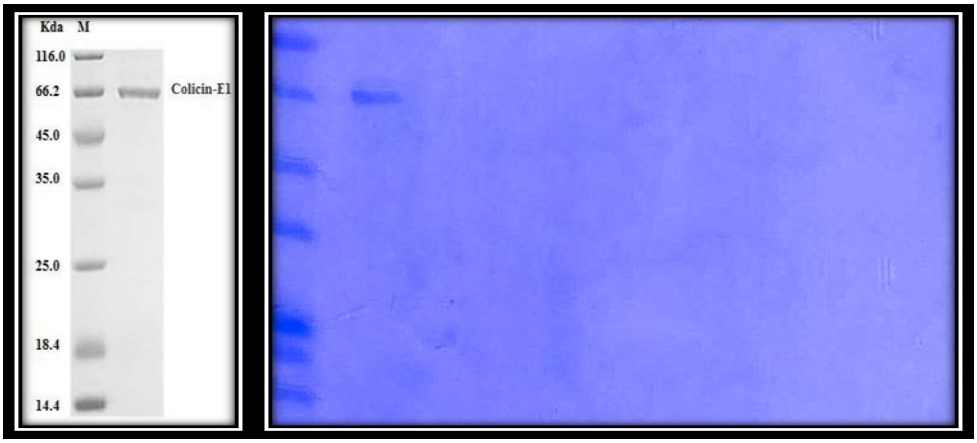

Figure (4-9): The figure shows the appearance of the molecular weight of colicin extracted from E.coli in the form of a packet using SDS PAGE polyacrylamide. Recombinant Escherichia coli Colicin-E1 (cea). CUSABIO, (CUBIO Innovation Center), Houston, USA. (Acrylamide 12.5%, 60 volt, 45 min)

4. Discussion

About 30-40% of pathogenic *E.coli* from various sources were found to synthesize colicin (Bradley 1991). After detection of colicin-producing isolates, mitomycin-C was used as an inducing agent and was used at a concentration of 0.001 mg. Mitomycin-C is one of the most effective methods for producing large amounts of colicin by inducing the pathogenic strain of bacteria with a suitable metabolic inhibitor (Géli and Bénédetti 1994). Mitomycin-C is very convenient to use and increases colicin production by 4-20 times compared to other inducibles such as ultraviolet (UV) radiation (Neff and Bernstein 1976). On the other hand, the medium used for bacterial growth with mitomycin-C led to increased colicin production. Ammonium sulfate was used instead of sonic extraction because (Makia, Ismail *et al*. 2013) indicated that colicin lost its activity using the latter methods, and it was found that extraction with salts with homogenization leads to an increase in specific starch. Also, the increase in salts may negatively affect the production process , where the colicin was precipitated at a saturation rate of 70% ammonium sulfate because (Borzenkov, Surovtsev et al. 2014) indicated that high saturation levels of 75-80% lead to protein denaturation. This study agree with study of (AL-Dhumaina 2009).

The best method to remove salts from colicin was dialysis with phosphate buffered slain. The buffer can be replaced with distilled water because it is a low concentration solution. The dialysis process was carried out by the exit of salts, ions and large molecules from the high concentration solution through the membrane (whose hole size was chosen according to the molecular weight of colicin) to the low concentration solution (outside the membrane) (Kelley 2004). The ultrafiltration process using flat membranes with a molecular weight of 10,000 kDa also provided the best results for purifying colicin. This study agree with (MURIANA and LUCHANSKY 1993). The purified colicin was detected by SDS PAGE polyacrylamide with the appearance of a single band indicating that it was pure colicin by matching its molecular weight with the molecular weights of standard proteins. It was noted that factors such as high voltage may affect the results of electrophoresis of colicin. The final product of pure colchicine was obtained in an amount of 2 grams as a product of a single bacterial isolate (*E.coli*). It can be used as an alternative to antibiotics after revealing its antibacterial effectiveness against many pathogenic bacterial isolates, as well as its application to cancer cell lines after taking into account the optimal conditions that affect its action (Yaghoubi, Khazaei *et al*. 2020). This study agree with (Yang, Johnson *et al*. 1992).

REFERENCES

1. AL-Dhumaina, T. F. H. (2009). "EXTRACTION AND PURIFICATION OF COLICIN PRODUCED BYESCHERICHIA COLI ISOLATED FROM URINARY TRACT INFECTION." Journal of Techniques **22**(4).
2. Borzenkov, V., et al. (2014). "Obtaining bacteriocins by chromatographic methods." Advances in Bioscience and Biotechnology **5**(05): 446-451.
3. Bourdineaud, J. P., et al. (1990). "In vivo properties of colicin A: channel activity is voltage dependent but translocation may be voltage independent." Proceedings of the National Academy of Sciences **87**(3): 1037-1041.
4. Bradley, D. E. (1991). "Colicinogeny of O55 EPEC diarrhoeagenic Escherichia coli." FEMS microbiology letters **90**(1): 49-52.
5. Burgess, R. R. (2009). "Protein precipitation techniques." Methods in enzymology **463**: 331-342.
6. Cascales, E., et al. (2007). "Colicin biology." Microbiology and molecular biology reviews **71**(1): 158-229.
7. Cotter, P. D., et al. (2013). "Bacteriocins—a viable alternative to antibiotics?" Nature Reviews Microbiology **11**(2): 95-105.
8. Dankert, J., et al. (1980). "Reversal by trypsin of the inhibition of active transport by colicin E1." Journal of bacteriology **143**(2): 594-602.

9. Fisher, C. L., et al. (1994). "The role of arginine 143 in the electrostatics and mechanism of Cu, Zn superoxide dismutase: computational and experimental evaluation by mutational analysis." Proteins: Structure, Function, and Bioinformatics **19**(1): 24-34.

10. Géli, V. and H. Bénédetti (1994). "Insertion of Proteins into Membranes A Survey." Membrane Biogenesis: 21-69.

11. Gratia, A. (1925). "On a remarkable example of antagonism between two strains of hummingbird." Comptes Rendus Biologies (CR BIOL) **93**: 1040-1042.

12. Hada, V., et al. (2023). "Nanoantibiotic effect of carbon-based nanocomposites: epicentric on graphene, carbon nanotubes and fullerene composites: a review." 3 Biotech **13**(5): 147.

13. Harman, G., et al. (1993). "Chitinolytic enzymes of Trichoderma harzianum: purification of chitobiosidase and endochitinase." Phytopathology **83**(3): 313-318.

14. Herschman, H. R. and D. R. Helinski (1967). "Purification and characterization of colicin E2 and colicin E3." Journal of Biological Chemistry **242**(22): 5360-5368.

15. Housden, N. G. and C. Kleanthous (2011). "Thermodynamic dissection of colicin interactions." Methods in enzymology **488**: 123-145.

16. Kelley, K. T. (2004). "How peritoneal dialysis works." Nephrology nursing journal **31**(5): 481.

17. Kirkup, B. C. and M. A. Riley (2004). "Antibiotic-mediated antagonism leads to a bacterial game of rock–paper–scissors in vivo." Nature **428**(6981): 412-414.

18. Kleanthous, C. and D. Walker (2001). "Immunity proteins: enzyme inhibitors that avoid the active site." Trends in biochemical sciences **26**(10): 624-631.

19. Lazdunski, C. J., et al. (1998). "Colicin import into Escherichia coli cells." Journal of bacteriology **180**(19): 4993-5002.

20. Leroy, F. d. r. and L. De Vuyst (1999). "Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin sakacin K." Applied and Environmental Microbiology **65**(3): 974-981.

21. Lü, X., et al. (2014). "Purification of novel bacteriocin produced by Lactobacillus coryniformis MXJ 32 for inhibiting bacterial foodborne pathogens including antibiotic-resistant microorganisms." Food control **46**: 264-271.

22. Makia, R. S., et al. (2013). "Detection of plasmid DNA profile in bacterial and fungal isolates from catheterized patients and its relation with antibiotic susceptibility." Journal of Biotechnology Research Center **7**(2): 52-62.

23. MURIANA, P. M. and J. B. LUCHANSKY (1993). Biochemical methods for purification of bacteriocins. Bacteriocins of lactic acid bacteria, Elsevier**:** 41-61.

24. Neff, D. N. and A. Bernstein (1976). "Purification and physical characterization of colicin 0 111." Archives of Biochemistry and Biophysics **176**(1): 144-153.

25. Neuhoff, V., et al. (1988). "Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250." Electrophoresis **9**(6): 255-262.

26. Richmond, V. L., et al. (1985). "Treatment of dialysis membranes for simultaneous dialysis and concentration." Analytical biochemistry **145**(2): 343-350.

27. Ryan, B. J., et al. (2023). Protein Extraction and Purification by Differential Solubilization. Protein Chromatography: Methods and Protocols, Springer**:** 349-368.

28. Schleif, R. F. and P. C. Wensink (2012). Practical methods in molecular biology, Springer Science & Business Media.

29. Simpson, R. J. (2010). "Rapid coomassie blue staining of protein gels." Cold Spring Harbor Protocols **2010**(4): pdb. prot5413.

30. Yaghoubi, A., et al. (2020). "The bacterial instrument as a promising therapy for colon cancer." International journal of colorectal disease **35**: 595-606.

31. Yang, R., et al. (1992). "Novel method to extract large amounts of bacteriocins from lactic acid bacteria." Applied and Environmental Microbiology **58**(10): 3355-3359.

32. Yu, A. C., et al. (2014). "Monitoring bacterial growth using tunable resistive pulse sensing with a pore-based technique." Applied microbiology and biotechnology **98**: 855-862.