

Molecular Characterization of Restriction Endonuclease Extracted from Local *E.coli* Isolate

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Abstract

An endonucleases enzyme named *EcoRHI* has been purified partially from one of pathogenic *E.coli* isolate about 87-fold with endonuclease recovery about 12.6% . The purification steps included ammonium sulfate precipitation with 50% saturation ratio, and the enzymatic solution reclaimed it through Sephadex G-100 column .The SDS-PAGE analysis that showed molecular weight of *EcoRHI* enzyme is 45 KDa. The purified endonuclease was able to digest λ -DNA into two fragments.

Introduction

Bacteria in their environment face many challenges , One of the most challenges is phages attack. To prevent this dramatic end ,bacteria have developed Restriction–Modification (R-M) system to destroy invading DNA. So, that means the Restriction enzymes play a good role as defense system in bacteria that protect host cells against foreign DNA, by cleaving incoming DNA that is recognized as foreign molecules , The restriction enzyme is not harmful to the host cell, because bacteria were developing modification system (Pingoud and Jeltsch, 2001 ; Bourniquel and Bickle, 2002).

R–M systems are mainly classified into four types I, II, III and IV depending on the recognition sequence, cutting position, cleavage requirements and structure. Type I R-M systems are multifunctional enzymes that can catalyze both restriction and modification processes . S-Adenosylmethionine (AdoMet) is the cofactor and methyl donor for the methyltransferase activity, the endonucleolytic activity requires ATP, AdoMet, and Mg^{2+} (Murray, 2000). Type II restriction endonucleases is a more simplified subunit organization. Restriction endonucleases are usually monomeric ,homodimeric or homotetrameric enzymes that cleave DNA within or close to their recognition sites , requiring only Mg^{2+} ions for their activity (Smith and Wilcox, 1970). Type III an endonuclease contains two different subunits or a single subunit. The enzymes require Mg^{2+} as a cofactor and ATP as an allosteric activator.

Modification-dependent restriction enzymes are a very diverse group, and in the current classification of nucleases fall either into the type IIM where ‘M’ stands for modification-dependent or type IV class (Roberts *et al.*, 2003).

Restriction enzymes have been purified and characterized in many parts of the World two decades ago (Marks *et al.*, 2003), but in Iraq, only a few attempts were made to purify these enzymes (Putrus, 1995), (Al-Khafagi, 1999) (Shikara *et al.* , 2009) from bacteria and (Shikara , 2008; 2010) from fungi.

Materials and methods

Bacterial strains, growth conditions, and reagents.

Lambda DNA was purchased from BIONEER, antibiotics for Antimicrobial Susceptibility Testing was purchased from Bioanalyse/Turkey, Wizard®Genomic DNA Purification Kit provided by Promega was used for DNA purification. The CelLytic B Plus Working Solution was prepared immediately for bacterial cells lysis purchased by Sigma Aldrich.

Activation of bacterial isolates

The bacterial isolates that were collected and activated by culturing them on nutrient broth at 37°C for 24 hrs.

Antibiotic susceptibility testing

Susceptibility of isolates to different antibiotics were tested following Kirby Bauer disc diffusion method using Muller Hinton Agar against selected antibiotics

Table(1): Antibiotics list

Antibiotics	Con. µg/disk	Supplied company
Amikacin (AK)	10	Bioanalyse/Turkey
Ampicillin(AM)	10	Bioanalyse/ Turkey
Gentamicin(CN)	10	Bioanalyse/ Turkey
Tetracycline(TE)	30	Bioanalyse/ Turkey
Doxycycline (DO)	30	Bioanalyse/ Turkey
Imipenem (IPM)	10	Bioanalyse/ Turkey
Nalidixic acid(NA)	30	Bioanalyse/ Turkey
Trimethoprim(TMP)	5	Bioanalyse/ Turkey

Screening for restriction endonuclease

Crude extract of 20 ml overnight grown cultures, was assayed for restriction endonucleases activity on un methylated λ DNA as a substrate. The cell lysis was carried out by using CellLytic B Plus kit that provided by Sigma-Aldirch , The digestion reaction was carried out at 37 °C for 1hr. reaction mixture containing the following composition: 1× of NE Buffer 2 , 0.1 mg/ml BSA, 1 µl of lambda DNA and 4 µl of crude extract with 55µl of reaction bufer. Each sample was loaded on 1% agarose gel.

Protein purification

The endonucleases enzyme was purified from on isolate of locally pathogenic *E. coli* by using some techniques such as precipitation by ammonium sulfate , Gel filtration and electrophoresis by using polyacrylamide gel.

The selection bacterial isolate was inoculated in 1000 ml of LB medium and grown at 37°C with vigorous fermentor. The cells were harvested by centrifugation (16.000 r.p.m for 10 min) at the end of the log phase ,then the cells resuspended in CellLytic B Plus cell lysis reagent (Sigma) containing protease inhibitor for cell lysis .The slurry was centrifuged at 16,000 r.p.m for 90 min at 4°C to remove cellular debris. Cleared lysate was adjusted to 50 mM NaCl and 1.5% streptomycin sulfate and stirred at 4°C for 30 min before centrifugation at 16,000 r.p.m for 20 min to remove nucleic acids and total protein content was spectrophotometrically determined at 280 nm.

The Ammonium sulfate was added as different saturation ratio (40,50,60,70 and 80)% gradually to 5ml of bacterial crude extract that prepared in ice bath until all amount of salt was dissolve , then the solution was centrifuged at 10.000 r.p.m for 10 min , discarded the supernatant and the precipitate was taken and dissolved in B-100 buffer (This buffer was prepared by mixing 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 100 mM NaCl and sterilized by Millipore filter) and dialyzed overnight against the same buffer at 4°C. after examined their ability to digest DNA , 50% ammonium sulfate saturation ratio was loaded onto sephadex G-100 gel was prepared based on the method that described by Stellwagen (1990), by mixing 15 gm of Sephadex G-100 beads 500ml of distal water and incubating in water bath (90°C) for 3hrs. and cooling at R.T. , then the gel was washed with 300 ml of B-100 buffer and mixed by glass rod ,the washing step was repeated twice , the babbles were removed by using vacuum pump. The gel was refill in glass column 1.5cm *50 cm in length , The column was balanced by adding 300 ml of B-100 buffer with flow rate 30ml/hr.

The enzyme was added to the gel gently and rinsed with B-100 buffer with flow rate 30ml/hr. , the fraction was collected with volume 3 ml for each fraction by fraction collector . And the protein concentration was followed by calculated spectrophotometric absorbance at 280 nm for each fraction, And drawn the relationship between them. All fractions that contain enzymatic activity were collected and concentrated by sucrose , then used the concentrated enzyme to digest λ DNA .

Polyacrylamide Gel Electrophoresis of REases

The REases were analyzed through 10% SDS-PAGE (Laemmli , 1970). Columns fractions were loaded on the gel and proteins after electrophoresis were visualized by Coomassie Brilliant Blue staining.

Results and Discussion

Antimicrobial Susceptibility Testing

The effects of different antibiotics on bacterial isolates were investigated and the antibiotic sensitivity was measured depending upon a diameter of inhibition zone (mm) according to (CLSI, 2014).

Escherichia coli is usually a commensal bacterium of humans and animals. Pathogenic variants cause intestinal and extraintestinal infections, including gastroenteritis, urinary tract infection, meningitis, peritonitis, and septicemia (Palmer *et al.*, 2010). *E. coli* is sometimes used as a sentinel for monitoring antimicrobial drug resistance in fecal bacteria because it is found more frequently in a wide range of hosts, acquires resistance easily

(Erb *et al.*,2007).

The result shows that all *E.coli* isolates of bacteria were completely resistant (100%) to ampicillin and piperacillin , the resistant to ampicillin was developed because β -Lactams antibiotics are widely used in human and veterinary medicine to treat human and animal infections, this result of completely resistant agree with those obtained by (Kadhim *et al.*, 2011).This widespread use of antibiotics could be associated with the selection of antibiotic resistance mechanisms in pathogenic and nonpathogenic isolates of *E. coli* (Sunde and Sorum, 1999; Andersen *et al.*, 2005). Whereas 50% of *E .coli* isolates in our study were resistant to Amikacin, Tetracycline, Doxycycline, Nalidixic acid, Trimethoprin/sulphamethoxazol. And 30 % of *E. coli* isolates were resistant to Gentamicin, and 20% of *E .coli* isolates were resistant trimethoprim. These results do not agree the results that obtained by (Kadhim *et al.*, 2011 ; Khudaier, 2012). All isolates (100%) were sensitive to Imipenem.

Table 1: The antibiotic sensitivity of *E. coli* isolates.

Antibiotics	<i>E.coli</i> Isolates									
	1	2	3	4	5	6	7	8	9	10
AK	R	I	I	S	R	R	R	R	S	S
AM	R	R	R	R	R	R	R	R	R	R
CN	R	R	R	I	S	S	S	S	S	S
TE	S	R	S	S	S	R	R	R	S	R
DO	R	R	R	R	S	S	S	S	S	R
TMP	I	I	I	I	S	I	I	I	R	R
IPM	S	S	S	S	I	S	S	I	S	S
NA	R	R	R	R	I	I	I	I	S	R
PRL	R	R	R	R	R	R	R	R	R	R
SXT	R	R	R	R	S	S	S	S	S	R

Genomic profile of bacterial isolates

For DNA extraction we used Wizard®Genomic DNA Purification Kit provided by Promega. By gel electrophoresis (0.7%), 8 isolate of *E. coli* were containing plasmid . This result agrees with result that obtained by (Kalantar *et al.*, 2011) who found from plasmid profiling of antibiotic resistant *E.coli* isolates revealed that the isolates contained various size plasmids.

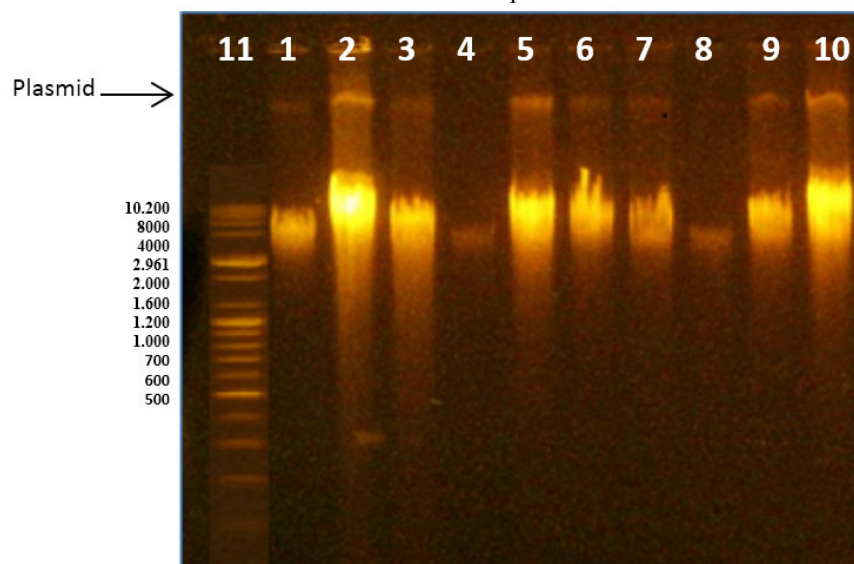


Figure (1): genomic profile of bacterial isolates 0.7% Agarose Gel Electrophoresis, lane 11 DNA marker (100, 200,300, 400,500, 600,700,800,900,1000, 1200, 1600, 2000, 2961,4000, 5007 ,5991, 8000, 10.200), 1-10 *E. coli* isolates.

Extraction and purification of restriction enzymes

Screening of restriction endonucleases

A large number of restriction–modification systems have been discovered and well characterized during the past decades, they occur ubiquitously among bacteria and their few phages (Roberts *et al.*, 2003).

For screening of restriction endonucleases the bacterial isolates were grown on LB at 37°C for 24 hrs. in vigorous fermentor. The cells were harvested by centrifugation (10,000 × g for 10 min) at the end of the log

phase, then the cells resuspended in CelLytic B Plus cell lysis reagent (Sigma) containing protease inhibitor for cell lysis (Chung *et al.*, 2011).without sonication. screening for restriction enzymes in 10 isolates of pathogenic *E. coli*. the study was showed there are no endonuclease activity for 1, 2, 3, 5, 6, 7, 9 and 10 of *E. coli* isolates and same endonuclease activity for 4 and 8 of *E. coli* isolates Figure (2) , this case agreement with Roberts and Halford (1993) they said that there are at least only one restriction enzyme for four bacterial isolates were examined for restriction enzyme activity. *E. coli* isolates were a good sources for restriction enzymes for all types (Meselson and Yuan ,1968; Mise and Nakajima, 1984 ; Kasarjian *et al.*, 2003 ;Kasarjian *et al.*, 2005 ;Lazim *et al.*, 2005 ;Siwek *et al.*, 2012).

To Indicate the recognition sequence we used all information about theoretical digestion of λ -DNA in REBASE, we found there are 23 restriction enzymes giving two fragment when using λ -DNA as a substrate but the enzymes that give cutting like our enzyme were *BmtI* and *NheI* .figure 3

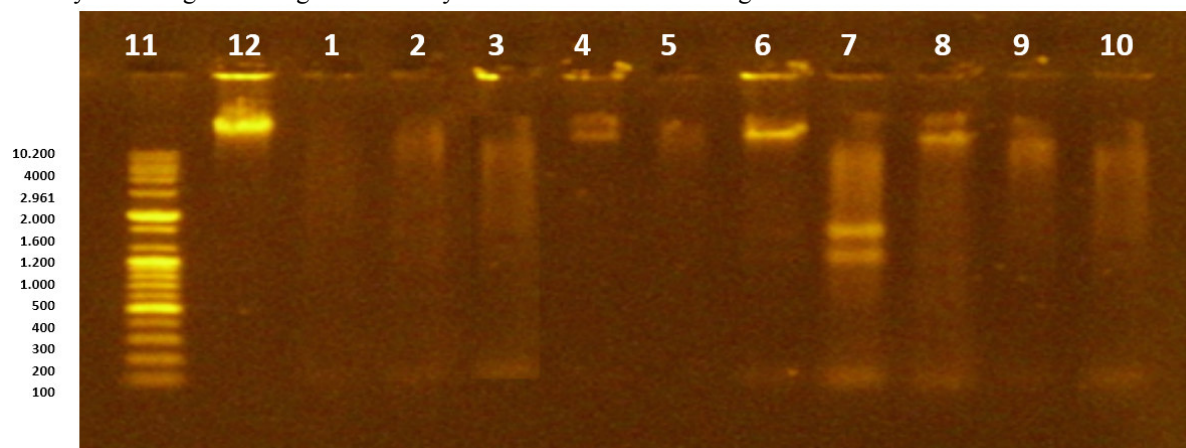


Figure (2): The endonucleases activity of 10 isolates of *E. coli* on to lambda DNA, 1% agarose gel. Lines 11 DNA marker 100bp plus, lane 12 uncut lambda DNA and lanes that labeled 1-10 are *E.coli* isolates .

E. coli isolate no.4 was selected to purified restriction enzymes based on the method that described by Al-Khafagi (1999) with some modifications .

All types of restriction enzymes were be active form in different ammonium sulfate precipitation ratio in our study we used ammonium sulfate in different saturation ratio (40 ,50 , 60 ,70 and 80) % , Then enzymatic solution was dissolved in B-100 buffer and dialyzed against the same buffer for 24 hrs. at 4°C, After incubation with λ -DNA for 1hr. 50% was only ammonium sulfate concentration that kept enzyme activity and stay enzyme to cut λ -DNA at the same form when examined as a crude , figure (4).

After testing a suitable ammonium sulfate ratio(50%) for enzymatic activity , No.4 *E. coli* isolate was cultured on 1000 ml of LB for using crude extract in next steps of purification.

From purification table(2) , volume of enzymatic extract was decreased with decreasing in protein concentration that mean in this step we discarded many unrequired proteins because of ammonium sulfate led to produce aqueous two phase system, lower phase contain enzyme with lower volume and higher concentration that agreement with Ingham (1990).

And then the enzymatic solution pass through sephadex G-100 column during this process we get three main protein peaks , After collect these fractions of each peak ,examined their ability to digest λ - DNA , after incubation with λ -DNA , the enzymatic activity found in third peak for (41,44,47) fractions. Figure (5). Al-Khafagi ,1999 got two main protein peaks when used Sephadex G-100,and only one DNases and RNases activity peak was be observed with Sephadex G-100 by Shikara *et al.*, 2009.

Protein content was estimated of each fraction by using absolute method that described by Whitaker and Granum in 1980. From purification table we observed the protein content was decreased rapidly after each purification step with increasing in purification fold.

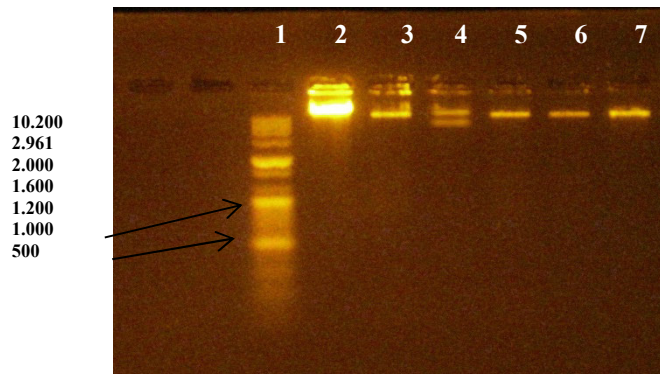


Figure (4): Ammonium sulfate ratio.

lane 1 DNA marker 100 bp plus , lane 2 uncut lambda DNA , lane 3 ,4 ,5 ,6 and 7 (40,50,60,70,and 80)% Ammonium sulfate saturation ratio respectively .

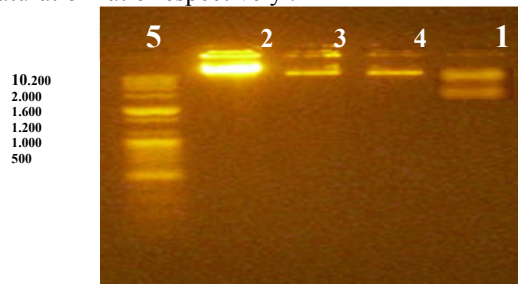


Figure (5): The enzymatic activity of each peak of protein solution fractions . lane 1 DNA marker 100 bp plus , lane 2 uncut lambda DNA, lane 3 ,4,5 endonuclease activity purified protein solution respectively .

Table (3): Purification steps of restriction enzyme.

Step	Total volume (ml)	Total protein (mg/ml)	activity (units/ml)	Specific activity (units/mg)	Total activity (unit)	Purification	Yield %
Crude extract	50	27	1421	52.62	71050	1	100
ammonium sulphate precipitation 50%	5	1.1	1100	1000	50005	19	77.4
Dialysis against B-100 buffer	12	0.7	900	1285.7	10800	23.4	15.2
Gel filtration with sephadex G-100	9	0.5	1000	4600	9000	87.4	12.6

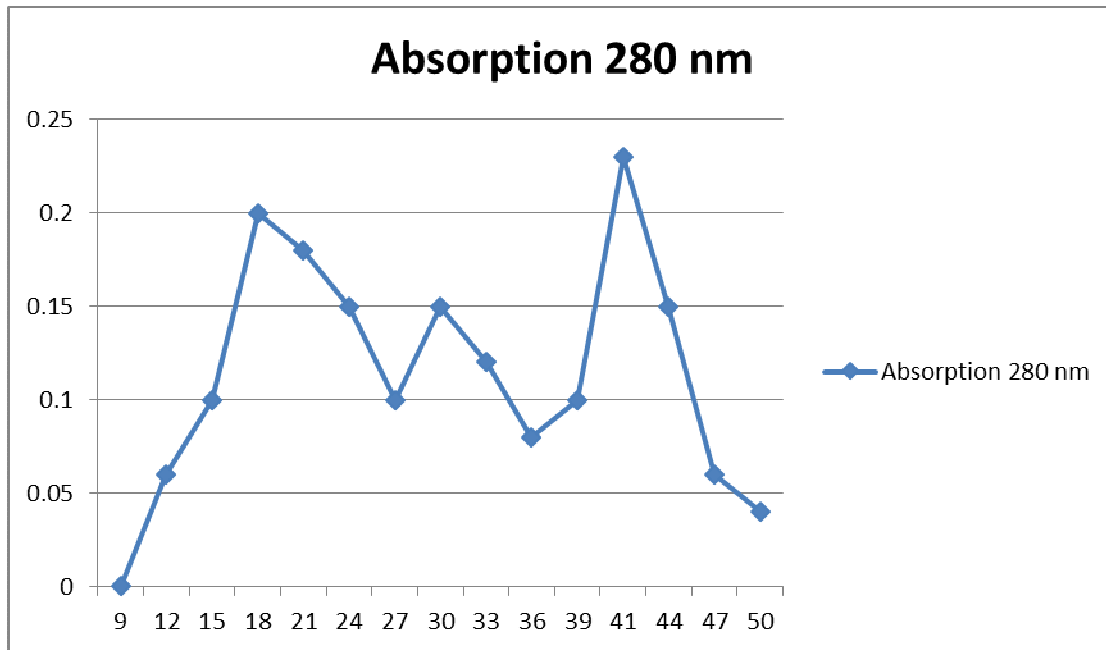
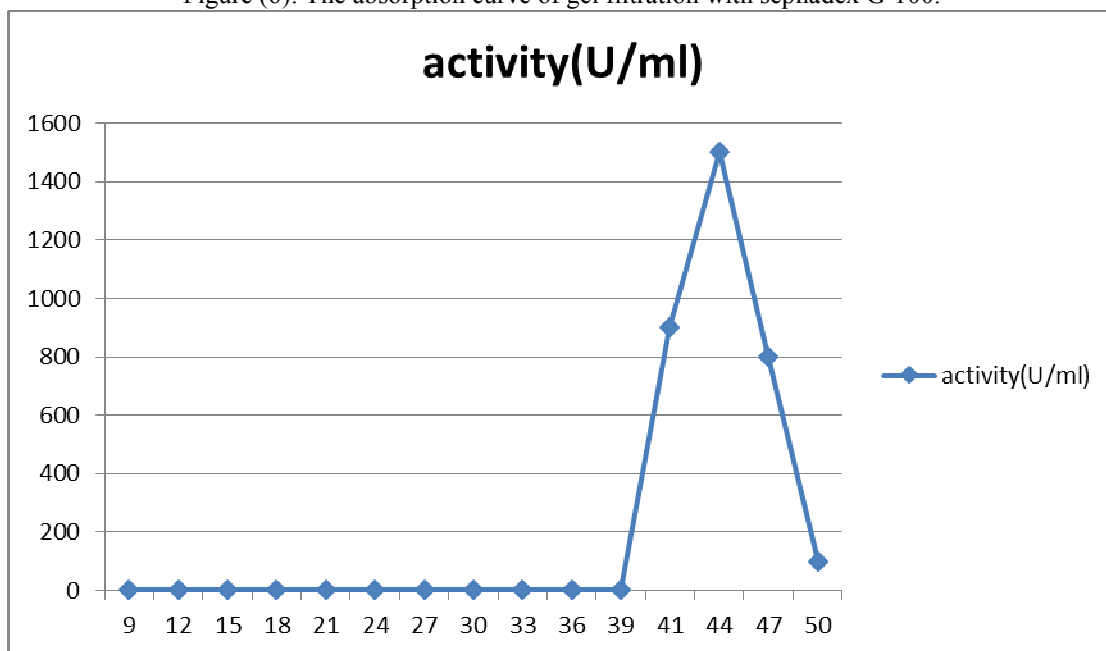


Figure (6): The absorption curve of gel filtration with sephadex G-100.



Figure(7):The activity curve of each fraction of enzymatic solution.

Detection the purity of restriction enzyme

After indication that third peak of protein fractions have ability to digest λ -DNA. The purity of enzyme was analyzed by 10% SDS-PAGE figure (8).

Lane 1 show the protein marker with molecular weight (14,20,30,45,66and 97)kda ,from this electrophorsis image we can see the protein bands were decreased after each purification steps that matched with decreasing of total protein content in purification table(table 3). After gel filtration with Sephadex G-100 we found only one protein band with molecular weight equal 45 kda that mean in this step we obtained high purified protein .

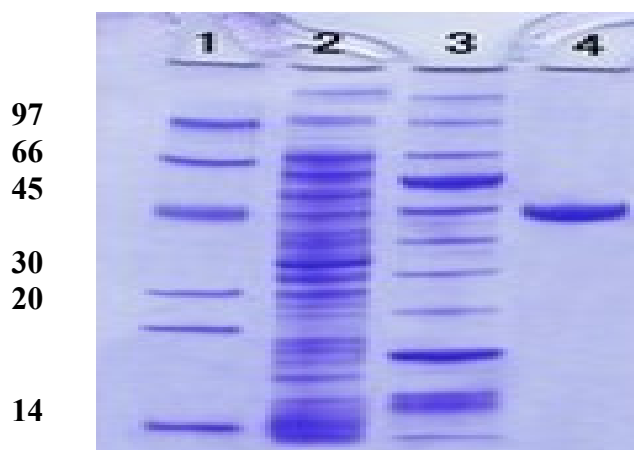


Figure (8): Polyacrylamide Gel Electrophoresis(SDS-PAGE).

Lane 1 protein marker, lane2 crude extract , lane 3 protein solution after ammonium sulfate precipitation, lane 4 protein solution after Gel filtration step .

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