A Restriction Enzyme from *Escherichia coli* Purification and General Properties

Mukaram Shikara* **Nadia Tariq Barakat** & Maysem Modaffer Al-Obaidy****

Received on:28/4/2008
Accepted on:4/12/2008

Abstract

An endonuclease restriction enzyme has been purified from *E. coli* about 40-fold with DNAse and RNAse recoveries of about 3%. The purification steps included precipitation of the enzyme with ammonium sulphate, and reclaimed it through Sephadex G-100 and DEAE-cellulose chromatography. The purified endonuclease was able to break lambda DNA into three bands. The enzyme has 5% of carbohydrate moiety which means it is a glycoprotein. Lastly, the comparison with other commercial restriction endonucleases proves that this enzyme is a restriction enzyme with enzymic activity dependent on Mg2+

Keywords: restriction enzyme, purification, E. coli, properties

أنزيم مقيد من بكتريا القولون التنقية والخواص العامة

الخلاصة

تمت تنقية أنزيم مقيد من بكتريا القولون حوالي 40-مرة مع استرداد مقداره 3% ، وتضمنت خطوات التنقية ترسيب الانزيم بواسطة كبريتات الأمونيوم ثم استرجاعه بواسطة كروماتوغرافيا الترشيح الهلامي (خلال عمودي سيفادكس-100 و دي-سليلوز)، وتم اختبار الأنزيم من خلال قطعه لدنا العاثية لامبدا إلى ثلاثة حزم، كما وجد 5% كربوهدرات مما يعني كونه بروتين سكري. أخيراً، ومع مقارنة الانزيم بانزيمات مقيدة تجارية، فأن الأنزيم أثبت أنه أنزيم مقيد يعتمد في نشاطه على أيون المغنيسيوم.

Introduction

Restriction enzymes are enzymes that cleave the sugar-phosphate backbone of DNA strands. They carried a host-defense function for the cell [1]. These enzymes recognize a specific DNA base sequence and cleave both strands of a double stranded DNA molecule at or near the recognition site. All restriction enzymes can be classified into three classes: I, II and III [2]. Type II is a sequence-specific and commonly known as restriction enzymes. In contrast with nonspecific endonuclea-ses, these enzymes generate reproducible nucleotide fragments from specific DNAs. They cleave double-stranded DNA by hydrolyzing two phosphodiester bonds (one per strand) within defined nucleotide sequences. Over 3330 enzymes have been discovered since the first report by Smith and collaborates [2] encompassing 228 distinct specificity.

Enzymes in the Type II restriction enzyme family are amenable to purification by chromatographic procedures. Ion exchangers at nearly neutral pH are used as separation matrices for extracts that have been freed of cellular nucleic acids. The affinity chromatography speeds the purification and protects against denaturation during fractionation [3]. Some enzymes required divalent ions such as Mg^{+2}, while others do not need this [4].

Restriction enzymes have been purified and characterized in many parts of the World two decades ago [2, 5, 6],

* **Biochemical Branch, Applied Science Department, University of Technology/ Baghdad**

** **The Iraqi Centre for Cancer Research and Medical Genetics/Baghdad**

https://doi.org/10.30684/etj.27.5.9
2412-0758/University of Technology-Iraq, Baghdad, Iraq

but in Iraq, only three attempts were made to purify these enzymes. Putrus [7] and Al-Khafagi [8] from bacteria and Shikara [9] from fungi. The present attempt aim to purify a restriction endonuclease from ***Escherichia coli***.

Materials And Methods

Purification of the enzyme

The purification of a restriction enzyme followed the procedure described by Smith and Wilcox [2].

E. coli were grown on McConckey Agar plate for 18-21 hrs at 37˚C. 0.5% NaCl solution (1:10 vol.) was added gradually to the bacterial cells. The pH brought to 8.0 with 0.5 M tris-base, then 1ml of lysozyme solution (25mg/ml) was added with continuous stirring for about 5 min. The mixture stands for 1 min, then the reaction was terminated with 50ml of ice-cold saturated ammonium sulphate solution (with vigorous stirring) to form 30% saturated fraction. The mixture was centrifuged at 3000 x g for 10 min. The supernatant was dialyzed against 1litre x 3 of Tris-HCl buffer, pH 8.0 for 24h and the volume **(Fraction I)** was measured, while the precipitant was discarded due to the presence of very little DNAse and RNAse (endonucleases) activities in it.

Solid ammonium sulphate was added to Fraction I to bring the saturation from 30% to 65%, and the supernatant was centrifuged as before, and the pellet resuspended in Tris-HCl buffer, pH 8.0, then dialysis against 1litre x 3 of the above buffer for 24h **(Fraction II),** but the supernatant decanted due to little endonuclease activity in it.

OD at 260 and 280nm of Fraction II was read, and the ratio was calculated to ensure the correct lysis step.

Fraction II was diluted with the above buffer to bring the solution to 43% $(NH_4)_2SO_4$ saturation with gradual addition of $(NH_4)_2SO_4$, then centrifuge and the pellet resuspended in the above buffer and dialyze **(Fraction III)**, while the supernatant decanted (due to little endonuclease activity).

Fraction III was found to has a high DNAse and RNAse activities, so it has been purified further by layered onto a 1.5 x 4 cm Sephadex G-100 mixed with 2:1 (w:w) glass beads (200 mesh). The column was washed with two column volume. A flow rate of 20ml.h^{-1} was used and 3 ml fractions-volume were collected. One-third of each fraction was kept for further analysis, and the rest were pooled together **(Fraction IV)**.

Fraction IV layered onto a 1.5 x 40cm DEAE-cellulose, pH 8.0 and washed with the above buffer, and the enzyme was eluted with 120ml of 0-0.6M NaCl in the above buffer. A flow rate of 30ml.h^{-1} was used and 2 ml fractions-volume were collected.

Zinc chloride was added to Fractions III and Fraction IV to precipitate the contaminations of ribonucleases (RNAses) from the enzyme.

Two-third of each fraction was pooled together **(Fraction V)** and rechromatographed again on 1.5 x 25 cm DEAE-cellulose column for further purification and desalt. A flow rate of 25ml.h^{-1} was used and 3 ml fractions-volume were collected. The peaked fraction (Fraction 65) is referred to as **(Fraction VI).**

All operations are carried out at 4°C, and DNAse and RNAse activities, protein and carbohydrate concentrations were determined for all fractions.

Acid-soluble Measurements

DNAse activity determined by measuring the amount of acid-soluble nucleotide liberated from DNA The incubation mixture contained 0.2mg of native DNA, 20um $MgSO_4$, 35um of Tris-HCl buffer, pH 8.1 and about 1 unit of enzyme in a total volume of 1ml.

RNAse activity determined by measuring the amount of acid-soluble nucleotide liberated from DNA The incubation mixture contained 0.2mM RNA, 20um $MgSO_4$, 35um of Tris-HCl buffer, pH 8.1 and about 1 unit of enzyme in a total volume of 1ml.

Different amounts (5-50ul) of 0.1 $ZnCl_2$ were added to the mixture to participate contaminants of RNAse.

The incubation mixture (for both DNAse and RNAse) is at 37°C for 1hr. 0.01% bovine serum albumin solution is added to aid in precipitating of DNA or RNA, before 1min of stopping the reaction with 4 ml 0.5M perchloric acid. The mixture was kept at 4°C for 10 min then centrifuged at 4000 x g for 10 min. The supernatants are read at 260nm and 280nm, respectively.

One unit of DNAse can catalyzes the release of 1umole of acid soluble nucleotide from native DNA in the presence of Mg^{+2} in 1hr at 37°C under the above conditions.

One unit of RNAse can catalyzes the release of 1umole of acid soluble nucleotide from RNA in the presence of Mg^{+2} in 1hr at 37°C under the above conditions.

In case of DNA-agarose gel electrophoresis, a unit activity is define as the ability of one volume of restriction enzyme to completely cut 1ug of lambda DNA at 37°C for 1hr under standard conditions

Determination Methods

Protein contents are determined by the methods of Lowry et al [10] using Bovine serum albumin as a standard.

Carbohydrates contents are determined by the method of Dubois et al [11] using glucose as a standard.

Agarose gel electrophoresis is used according to the method described by Maniatis et al [12]. SDS-polyacrylamide gel electrophoresis was used according to the method described by Blackshear [13].

Lambda DNA 32300 KD, size 48502bp, concentration 250mg.ml^{-1}
5 A_{260} units (1ml) were obtained from Roche Molecular Biochemicals, Germany

The purification-fold was collected by dividing (the specific activity of each fraction) on (the specific activity of fraction I), while the percentage of yield was collected by multiplying (the total activity of each fraction) by 100 and then divided on (the total activity of Fraction I) (5, 7).

Results

Tables 1 and 2 show the steps of purification of the restriction enzyme.

E. coli were grown on McConckey Agar for 16-18hr. Lysis of bacteria with lysozyme results in viscous suspension containing broken membranes, short chains of mucopolysaccharides, and all the intracellular components.

Lysis with lysozyme is a very important step, but must be terminated after 5-6 min. If the reaction is allowed to continue, the DNA will be dissociated from the membranes and form long slimy strands attached to the stirring rod. The suspension (with no DNA release) will be thixotropic gel, somewhat like custard pudding, but not slimy or gooey. For that reason, ammonium sulphate up to 30% was added to stop the reaction. Cell membranes, ribosomes, most of cellular DNA and a number of proteins become insoluble and precipitate by centrifugation at 3.000 x g. The formation of air bubbles during ammonium sulphate fractionation must be avoided since it might cause the denaturation of the enzyme due to surface inactivation [14, 15].

If the decanted supernatant volume after the addition of ammonium sulphate (to stop the reaction) is less than 100 ml, the lysis will be improper; and if the 260/280 ratio for $(NH_4)_2SO_4$ fraction is below 0.75, the lysis step is improper also [15, 16].

Several trials were conducted until the concentration of 10ul of 0.1M $ZnCl_2$ was found to be suitable to precipitate the contaminants of RNAse without decreasing DNAse activity.

Since most of DNAse and RNAse activities are found in Fraction III, it was used for further purification by applying to a Sephadex G-100-glass beads column, and a peak of activity

was observed between fractions 47-67 (Fig.1).

One-third of each fraction was kept for further research, and two-third were pooled together (**Fraction IV**) and loaded into DEAE-cellulose column. Two peaks of activity were observed. The first peak was in the wash region between fractions 7-17 contained very little activity, while the second peak's fractions, which were eluted between 0.1-0.2M NaCl , between 44-56 with a simple shoulder between fractions 57-70 (Fig.2).

One-third of each fraction from the second peak was kept for further experiments, and two-third were pooled together (**Fraction V**) and dialyzed for 5 h with two changes against 1 liter of Tris-HCl buffer, pH 8.0 and rechromatographed on DEAE-cellulose column. Two peaks were observed also, a peak between 10-30 fractions with very little activity, and the other peak eluted between 0.09-0.18M between fractions 60-70. (Fig.3).

Fraction 65 (from rechromatographed DEAE-cellulose column) has the highest DNAse and RNAse activities, for that reason it is referred to as **(Fraction VI)**. Fraction VI is treated with lambda DNA which was completely cut to three bands (Fig. 4). This means that the purified enzyme is an endonuclease and a restriction enzyme [16, 17].

An enzymic activity is dependent on the presence of Mg^{+2}. The purified endonuclease was optimally active at 20uM Mg^{+2}. The activity was reduced to about 80% in the absence of Mg^{+2}.

The presence of EDTA and glycerol interfered with lambda DNA digestion pattern by the enzyme, so even they were recommended as stable materials, they were not use in this research (18).

Discussion

A restriction enzyme has been purified from *E. coli* about 40-fold with DNAse and RNAse recoveries of 2.95% and 2.77% respectively. It contained 0.5% of carbohydrate moiety, which means the enzyme is a glycoprotein. The optimal pH is 8.0-8.2, and is stable for 2h at room temperature, 48h at 4C and more than one month at –4C. The addition of magnesium ion will increase its activity more than 80-fold.

The adsorption of the second peak to DEAE-cellulose column might suggest the presence of one form of the enzyme with low isoelectric point. The first peak suggests the presence of a form of the enzyme with a high isoelectric point .

In comparison with the three restriction endonucleases purified at Iraq, Putrus [7] has 40-fold purification with recovery of 50%, Al-Khafagi [8] has 188-fold purification with recovery of 20% and Shikara [9] has 419-fold purification with a recovery of 40.6%. The purified fraction (Fraction VI) was able to break lambda DNA into three bands, so this enzyme is a restriction enzyme. Each band was cut and run in SDS-polyacrylamide gel. No clear band can be shown, but when each lane of the gel sliced into small parts, and each of them were eluted with buffer. Each band shows a single peak of endonuclease activity.

A limited comparison with two other standard enzymes (EcoR1 and AatII) has been satisfactory since all restrict lambda DNA (Fig. 4).

The breakage of lambda DNA into three bands (as far as one can see from the gel) is a characteristic shared with more than 30 restriction enzymes (2, 4, 19, 20), so more research must be done in the future to identify the characteristic of this enzyme.

Most restriction enzymes reported in the purified form are composed of two equal subunits with molecular weights of 20000-25000 or single polypeptides with molecular weights of 30000-35000. Both previous researchers claimed that their purified enzymes consist of a single polypeptide chain with very little proof (4, 7, 19). The presence of a single polypeptide band in

SDS-polyacrylamide gel needs not to prove that this band is connected with a restriction enzyme activity. It is very possible that a very faint band (or bands) can not be seen by naked eyes is responsible for activity. From our results with SDS-polyacrylmide gel electrophoresis, the researchers cannot form an opinion about this until future research is done (20). The purified enzyme is dependent on Mg^{+2} as several endonucleasess, most prominent of them *Hemophilus influenza* purified restriction enzyme [2].

References

[1]- Devor, E.J., "A brief history of the RFLP. In. Molecular applications in Biological Anthropology"., Cambridge University Press, pp. 1-18, 2005.

[2]-Smith HO and Wilcox, KW., "A restriction enzyme from *Hemophilus influenzae* I. Purification and General properties"., J. Mol. Biol. 51: 379-392. 1970.

[3]- Cooney, C.A., "The restriction enzyme Ehel (GGC/GCC) is sensitive to CPG methylation"., Nucleic Acid Res., vol. 18, no. 12, pp. 3667-3675, 1990.

[4]- Halford, S.E., "Hopping, Jumping and looping by restriction enzyme"., Biochemical Society, pp. 363-373, 2000 .

[5]- Pingoud A., and Jeltsch, A., "Structure and function of type II restriction endonucleases"., Nucleic Acids Research, 29: 3705-3727, 2001.

[6]- Marks, P., McGeehan, J., Wilson, G., Errington, N. and Kneale, G., "Purification and characterization of a novel DNA methyltranferases", Nucleic Acids Research, 31: 2803-2810, 2003.

[7]- Putrus, S.G., "Extraction and characterization of an isoschizomer for the restriction enzyme Hind III from locally isolated *Haemophilus influenzae*"., M.Sc. thesis, College of Science, University of Baghdad, 1995.

[8]- Al-Khafagi, K.A.M., "Extraction of a restriction enzyme from a local isolate of the thermophilic bacteria *Bacillus stearothermophilus"*., M.Sc. thesis, College of Science, University of Baghdad, 1999.

[9]- Shikara, M., "Purification of a restriction enzyme from *Aspergillus niger* and some general properties". Al-Mustansiriyah J. of Sci., vol. 19, No.3, 2008.

[10]- Lowry, O.H., Rosebrough, N.J., Furr, A.L. and Randall, R.J., "Protein measurement with the Folin phenol reagent"., J. Biol. Chem. 193: 1209-1220, 1951.

[11]- Dubois, M, Gilles, K.A., Hamilton, J.K., Rebers, R.A. and Smith, F., "Calorimetric method for determination of sugars and related substances"., Anal. Chem. 28: 350-359, 1956.

[12]- Maniatis, T. Fritseh E.F. and Sambrook J., "Molecular Cloning. A laboratory Manual"., Coldspring Harbor Laboratory, New York, 1982.

[13]- Blackshear, P.J., "Systems for polyacrylamide gel electrophoreeis In: Methods of Enzymology". (Ed. Jackohy W. R.). Academy Press INC, Harcourt Brace Jovanovich Publishers, Vol. 104: 239-255, 1984.

[14]- Oliver, S.G. and Ward, J.M., "A dictionary of genetic engineering, Cambridge University Press, 1985.

[15]- Thach, R.E. and Newburger, M.R., "Research techniques in Biochemistry and Molecular Biology", 9th Ed. W.A. Benjamin, California, London, pp.14-32, 1995.

[16]- Cummings, O.W., King, T.C., Holden, J.A. and Low, R.., "Purification and characterizeation of the potent endonuclease

in extracts of Bovine heart mitochondria", J. Biol. Chem., 262: 2005-2015, 1987.

[17]- Wong, J., Hutchison, S.B., and Liem, R.K., "An isoelectric variant of the 150,000-dalton neurofilament polypeptide. Evidence that phosphorylation state affects its association with the filament"., J. Biol. Chem., 259: 10867 – 10874, 1984.

[18]-. Farias, R. N. et al., "Relationship between isoelectric point of native

[19]- Takahashi M. et al., "Developmental changes in the isoelectric variants of rat hepatic hydroxysteroid sulpho-transferase"., Biochem. J. 293, 795-800, 1993.

[20. Xiao-Hong, N. X. and Yeung, E. S., "Long-range Electrostatic Trapping of Single-Protein Molecules at a Liquid-Solid Interface". Science, 281: 1650-1653, 1998.

Table 1. Purification Steps of the enzyme: DNAse activity

Fraction	Total Volume (ml)	Total Activity (units)	Total protein (mg)	Specific Activity (unit/mg)	Purification	Yield %
I	110	8126	478	17.0	1	100
II	87	6613	210	31.50	1.85	81.38
III	75	4549	102	44.60	2.62	55.98
IV	48	2229	12.9	172.79	10.16	27.43
V	18	1254	5.5	228	13.41	15.43
VI (Fr. 65)	3	240	0.34	705.88	41.52	2.95

Table 2. Purification Steps of the enzyme: RNAse activity

Fraction	Total Volume (ml)	Total Activity (units)	Total protein (mg)	Specific Activity (unit/mg)	Purification (fold)	Yield %
I	110	8126	478	17.0	1	100
II	87	6613	210	31.50	1.85	81.38
III	75	4549	102	44.60	2.62	55.98
IV	39	3135	12.9	243.02	14.30	38.58
V	22	1758	5.5	319.65	18.80	21.63
VI (Fr. 65)	3	201	0.34	591.18	34.78	2.47

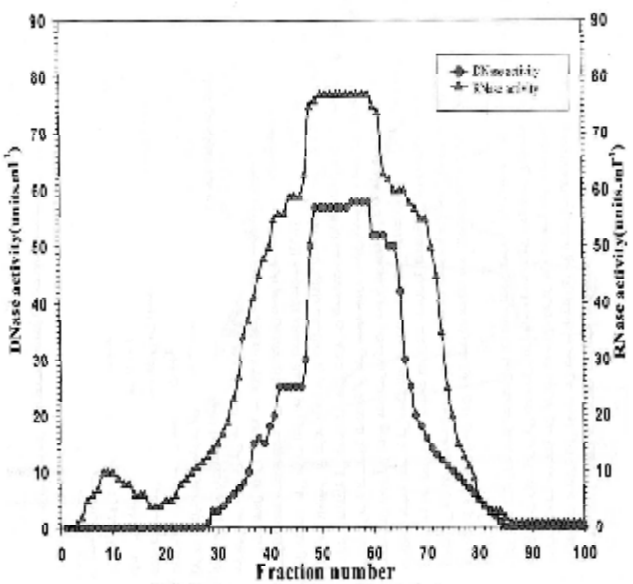


Fig 1. Chromatography of 43 % $(NH_4)_2SO_4$ fraction on Sephadex G-100-glass beads column

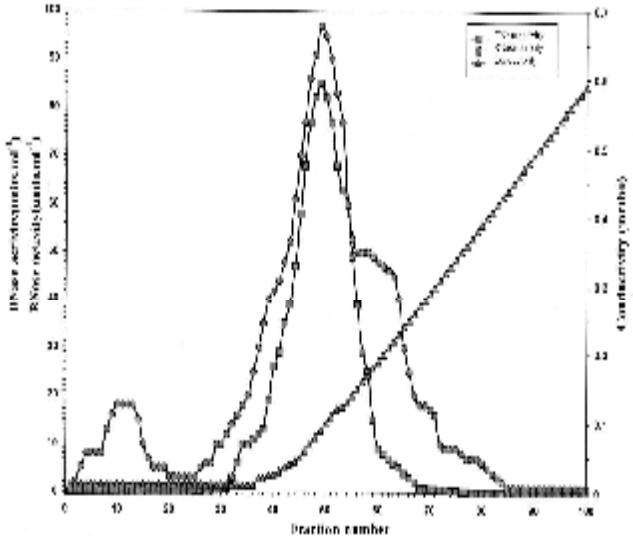
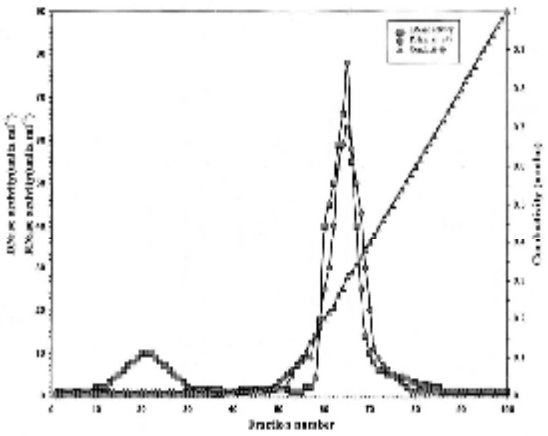


Fig 2. Chromatography of Sephadex G-50 pooled fractions on DEAE-Cellulose column



65 65 AatII EcoR1

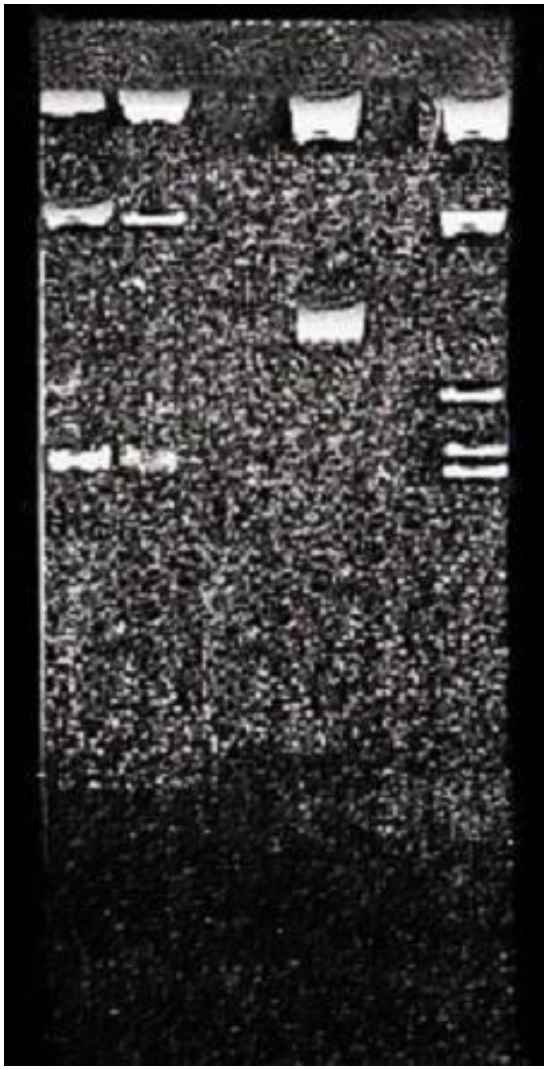


Fig. 4: Agarose gel electrophoresis treated with ethidium bromide.
Lanes 1 and 2 are lambda DNA restricted (cut) by fraction 65 from DEAE-cellulose
Lane 3 is lambda DNA restricted (cut) by AatII restriction enzyme
Lane 4 is lambda DNA restricted (cut) by EcoR1 restriction enzyme