Purification of a Restriction Enzyme from Aspergillus niger and Some General Properties

Mukaram Shikara
Chemical Biotechnology Division, Applied Sciences Department, University of Technology

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ABSTRACT

A restriction enzyme has been purified from 7-day Aspergillus niger culture by a procedure that included the recovery of the enzyme by ammonium sulphate, DEAE-cellulose and Sephadex G-100. The purification procedures were complicated with the presence of carbohydrates and polyphenolic pigments. The enzyme has been tested against lambda DNA and comparison with other standard restriction enzymes. The enzyme has been stable with 419-fold purification with the recovery of 40%.

INTRODUCTION

Restriction enzyme systems are aimed to destroy foreign DNA without destroying their own bacterial or fungal DNA [1]. These systems consist of two enzymatic activities: a modification methyltransferase that recognizes a specific DNA sequence, its recognition site, and methylates particular bases in that sequence, one in each strand; and a restriction endonuclease that recognizes the same sequence as the methyltransferase and, if the site is not methylated, proceeds to cleave the DNA foreign DNA entering the cell. [2]. Sequences of bases are short (usually 4-6 bases long). The sequence of bases in genetic material is not randomly distributed as genes are related and sequences conserved this specific site recognition lowers the number of cutting sites on a genome considerably. On a bacterium with a small chromosome, there may only be one or two such cutting sites [3].

The key feature of restriction endonucleases is not their ability to cleave DNA at their recognition sequences, but rather their ability to avoid cleaving DNA at any other sequence. The ability of these enzymes
promoted extensive screening of bacteria and fungi by biochemical assays and by genome analyzes [4].

The wide range of different restriction enzymes and therefore different cutting sites enables the researcher to manipulate DNA in such a simple yet specific way. Two separate attempts to purified restriction endonucleases were done from bacteria [5] [6]. The present attempt aim is to purify a restriction endonuclease from Aspergillus niger, a model genetic organism and an important producer of industrial enzymes in order to produce a line of production from this important enzyme.

**MATERIALS AND METHODS**

**Steps of Purification**

Aspergillus. niger has been maintained on agar petridishes which were stored at 4°C. A growing colony was cut of approximately 3cm² from the medium are ground in liquid medium to break cell walls. Then, cultivated as previously mentioned [7] at 30°C for seven days in a 6 liter liquid medium (500ml lots to 1 liter flasks) containing 6% glucose, 2% peptone, 0.8% sodium nitrate, 1% KH₂PO₄, 1% MgSO₄, 0.5% MnCl₂, 0.1% CuSO₄, 0.01% FeSO₄, 0.1 ZnSO₄, 0.04% Na₂B₄O₄ in distilled water with shaking. The press-dried mycelia (40 g/l medium) were suspended in 0.10M Tris-HCl buffer, pH 8.0 and mixed in equal weight with glass beads (20 mesh) and homogenized in an ice-cold Warning blender for 15 min. The homogenate centrifuged at 3000 x g for 30 min at 4°C and the supernatant has been used as the "crude extract". Solid ammonium sulphate was added to form 0-35% and 35-80% saturation fractions respectively. The use of batch-wise Shepadex G-50 with both fractions 3:1(v/v) led to the absorption of most polyphenolic pigments and a good deal of salts. After centrifugation at 4000 x g for 15 min, both were dialyzed against 1 x 5 liter of the above buffer for 24 h, and measured for DNase activity.

35-80% saturation fraction was found to have a high DNase activity, so it has been purified further by layered onto a 1.5 x 40cm DEAE-cellulose, pH 8.0. The column was washed with two column volume, then the enzyme is eluted with 120ml of 0-0.6M NaCl in the above buffer. A flow rate of 40ml.h⁻¹ was used and 2 ml fractions-volume were collected.

Two-third of each DEAE-cellulose active fractions were pooled together and layered on 1.5 x 25 cm Sephadex G-100 to further purification and desalt. A flow rate of 25ml.h⁻¹ was used and 3 ml fractions-volume were collected.
All operations were carried out at 4°C. DNase activity, protein and carbohydrate concentrations were determined for all fractions.

DNase activity determined by measuring the amount of acid-soluble nucleotide liberated from DNA. The incubation mixture contained 0.2mg of native DNA, 20um MgSO4, 35um of Tris-HCl buffer, pH 8.1 and about 1 unit of enzyme in a total volume of 1ml. The incubation period is 1h at 37°C. 0.01% bovine serum albumin solution to aid in precipitating the DNA before 1min of stopping the reaction with 4 ml 0.5M perchloric acid. The mixture was kept at 4C for 10 min then centrifuged at 4000 x g for 10 min. The supernatant was removed and read at 260nm.

One unit of the enzyme can catalyzes the release of 1umole of acid soluble nucleotide from native DNA in the presence of Mg in 1hr at 37°C under the above conditions.

In case of DNA-agarose gel electrophoresis, a unit activity is defined as the ability of one volume of restriction enzyme to completely cut 1ug of lambda DNA at 37°C for 1hr under standard conditions [8].

Protein contents were determined by the methods of Lowry et al [9] using Bovine serum albumin as a standard.

Carbohydrates contents were determined by the method of Dubois et al [10] (1956) using glucose as a standard.

Agarose gel electrophoresis was used according to the method described by Maniatis et al (1982) [11]

Acrylamide gel electrophoresis was used according to the method described by Blackshear (1984) [12]

Lambda DNA 32300 KD, size 48502bp, concentration 250mg.ml⁻¹

Standard restriction enzymes (EcoRI and AatII) were obtained from Sigma Chemical Company. Aat II from Acetobacter aceti (activity 500-5000 units.ml⁻¹) and EcoRI from Escherichia coli (activity 10000 units.ml⁻¹).

RESULTS AND DISCUSSION

Aspergillus niger were grown in a nitrogen limited media for seven days and 16g dry weight/100ml media was obtained. The purification procedures of enzyme in general and restriction enzyme in particular have been complicated by the presence of carbohydrates excreted from the fungal cells during submerged growth [13, 14]. In order to prevent excessive production of carbohydrates, the walls of the fungal cells were breakdown in liquid nitrogen before culturing (see Methods).
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The presence of polyphenolic pigments has been noticed in crude extract and ammonium sulphate fractions and proteins are likely to be absorbed by these pigments. The use of batchwise Sephadex G-50 during 35-80% (NH₄)₂SO₄ fraction and before dialysis has been successful in removing many inactive proteins, and to the absorption of most pigments into Sephadex particles, so the concentration of protein decreases one tenth of the original concentration in the crude extract.

Since most of DNase activity was found in the desalted (NH₄)₂SO₄ fraction, so it was used for further purification. This fraction has been applied to a DEAE-cellulose column. Two peaks of activity were observed. The first peak was in the wash region between fractions 47-77 contained very little activity, while the second peak’s fractions, which were eluted between 0.1-0.2M NaCl, between 147-178 with a simple shoulder between fractions 139-146. The highest points of the second peak were 156, 157, 158, 159, 160, 161, 162, and 163 (Fig.1). The adsorption of the second peak to the column might suggest the presence of one form of the enzyme with low isoelectric point. The first peak suggests the presence of some form of the enzyme with a high isoelectric point.

Most polyphenolic compounds present in ammonium sulphate fraction were absorbed to DEAE-cellulose and separated from the enzyme.

All peaked fractions from the second peak were dialyzed individually for 5 h with two changes against 1 liter of Tris-HCl buffer, pH 8.0. One-third of each fraction was kept for further experiments, and two-third were pooled together and loaded into Sephadex G-100 (see Method). A peak (fractions 27, 28 and 29) with very well structured shoulder (fractions 23-26) was obtained. The peaked fractions were freeze for further experiments (Fig.2). Table 1 shows the steps of purification of the enzyme.
### Table -1. Purification Steps of the enzyme

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Volume (ml)</th>
<th>Activity (units/ml)</th>
<th>Total Activity (units)</th>
<th>protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Specific Activity (unit/mg)</th>
<th>Purification</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>600</td>
<td>0.908</td>
<td>545</td>
<td>0.103</td>
<td>61.8</td>
<td>8.81</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>35-80% (NH₄)₂SO₄ Fraction</td>
<td>30</td>
<td>16.50</td>
<td>495</td>
<td>0.09</td>
<td>2.7</td>
<td>183.3</td>
<td>20.8</td>
<td>90.82</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>16</td>
<td>29.987</td>
<td>479.8</td>
<td>0.015</td>
<td>0.25</td>
<td>1919.2</td>
<td>217.8</td>
<td>88.04</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>9</td>
<td>24.60</td>
<td>221.4</td>
<td>0.0066</td>
<td>0.06</td>
<td>3690</td>
<td>418.8</td>
<td>40.6</td>
</tr>
</tbody>
</table>

In view of their very low concentration of protein, the pooled DEAE-cellulose fractions and the peak fractions from Sephadex G-100 were treated with trichloroacetic acid and the precipitated proteins were redissolved and loaded onto SDS-polyacrylamide gels (Fig. 3). The pooled DEAE-cellulose gives 2-3 clear bands in the region of 30000 and a very heavy band (or several bands close together) in 15000 region. All the three fractions from Sephadex G-100 showed similar profile, but the band (or bands) in 15000 region in fraction 27 showed a great similarity with DEAE-cellulose pooled fraction. These results are conflicted with the fact that only one single broad peak of activity appeared (Fig.2). This suggest two explanations: the first was that the activity has been associated with a minor polypeptide which was too faint to be detected by stain, and the second explanation was that the peak of activity was made up of two or more overlapping peaks (see discussion).

Pooled fractions of DEAE-cellulose and the three main fractions from Sephadex G-100 were treated with lambda DNA. Fig. 4 showed that lambda DNA has been completely cut to smaller sized, which means that the purified enzyme is an endonuclease and a restriction enzyme [15].
The researcher purified a restriction enzyme from \textit{A. niger} 419-fold with DNase recovery of 40.6%. The purification of such enzymes is limited due to the presence of carbohydrates and polypeptide compounds in excess in fungi.

The use of Sephadex G-50 during ammonium sulphate fractionation and the breakage of cell wall with liquid nitrogen have been two successful steps to remove most of the carbohydrates and polyphenolic compounds.

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. The suggestion (from results) that two (or more) polypeptides overlapping each other do not agree with the results obtained from DNA-agarose electrophoresis, since the pooled DEAE-cellulose fraction and the three fractions from Sephadex-G-100 gave the same profile. Also, fraction 27 from Sephadex G-100 showed a similar profile to pooled DEAE-cellulose fraction (Fig. 4). This will give strength to the hypothesis that the endonuclease activity is associated with a minor polypeptide which was too faint to be detected is more plausible explanation. Further research will be done to clarify this problem.

Since all purified fractions were able to break lambda DNA into smaller sizes, so this enzyme is a restriction enzyme. A comparison between this purified enzyme and two standard restriction enzymes (AatII and EcoRI) obtained from (Sigma) showed some similarity. The results are not conclusive since the breakage of lambda DNA into 7-9 bands (as far as one can see from the gel) is a characteristic shared with more than 30 restriction enzymes, and due to purification of the enzyme. More research must be done.

The purified enzyme contained 0.3\% of carbohydrate moiety, which means the enzyme is a glycoprotein. Preliminary experiments show that its optimal pH is 8.0-8.2, and is stable for 2h at room temperature, 48h at 4\(^\circ\)C and more than one month at –4\(^\circ\)C. The addition of magnesium ion will increase its activity more than 80-fold. Hopefully successive experiments will confirm this.
Fig-1: DEAE – Cellulose chromatography of 35-80% ammonium sulphate fraction from the crude extral of A.niger
Fig-2: Sephadex G-100 chromatography on the second peak eluted from DEAE – Cellulose
Fig-3: SDS-polyacrylamide Electrophoresis of 30-80% Saturated (NH₄)₂SO₄ fraction, Sephadex G-100 fractions (27, 26 and 29) with standards.
Fig-4: Agarose gel Electrophoresis of Undigested Lambda DNA, 30-80% Saturated (NH₄)₂SO₄ fraction, Sephadex G-100 fractions (27, 26 and 29), and two standard restriction enzymes: EcoR I and Aat II.
REFERENCES


