A rapid method for preparation of calf spleen exonuclease

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Received 13 July 1977

ABSTRACT

Chromatography on Concanavalin A Sepharose is a simple procedure for partial purification of spleen phosphodiesterase. This procedure removes much, but not all, of the ribonuclease activity found as contaminant in most spleen phosphodiesterase preparations.

INTRODUCTION

Spleen phosphodiesterase is an exonuclease degrading RNA and DNA from the 5'-terminus sequentially to yield nucleoside 3'-phosphates (1). As an exonuclease this enzyme is a very useful tool in nucleic acid sequence analysis (2,3). However, as shown earlier (4), most preparations of this enzyme are contaminated with varying amounts of endonuclease activity; this greatly limits their application. A number of nucleases are known to be glycoproteins (5,6) for which easy purification schemes exist based on their affinity for lectins (e.g. Concanavalin A). Therefore we were interested to see whether Concanavalin A affinity chromatography could be used to purify spleen phosphodiesterase if this enzyme were a glycoprotein. The work described in this paper shows that chromatography on Concanavalin A Sepharose can be used as an easy step to purify this enzyme to a degree not obtained previously and to remove simultaneously a substantial amount of contaminating endonuclease.

MATERIALS AND METHODS

General. Freshly frozen calf spleens were obtained from the local slaughterhouse. Spleen phosphodiesterase was a product of Worthington Biochemical Corp. Methyl-α-D-mannopyranoside was from Calbiochem. Concanavalin A covalently bound to Sepharose 4B was obtained from Pharmacia. 2'-Deoxythymidine 3'-p-nitrophenylphosphate (monosodium salt) was a product of Boehringer-Mannheim. The ribooligonucleotides (Ap)gA and (Up)7U were gifts...
of Dr. H.G. Khorana. They were phosphorylated at the 5'-hydroxyl with polynucleotide kinase (kindly given by Dr. Sekiya) and $\gamma$-[\textsuperscript{32}P]ATP (specific activity 50 C1/nmole). Reaction with polynucleotide phosphorylase, $\alpha$-[\textsuperscript{32}P]GDP (7) and T1 RNase (3) yielded (Ap)\textsubscript{g}Ap*Gp* and (Up)\textsubscript{g}Up*Gp*. The phosphorylated oligonucleotides were purified by homochromatography on thin layer DEAE-cellulose plates (Analtech Inc.) at 65°C using 50 mM or 75 mM KOH digested homomixture (9).

**Assay for Spleen Phosphodiesterase.** This was carried out according to Razzell and Khorana (1) using thymidine 3'-p-nitrophenylphosphate as substrate. The usual reaction mixture (0.03 ml) contained 0.25 mg thymidine 3'-p-nitrophenylphosphate and 0.01-1.0 units of enzyme in 0.2 M sodium acetate (pH 6.0) - 0.01% Tween 80. After incubation at 37° the reaction was terminated by addition of 0.1 M sodium hydroxide (1 ml). The absorbance at 400 nm is a measure of the enzyme activity. One unit of spleen phosphodiesterase is defined as the amount of enzyme which hydrolyzes one mg of substrate per hour (1).

**Enzyme Preparation.** The procedure of Hilmoe (10) was followed up to the acetone powder extract step. All steps were performed at 4°C. Five frozen spleens (approx. 200 g) were partially thawed, the outer membranous tissue was removed, and the spleens cut into small pieces. They were homogenized for 2 min in a Waring blender in two equal batches, each suspended in 300 ml of 11% sucrose and 100 g of crushed ice. The resulting mixture was centrifuged for 10 min at 1500 x g. The pH of the supernatant (880 ml) was adjusted to 5.1 by addition of 2 M acetic acid (6.5 ml). The precipitate was collected by centrifugation for 30 min at 1500 x g, washed with 8.5% sucrose and again centrifuged. The precipitate was suspended in five volumes of cold acetone and the mixture was homogenized for 15 seconds in a Waring blender. The suspension was filtered on a Buchner funnel, the precipitate washed several times with cold acetone and dried under vacuum to yield approximately 25 g of "acetone powder". About two-thirds of the acetone powder was extracted with 280 ml of 0.2 M sodium acetate (pH 6) in a Waring blender and the mixture centrifuged. The supernatant (275 ml) was made 0.01% in Tween 80 ("acetone powder extract") and used for Concanavalin A Sepharose chromatography.

**RESULTS AND DISCUSSION**

A trial experiment revealed that spleen exonuclease binds well to Concanavalin A Sepharose; thus this material may be used for purification of
the enzyme. Both, commercial spleen phosphodiesterase and enzyme freshly prepared from calf spleens, were chromatographed on Concanavalin A Sepharose under somewhat different conditions. Figure 1 shows the elution profile of protein and enzyme activity obtained when the commercial enzyme preparation was chromatographed and eluted by step changes in buffers. The enzyme activity emerged in two peaks, the material was pooled separately (Pool A and Pool B) as indicated in Figure 1. The recovery in this step was only about 30% (Table I), an indication that the enzyme was fairly labile.

Figure 2
Concanavalin-A Sepharose Chromatography of Spleen Exonuclease.

Acetone powder extract (50 ml) (see Materials and Methods) was applied to a ConA-Sepharose column (1 x 20 cm) preequilibrated with 0.2 M sodium acetate (pH 6) - 0.01% Tween 80. After washing with 20 ml of the same solution the enzyme was eluted with a gradient (total volume 100 ml) from 0 - 0.4 M methyl-mannopyranoside in 0.2 M sodium acetate (pH 6) - 0.001% Tween 80 - 1.0 M sodium chloride. The gradient was started at fraction 16. Fractions were pooled as indicated and dialyzed twice against distilled water. The sample was then lyophilized and dissolved in 0.5 ml of 0.05 M ammonium acetate (pH 5.7) - 0.01% Tween 80.
We then applied this procedure to the purification of spleen exonuclease from fresh spleens. "Acetone powder extract" was prepared from fresh frozen spleens as described by Hilmoe (10) and subjected to Concanavalin A Sepharose chromatography using a linear gradient of methyl-mannopyranoside for elution. This step results in a twenty-fold purification of the enzyme (Figure 2 and Table I).

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Purification of Spleen Exonuclease</th>
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<tbody>
<tr>
<td></td>
<td>Volume (ml)</td>
</tr>
<tr>
<td>Expt. 1 Worthington Enzyme</td>
<td>1.0</td>
</tr>
<tr>
<td>Con-A Sepharose Pool A</td>
<td>3</td>
</tr>
<tr>
<td>Pool B</td>
<td>5</td>
</tr>
<tr>
<td>After lyophilization &quot;Fraction A&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>&quot;Fraction B&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>Expt. 2 Acetone powder extract</td>
<td>50</td>
</tr>
<tr>
<td>Con-A Sepharose Pool C</td>
<td>21</td>
</tr>
<tr>
<td>After lyophilization &quot;Fraction C&quot;</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The great lability of the enzyme posed a major problem in the purification. As pointed out by Razzell and Khorana (1) the addition of the detergent Tween 80 to all buffers alleviated this problem to some extent. Glycerol did not stabilize the enzyme. During the concentration of the column fractions there was a great loss of enzyme activity. Lyophilization turned out to be the least destructive concentration method in our hands; dialysis against polyethylene glycol, even in the presence of glycerol, led to loss of activity. Despite these losses in the concentration step the simple chromatographic procedure outlined above results in an enzyme of high specific activity with a good yield compared to previous preparations (1,4).

In order to assess the exo- and endonuclease activities contained in our enzyme preparation we digested various oligoribonucleotides containing a radioactive 3'- or 5'-phosphomonooester group. In the first experiment

3514
(Up)₇Up*Gp*, labeled in the two 3'-terminal phosphates, was used to test the flowthrough peak (Fraction I) and the enzyme peak (Fractions A and B, Figure 1). As can be seen in Figure 3 there is some endonucleolytic degradation of the radioactive oligonucleotide by Fraction I, while the spleen phosphodiesterase preparation (Fractions A and B) rapidly degraded it in an exonucleolytic fashion.

In a second experiment, we compared the two enzyme preparations (Figures 1 and 2). In order to test rigorously for endonuclease contamination, we used ten times the normal enzyme/substrate ratio. As can be seen (Figure 4) each preparation gave a very rapid degradation of the normal substrate, (Ap)₉Ap*Gp*, at this concentration. But treatment of p*(Up)₇U, which should be very resistant to 5'-exonucleolytic cleavage (1), with our enzyme revealed the presence of a contaminating endonuclease. The contamination is less noticeable in the enzyme prepared from fresh spleens (Fraction C). These results indicate that our method for spleen phosphodiesterase purification eliminates some, but not all, contaminating endonuclease activity.

As expected our enzyme preparations were not pure; SDS-polyacrylamide gel electrophoresis revealed eight protein bands. Since it was not possible to assay for phosphodiesterase activity after elution from the gel, and thus determine the molecular weight of the enzyme, we analyzed our preparation by gel filtration on Sephadex G-100. Enzyme activity eluted just...
Figure 4
Chromatography of spleen exonuclease digest of radioactive oligonucleotides.
10,000 cpm of the radioactive oligonucleotides indicated in the figure (+2 μg carrier RNA) were incubated with our enzyme preparations at a high enzyme/substrate ratio. The origin of the enzyme fractions is described in Table 1. 50mM KOH digested homomixture was used for chromatography.
ahead of the hemoglobin marker (data not shown). This would indicate a molecular weight in excess of 64,000 daltons but it must be noted that molecular weight estimations for glycoproteins may give anomalous results when standard techniques are applied (12).

If further purification of spleen phosphodiesterase is deemed necessary, DEAE-Sephadex (4) or phosphocellulose (11) chromatography have been shown to be quite useful, albeit with significant loss in total activity.

*This work was supported by grants from the National Institutes of Health (GM 22854 and HD 09167).

REFERENCES
7. Since α-[32P]GDP is not commercially available we prepared it by hydrolysis of α-[32P]GTP. To an aqueous solution (~0.2 ml) of α-[32P]GTP (1 mCi, 10 nmoles) was added an equal volume of 0.2 M sodium acetate (pH 5.0) - 0.04 M CuCl₂ and the mixture incubated for 45 min at 80°C (7). Products were separated by electrophoresis on DEAE paper at pH 1.7 and eluted with 30% triethylammonium bicarbonate. The yield of α-[32P]GDP was typically ~30%.