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ISOLATION AND PURIFICATION OF A DIISOPROPYL PHOSPHOROFLUORIDATE HYDROLASE FROM THERMOPHILIC BACTERIA

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Diisopropylphosphorofluoridate (DPF) hydrolase from thermophilic bacteria has been partially purified by using ammonium sulfate precipitation. This procedure was then followed by various column chromatography isolation techniques - DE-52, phenyl boronate agarose, and phenyl sepharose CL-4B. Further work is needed to purify the enzyme to homogeneity.

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Preface

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ISOLATION AND PURIFICATION OF A DIISOPROPYL PHOSPHOROFLUORIDATE HYDROLASE FROM THERMOPHILIC BACTERIA

1. INTRODUCTION

Organophosphate detoxicating hydrolase - also known as phosphorylphosphatase - was first described by Mazur[1] in 1946. Up to now the enzyme has not been isolated to homogeneity. Therefore, nothing is known either about its natural substrate or about its physiological role. Two types of phosphoryl-phosphatases are known. One is called Mazur-type phsophoryl-phosphatase from mammalian and bacterial sources, and its activity is stimulated by manganese. Another type, called 'squid-type' is more stable, but its activity is not stimulated by manganese[2,3].

The specific phosphorylphosphatase currently worked on in my laboratory is DFPase (EC 3.8.2.1) detoxifies diisopropyl-phosphorofluridate (DFP) to a non-toxic diisopropylphosphoric acid and an inorganic fluoride ion. The toxicity of DFP is due to its potency as an antiacetylcholinesterase compound by irreversible bonding to acetylcholinesterase. The aim of this research is to isolate DFPase from thermophilic bacteria. The reason to use thermophilic bacteria is in the hope that the enzyme will be relatively more stable at room temperature than the same enzyme from other sources. If so, its potential as a detoxifying agent will be greater.

2. MATERIALS and METHODS

The following reagents were obtained from commercial sources: DE-52 (Sigma), Matrex gels (blue A, red A, orange A, green A, or blue B kit, Amicon), Matrex gels (PBA-10, PBA-30, PBA-60, Amicon), DFP (Sigma), phenyl-sepharose (Sigma). All enzyme assay reagents were from Sigma. All SDS gel electrophoresis and protein assay reagents were from Bio-Rad.

[1] Mazur A. (1946) An enzyme in animal tissue capable of hydrolyzing the phosphorus fluorine bond of alcyl fluorophosphatases. J. biol. Chem. 164: 271-289.

[2] Hoskin F.C.G. and Prusch R.D. (1983) Characterization of a DFP-hydrolyzing enzyme in squid posterior salivary gland by use of soman DFP and manganous ion. Comp. biochem. Physiol. 75: 17-20.

[3] Chemnitius J-M., Losch H., Losch K. and Zech R. (1983) Organophosphate detoxicating hydrolases in different vertebrate species. Comp. biochem. Physiol. 76: 85-93.

Enzyme Assay:

A DFP solution was prepared as follows: 100 ml of a modified Hoskins[2] buffer (400 mM NaCl, 50 mM KCl, 5 mM Bis-Tris propane, pH 7.2) was added to 40 ul DFP just before enzyme assay. Enzyme activity was measured by the amount of F- ion liberated with a fluoride sensitive electrode and continuously recorded on a Corning thermal printer. The electrode was dipped into a double-walled beaker on a magnetic stirrer thermoctatized to 28 C with a circulating water bath. 3 ml DFP solution were then added, along with 36 ul 2.5% Mn++. The enzymatic reaction was started by adding 0.05 to 0.1 ml sample solution. One unit (IU) of DFPase activity is that amount of enzyme which hydrolyzes 1 umole of DFP/min at the assay conditions.

Purification of DFPase from thermophilic bacteria:

All column chromatography steps were performed at room temperature and enzyme activity was assayed with the standard assay described above throughout the purification. The following buffers were used: buffer A - 12.5 mM Imidazole, 15 mM NaCl, pH 7.0; buffer B - 12.5 mM Imidazole, 100 mM NaCl, pH 7.0; buffer C - 12.5 mM Imidazole, 500 mM NaCl, pH 7.0; buffer D - 50 mM Imidazole, 50 mM NaCl, 10 mM Mn++, pH 7.8; buffer E - 75 mM Imidazole, 75 mM NaCl, 20 mM EDTA, pH 7.8; buffer F - 1 M (NH)2SO , 25 mM Tris, pH 7.5; buffer G - mM Tris, pH 7.5.

Preliminary Experiment:

Step 1 - Preparation of cell homogenates from thermophilic bacteria:

Thermophilic bacteria, 50-80 grams, were grown, harvested, and French pressed by Dr. Joseph DeFrank of the Biotechnology Division of CRDEC. The suspension was centrifuged for 20 minutes at 15,000 rpm in a Servall RC-2 centrifuge to eliminate cell debris. The supernatant was then precipitated with 30% (final concentration) ammonium sulfate and was centrifuged at 15,000 rpm for 20 minutes. The supernatant was then adjusted to 70% ammonium sulfate concentration. After half an hour, it was centrifuged in the same manner as above. The pellet was collected and redissolved in buffer A.

Step 2 - DE-52 column chromatography:

The redissolved 30-70% ammonium sulfate precipitate was dialyzed overnight against 4 l of buffer A. It was then centrifuged at 15,000 rpm for 20 minutes. The supernatant was loaded onto a DE-52 column (1 x 30 cm) previously equilibrated with buffer A. The sample was 1.5 times larger than the bed volume of the resin. The run-through fraction had very little

enzymatic activity. After washing the column with buffer until very low protein was eluted, the column was eluted with 150 ml buffer B and 150 ml buffer C in a linear gradient. The eluted fractions were assayed for enzymatic activity. The fractions with activities were collected and pooled. The proteins were precipitated with 90% ammonium sulfate as means of concentrating the sample.

Step 3 - Gel filtration chromatography on Sephadex G-150 (medium grade):

After centrifugation, the above pellet was dissolved in the buffers needed for the following preliminary experiment with the dye-ligand screening kit and with the phenyl boronate agarose kit (see Table 1 for the buffers used for the kits).

Step 4 - Column chromatography of dye-ligand binding kit:

The fraction with enzyme activity from Step 3 above was pooled and applied on the dye-ligand binding kit with three different buffer systems (see Table 1).

Step 5 - Phenyl boronate -10, -30, -60 kit column chromatography:

The enzyme fraction from Sephadex G-150 was loaded onto the three columns (2 ml bed volume) kit in two different buffer systems (with and without Mn++).

Results of the Preliminary Experiment:

Two peaks from DE-52 had enzymatic activity. The run-through fraction from the dye-ligand binding column kit had most if not all of the enzymatic activity, indicating there was no binding of the enzyme protein by the dye-ligand (see Table 1). However, there was binding of the enzyme to the phenyl boronate agarose -30 and -60 columns (see Table 1). From these preliminary experiments, the method of purification was modified as shown in Figure 1, flow diagram.

Steps 1 and 2:

These were the same as in the above preliminary experiment.

Step 3 - Phenyl boronate agarose (PBA-60) column chromatography:

Peak I from DE-52 (see Figures 1 and 2) was precipitated with 90% ammonium sulfate. The pellet was collected after centrifugation and was redissolved into buffer A plus 10 ml Mn++. It was dialyzed against the same buffer overnight. The dialyzed fraction was then centrifuged and the sample was then

Table 1. Preliminary Experiment with Dye-Ligand Binding and PBA Kits.

Dye-ligand binding kit:	Enzyme fractions from Sephadex Cr-150 in the following buffers		
	0.05 M Tris pH 8.0	0.02 M Tris + 10 mM Mn++ pH 7.2	0.01 M Phosphate pH 7.2
	% Recovery of DFPase activity as compared with controls		
Control	100%	100%	100%
Blue A	100%	73%	77%
Blue B	100%	90%	100%
Green A	100%	86%	100%
Red A	100%	83%	77%
Orange A	100%	86%	92%

PBA (Phenyl Boronate Agarose) -10, -30, -60 Matrix Gel Kit:

	Enzyme fraction from Sephadex Cr-150	
	0.01 M Phosphate pH 7.2	0.02 M Tris + 10 mM Mn++ pH 7.2
	% Recovery of DFPase activity as applied to columns	
PBA-10	60%	66%
PBA-30	38%	17%
PBA-60	25%	10%

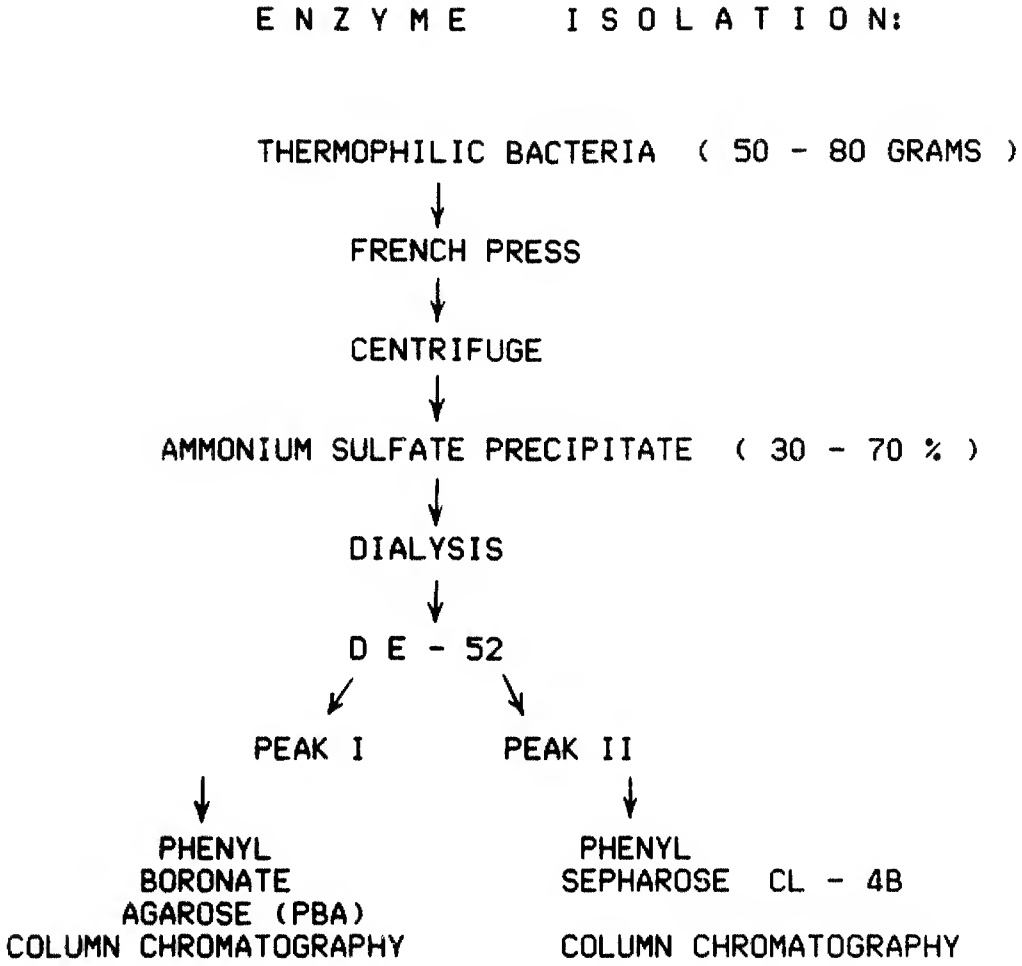

Figure 1. Flow diagram of the procedure for the purification of DFPase from thermophilic bacteria.

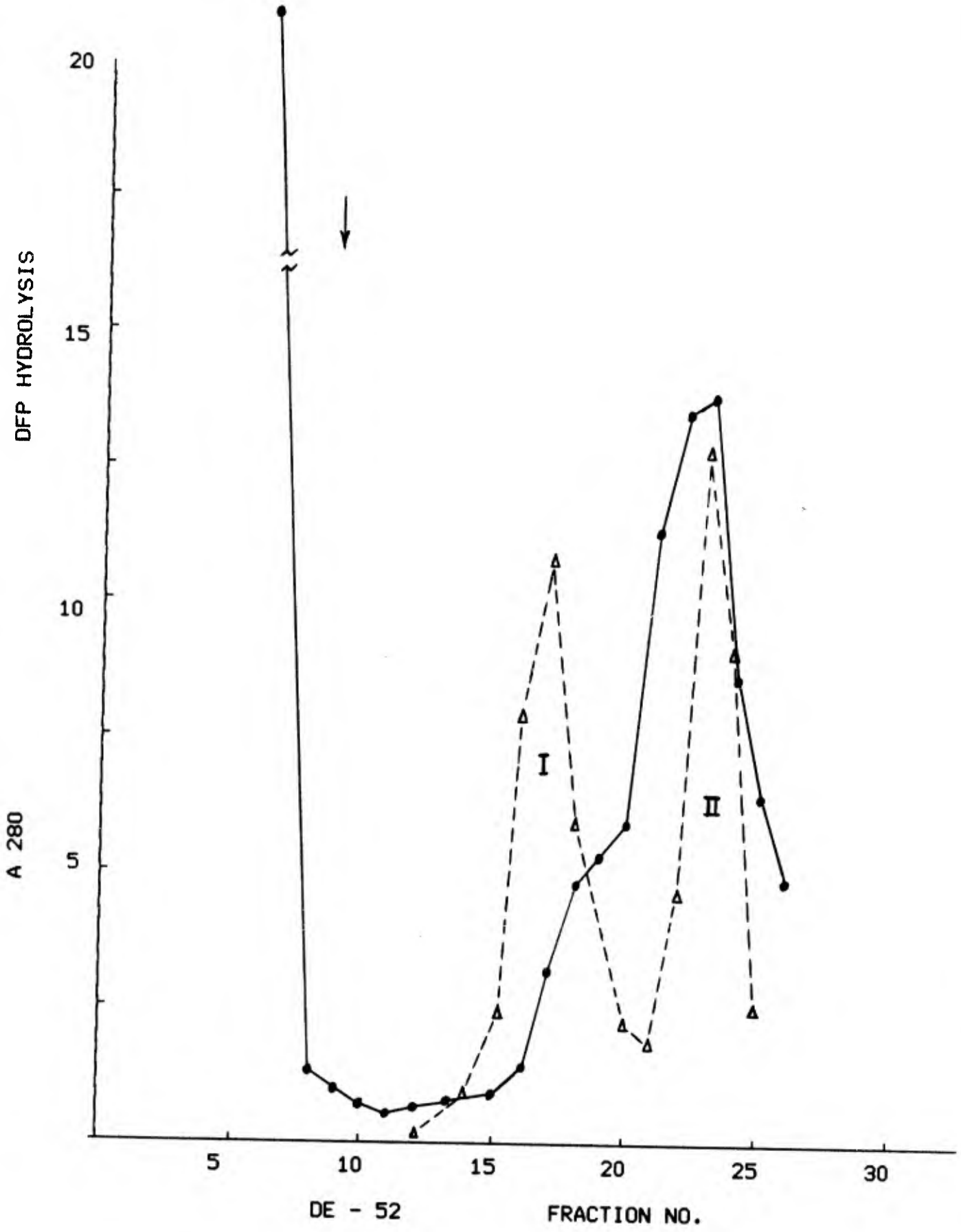

Figure 2. Column Chromatography
Arrow indicates start of the linear gradient

applied onto a 0.7 x 10 cm phenyl boronate agarose-60 (PBA-60) column (2-3 ml bed volume). The column was washed with buffer A plus 10 mM Mn++ until no more protein occurred in the eluted fraction. The column was eluted with buffer D followed by buffer E.

Step 4 - Phenyl sepharose CL-4B column chromatography:

Peak II from Step 2 (see Figure 2) was precipitated with 90% ammonium sulfate. After centrifugation, the pellet was redissolved into buffer F. It was then applied onto a 1 x 15 cm phenyl sepharose CL-4B column previously equilibrated with buffer F. The column was then washed with buffer F until no more protein was eluted. The linear gradient was then started with 75 ml buffer F and 75 ml buffer G.

Step 5 - SDS gel electrophoresis:

The highest activity fraction from Step 3 was subjected to SDS gel electrophoresis[4]. Samples, after ammonium sulfate precipitation, were redissolved in buffer A. The samples were mixed with one-half to equal volumes of SDS-PAGE sample buffer with beta-mercaptoethanol, boiled for 8 minutes and run on a 7.5% SDS gel electrophoresis. The gels were then stained with coomassie brillant blue G-250.

Protein Determination - Protein was measured using a Bio-Rad system.

3. RESULTS and DISCUSSION

Thermophilic bacteria were chosen as the source of DFPase since they are readily available and hopefully will be more stable at room temperature. For the procedure to be successful, a number of points have to be addressed as follows:

Step 1 - After 30-70% ammonium sulfate precipitation, the dialysis against buffer A should not be too long (i.e. two days) or else some enzyme activity will be lost. However, the dialysis also can't be too short or the enzyme will not bind to the DE-52 column.

Step 2 - The product from step 1, after dialysis, was a cloudy (even after centrifugation), pinkish solution. However, all the cloudy, pinkish solution passed right through the column, leaving a dark, brown band near the top of the column. Since there were much more contaminating proteins than the DFPase protein, a small column was sufficient to bind all the enzyme protein. In terms of protein content, the run-through fraction had about 82% of the total applied protein. A linear salt

[4] Laemmli U.K. (1970) Nature (Lond.) 227: 680-5.

gradient (0.1 - 0.5 M NaCl) eluted two peaks with DFPase activity (Figure 2). The first DFPase peak (Peak I) has lower protein content than Peak II. Peak II contains the majority of the protein bound to the column. Therefore, Peak I has a higher specific activity than Peak II.

Step 3 - Affinity chromatography on PBA-60 column:

Peak I from DE-52 must be dialyzed against buffer A plus 10 mM Mn++. A low ionic strengh buffer and Mn++ are essential for binding. Furthermore, the interaction of the enzyme to PBA is strong. The column can be washed with 6 times more concentrated buffer in the presence of 10 mM Mn++ without eluting the enzyme. The enzyme can be elulted in a broad peak in the absence of Mn++ and in the presence of EDTA.

Step 4 - The result of chromatography with phenyl sepharose CL-4B is shown in Figure 3. This column chromatography achieved a 5-fold increase in specific activity.

Step 5 - There are four bands from SDS gel electrophoresis (lane b, Figure 4) of Peak I from DE-52 column. The most intensified protein (#1, lane b) has a molecular weight between 43K and 67K daltons. This protein band decreases in intensity after PBA-60 column chromatography (gel electrophoresis results not shown), but specific activity is at least 10 times increased, indicating that this protein band (#1) is not the enzyme protein. Therefore, one or both protein bands (#3 and #4, lane b) between 30K and 43K must be enzyme protein. This is in agreement with Hoskins [2] who reported that DFPase is a dimer with a molecular weight of 62,000 daltons. The purest DFPase fraction, which occurred after PBA-60 column chromatography treatment, had a specific activity of 8.22 IU/mg protein. Further work is needed to purify this enzyme to complete homogeneity.

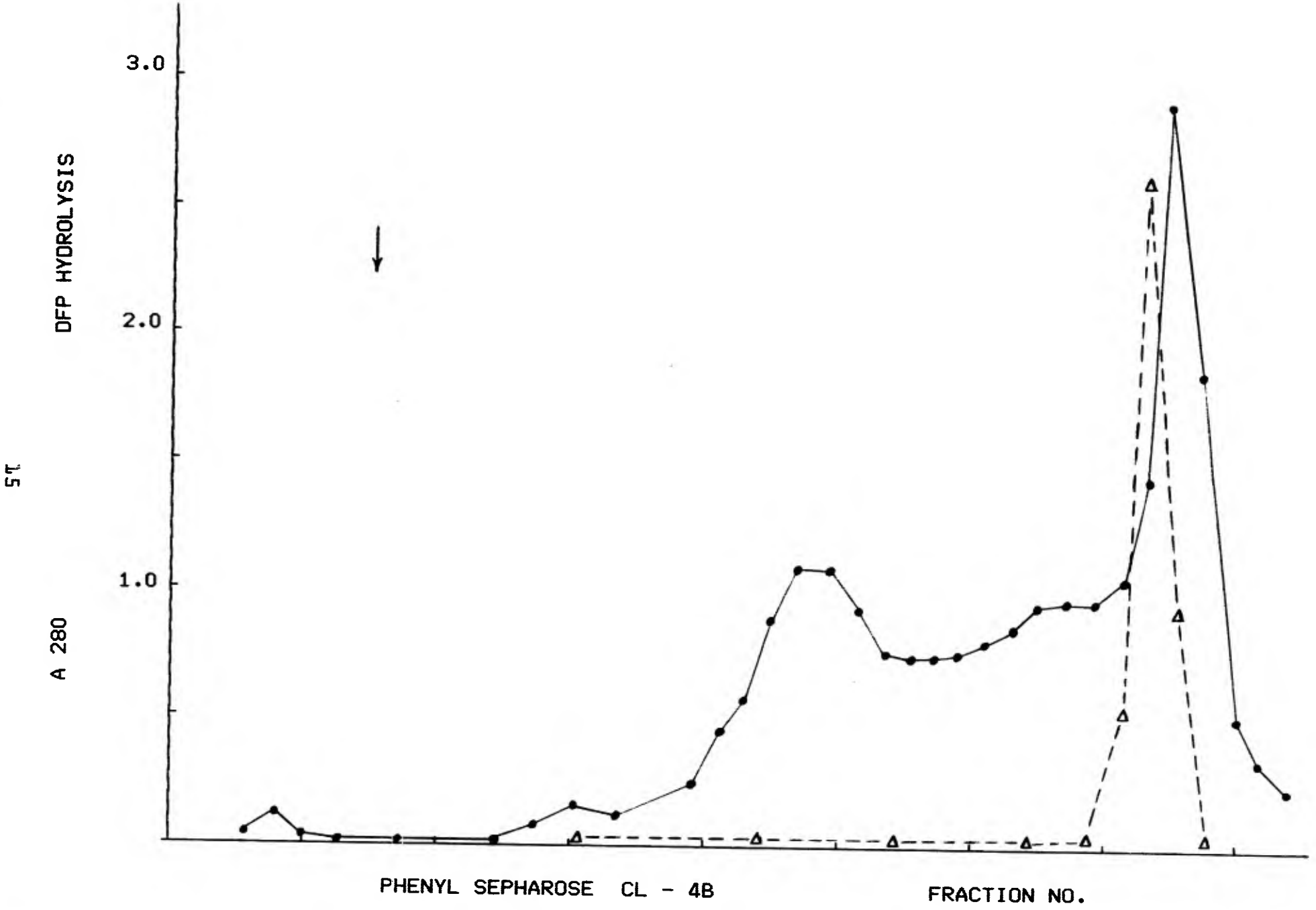

Figure 3. Phenyl Sepharose CL-4B Column Chromatography
Arrow indicates start of the linear gradient

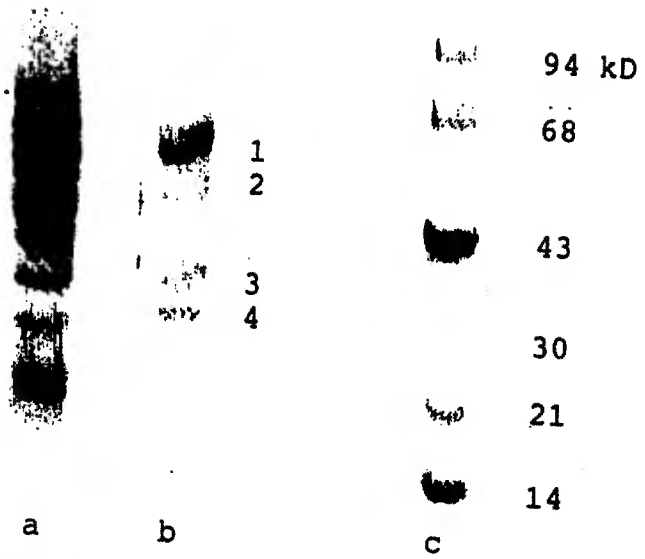

Figure 4. SDS Gel Electrohoresis

Lane a sample before DE-52 column
Lane b Peak I from DE-52 column
Lane c molecular weight standards (given in kilodaltons)