Low Mr acid Phosphatase Purification from Sheep Brain by Affinity Chromatography

ABDUR RAHIM KHAN AND AHMAD SAEED
Department of Chemistry, Gomal University, D.I. Khan, Pakistan

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Summary: The low molecular weight (Mr) acid phosphatase from sheep brain was purified to homogeneity using affinity chromatography on p. aminobenzylphosphonic acid-agarose to obtain the enzyme with specific activity, 25 U/mg of protein and yield of about 6%. The low molecular weight was estimated to be 16,000 by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Introduction

Acid phosphatases are ubiquitous in nature which have been studied in plants and animals [1]. Mammalian tissues contain at least two types of acid phosphatase forms of Mr, 90,000 - 100,000 and 13400 - 30,000 which can be separated by gel chromatography [2-4]. These high and low molecular weight acid phosphatases can be distinguished on the basis of its localization within the cell, substrate requirements, optimal pH, sensitivity to activators or inhibitors and kinetic parameters.

The low molecular weight acid phosphatases have been purified from various tissues and extensively characterized [5-9].

Recently we have also reported purification of low molecular weight acid phosphatase from sheep brain [10] by a procedure which includes $(NH_4)_2SO_4$ fractionation, chromatography on SP-Sephadex C-50 and gel filtration on Ultrogel ACA-54 column to specific activity of 6.6 U/mg of protein with overall recovery of 2.5%.

In present study we have employed a modified procedure of purification involving the use of p.aminobenzylphosphonic acid as ligand [11] in affinity gel for affinity chromatography which is found to be critical for enzyme homogeneity as well as for high specific activity.

Results and Discussion

Acid phosphatase was purified from sheep brain by a method that was the modification of procedure described earlier [10].

Step 1

Sheep brain (1 kg) was minced and then stirred with 3L of 0.01M acetate buffer, pH 5.0 containing 1mM EDTA and 0.1mM PMSF for 30 minutes. The mixture was homogenised in an ordinary blender for 2 minutes and then centrifuged at 2700g for 60 minutes. The supernatant was filtered over glass wool.

Step 2

Solid ammonium sulphate was added to 30% saturation, then centrifuged at 2700g for 60 minutes. The supernatant was 60% saturated with ammonium sulphate and centrifuged again at 2700g for 60 minutes. The precipitate was dissolved in 400ml of 0.01M acetate buffer, pH 4.9 containing 1mM EDTA and 0.1mM PMSF and dialysed against 5L of 0.01M acetate buffer, pH 5.1 containing 1mM EDTA and 0.1mM PMSF.

Step 3

The dialysed sample was centrifuged at 10,000g for 30 minutes and the supernatant was applied to SP-Sephadex C-50 column (6.5 x 40 cm) equilibrated with dialysis buffer. The column was washed with 2.5L of the same buffer until the effluent optical density at 280 nm was less than 0.1; the enzyme was then eluted with 3.6L of 0.3 M sodium hydrogen phosphate adjusted to pH 5.5 containing 1mM EDTA and 0.1mM PMSF (Fig. 1). The most active fractions were pooled and precipitated by adding solid ammonium sulphate to 70% saturation. The precipitate was collected by centrifugation at 10,000g for 30 minutes and

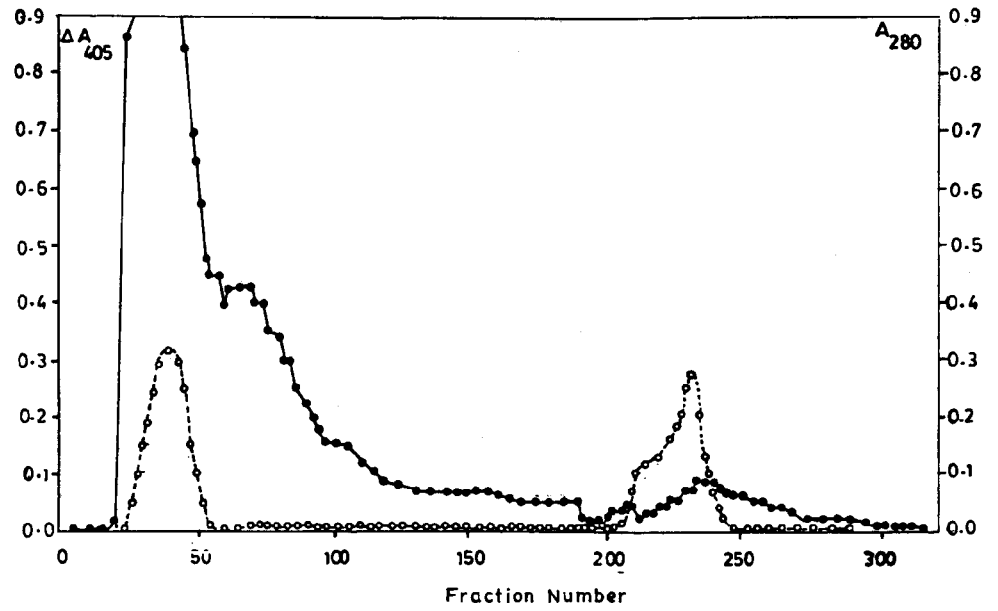

Fig. 1: Elution profile of SP-Sephadex C-50 column (6.5 x 40 cm). Flow rate was 200 ml/h. 18 ml fractions were collected and assayed for absorbance at 280 nm (●—●) and acid phosphatase activity (o-o) at 405 nm.

dissolved in 10ml of 0.01M acetate buffer pH 5.1 containing 1mM EDTA and 0.1mM PMSF.

Step 4

The enzyme was applied to Sephadex G-75 column (3 x 90 cm) equilibrated and eluted with 0.01M acetate buffer pH 5.5 containing 1mM EDTA, 0.1mM PMSF and 0.1M NaCl. The elution profile is shown in Fig. 2. The active fractions were combined and the enzyme was concentrated by Amicon ultrafiltration using YM 5 membrane to 10 ml.

Step 5

The enzyme from step 4 was dialysed overnight against 1L of 0.2M sodium citrate buffer, pH 6.5 containing 1mM EDTA and 1mM DTT. Then it was applied to column (0.9 x 14 cm) of p. aminobenzylphosphonic acid - Sepharose that was equilibrated in the same buffer. The column was extensively washed with the same buffer to remove unbound proteins and the enzyme was eluted by a 0 to 0.1M sodium hydrogen phosphate linear gradient in the same buffer (total volume of

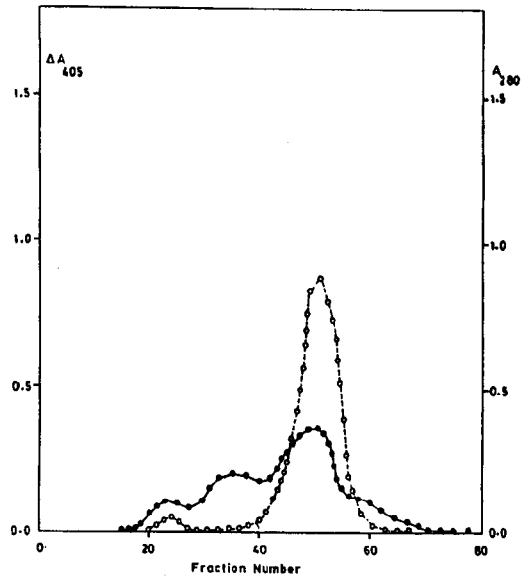

Fig. 2: Gel chromatography on Sephadex G-75 column (3 x 90 cm). Flow rate was 19ml/h. 14 ml fractions were collected and assayed for absorbance at 280 nm (●—●) and acid phosphatase activity (o-o) at 405 nm.

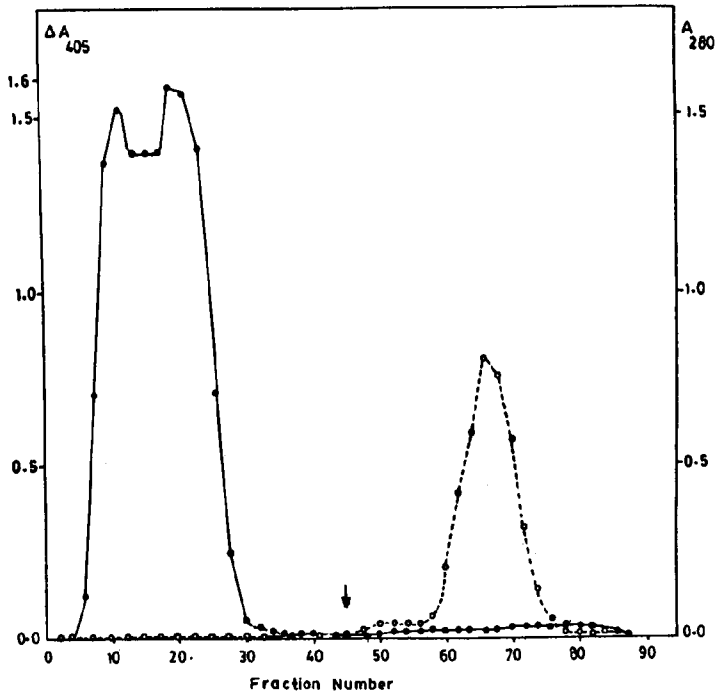

Fig. 3: Affinity chromatography on p.amino benzyl-phosphonic acid -agarose column (0.9 x 14 cm). Flow rate was 15ml/h and 1 ml fractions were collected. Arrow indicates the start of elution with linear gradient. The absorbance at 280 nm (●—●) and acid phosphtase activity (o-o) at 405 nm.

100ml). Figure 3 shows the chromatogram; the active fraction pooled and concentrated by ultrafiltration. A summary of the purification of low molecular weight acid phosphatase from one kg of sheep brain is presented in table-1.

Twenty seven fold purification was achieved on SP-Sephadex C-50 column giving 32% of enzyme recovery. A further eight fold purification was done by Sephadex G-75 chromatography with overall recovery of 20%. The enzyme was further purified to homogeneity by this affinity chromatography. 1.3 mg of enzyme was obtained from 1kg of sheep brain which was purified 1400 fold with overall recovery 6%. This is far better than the earlier result published [10].

The homogeneity of the enzyme was checked on 12.5% SDS-polyacrylamide gel electrophoresis. Single band was detected by silver staining. The molecular weight obtained by SDS-PAGE was 16,000.

Table-1: Purification of sheep brain low Mr acid phosphatase.

Step	Vol (ml)	Act U/ml	T.Act	Prot (mg/ml)	S.A.	P.F.	%age Yield
1. Crude Extract	2400	0.233	555.2	12.6	0.0180	-	100
2. 30% $(NH_4)_2SO_4$ saturation	2490	0.148	368.5	7.9	0.0187	-	65.9
3. 60% $(NH_4)_2SO_4$ saturation	480	0.705	338.4	14.7	0.0479	2.6	60.5
4. SP-Sephadex C-50	22	8.199	180.4	16.6	0.494	26.8	32.2
5. Sephadex G-75	8	14.2	113.6	3.85	3.688	204	20.4
6. Affinity chromatography on p-aminobenzyl-phosphonic acid agarose	2	16.1	32.2	0.646	24.92	1384	5.8

Vol; volume, Act; Activity, T.Act; Total Activity, Prot; Protein, S.A.; Specific Activity, P.F; Purification factor.

Thus coupling of p.aminobenzylphosphonic acid to agarose yielded an effective and specific affinity medium. By means of affinity chromatography using this medium low Mr acid phosphatases were also purified from bovine brain [5], rat liver [9] and human erythrocytes [11]. A spacer arm between p.aminobenzylphosphonic acid and Sepharose was not needed for proper binding of

acid phosphatase, probably of small size of the enzyme (Mr = 14-18 KDa). This is advantageous because the use of a spacer may lead to non specific adsorption of desired protein. The effect of introducing a spacer between p.aminobenzylphosphonic acid and Sepharose was studied with Sepharose coupled to. 4-(4'-amino-2'-methyl phenyl azo) benzylphosphonic acid. Acid phosphatase was retained and eluted as for p.aminobenzylphosphonic acid-Sepharose, but the binding was less specific in that other protein was adsorbed as well [11].

Experimental

Materials

Fresh sheep brain was obtained from the local slaughterhouse. P-nitrophenyl phosphate from Merck Co, SP-Sephadex C-50 and Sephadex G-75 were purchased from Pharmacia; All other reagents used were of the highest purity commercially available.

Methods

Enzyme assay

Acid phosphatase activity was determined on p-nitrophenyl phosphate as previously described [10]. One unit of activity was defined as the amount of enzyme required to produce 1 μmol of p.nitrophenol per minute and specific activity was expressed as units per mg of protein.

Protein determination

Protein concentration was determined by the biuret method according to Beisenherz *et al* [12].

SDS - polyacrylamide gel electrophoresis

Purified preparation of acid phosphatase was subjected to electrophoresis in SDS polyacrylamide gel [13], 12.5% and protein was visualised by silver staining method [14].

Affinity gel synthesis

1. Cyanogen bromide activation

70 ml Sepharose 4B was washed with 180 ml water, followed by washing with 90 ml acetone-water mixture (3:7) and 90 ml acetone - water

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Fig. 4: SDS - polyacrylamide gel electrophoresis of the purified low Mr acid phosphatase from sheep brain, Lane 1; The standard proteins used were phosphorylase b (Mr = 97,400); bovine serum albumin (Mr = 66,200); ovalbumin (Mr = 42,700); carbonic anhydrase (Mr = 31,000); soyabean trypsin inhibitor (Mr = 20,500); α - lactalbumin (14,500), Lane 2.

mixture (6:4) on sintered funnel. The gel was suspended in 100 ml of acetone-water mixture (6:4) and cooled to -15°C in a container containing ice and pure sodium chloride. Sepharose was activated by CNBr method [15]. CNBr solution was added (200 mg/ml Sepharose) slowly and stirred for 2-3 minutes. The activated gel was washed with 900 ml of cold water followed by washing with 180 ml of sodium borate buffer of pH 9.2.

2. Coupling the ligand

0.2M solution of p.aminobenzylphosphonic acid was prepared in 0.1M borate buffer pH 9.2, mixed with 1.5 vol. of dimethyl formamide. Activated gel was coupled to the ligand by mixing 1 vol. of the gel with 2 vol. of solution of ligand. The mixture was stirred gently at 4°C for 20h.

3. Washing the gel

To remove the excess of uncoupled ligand, gel was washed with excess of cold water and then washed alternatively with 0.1M sodium bicarbonate buffer pH 9.0, 0.1M acetate buffer pH 4.0 and 0.2M sodium citrate buffer pH 6.0.

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