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PROTAMINE-AGAROSE AND NON-CHARGED ALKYL DERIVATIVES OF AGAROSE IN THE PURIFICATION OF RAT-LIVER PHOSPHOPROTEIN PHOSPHATASES

VINCENT P.K. TITANJI [a] and SVEN PÅHLMAN [b]

[a] *Institute of Medical and Physiological Chemistry, Box 575, Uppsala University, Uppsala, and* [b] *Institute of Biochemistry, Box 576, Uppsala University, Uppsala (Sweden)*

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Summary

1. Protamine-agarose and hydrophobic interaction chromatography were found to be effective in the purification of phosphoprotein phosphatase(s) (phosphoprotein phosphohydrolase, EC 3.1.3.16) of rat-liver. The phosphoprotein phosphatases of rat-liver cytosol were first resolved into three fractions, termed A, B and C, in order of elution from DEAE-cellulose. Whereas all fractions displayed activity towards [^{32}P]phosphoprotamine, only fractions B and C displayed appreciable activity towards [^{32}P]phosphopyruvate kinase. Since fraction B exhibited the most stable properties and the highest recovery of enzymatic activity towards [^{32}P]phosphoprotamine and [^{32}P]phosphopyruvate kinase, it was selected for further purification. The method developed involves sequential chromatography of fraction B on Sephadex G-200, protamine-agarose, histone-agarose and then again on Sephadex G-200 as a final step. A 400-fold enrichment in the phosphoprotamine phosphatase activity of fraction B was obtained. Purified fraction B also displayed substantial phosphatase activity towards [^{32}P]phosphopyruvate kinase and [^{32}P]phosphohistones. An apparent molecular weight of about 250 000 was estimated for purified fraction B on a calibrated Sephadex G-200 column. The present data indicate that rat-liver cytosol contains multiple forms of phosphoprotein phosphatases and suggest a technique which might be applied for the further purification of at least fraction B.

2. In a separate approach, a combination of pentyl-agarose and protamine-agarose chromatography was shown to be a convenient method for the enrichment (up to 20-fold) of phosphoprotein phosphatase activity from crude liver extracts.

Introduction

In 1969 Meisler and Langan [1] demonstrated the existence in rat liver of two phosphoprotein phosphatases (phosphoprotein phosphohydrolase, EC 3.1.3.16) which dephosphorylated phosphohistones and phosphoprotamines. Rat-liver phosphoprotein phosphatase preparations active on phosphohistones and phosphoprotamine have also been shown to dephosphorylate and reactivate phosphopyruvate kinase [2,3].

A persistent problem in elucidating the role of phosphoprotein phosphatases has been their purification. Whereas what are considered to be dissociated catalytic subunits of phosphoprotein phosphatase(s) (M_r = 30 000—35 000) have been obtained in homogeneous form from several tissues [4—6], higher molecular weight forms of the enzyme have only been purified to approx. 60-fold [1,7]. A greater understanding of the properties of phosphoprotein phosphatases obviously requires the further purification of the higher molecular weight forms of the enzymes. However, purification of these forms has been difficult, partly due to the lack of suitable techniques and partly due to the instability of the enzyme forms.

In the present investigation a procedure, involving DEAE-cellulose, Sephadex G-200, protamine-agarose and histone-agarose chromatography for the partial purification of a phosphoprotein phosphatase, termed fraction B, has been developed, resulting in a 400-fold enrichment of enzymatic activity. Some kinetic properties of this fraction have been investigated with phosphoprotamine and phosphopyruvate kinase as substrates. Data are presented showing that hydrophobic interaction chromatography on pentyl-agarose followed by protamine-agarose chromatography is convenient for the partial purification of phosphoprotein phosphatases from small samples of crude liver extracts. A preliminary report of these findings has appeared [8].

Experimental

Protein was determined according to the method of Lowry et al. [9] in crude extracts and from the absorbance at 280 nm in purified fractions assuming $E^{1cm}_{0.1\%} = 1.0$. In the following description buffer A, containing 10 mM imidazole · HCl, pH 7.5, 15 mM 2-mercaptoethanol and 2.5 mM $MgCl_2$ was often used, unless otherwise stated.

Alkyl-agarose was synthesized by a slight modification of the method of Rosengren et al. [10]. After exhaustive washing with deionized water on a glass funnel, the agarose was packed in a glass column with an inner diameter of 5 cm. Excess water was removed from the top of the column and the volume of the settled gel was measured. In order to obtain reproducible coupling-yields, it was necessary to wash the agarose (Sepharose 4B, Pharmacia, Uppsala, Sweden) extensively with dioxane. Therefore, one volume of dioxane was passed through the gel and the water content in the effluent monitored according to a description of the method of Fischer [11]. Four volumes of dioxane proved sufficient to remove excess water. The gel was then removed and coupled as described previously [10].

Protamine sulphate (Sigma) was linked to CNBr-activated agarose (Sepharose

4B) according to standard descriptions [12]. 1 g CNBr, dissolved in water, was used for activation of 10 ml of settled agarose. 5 or 10 mg of protamine dissolved in 20 ml coupling buffer (0.1 M $NaHCO_3$ in 0.5 M NaCl) were added to 10 ml of settled CNBr-activated agarose and allowed to react at 4° C for about 14 h. The gel was washed extensively with the coupling buffer and then deionized water. Histone-agarose was prepared by a similar procedure except that 70 mg mixed calf-thymus histone (Sigma type IIA) in 40 ml coupling buffer were added to 20 ml packed CNBr-activated agarose [13].

Hydrophobic interaction chromatography. Alkyl-agarose columns (1 × 9 cm) were equilibrated with buffer A containing 3 M NaCl. The cell-sap (in amounts specified under the legend to Fig. 6 and Table III) was diluted 4-fold with buffer A containing 4 M NaCl and applied to the column at a flow rate of 20 ml/h. Fractions of 3 ml were collected. The column was washed with the starting buffer until no absorbance at 280 nm was detectable. The column was then eluted with buffer A.

Enzyme assays. Phosphoprotein phosphatase activity was assayed according to a method previously published [3]. The final reaction mixture (40 or 100 μl) contained 50 mM Tris · HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 2.5 mM $MnCl_2$ and the respective phosphoproteins: 5 μM [^{32}P]-phosphopyruvate kinase, 20 μM [^{32}P]phosphohistones or 60 μM [^{32}P]phosphoprotamine. The reaction mixture for the dephosphorylation of pyruvate kinase contained, in addition, 5% glycerol and 0.05 mM fructose 1,6-disphosphate to stabilize the enzyme. The reaction was started by addition of the protein phosphatase and was allowed to continue at 30° C for 5—10 min. The release of [^{32}P]orthophosphate was linear for at least 15 min at the enzyme concentrations used. One unit of protein phosphatase is defined as the amount of enzyme which catalyses the release of 1 nmol of orthophosphate per min under these conditions. The substrate is expressed as the concentration of the [^{32}P]-phosphate moiety in the respective phosphoproteins. The specific radioactivity of the [^{32}P]phosphoproteins varied between 20 and 100 cpm/pmol.

Purification of phosphoprotein phosphatase fraction B. Freshly excised livers (71 g) from male Sprague-Dawley rats weighing 300—350 g were homogenized in 3 vols. of 250 mM sucrose containing 15 mM 2-mercaptoethanol, 1 mM EDTA (pH 7.0, NaOH) and 0.1 mM phenylmethylsulfonyl fluoride. A Potter-Elvehjem glass-homogenizer, fitted with a teflon pestle, was used and homogenization was performed with six strokes at 940 rev./min in 30 s. This and other steps were performed at 0—4° C. The homogenate was centrifuged at 16 000 × *g* for 20 min and the supernatant obtained was further spun at 46 000 × *g* for 2 h. The post-microsomal supernatant was filtered through glass wool to remove floating fat. The supernatant was applied to a DEAE-cellulose column (Whatman DE-52) (3.2 × 20 cm) equilibrated and eluted with buffer A containing 40 mM NaCl. The column was washed at a flow rate of 200 ml/h for 5—6 h and the enzymes were eluted at a flow rate of 60 ml/h using a linear gradient from 40 to 350 mM NaCl in buffer A (500 + 500 ml). Fractions of 10 ml were collected and assayed for protein phosphatase activity. Three phosphoprotein phosphatase fractions, termed A, B and C in order of elution, were obtained, as shown in Fig. 1.

Because of its greater yield and stability, phosphoprotein phosphatase frac-

tion B was selected for further purification. In the following experiment the amount of rat liver used was increased to 146 g in order to obtain a greater amount of fraction B. Solid $(NH_4)_2SO_4$, to a final concentration of 70%, was added slowly with constant stirring to 12 000 units of pooled phosphoprotein phosphatase B from the DEAE-cellulose step. After stirring for another 15 min at 4°C, the solution was centrifuged at 16 000 × *g* for 20 min. The precipitate was dissolved in buffer A containing 40 mM NaCl, dialysed against the same buffer then insoluble material was removed by centrifugation at 16 000 × *g* for 20 min. The enzyme was further concentrated in a collodion bag to about 5 ml and then chromatographed on a Sephadex G-200 column (2.5 × 57 cm) equilibrated and eluted with buffer A containing 0.1 M NaCl at a flow rate of 16 ml/h. Fractions of 2.7 ml were collected and those with a specific activity exceeding 20 units per mg protein were precipitated with $(NH_4)_2SO_4$ (up to 70%) as described above. The precipitate was collected by centrifugation at 16 000 × *g* for 20 min and chromatographed on the same Sephadex G-200 column as described above. After Sephadex G-200 chromatography the activity emerged as a single well-defined peak (not illustrated). All fractions showing more than 50 units per mg protein were pooled and applied to a protamine-agarose column (2.5 × 5.3 cm) (Fig. 2). The column was equilibrated with buffer A and sequentially eluted with portions of the same buffer containing 0.25, 0.5, and 1 M NaCl. Most of the activity was recovered in the fractions eluted with 0.5 M NaCl. The fractions eluted with 0.5 M were pooled and solid $(NH_4)_2SO_4$ was added as above. The mixture was then stirred on an ice-bath for another 15 min and subsequently centrifuged at 16 000 × *g* for 30 min. The precipitate was carefully dissolved in 5 ml of buffer A containing 20% sucrose, dialysed against the same buffer, and chromatographed on a histone-agarose column (1.5 × 10 cm) equilibrated with buffer A containing 20% sucrose and 40 mM NaCl. The column was washed with about 150 ml of the starting buffer. The elution was continued with a 200 ml linear gradient of 40—500 mM NaCl in buffer A containing 20% sucrose. The flow rate was 16 ml/h and fractions of 3.5 ml were collected and tested for enzymatic activity (Fig. 3). After this step, the protein concentration was very low and the fractions were kept immersed in an ice-bath. Samples were diluted and used for enzyme analysis. The fractions containing the highest specific activity were immediately concentrated at 2°C in a collodion bag to about 0.5 ml. The enzyme was then chromatographed on a Sephadex G-200 column (1.6 × 40 cm) equilibrated and eluted with buffer A containing 20% sucrose and 0.1 M NaCl (Fig. 4). Although the enzyme seems to elute at the same volume as catalase the apparent molecular weight as estimated on a larger column (2.5 × 57 cm) is about 250 000 (results not illustrated). The pooled fractions were concentrated in a collodoin bag to 1 ml and stored in 0.1-ml portions at −25°C where activity was retained undiminished for at least 2 weeks. The entire procedure usually took about 14 days. The procedure up to and including the step of the first Sephadex G-200 chromatography could be used to partially purify phosphoprotein phosphatases fraction A and C approx. 14- and 4-fold, respectively. Attempts to purify these fractions further were frustrated by their relative lability. Except where specifically mentioned these fractions were not investigated further in this report.

Results

Purification of phosphoprotein phosphatase fraction B

The results presented in Fig. 1 show that rat-liver phosphoprotein phosphatases could be resolved into three fractions termed A, B and C according to order of elution from DEAE-cellulose. Earlier, it was briefly reported [8] that phosphoprotein phosphatase fractions B and C represent the main activities towards pyruvate kinase, whereas the activity of phosphatase fraction A towards this substrate is negligible. The data of Fig. 1 are in agreement with those findings [8]. Furthermore it can be seen that [^{32}P]phosphoprotamine and [^{32}P]phosphohistones are good substrates for phosphoprotein phosphatase fraction A. Not shown on this figure, but repeatedly confirmed in separate experiments, was the observation that all three phosphoprotein phosphatase fractions could dephosphorylate rabbit skeletal muscle [^{32}P]phosphorylase *a* [5] and furthermore, that [^{32}P]phosphorylase *a* phosphatase roughly co-purified with [^{32}P]phosphoprotamine phosphatase through the step of DEAE-cellulose chromatography. No further attempts were made to characterize the phosphorylase *a* phosphatase activity of the separated fractions in the present investigation.

Under the conditions chosen for chromatography and upon storage at 2° C fraction B exhibited the most stable activity. Since it also represented the highest recovery of [^{32}P]phosphoprotamine phosphatase activity and substantial recoveries of [^{32}P]phosphopyruvate kinase phosphatase (Fig. 1), it was chosen for further studies.

The results of a typical preparation of phosphatase fraction B are shown in Table I. Protamine-agarose chromatography was particularly effective, yielding a more than 3—4 fold purification (Fig. 2). This step could also be used immediately after the DEAE-cellulose chromatography (Fig. 1, Table I), in

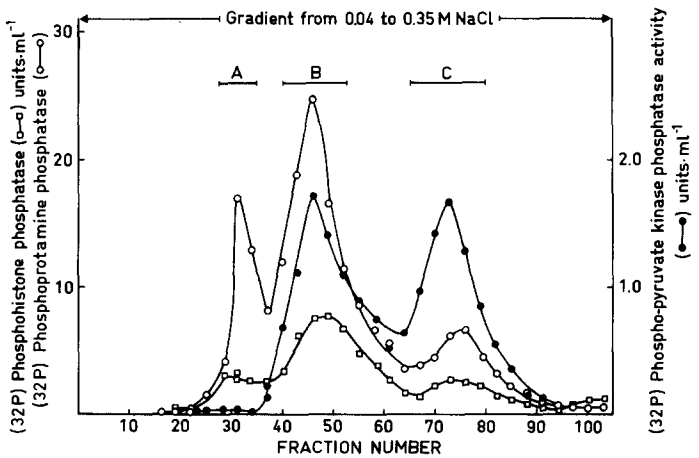

Fig. 1. Typical separation of rat-liver phosphoprotein phosphatases by chromatography on DEAE-cellulose. In cell-sap obtained from 71 g liver, the total recoveries of [^{32}P]phosphoprotamine, [^{32}P]phosphopyruvate kinase and [^{32}P]phosphohistones phosphatase activities were 13 900, 1450, and 2460 units, respectively. Samples from the fractions indicated were appropriately diluted and tested for the dephosphorylation of the substrates indicated. Other details are given under Experimental in the text.

TABLE I

PURIFICATION OF PHOSPHOPROTEIN PHOSPHATASE FRACTION B

Cell-sap from 146 g liver was fractionated by DEAE-cellulose chromatography as described in the legend of Fig. 1 and under Experimental. The amount of phosphatase fraction B used for further purification was 12 000 units in terms of [^{32}P]phosphoprotamine phosphatase activity. This value was taken to be 100% in the calculation of the recovery of enzymatic activity. At each step, the pooled peak fractions, were assayed for phosphatase activity towards 20 μM [^{32}P]phosphohistones, or 5 μM [^{32}P]phosphopyruvate kinase and the rates obtained compared with the values obtained with 60 μM [^{32}P]phosphoprotamine as a substrate. It has been checked in separate experiments that at each step of the procedure phosphatase activity towards all the aforementioned substrates is recovered at approximately the same area of the chromatogram and that no further phosphatase peaks are exhibited except those shown on the figures described in Experimental. Not shown on this table, is the observation that the activity ratios in phosphatase fraction B, seen after the Sephadex G-200 I chromatography (see Experimental), are roughly equal to those shown for Sephadex G-200 II in this table. The activity ratios for phosphatase fractions A and C, purified through the stage of DEAE-cellulose, can be calculated directly from the data of Fig. 1. Additional explanations are given in the text.

step	[^{32}P]Phosphoprotamine phosphatase activity			Activity ratios	
	Units	Units/mg protein	Yield (%)	[^{32}P]-Phosphoprotamine / [^{32}P]-Phosphopyruvate kinase	[^{32}P]-Phosphoprotamine * / [32P]-Phosphohistone
Cell-sap (total phosphatase)	59 520	4.8	—	12.2	2.1
DE-52 (phosphatase B)	12 000	18	100	18.4	2.5
Sephadex G-200 II	4 797	83	39.9	30.6	1.7
Protamine-agarose	4 039	260	33.6	20.9	1.6
Histone-agarose	769	1922	6.4	49.4	3.9
Sephadex G-200 III	891	2158	7.4	22.6	2.1

* This ratio as determined in cell sap varied from 2 to 6 depending on the batch of [^{32}P]phosphohistones used.

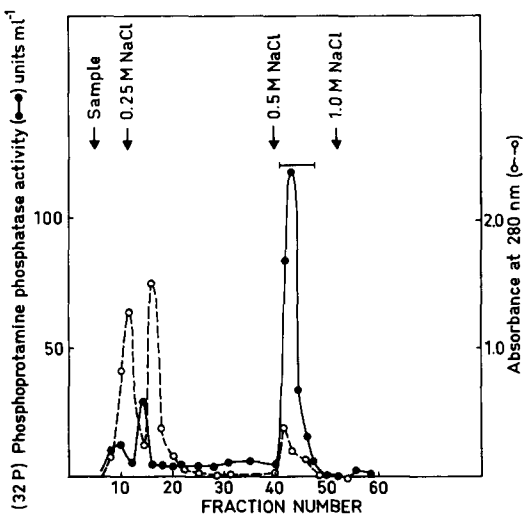

Fig. 2. Chromatography of phosphoprotein phosphatase fraction B on protamine-agarose. The column (2.5 X 5.3 cm) was equilibrated and eluted at a flow rate of 50 ml/h with buffer A; 15-ml fractions were collected. The horizontal bar indicates the fraction used for further purification. The material used had been purified through the step of Sephadex G-200 II (see Table I). For further explanation, see Experimental and Results.

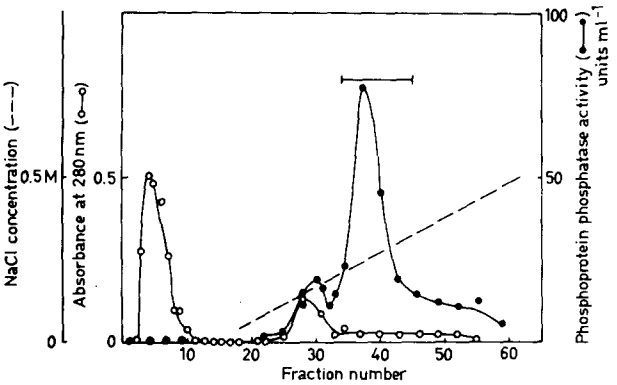

Fig. 3. Histone-agarose chromatography of phosphoprotein phosphatase B. A histone-agarose column (1.5 X 10 cm) was equilibrated with buffer A containing 40 mM NaCl and 20% sucrose. The enzyme pool from the protamine-agarose column was applied to the histone-agarose column. Fractions 1—18 were 10 ml each, the others were 3.5 ml. The horizontal bar indicates the fractions used for further purification. Activity was detected with [^{32}P]phosphoprotamine as a substrate. The material used had been purified through the step of protamine-agarose chromatography.

which case, a more than 15-fold purification was obtained, usually with better than 50% recovery of the enzymatic activity. However, it was preferable to employ protamine-agarose chromatography after removal of some inert protein by the Sephadex G-200 chromatography. The performance of the protamine-agarose depends not only on the degree of substitution, but also on the phosphoprotein phosphatase fraction used. Gels made by adding different amounts of protamine per ml CNBr-activated agarose were tested. The best purification and recovery of enzymatic activity was obtained with beds made by adding 0.5 mg protamine per ml settled CNBr-activated agarose. With highly substituted protamine-agarose (made by adding 4 mg protamine per ml CNBr-activated

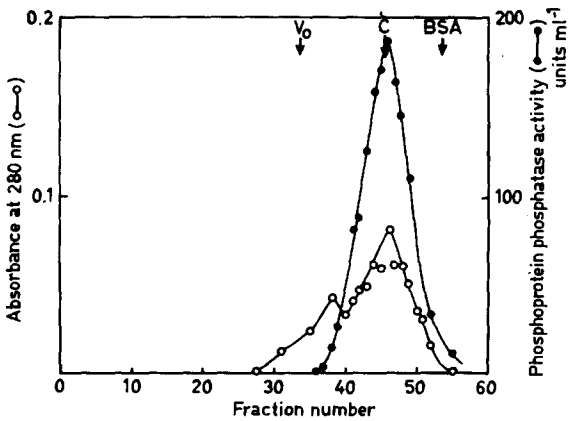

Fig. 4. Chromatography of phosphoprotein phosphatase B on Sephadex G-200. The enzyme used was the pooled fractions from the histone-agarose column of Fig. 3. The void volume (V_0) of the colum (as determined with blue dextran) was 15.7 ml and the total volume (V_t) was 45 ml. The elution volumes (V_e) of catalase (C) and bovine serum albumin (BSA) determined in a separate experiment on the same column were 23.2 and 28.4 ml, respectively. Phosphoprotamine phosphatase activity (•———•) had a V_e of 23.6 ml on this column. o———o, absorbance at 280 nm.

agarose) desorption was difficult and enzymatic activity was eluted with low recovery over a wide range of NaCl concentrations.

In order to obtain good recoveries of enzymatic activity, it was necessary to carry out the purification at 0—4°C and to include 20% sucrose in the buffers during the final steps of the procedure. As can be seen on Table I, a 400-fold enrichment of the activity towards [^{32}P]phosphoprotamine was obtained.

Fig. 4 shows that purified phosphatase B emerges from the column immediately preceding catalase (240 000). This elution position is consistent with the apparent molecular weight of approx. 250 000 found for less purified material from DEAE-cellulose step. The apparent molecular weights of phosphatase fractions A and C were 250 000 and 140 000, respectively.

The specific activity of about 2000 units per mg protein determined for purified phosphatase B is higher than the values reported previously [1,2]. The recovery of enzymatic activity was fairly low with most of the losses usually encountered during the concentration before gel chromatography and during the last ammonium sulphate precipitation (see Experimental).

Hydrophobic interaction chromatography of rat-liver phosphoprotein phosphatases

Experiments were carried out to investigate the conditions for the use of hydrophobic interaction chromatography in the purification of rat-liver phosphoprotein phosphatases. At high ionic strength (3 M NaCl in buffer A) alkyl-agarose columns with four to seven carbon atoms in the alkyl groups were found to bind more than 80% of the phosphoprotein phosphatase activity of rat-liver cell-sap. Shorter carbon chains were not tested.

Upon decreasing the ionic strength, 45—75% of the original activity could be desorbed from butyl-, pentyl- and hexyl-agarose (Fig. 5). The recovery of enzymatic activity from heptyl-agarose was about 10—20%, which was similar to the recovery from octyl-agarose. With dekyl-agarose, the adsorption was complete and irreversible (results not illustrated). Under similar starting conditions (Fig. 5), unsubstituted agarose did not retain phosphoprotein phosphatase activity.

Optimal recovery of phosphoprotein phosphatase activity was obtained using butyl- and pentyl-agarose with a degree of substitution [10] in the range 50—110 mmol alkyl residues per mol hexose (data not illustrated). It is also shown in Fig. 5 that phosphoprotein phosphatase could be enriched 5-fold as compared with the specific activity in cell-sap, which was 3 units per mg protein in these experiments. These observations suggested an alternative approach for the purification of the enzymes. Starting with cell-sap it was possible to obtain a preparation with a specific activity of 60 units per mg protein by chromatography on pentyl-agarose followed by protamine-agarose (Table II). The amount of starting material was limited by the capacity of the pentyl-agarose, which was about 5—6 mg protein per ml settled gel.

We have so far been unable to employ pentyl-agarose chromatography at the latter stages of the purification of phosphatase fraction B. For example, although a 2-fold enrichment could be obtained by starting with phosphatase fraction B from the DEAE-cellulose step (Fig. 1), in most experiments, severe losses of enzymatic activity were observed, presumably because of sample dilu-

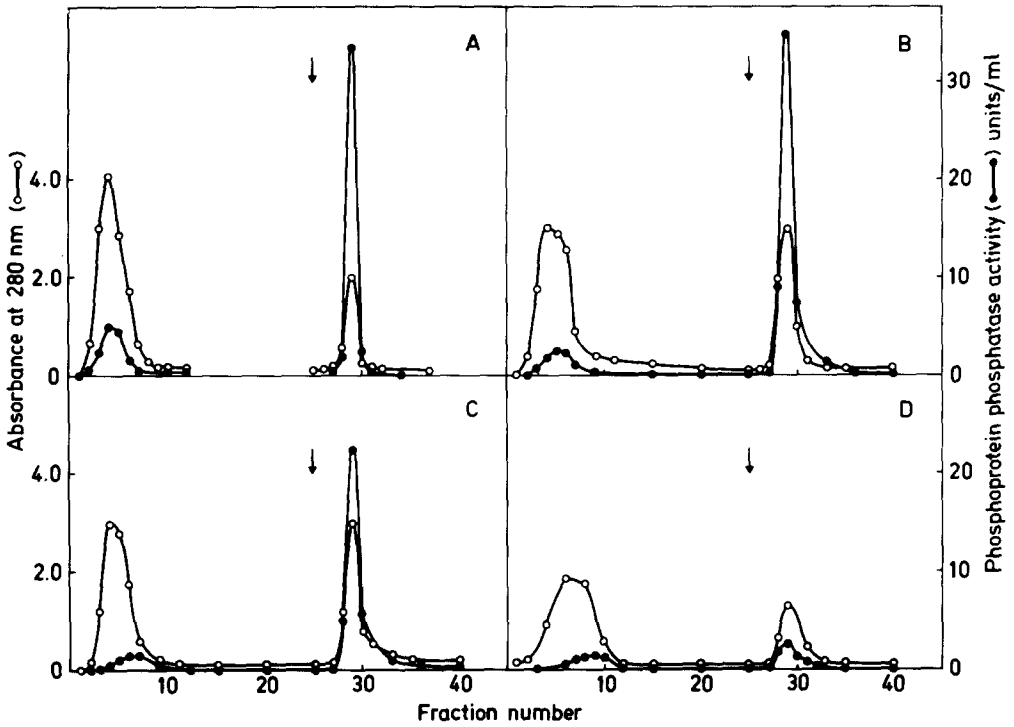

Fig. 5. Chromatography of phosphoprotein phosphatases from rat-liver cell-sap on alkyl-agarose. Panels A, B, C and D refer to butyl-, pentyl-, hexyl- and heptyl-agarose, respectively. 264 units (95.5 mg protein) were applied in experiments A, B and C, while 200 units (70.3 mg protein) were used for experiment D. The recoveries of enzymatic activity eluted with buffer A (↓) were, respectively, 60, 70 and 45% for experiments A, B and C and 10% for experiment D. The degree of substitution [10] of the gels used was similar, i.e. about 80 mmol residues/mol hexose. Other details are given under Experimental.

TABLE II

PURIFICATION OF PHOSPHOPROTEIN PHOPHATASE(S) OF CELL-SAP BY CHROMATOGRAPHY ON PENTYL-AGAROSE FOLLOWED BY PROTAMINE-AGAROSE

Cell-sap, containing 382 units of phosphoprotein phosphatases and 127 mg protein was chromatographed on pentyl-agarose columns as described in Experimental. After dialysis against buffer A, the enzyme was further purified by chromatography on a protamine-agarose column (1.0 X 6.4 cm), equilibrated and eluted as described for the purification of phosphatase fraction B (see the legend of Fig. 2 and Experimental). Enzymatic activity was detected with 60 μM [^{32}P]phosphoprotamine as substrate. Details are given in the text and under Experimental.

Step	Phosphoprotamine phosphatase activity		
	Units/mg protein	Units	Yield
Cell-sap	3	382	100
Pentyl-agarose	16	132	34
Protamine-agarose	60	103	27

tion during elution. Evidently, it is more convenient to use this step with the crude extracts, as illustrated in Table II and Fig. 5. As illustrated in Table II the combination of pentyl-agarose followed by protamine-agarose chromatography appears to be suitable for the rapid enrichment of samples of phosphoprotein phosphatase(s), since after these steps contaminants such as ATPases and β-glycerophosphatases are essentially removed from the preparation.

Kinetic studies

Table I shows that the activity towards [^{32}P]phosphopyruvate kinase, [^{32}P]-phosphohistone and [^{32}P]phosphoprotamine were present at all stages of the purification of phosphoprotein phosphatase fraction B. However the activity ratios were not constant during the purification and the reason for this observation remains unclear. Some of the kinetic properties of purified phosphatase fraction B were investigated. With [^{32}P]phosphoprotamine as a substrate the apparent K_m value for the purified fraction B was about 0.01 mM, the value observed for pyruvate kinase was 0.02 mM. The apparent K_m for the dephosphorylation of [^{32}P]phosphohistones was about 0.05 mM. In all cases the K_m determinations were carried out under the conditions of the standard assay (ref. 3 and Experimental).

The effects of selected ligands on the dephosphorylation of [^{32}P]phosphopyruvate kinase is shown on Table III. As was observed earlier with another phosphoprotein phosphatase preparation [3] 2.5 mM $MgCl_2$ appeared to remove most of the inhibition induced by 1 mM ATP. Although 2.5 mM $MgCl_2$ or 2.5 mM $MnCl_2$ stimulated the phosphatase activity from the DEAE-cellulose about 2—3-fold, stimulation by divalent cation appeared to be less pronounced with purified phosphatase fraction B. The reason for this apparent discrepancy remains to be determined. However, as expected NaF and KH_2PO_4 were inhibitory. The partial inhibition of phosphatase fraction B by EDTA indicates that added divalent cations are not absolutely required for enzymatic activity. Similar effects of the selected ligands (Table III) have been reported as being common with respect to several preparations of phosphoprotein phosphatases [3,6, 7].

TABLE III

EFFECTORS OF PURIFIED PHOSPHOPROTEIN PHOSPHATASE FRACTION B

Incubation was carried out in the standard reaction mixture containing 5 μM [^{32}P]phosphopyruvate kinase as a substrate, and 0.04 μg purified phosphatase fraction B (see Table I). Divalent cations were not included in the reaction mixture, except where indicated. The mean ± S.D. of four determinations are given. Other details are given in the text.

Effector	Phosphatase B activity (pmol released/5 min)
1. No additions	16.1 ± 0.3
2. 2.5 mM EDTA	11.0 ± 1
3. 2.5 mM $MgCl_2$	17.1 ± 3.2
4. 2.5 mM $MnCl_2$	15.8 ± 1.7
5. 5 mM potassium phosphate, pH 7.5	8.8 ± 0
6. 25 mM NaF	5.0 ± 0.5
7. 1 mM ATP	5.9 ± 0.9
8. 1 mM ATP + 2.5 mM $MgCl_2$	12.5 ± 0.1

Discussion

In the present investigation a procedure has been developed which permits a 400-fold enrichment of phosphatase fraction B. This fraction represents the main activity towards phosphoprotamine and displays phosphopyruvate kinase phosphatase activity (Table I, Fig. 1). The further purification of the enzyme has proceeded beyond the degree reported by others [1,7] and has been achieved without converting the enzyme to a smaller form, as was the case with other phosphoprotein phosphatases of similar specific activity [4–6]. One unique step in this procedure was the application of protamine-agarose chromatography (Fig. 2). Another effective step, used in a separate approach, was hydrophobic interaction chromatography on non-charged derivatives of agarose (Fig. 5 and Table II). The salt dependence observed (Fig. 5) is expected of hydrophobically interacting proteins [10,14,15]. The effectiveness of histone-agarose chromatography in the purification of fraction B (Table I, Fig. 4) confirms and extends the observation of others [5,13], that this method can be used in the purification of phosphoprotein phosphatases from several sources. The procedures investigated seem to provide a foundation for the further purification of, at least, phosphatase fraction B (Table I).

The removal by Mg^{2+}, of the inhibition induced by ATP is in agreement with previous results [3] indicating that these ligands can modulate the dephosphorylation of phosphopyruvate kinase in vitro. In fact the pattern of the regulation of phosphatase fraction C from the DEAE-cellulose chromatography by the ligands listed in Table III was essentially similar to those seen with phosphatase fraction B from the same step (results not illustrated) and the apparent K_m for the dephosphorylation of phosphopyruvate kinase fraction C was also about 0.02 mM. These observations support the close similarity of the regulatory properties of those phosphoprotein phosphatase preparations which use phosphopyruvate kinase in vitro as a substrate.

Others have suggested that multiple forms of liver phosphoprotein phosphatases can be produced by proteases which are released from lysosomes during vigorous homogenisation of the tissue [16]. In the present investigation livers were homogenized gently to minimise lysosomal damage and in the presence of phenylmethylsulfonyl fluoride in an attempt to inhibit proteolysis. Although complete inhibition of proteolytic enzymes could not be guaranteed, their effects were considered to be minimal since we could not generate lower molecular weight forms of the enzymes by storing the homogenates for more than 6 h before chromatography on DEAE-cellulose. Furthermore, the pattern displayed on Fig. 1 was highly reproducible in repeated experiments.

The multiple forms of phosphoprotein phosphatases observed in this and other reports [1,7,18] are consistent with the diversity of the phosphoprotein substrates. The finding that most of the phosphopyruvate kinase phosphatase activity resides in fractions B and C supports the hypothesis that these fractions may be involved in vivo in the dephosphorylation of phosphorylated pyruvate kinase.

Acknowledgements

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