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Chromatographic separation of four Ser/Thr–protein phosphatases from solubilized ciliary membranes of *Paramecium tetraurelia* by heparin–Sepharose

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ABSTRACT

Chromatography of deoxycholate-solubilized proteins from *Paramecium* ciliary membranes on heparin–Sepharose resolved three peaks of protein phosphatase activities: one type 2A-like and a type 2C phosphatase in the flow-through fractions, another type 2A-like enzyme in the 0.1 *M* NaCl eluate and type 1 protein phosphatase in the 0.5 *M* NaCl eluate. The differential sensitivity of the two type 2A-like phosphatases to heparin and protamine further substantiated the existence of distinct isozymes. Once solubilized, none of these ciliary phosphatases required detergent to remain soluble. The molecular mass as determined by chromatography on Superose 6 was in the range 30 000–45 000 dalton for all four protein phosphatases.

INTRODUCTION

The protozoon *Paramecium* is used as a model organism to study signal reception and transduction at a unicellular level (for a review, see ref. 1). The availability of mutants with electrophysiologically characterized defects makes this ciliate a valuable tool for basic biochemical and pharmacological research on excitation-related processes [2,3].

Recently, regulation of voltage-dependent Ca-channels localized in the ciliary membrane of *Paramecium* by phosphorylation and dephosphorylation was suggested [4]. So far, cAMP-, cGMP- and Ca^{2+}-stimulated protein kinases and type 1, 2A-like and 2C protein phosphatases have been identified in the cilia from *Paramecium* [1,4,5].

This paper describes the resolution of the ciliary type 2A-like phosphatase activities by column chromatography. Heparin–Sepharose proved suitable for separating four protein phosphatases into three peaks of activity. The molecular mass of these enzymes was subsequently determined by fast protein liquid chromatography (FPLC) on Superose 6.

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EXPERIMENTAL

Cell culture and tissue preparation

Paramecium tetraurelia wild-type 51s was mass cultured axenically in 20-l bioreactors as described [5]. Cells at the early stationary phase (30 000/ml) were harvested in a cream separator. Cilia were removed from the cells by Ca-shock and purified by differential centrifugation [6]. The purity of the cilia was routinely checked by phase contrast microscopy.

Ciliary protein phosphatases were solubilized by adding 1 part of 2% deoxycholate [dissolved in 50 m*M* Tris–HCl (pH 7)] to 9 parts of a suspension of cilia (2 mg/ml) in 10 m*M* 3-(N-morpholino)propanesulphonic acid–Na (pH 7.5). After being vortex mixed and allowed to stand on ice for 30 min, the suspension was centrifuged for 1 h at 100 000 × *g*. The supernatant was used as a source of solubilized phosphatase activities and stored at −70°C.

Preparation of ^{32}P-labelled protein substrates

^{32}P-labelled phosphorylase *a* was prepared by phosphorylation with phosphorylase kinase [7], and casein by phosphorylation with cAMP-dependent protein kinase [8].

Protein phosphatase assays

Incubations (30 μl) contained 50 m*M* Tris–HCl (pH 7), 0.1 m*M* EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.6 mg/ml bovine serum albumin, ^{32}P-labelled substrate and enzyme. The substrate concentrations were 10 μ*M* for phosphorylase *a* or 1 μ*M* for phosphocasein. Caffeine (5 m*M*) was present when phosphorylase *a* was used as substrate [7], while assays for protein phosphatase 2C (using phosphocasein as a substrate) contained 20 m*M* magnesium acetate [8]. After 10 min at 30°C, the reactions were terminated and the solutions analysed as described [7,8]. Release of radioactivity was restricted to 30% of the input to ensure linear reaction rates. One unit of activity was defined as the amount which catalysed the release of 1 μmol phosphate/min.

Affinity chromatography on heparin–Sepharose

Solubilized phosphatases from ciliary membranes were applied to heparin–Sepharose. Up to 30 mg of protein in 50 ml could be successfully applied at once to a 10-ml column. The column was developed successively with buffer alone and with buffer containing 0.1 and 0.5 *M* NaCl. Further experimental details are given in the legend to Fig. 1.

Size-exclusion chromatography

Analyses were carried out on a Pharmacia (Uppsala, Sweden) automated FPLC system using a Superose 6 HR column (30 × 1.0 cm I.D.). The system was equipped with an LCC-500 gradient programmer, two P-500 dual piston pumps, an MV-7 automated injection valve, solvent mixer, prefilter, sample loop (0.5 ml), a UV-M monitor and a recorder.

RESULTS AND DISCUSSION

General purification problems

Membrane-bound mammalian protein phosphatase type 1 can be released by treatment with 1 *M* NaCl [9]. For solubilization of protein phosphatases localized in the ciliary membrane from *Paramecium*, however, detergent was required. At a protein concentration of up to 2 mg/ml, 0.2% deoxycholate released 90% of total particulate phosphatase activity into the supernatant. Although affinity chromatography is commonly used as one of the last steps in enzyme purification, in this work heparin–Sepharose (Pharmacia) was chosen at the beginning for the following reasons: (a) concentration and partial purification by precipitation of phosphatases with ammonium sulphate was impossible because, owing to the presence of detergent, a "floating pellet" was obtained; (b) chromatography on DEAE and Mono Q ion exchangers was precluded because of the anionic nature of deoxycholate; the anionic detergent could not be replaced by neutral detergents, such as Nonidet P40, Triton X-100 or Lubrol PX, without a substantial reduction in yields; (c) cation-exchange chromatography (Mono S) was unsuccessful as virtually all of the phosphatase activity was already recovered in the flow-through fractions; and (d) gel permeation chromatography was not practicable owing to the large starting volume of usually 50 ml.

Separation of Ser/Thr–protein phosphatases on heparin–Sepharose

Type 2A phosphatases from mammalian cells do not bind to heparin–Sepharose at 0.1 *M* NaCl whereas type 1 enzymes are retained and can be eluted with 0.5 *M* NaCl [10]. The same is true for the protein phosphatases from *Paramecium* cilia, cytosol and cell membranes [4]. By modifying this procedure we succeeded in further separating the protozoan type 2A-like phosphatase activities.

Solubilized ciliary phosphatases were applied to heparin–Sepharose in the absence of salt; 55% of the applied protein did not bind to the column material, 45% eluted with 0.1 *M* NaCl and only 5% was recovered in the high salt fraction (Fig. 1A). Phosphorylase phosphatase activity measured in the absence of divalent cations was found in the flow-through fractions (34% of total activity), the 0.1 *M* NaCl eluate (11%) and the 0.5 *M* NaCl eluate (59%, Fig. 1B). The dephosphorylation rate in any of these fractions was not affected by addition of up to 1 m*M* Ca^{2+}. The recovery of enzyme activity after correction for inhibition by salt was routinely 95–100%. In order to rule out that the binding capacity of heparin–Sepharose had been exceeded during the first column run, we rechromatographed the flow-through fractions on new material. All of the phosphatase activity was again recovered in the flow-through. Therefore, we conclude that the binding capacity during the first run was already sufficient.

The protein phosphatase activities in the flow-through and 0.1 *M* NaCl eluate were both classified as type 2A-like enzymes with regard to substrate specificity, divalent cation requirement and insensitivity toward inhibitor proteins I_1 and I_2 (for a phosphatase review, see ref. 9). However, these two type 2A-like phosphatases were clearly different in their responses to heparin and protamine (Fig. 2). The phosphatase activity in the flow-through was unaffected by protamine and only slightly activated by heparin. In contrast, the enzyme activity which was eluted by 0.1 *M* NaCl was potently

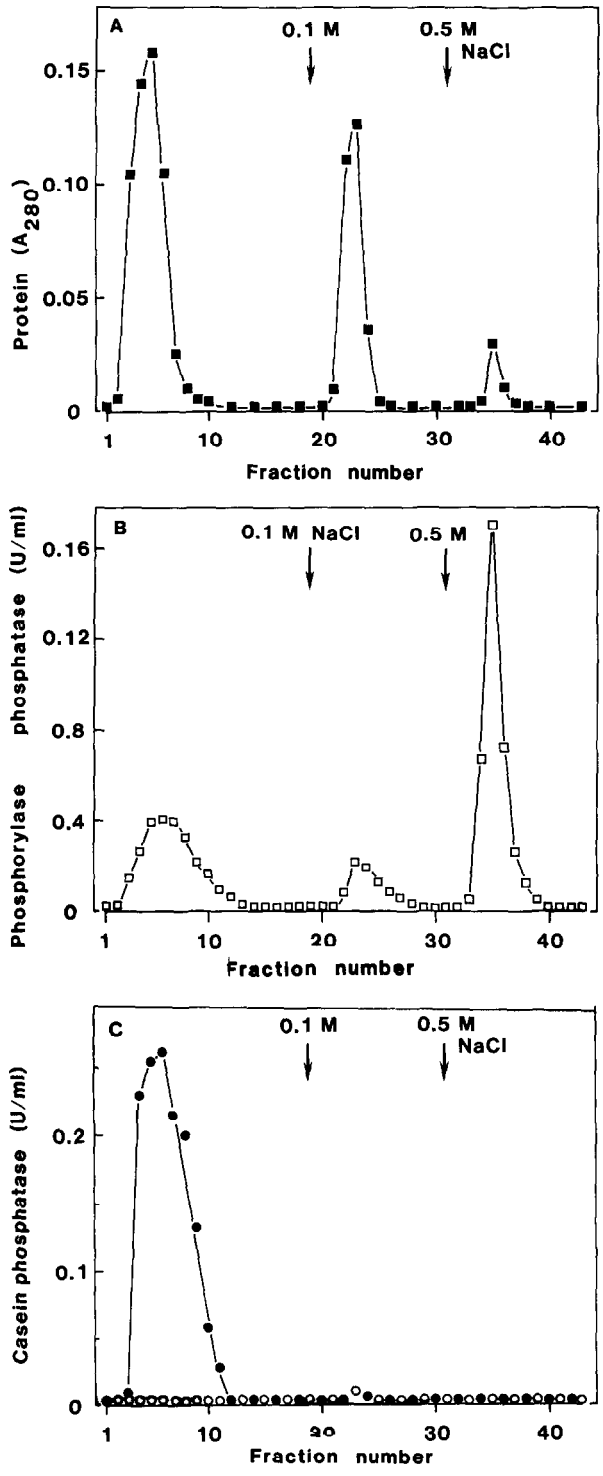

Fig. 1. Chromatography of ciliary protein phosphatases on heparin–Sepharose. The 100 000 *g* (1 h) supernatant fraction of 0.2% deoxycholate-treated cilia (6.7 mg, 10 ml) was applied to the column (4.8 × 1.6 cm I.D.) equilibrated in 20 m*M* Tris–HCl (pH 7)–0.1 m*M* EGTA–0.1% (v/v) 2-mercaptoethanol–0.1 mM phenylmethylsulphonyl fluoride–1 m*M* benzamidine–5% (v/v) glycerol. Arrows indicate the positions at which the column was eluted with equilibration buffer plus 0.1 or 0.5 *M* NaCl. The flow-rate was 3 ml/min and fractions of 3 ml were collected. (A) Absorbance at 280 nm; (B) phosphorylase phosphatase activity in the absence of divalent cations and in the presence of 0.1 m*M* EGTA; (C) casein phosphatase with (○) 0.1 m*M* EGTA or (●) 0.1 m*M* EGTA plus 10 m*M* Mg^{2+}.

inhibited by protamine and almost doubled by heparin (Fig. 2). This indicates that two distinct isozymic forms of phosphatase type 2A were efficiently and easily separated by this method.

The phosphatase activity in the 0.5 *M* NaCl eluate could be ascribed to a type 1 enzyme according to standard classification criteria, *e.g.*, inhibition by inhibitor proteins I_1 and I_2 and okadaic acid [4], inhibition by heparin and protamine (Fig. 2) and preferential dephosphorylation of the β-subunit of phosphorylase kinase [4]. As 0.5 *M* NaCl inhibited type 1 activity by about 50%, the salt concentration necessary for elution was reduced in subsequent experiments; 0.3 *M* NaCl was found to be sufficient for quantitative elution of this phosphatase type 1 activity from heparin–Sepharose (data not shown). None of the other protein phosphatases was affected by 0.3 *M* NaCl and inhibition of the type 1 enzyme was reduced to 20%. Hence dialysis of the fractions which resulted in complete removal of salt inhibition could be omitted in daily routine application. The heparin–Sepharose chromatography resulted in a 100-fold purification of the type 1 phosphatase. However, on silver-stained sodium dodecyl sulphate gels more than 20 discrete protein bands were clearly visible, indicating the need for further purification.

Using phosphocasein as a substrate in the presence of 10 m*M* Mg^{2+}, a single peak of casein phosphatase activity was detected in the flow-through fractions. Dephosphorylation of casein, in contrast to phosphorylase *a*, had an absolute requirement for Mg^{2+}, indicating that it was indeed catalysed by a type 2C protein

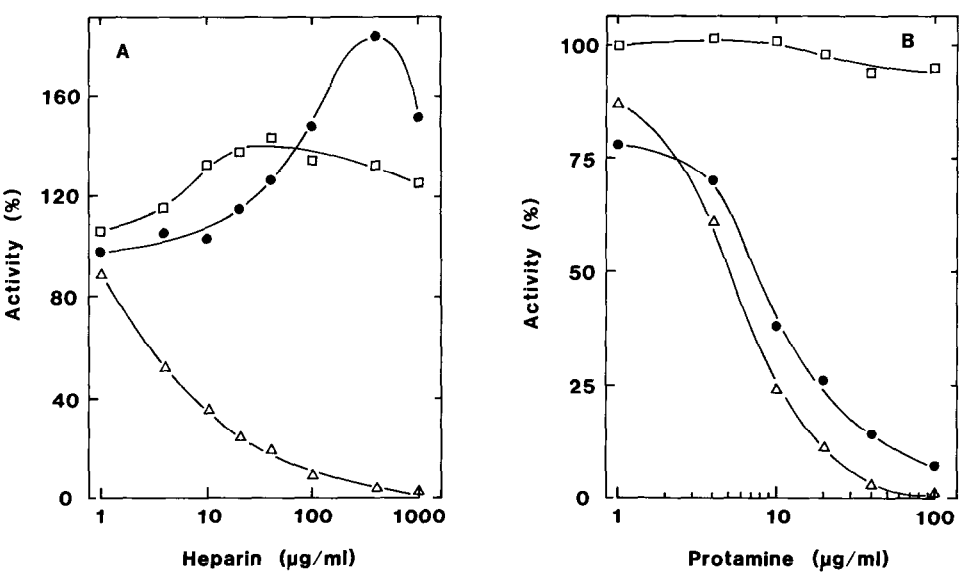

Fig. 2. Effect of (A) heparin and (B) protamine on phosphorylase phosphatase activities from the solubilized ciliary membranes of *Paramecium*. Flow-through fractions from (□) heparin–Sepharose, (●) 0.1 *M* NaCl and (△) 0.5 *M* NaCl eluates were assayed. Salt-containing fractions were dialysed prior to testing. Activities are expressed as a percentage of values obtained in the absence of heparin and protamine, respectively. Assays were carried out in the absence of Mg^{2+} to avoid interference from type 2C enzyme activity.

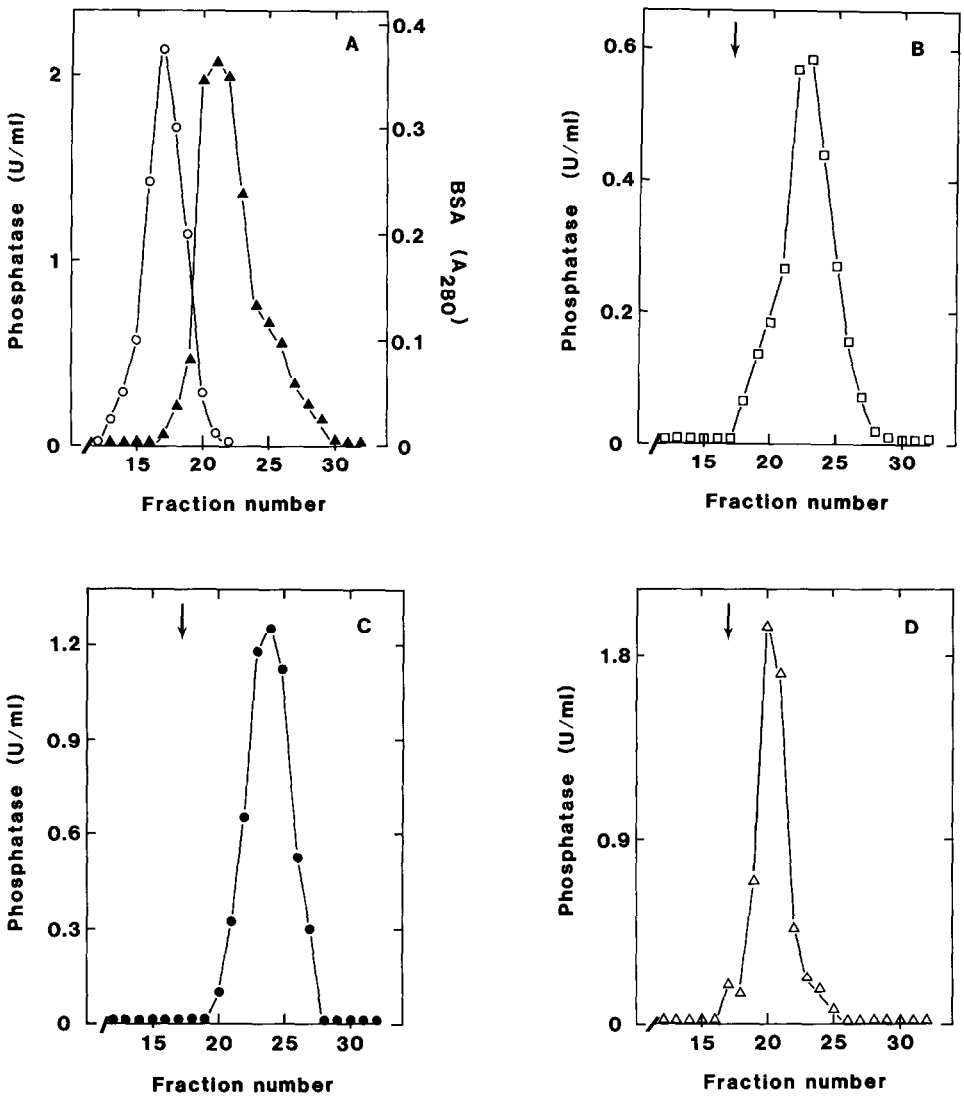

Fig. 3. Gel filtration of ciliary protein phosphatases on Superose 6. The column (23 ml) was equilibrated and run with 20 m*M* Tris–HCl (pH 7)–0.1 m*M* EGTA–0.1% (v/v) 2-mercaptoethanol–0.1 m*M* phenylmethylsulphonyl fluoride–1 m*M* benzamidine at a flow-rate of 0.3 ml/min. Fractions of 0.5 ml were collected. Sample input volume, 0.5 ml. (A) Chromatography of (○) bovine serum albumin (66 000 dalton) and (▲) catalytic subunit of protein phosphatase type 1 isolated from rabbit liver (37 000). The marker proteins were detected by absorbance at 280 nm and phosphorylase phosphatase activity, respectively. The arrows in B–D denote the position of bovine serum albumin. In B–D fractions from deoxycholate-solubilized ciliary phosphatases after chromatography on heparin–Sepharose (see Fig. 1) were applied to Superose 6. (B) Heparin–Sepharose flow-through; (C) heparin–Sepharose 0.1 *M* NaCl eluate; (D) heparin Sepharose 0.5 *M* NaCl eluate.

phosphatase. No type 2C activity was detectable in either the 0.1 or 0.5 *M* NaCl eluates (Fig. 1C).

Gel filtration of heparin–Sepharose fractions on Superose 6

The molecular mass of the free catalytic subunits from mammalian protein phosphatases types 1 and 2A are almost identical, *i.e.*, 35 000 and 37 000 dalton, respectively [9]. The size of the holoenzymes, in contrast, is much larger (> 150 000 dalton) [9].

To determine the molecular mass of solubilized ciliary phosphorylase phosphatases from *Paramecium*, aliquots of those fractions with the highest activity from heparin–Sepharose flow-through and 0.1 and 0.5 *M* NaCl eluates were directly applied to Superose 6, respectively. For calibration, bovine serum albumin was run on the same column under otherwise identical conditions (Fig. 3A). Further, to compare directly the chromatographic behaviour of the protozoan enzymes with those from mammalia n tissue, the catalytic subunit of phosphatase type 1 from rabbit liver was also passed through the same column (Fig. 3A).

All phosphorylase phosphatases originating from *Paramecium* cilia chromatographed on Superose 6 at positions corresponding to molecular masses of 30 000–45 000 dalton (Fig. 3B–D). The type 2A-like phosphatase activity from heparin–Sepharose flow–through reproducibly revealed a pronounced shoulder on the ascending part of its elution profile on Superose 6 (Fig. 3B). This effect may be due to micelle formation, as deoxycholate used for solubilization of membraneous phosphatases is not retained by heparin–Sepharose and was, therefore, applied to Superose 6 concomitantly with the phosphatase present in the heparin–Sepharose flowthrough fractions. The other type 2A-like phosphatase present in the 0.1 *M* NaCl eluate from heparin–Sepharose no longer contained any deoxycholate and chromatographed on Superose 6 as a sharp and symmetrical peak (Fig. 3C). *Paramecium* type 1 phosphatase present in the heparin–Sepharose 0.5 *M* NaCl fractions eluted at the same position on Superose as did the catalytic subunit from rabbit liver type 1 phosphatse (compare Fig. 3A and D).

So far, heparin–Sepharose has been established for the separation of mammalian types 1 and 2A protein phosphatases. It is commonly used in the presence of 0.1 *M* NaCl [11,12]. As is obvious from the data presented here, running heparin–Sepharose in the absence of NaCl prior to salt step elution results in the separation of two phosphatases that otherwise would have coeluted in the 0.1 *M* NaCl flow-through. Therefore, this procedure seems to be very valuable for the efficient and rapid separations of closely related Ser/Thr–protein phosphatases. Further studies with phosphatases from sources other than *Paramecium* cilia will be necessary in order to clarify whether subtypes of 2A-like phosphatase are ubiquituous in nature.

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