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SYNTHESIS OF ATP–POLYETHYLENE GLYCOL AND ATP–DEXTRAN AND THEIR USE IN THE PURIFICATION OF PHOSPHOGLYCERATE KINASE FROM SPINACH CHLOROPLASTS USING AFFINITY PARTITIONING

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SUMMARY

ATP was covalently bound to the polymers dextran of molecular weight 500 000 and polyethylene glycol (PEG) of molecular weight 7000–9000. The degree of substitution (the ATP:polymer molar ratio) was varied by using different concentrations of ATP in the synthesis of ATP–dextran. The partitioning of the ATP–dextran derivative in an aqueous two-phase system containing dextran and PEG was found to depend on the degree of substitution. The potential of the ATP–polymer derivatives obtained for the extraction of phosphoglycerate kinase (E.C. 2.7.2.3) from crude spinach leaf and chloroplast extract using affinity partitioning was studied and compared with the corresponding polymer derivatives of the reactive dye Procion Turquoise MX-G. Both ligands, whether bound to dextran or PEG, affected the target enzyme, phosphoglycerate kinase, but ATP showed a higher specificity. ATP bound to dextran was found to be the most powerful derivative and a rapid extraction procedure involving three main steps, precipitation with PEG, affinity partitioning with ATP bound to dextran as ligand and ion-exchange treatment, was designed. This procedure is rapid, easy to scale up and yields an enzyme preparation that is 80% pure.

INTRODUCTION

When two suitable polymeric substances (differing in molecular structure) are mixed in water at sufficiently high concentrations (3–8%), two phases are formed where the polymers are enriched in opposite phases. Because of the high water content of both phases, proteins can be included in the two-phase systems. The partitioning of proteins between the phases depends on a number of variables that can be adjusted to make these two-phase systems useful for protein purification[1]. One way of significantly and selectively influencing the partitioning is to introduce biospecific ligands that are covalently bound to one of the phase-forming polymers. This technique has been named affinity partitioning[1,2].

ATP has been used as a biospecific ligand in affinity chromatography but so far, to our knowledge, not in affinity partitioning. Chloroplast phosphoglycerate kinase,

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an enzyme in the Calvin cycle (reductive pentose phosphate pathway), has been purified from spinach by affinity chromatography using ATP as ligand[3,4].

In this work, ATP was covalently bound to the two phase-forming polymers dextran and polyethylene glycol (PEG). As an illustation of the usefulness of ATP–polymer derivatives in affinity partitioning, the extraction of phosphoglycerate kinase from crude spinach leaf and chloroplast extract was compared with the corresponding derivatives of a reactive dye, Procion Turquoise MX-G. These dyes are widely used as ligands for nucleotide-binding enzymes[5]. ATP bound to dextran was found to be the most powerful derivative and was used for the batch extraction of phosphoglycerate kinase.

EXPERIMENTAL

All manipulations were carried out at 4 ± 1°C unless indicated otherwise.

Materials

Dextran T500 ($\bar{M}_r$ = 500 000), Sephadex G-25 (fine) and DEAE-Sephadex A-50 were purchased from Pharmacia (Uppsala, Sweden) and polyethylene glycol (PEG) with M_r = 7000–9000 (Carbowax 8000) was obtained from Union Carbide (New York, NY, U.S.A.). Celite was a product of Johns-Manville (Denver, CO, U.S.A.) and glass-fibre filters of Whatman (Maidstone, U.K.). Tetrabutylammonium hydroxide, 2,2,2-trifluoroethanesulphonyl chloride (tresyl chloride) and adipic acid dihydrazide were obtained from Fluka (Buchs, Switzerland). Na_2ATP was supplied by BDH (Poole, U.K.) and other biochemicals by Sigma (St. Louis, MO, U.S.A.). The water was singly distilled and then passed through a mixed ion exchanger. All other chemicals were of analytical-reagent grade.

Spinach (*Spinacia oleracia* L., cv. Medania; Weibulls, Sweden) was grown hydroponically as described previously[6]. A 0.5 *M* stock solution of tetrabutylammonium phosphate was prepared by adjusting the pH of 0.5 *M* phosphoric acid to 7.7 with 40% tetrabutylammonium hydroxide. When such a solution is used to prepare the two-phase systems in this work the pH on dilution will be 7.5.

Synthesis of ATP–polymer derivatives

Periodate-oxidized ATP was bound to dihydrazide-substituted PEG and dextran by condensation with the hydrazo group. The final products were analysed by gel filtration on a Sephadex G-25 column (20 × 1.5 cm I.D.) using 50 m*M* Tris–HCl (pH 7.5) as eluent. The effluent was monitored with an Optilab flow-through refractometer and a LKB Uvicord-S (280 nm filter) ultraviolet monitor in order to detect the polymers and the ligand separately. The amount of ATP bound to dextran was determined enzymatically by using the ATP–dextran derivative instead of free ATP in a phosphoglycerate kinase assay system[7]. The amount of bound nucleotide (ATP + ADP + AMP) was determined photometrically at 259 nm using a molar absorption coefficient of 15 400 l mol^{-1} cm^{-1} [8]. The amount of bound ATP was found to be 90% of the amount of bound nucleotide. The amount of ATP bound to PEG was obtained by correcting the measured absorbance value according to the above.

Tresyl-PEG. Tresyl activation was performed essentially as described elsewhere[9]. A 10-g amount of PEG was dissolved in 25 ml of dichloromethane containing

0.34 ml of triethylamine. The reaction mixture was cooled on ice and 0.300 g of 2,2,2-trifluoroethanesulphonyl chloride was added slowly. The mixture was stirred for 30 min on ice, 1 h at 4 ± 1°C and 24 h at room temperature. The solvent was removed by flushing with nitrogen. The residue was recrystallized three times from 100 ml of ice-cold ethanol.

Tresyl-dextran. Dextran (5 g) was dissolved in 25 ml of dimethyl sulphoxide, then 1 ml of triethylamine and 5 ml of dichloromethane were added slowly. The mixture was cooled on ice and 0.35 g of 2,2,2-trifluoroethanesulphonyl chloride was added slowly while stirring the mixture. The reaction mixture was stirred for 30 min on ice, 1 h at 4 ± 1°C and 24 h at room temperature. The reaction was terminated by precipitating the dextran with 50 ml of dichloromethane. The precipitate was washed and kneaded with several portions of dichloromethane.

Adipoyldihydrazo-PEG. Tresyl-PEG (10 g) was dissolved in 75 ml of 0.2 *M* carbonate buffer (pH 9.9) containing 0.5 *M* adipic acid dihydrazide. The reaction was conducted by stirring the mixture for 24 h at room temperature and was terminated by freeze-drying. The lyophilizate was extracted with chloroform and filtered through glass-fibre filters, first Whatman GF/C and then GF/F. The filtrate was clarified by passing it through a Celite column (20 × 2.5 cm I.D.) and the solvent was removed by evaporation. The yield was 2.3 g.

Adipoyldihydrazo-dextran. Tresyl-dextran (5 g) was dissolved in 50 ml of 0.2 *M* carbonate buffer (pH 9.9) containing 0.5 *M* adipic acid dihydrazide. The reaction mixture was stirred for 24 h at room temperature. After completion of the reaction, free adipic acid dihydrazide was removed by dialysing the reaction mixture against distilled water for 24 h. The dialysate was finally freeze-dried. The yield was 2.5 g.

Activation of ATP. ATP was activated by oxidation with sodium metaperiodate, essentially as described by Kuntz and Krietsch[4]. Briefly, 300 m*M* ATP (pH 8) and 300 m*M* sodium metaperiodate (pH 6) were mixed in equal volumes and the resulting mixture was stirred, while protecting it against light, for 2 h on ice. The reaction mixture was used directly in the coupling procedure.

ATP–adipoyldihydrazo-PEG. Adipoyldihydrazo-PEG (2.0 g) was dissolved in 10 ml of acetate buffer (pH 5.0) and activated ATP was added to give a final concentration of 50 m*M*. The coupling reaction was conducted by stirring the mixture for 24 h at room temperature. The reaction was terminated by freeze-drying, and the product formed was extracted with dichloromethane. The solid particles in the slurry were allowed to sediment, then the supernatant was collected and filtered. The solvent was finally removed by evaporation. The yield was 1.0 g and the degree of substitution was 0.06 mol of ATP per mole of PEG.

ATP–dipoyldihydrazo-dextran. Two couplings of ATP were conducted in order to give one low-substituted and one high-substituted end-product. In each coupling 2.0 g of adipoyldihydrazo-dextran were dissolved in 10 ml of 100 m*M* acetate buffer (pH 5.0). Activated ATP was then added to give final concentrations of 10 and 50 m*M*, respectively. The reaction mixtures were stirred for 24 h at room temperature and subsequently dialysed against distilled water for a further 24 h at 4 ± 1°C. After completing the dialysis, the solutions were freeze-dried. The yield was about 2 g in each instance and the degrees of substitutions were 4 and 40 mol of ATP per mole of dextran, respectively.

Synthesis of dye–polymer derivatives

Procion Turquoise MX-G was coupled to PEG as described by Johansson[2] and to dextran as described by Johansson and Andersson[10]. The amount of bound dye was determined photometrically at 620 nm using a molar absorption coefficient of 51 200 $l\,mol^{-1}\,cm^{-1}$. The degree of substitution was 1 mol of dye per mole of PEG and 6 mol of dye per mole of dextran, respectively.

Preparation of leaf extracts

Whole, freshly harvested leaves were homogenized in a household centrifugal juicer (Multipress MP50, type 4154; Braun, Frankfurt, F.R.G.) supplemented with filter-paper and the resulting solution was centrifuged (Sorvall SE-12) at 20 000 rpm (41 545 *g* at r_{max}) for 10 min. The pH of the clear, green supernatant was adjusted to 7.5 with sodium hydroxide and used without further manipulations.

Preparation of chloroplast extracts

Intact chloroplasts, prepared as described previously[6], were suspended in 25 m*M* tetrabutylammonium phosphate–1 m*M* 2-mercaptoethanol (pH 7.5) and treated in a Potter–Elvehjem glass–PTFE homogenizer on ice (20 strokes by hand) followed by centrifugation as above. The resulting clear supernatant showed a slight yellowish green colour and was used without further manipulations.

Aqueous two-phase systems

The two-phase systems were prepared from aqueous stock solutions of dextran (23.3%, w/w), PEG (40.0%, w/w), buffer, salts, sample solution and, when applicable, ligand–dextran derivative (2.75%, w/w) or ligand–PEG derivative (4.00%, w/w). The systems were equilibrated by mixing and samples from the upper and lower phases were withdrawn and analysed for phosphoglycerate kinase activity and protein content[2]. The partition coefficients, K, of the enzyme activity and protein is defined as the ratio between the concentrations of the component in the upper and lower phases, respectively. The separation factor, β, is defined as the ratio between the partition coefficients of phosphoglycerate kinase and total protein.

Precipitation with PEG

Chloroplast extracts were prepared as described above except that the isolated chloroplasts were suspended in 25 m*M* sodium phosphate–0.1 m*M* magnesium chloride–0.1 m*M* Na_2EDTA–1 m*M* 2-mercaptoethanol (pH 7.5) (NaPB) before treatment in a Potter–Elvehjem homogenizer. Appropriate weights of 50% (w/w) PEG in NaPB were added to chloroplast extracts in plastic micro-centrifuge tubes and the total weight of each sample was adjusted to 1.00 g with NaPB. The solutions were mixed with a vortex mixer, incubated for 1 h and centrifuged (Sorvall SS-34 with adapters) at 20 000 rpm (15 000 *g*) for 1 h. The supernatants were poured off, the tubes were flicked in order to remove as much liquid as possible and the pellets were dissolved in 1.0 ml of NaPB. The supernatants and the dissolved pellets were analyzed for enzyme activity and total protein content.

Purification of phosphoglycerate kinase from chloroplasts

A crude chloroplast extract was prepared by a simplified procedure involving

homogenization and a rapid centrifugation as described previously[6] except that the pelleted chloroplasts were suspended in 25 m*M* tetrabutylammonium phosphate–1 m*M* 2-mercaptoethanol (pH 7.5) and treated with a tight-fitting Dounce homogenizer. The extract was mixed with solid, ground PEG with gentle stirring until the concentration of the polymer was 30% (w/w) and centrifuged (Sorvall SS-34) at 20 000 rpm (48 200 *g* at r_{max}) for 1 h. The pellet was discarded and the supernatant, containing the main part of the phosphoglycerate kinase activity, was used as a 30% (w/w) PEG stock solution in the preparation of a two-phase system with the following final concentrations: 10.4% (w/w) dextran, 5.0% (w/w) PEG, 50 m*M* tetrabutylammonium phosphate (pH 7.5), 0.1 m*M* magnesium chloride, 0.1 m*M* Na_2EDTA and 1 m*M* 2-mercaptoethanol; 4% of the total dextran was low substituted ATP–dextran (4 mol of ATP per mole of dextran). The system was mixed and the phases were separated. The upper phase, enriched in the bulk proteins, was discarded and the lower phase, enriched in phosphoglycerate kinase activity, was washed with pure upper phase. The new lower phase was diluted with 40 m*M* Tris–HCl–5 m*M* magnesium chloride–1 m*M* 2-mercaptoethanol (pH 7.5) (Tris buffer) containing 4 m*M* Na_2ATP and 4 m*M* sodium hydrogensulphite in such a manner that the final concentrations of Na_2ATP and sodium hydrogensulphite were 3 m*M* each. After incubation for 5 min more Tris buffer was added to give a total dilution of 14-fold, which means that the dextran concentration had decreased to 1.6%. The resulting solution was passed through a bed of DEAE-Sephadex A-50 (18 × 2.5 cm I.D.). The bed was washed with Tris buffer until all of the dextran had eluted, as determined with a polarimeter, and then the adsorbed protein was eluted with Tris buffer containing 0.4 *M* sodium chloride.

Assays

Phosphoglycerate kinase activity was measured according to Kuntz and Krietsch[4] except that bovine serum albumin and dithioerythritol in the dilution buffer were omitted and the reaction was monitored at 340 nm using a molar absorption coefficient for NADH of $6.22 \cdot 10^3$ l mol^{-1} cm^{-1}. The initial velocity was determined and the concentration of 3-phosphoglycerate was 3 m*M*. The measured activities were corrected for the inhibitory effect caused by the polymer ligands. Protein was analysed according to Bradford[11] using calibration graphs obtained with bovine serum albumin. Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis was run and analysed as described by Johansson and Joelsson[12].

RESULTS AND DISCUSSION

Properties of ATP–polymer derivatives

The final synthesis products consisted of one fraction and contained no free nucleotide as judged by gel filtration (data not shown). The moles of ATP bound per mole of polymer (the degree of substitution) was determined photometrically and enzymatically. The ATP–PEG derivative was found to contain 0.06 mol of bound ATP per mole of PEG. For the ATP–dextran derivative the degree of substitution was varied by using different concentrations of periodate-oxidized ATP in the coupling to adipoyldihydrazo-dextran. The ATP–dextran derivatives obtained were included (2% of the total dextran) in phase systems of the same composition as in the extraction experiments below (see Figs. 3 and 4) containing 50 m*M* tetrabutylammonium

phosphate as buffer. The partition coefficients of the derivatives were calculated from meaurements of the absorption at 259 nm in the upper and lower phases, respectively. The log K of the low-substituted derivative (4 mol of ATP per mole of dextran) was -0.45 and that of the high-substituted derivative (40 mol of ATP per mole of dextran) was -0.30.

This increase in the partition coefficient, due to an increase in the degree of substitution, has also been found for the reactive dye Procion Yellow HE-3G bound to dextran and was explained by the presence of aromatic elements in the dye (which are known to be better solvated in PEG solutions than in pure water) and the effect of phosphate buffer on negatively charged polymers[13]. When the ATP–dextran derivative is used for affinity partitioning (in a dextran–PEG two-phase system), the capacity to enrich the target enzyme in the lower phase will increase with a decreasing partition coefficient of the derivative. Hence, in order to optimize (the effectiveness of) the extraction, a derivative with a low degree of substitution should be used.

The ATP–polymer derivatives were capable of replacing ATP in the phosphoglycerate kinase assay, which shows that they are enzymatically active. Both derivatives partly inhibited the activity of phosphoglycerate kinase (from spinach leaf extract) at concentrations corresponding to those used in the extraction experiments below. Both derivatives reduced the enzyme activity by around 10% (Fig. 1).

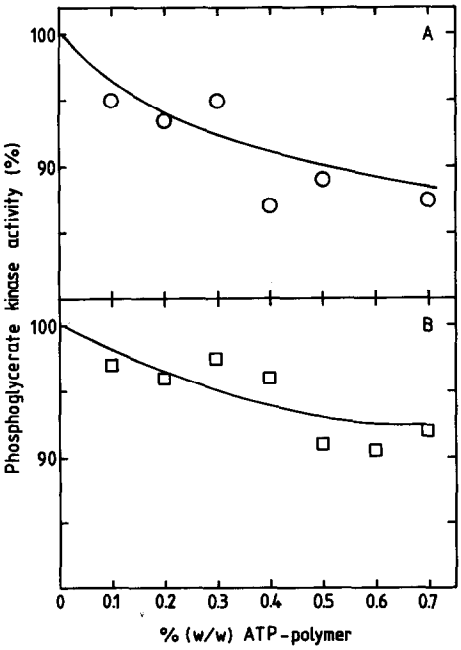

Fig. 1. Influence of ATP–polymer derivatives on the activity of phosphoglycerate kinase from spinach leaf extract. The extract was incubated at the indicated concentrations of derivative for at least 40 min at 4 ± 1°C, diluted 10-fold whereupon the enzyme activity was measured as described under Experimental. Derivatives: (A) ATP–PEG; (B) ATP–dextran. The data points represent averages of duplicate determinations. No statistical calculations were made to determine the best fit.

Extraction of phosphoglycerate kinase

The usefulness of affinity partitioning for enzyme purification can be evaluated by comparing the partitioning of the target enzyme with that of total protein. When the affinity ligand is bound to PEG, PEG–dextran two-phase systems containing sodium phosphate buffer has been used to achieve an enrichment of the target enzyme in the upper phase and total protein in the lower phase[10]. For PEG–dextran systems containing ligand bound to dextran, tetrabutylammonium phosphate was tested as a replacement for sodium phosphate in order to enrich the total protein in the upper phase. A maximal partition coefficient of total protein was achieved by using tetrabutylammonium phosphate concentrations above 25 m*M* (Fig. 2).

Fig. 3A and C show the affinity partitioning of spinach leaf extract using increasing concentrations of ATP–polymer derivatives. For comparison, the same experiment was performed with the reactive dye Procion Turquoise MX-G as ligand instead of ATP (Fig. 3B and D). This, together with Procion Orange H-ER, was the most effective of the reactive dyes tested when the nucleotide-binding enzymes of glycolysis was extracted from yeast[14]. Both ligands, wether bound to PEG or dextran, affected the target enzyme, phosphoglycerate kinase. However, ATP showed a higher specificity, that is, it did not change the partitioning of total protein to the same extent as the dye did. The best separation between the target enzyme and total protein was achieved using ATP bound to dextran (Fig. 3C). When chloroplast extract was used instead of leaf extract as the starting material, the separation was further enhanced (Fig. 4).

Precipitation with PEG

The precipitation of phosphoglycerate kinase and total protein, when chloroplast extract was treated with increasing concentrations of PEG, is shown in Fig. 5. At PEG concentrations above 30% (w/w) the percentage of precipitated total protein reaches a plateau value of 60. The percentage of precipitated phosphoglycerate kinase

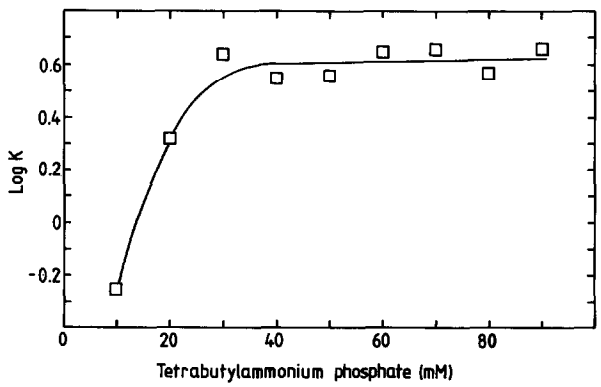

Fig. 2. Partitioning of total protein from spinach leaf extract as a function of the concentration of tetrabutylammonium phosphate. System: 8% (w/w) dextran–6% (w/w) PEG–tetrabutylammonium phosphate as indicated (pH 7.5)–0.1 m*M* magnesium chloride–0.1 m*M* Na_2EDTA–1 m*M* 2-mercaptoethanol–leaf extract. Temperature: 4 ± 1°C.

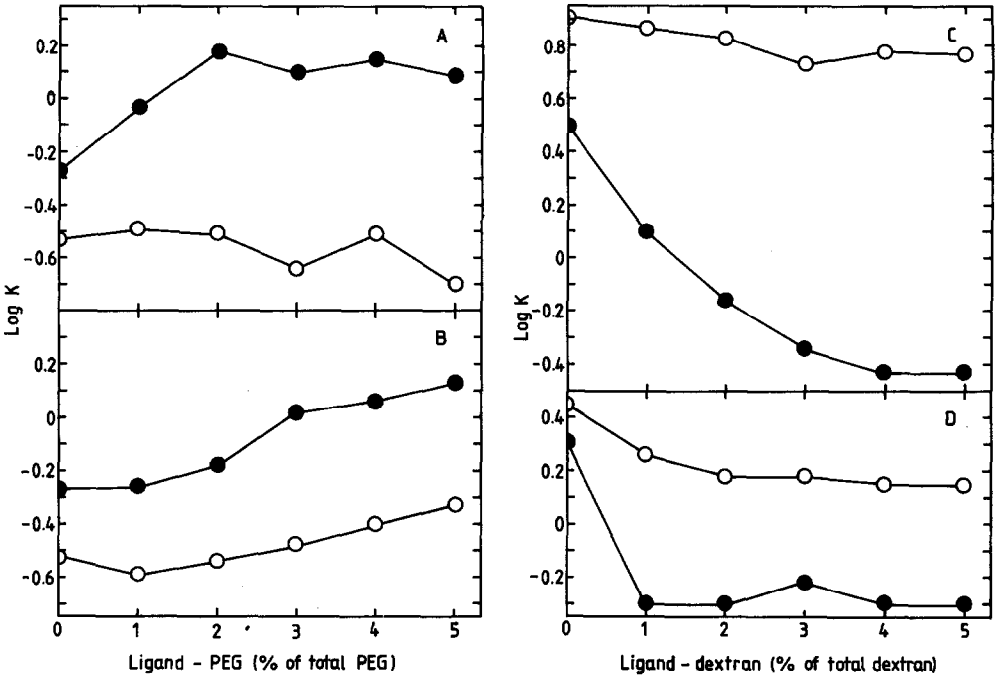

Fig. 3. Partitioning of phosphoglycerate kinase and total protein from spinach leaf extract as a function of the concentration of ligand–polymer derivative. System: 8% (w/w) dextran–6% (w/w) PEG–buffer as indicated (pH 7.5)–0.1 m*M* magnesium chloride–0.1 m*M* Na_2EDTA–1 m*M* 2-mercaptoethanol–leaf extract. The measured activities were corrected for the inhibitory effect caused by the polymer ligands. Temperature: 4 ± 1°C. Buffer: (A and B) 25 m*M* sodium phosphate; (C) 50 m*M* tetrabutylammonium phosphate; (D) 25 m*M* tetrabutylammonium phosphate. Derivatives: (A) ATP–PEG; (B) Procion Turquoise–PEG; (C) ATP–dextran; (D) Procion Turquoise–dextran. Curves: ●, phosphoglycerate kinase; ○, total protein.

increases linearly between 25 and 40% (w/w) PEG. At a PEG concentration of 30% (w/w), 55% of the total protein and less than 15% of phosphoglycerate kinase are precipitated.

Purification of phosphoglycerate kinase from chloroplasts

Using the data presented in Figs. 2–5, a procedure for the extraction of phosphoglycerate kinase from crude chloroplast extract was designed. The procedure, summarized in Table I and Fig. 6, involves three main steps: precipitation with PEG, affinity partitioning with dextran-bound ATP and ion-exchange chromatography. The main function of the last step is to remove the polymers (mainly dextran) from the extracted protein. The final preparation is 80% pure as judged by scanning of a SDS-polyacrylamide gel (Fig. 6B) and its specific activity. The molecular weight was determined to be 46 000 ± 1000, which is identical with the value reported by Kuntz and Krietsch[4]. The decrease in yield after the final ion-exchange treatment may be explained by the removal of the polymers, which are known to increase enzyme activity[15]. A final recovery of more than 100% has been explained by the presence of tannin-like substances that act as phosphoglycerate kinase inhibitors when the starting

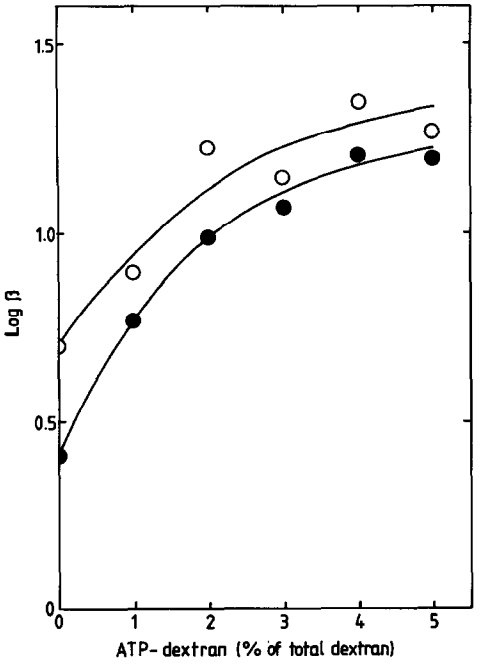

Fig. 4. Partitioning of extracts from spinach leaf and chloroplasts as a function of the concentration of ATP–dextran derivative. Experimental conditions as in Fig. 3. β = Separation factor = ratio between the partition coefficients of phosphoglycerate kinase and total protein. Curves: ●, leaf extract; ○, chloroplast extract. No statistical calculations were made to determine the best fit.

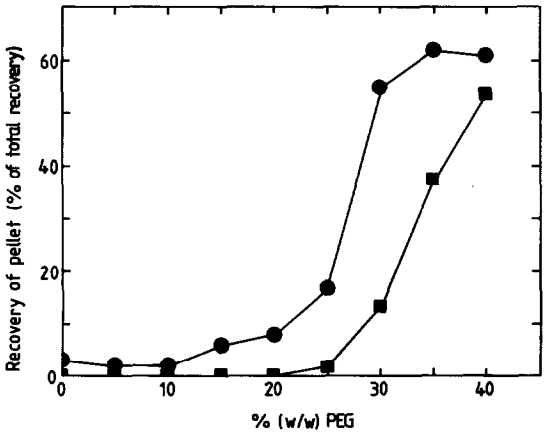

Fig. 5. Precipitation of phosphoglycerate kinase and total protein in spinach chloroplast extract by PEG. Temperature: 4 ± 1°C. Curves: ■, phosphoglycerate kinase; ●, total protein.

TABLE I

PURIFICATION OF CHLOROPLAST PHOSPHOGLYCERATE KINASE FROM 300 g OF DERIBBED SPINACH LEAVES

Experimental conditions as described under Experimental. U = units of phosphoglycerate kinase activity defined as the amount of enzyme phosphorylating 1 μmol/min of 3-phosphoglycerate at 25°C.

Fraction	*Volume (ml)*	*Total protein (mg)*	*Total apparent activity (U)*
Chloroplast (stroma) extract	13.5	198	123
30% (w/w) PEG supernatant	14.7	15	589*
Affinity partitioning:			
First lower phase	46	3.2	540*
Second, washed lower phase	38	0.8	467*
DEAE-Sephadex A-50 eluate	6	0.4	136**

* Greater than the initial value because of enhancing effect of polymers on enzyme activity.
** Increase over the initial value because of removal of inhibitory (tannin-like) substances.

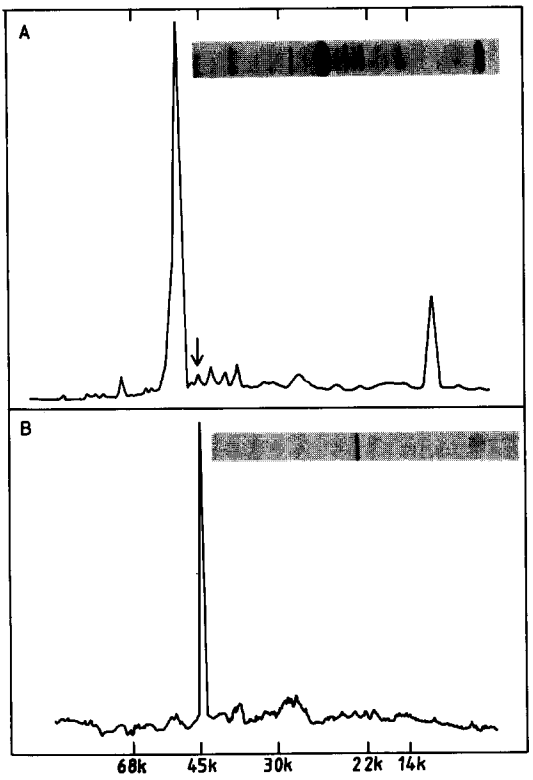

Fig. 6. Polypeptide pattern obtained by SDS-polyacrylamide gel electrophoresis, stained with Coomassie Brilliant Blue R and analysed by photometric scanning. Molecular weights (× 1000) are indicated. (A) Chloroplast (stroma) extract, band corresponding to phosphoglycerate kinase indicated by arrow; (B) DEAE-Sephadex A-50 eluate.

material was spinach leaf extract[4]. If these substances were present in the spinach chloroplast extract, they were then removed during the affinity partitioning and the final ion-exchange treatment.

The results obtained in this work demonstrate that ATP bound to dextran and PEG can be used for enzyme purification from crude extracts using affinity partitioning. They also show that suitable two-phase systems for preparative extraction can be designed. Determination of extraction curves, such as those in Figs. 3 and 4, in combination with analysis of the influence of the ligand–polymer derivative on the activity of the target enzyme (Fig. 1), is a fruitful strategy in formulating a two-phase system for the extraction. The last, and most time-consuming, step in the extraction procedure is the ion-exchange step, which can also be carried out as a batch procedure. The time for this three-step procedure can be considerably reduced by using centrifugal techniques for the separation of the phases and removal of the ion exchanger. The capacity can be increased many times by using suitable centrifugal separators. It is likely that the described procedure for coupling of ATP to the polymers could be adapted for other nucleotides[16], which will further increase the range of suitable target enzymes for purification.

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REFERENCES

1 G. Johansson, in H. Walter, D. E. Brooks and D. Fischer (Editors), *Partitioning in Aqueous Two-Phase Systems*, Academic Press, New York, 1985, Ch. 6.
2 G. Johansson, *Methods Enzymol.*, 104 (1984) 356.
3 G. W. K. Kuntz, S. Eber, W. Kessler, H. Krietsch and W. K. G. Krietsch, *Eur. J. Biochem.*, 85 (1978) 493.
4 G. W. K. Kuntz and W. K. G. Krietsch, *Methods Enzymol.*, 90 (1982) 103.
5 P. D. G. Dean and D. H. Watson, *J. Chromatogr.*, 165 (1979) 301.
6 L.-O. Persson, *Photosynth. Res.*, 15 (1988) 57.
7 H. U. Bergmeyer, *Methoden der Enzymatischen Analyse*, Vol. II, Verlag Chemie, Weinheim/Bergstrasse, 2nd ed., 1970, p. 2021.
8 H. U. Bergmeyer, *Methoden der Enzymatischen Analyse*, Vol. I, Verlag Chemie, Weinheim/Bergstrasse, 2nd ed., 1970, p. 489.
9 K. Nilsson and K. Mosbach, *Biochem. Biophys. Res. Commun.*, 102 (1981) 449.
10 G. Johansson and M. Andersson, *J. Chrbmatogr.*, 303 (1984) 39.
11 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
12 G. Johansson and M. Joelsson, *Enzyme Microb. Technol.*, 7 (1985) 629.
13 G. Johansson and M. Joelsson, *J. Chromatogr.*, 393 (1987) 195.
14 G. Johansson and M. Andersson, *J. Chromatogr.*, 291 (1984) 175.
15 V. Jancsik, T. Keleti, G. Y. Biczok, M. Nagy, Z. Szabo and E. Wolfram, *J. Mol. Catal.*, 1 (1975/76) 137.
16 M. Wilchek and R. Lamed, *Methods Enzymol.*, 34 (1974) 475.