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Note

Use of an activated nylon membrane (Immunodyne) as an affinity adsorbent for the purification of phosphofructokinase and phosphoglycerate kinase from yeast

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Affinity chromatography has been widely applied to the isolation of biological macromolecules either as a single-step procedure or in combination with other purification techniques[1,2]. In principle, a ligand immobilized to a solid matrix interacts with binding sites on proteins such as active or regulatory sites of enzymes, therefore discriminating between proteins exhibiting such binding sites or not. Further conditions for the subsequent desorption of bound proteins can be chosen which allow a selective elution.

Despite its simple concept and its successful application, problems are often connected with affinity chromatography such as the rigidity and physical shape of the affinity matrix used, its insolubility and chemical stability, the ease of derivatization, its reuseability and, of course, the overall costs.

This paper demonstrates the reversible binding of yeast phosphofructokinase (EC 2.7.1.11) and yeast phosphoglycerate kinase (EC 2.7.2.3) to commercially available Immunodyne nylon membrane and the use of this membrane as an affinity matrix for the enrichment of these two enzymes from yeast cell homogenates. The technique applied is extremely simple and does not require sophisticated equipment.

EXPERIMENTAL

Immunodyne, Biodyne A and Loprodyne nylon membranes were obtained from Pall Filtrationstechnik (Dreieich, F.R.G.). The membranes were cut with scissors to the appropriate size. Fructose-6-phosphate, 3-phosphoglycerate, ATP, AMP, NAD, NADP and auxiliary enzymes (aldolase, glycerolphosphate dehydrogenase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase) were obtained from Boehringer (Mannheim, F.R.G.). Reagents for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), phenylmethylsulphonyl fluoride (PMSF) and molecular weight markers were purchased from Serva (Heidelberg, F.R.G.). Buffer substances were of analytical-reagent

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grade from Sigma (St. Louis, MO, U.S.A.) and VEB Laborchemie (Apolda, G.D.R.). Baker's yeast was supplied by VEB Backhefe (Leipzig, G.D.R.).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE was carried out at 8% (w/v) acrylamide according to Laemmli[3]. Treatment with SDS prior to electrophoresis was done by mixing equal volumes of sample and incubation buffer [110 m*M* Tris–HCl (pH 6.8), 16% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue] and subsequent heating of the mixture at 100°C for 10 min.

Enzyme assays

Phosphofructokinase activity was assayed at 3.0 m*M* fructose-6-phosphate, 0.6 m*M* ATP and 1 m*M* AMP in imidazole buffer (pH 7.2) according to Hofmann and Kopperschläger[4].

Phosphoglucose isomerase activity was assayed as described by Clifton *et al.*[5] at 1.4 m*M* fructose-6-phosphate in triethanolamine buffer (pH 7.6).

Phosphoglycerate kinase activity was determined at 6 m*M* 3-phosphoglycerate and 1 m*M* ATP in triethanolamine buffer (pH 7.6) according to Kulbe and Bojanovski[6].

Protein determination

Protein concentration was determined according to Bradford[7] using dried bovine serum albumin for calibration.

Preparation of yeast cell homogenates and extracts

Yeast cells were washed several times with buffer A [50 m*M* sodium phosphate (pH 7.2), supplemented prior to use with 0.5 m*M* PMSF and 1 m*M* 2-mercaptoethanol]. A 1-g portion of the cell pellet after the last centrifugation was swirled with 3 g of acid-washed glass beads (0.5 mm diameter) in 1 ml of buffer A for five 1-min cycles. The tubes were kept on ice during 30-s interruptions. The supernatant above the fast-settling glass beads was used as a cell homogenate. It was centrifuged at 25 000 *g* to remove cell debris to give a cell-free extract.

Preparation and handling of the nylon membranes

The membranes were cut into 50 × 110 mm pieces and transferred to polyethylene bottles (diameter 45 mm, height 70 mm). The bottles were filled with 10 m*M* sodium hydroxide solution, which causes the membranes to adhere loosely to the wall of the bottles. After 1 h the bottles were emptied, and 5 ml of fresh 10 m*M* sodium hydroxide solution were added; 5 ml was always the standard volume used in washing and regeneration steps. The bottles were then fixed with rubber bands to a rotating shaft in horizontal positions (Fig. 1). This arrangement allowed the use of small volumes and utilization of the whole membrane area. To replace solutions the bottles could be easily removed and refixed and it was also very convenient to handle several bottles simultaneously. The membranes were treated in this way three times with 10 m*M* sodium hydroxide solution for 60 min each time and then equilibrated with buffer A. Binding of proteins to the nylon filters was achieved by incubation of the cell homogenates or extracts with the membranes with continuous rotation for the

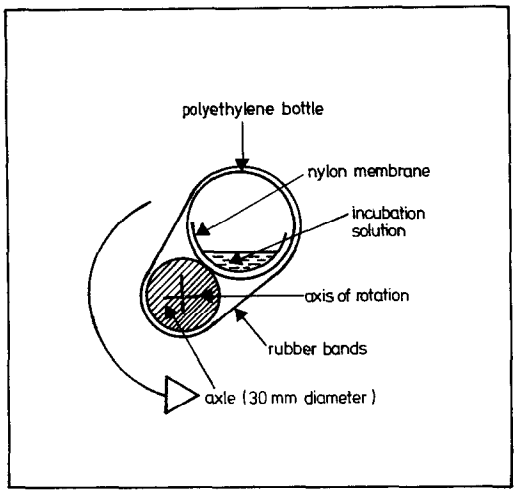

Fig. 1. Treatment of the nylon filters with a small volume of incubation solution within a bottle fixed to a rotating shaft.

indicated times. Subsequently the membranes were washed six times with buffer A. Desorption was performed by exposing the membranes with the desorption buffers indicated in the text. All incubations were performed at cold room temperature (4–6°C).

To regenerate the membranes they were washed three times before each use with 10 m*M* sodium hydroxide solution in 15-min cycles and re-equilibrated. The membranes were stored in 15% methanol for periods up to 1 week or air-dried after regeneration and equilibration with water.

RESULTS AND DISCUSSION

Immunodyne membranes are pre-activated nylon membranes developed for the covalent fixation of proteins via hydroxyl, carboxyl or amino groups (see the manufacturer's instructions for the use of these membranes). An unexpected property of this membrane after alkaline inactivation of the reactive group is its ability for selective binding of phosphofructokinase and phosphoglycerate kinase from a yeast cell extract (Fig. 2). During incubation of the yeast extract with the membrane, the phosphofructokinase activity decreased within 2 h from 7.5 to 0.4 U/ml and the phosphoglycerate kinase activity decreased from 83 to 50 U/ml. In contrast, the phosphoglucose isomerase activity remained constant over the incubation period, indicating the selectivity in the binding of phosphofructokinase and phosphoglycerate kinase. The total protein content in the extract decreased within the first 30 min of incubation from 16.6 to 15 mg/ml and remained at this level during further incubation. In a control incubation of the extract without the membrane (data not shown), no inactivation of the two enzymes was observed, indicating that the decrease in the activities of phosphofructokinase and phosphoglycerate kinase is due to adsorption of the two proteins to the membrane.

The binding of phosphofructokinase is restricted to the Immunodyne membrane

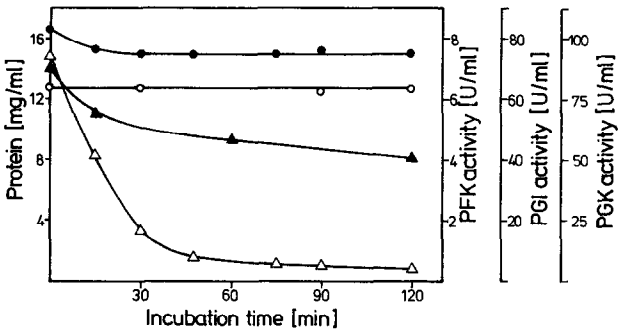

Fig. 2. Incubation of yeast cell extract with the Immunodyne membrane. Aliquots of 3 ml of yeast cell extract were rotated with the Immunodyne membrane and aliquots were taken at the indicated times for the determination of total protein content (●) and the activities of phosphofructokinase (PFK) (△), phosphoglycerate kinase (PGK) (▲) and phosphoglucose isomerase (PGI) (○).

(Table I). Incubation of a yeast cell homogenate with different nylon membranes (Immunodyne, Biodyne, Loprodyne) caused a decrease in the phosphofructokinase activity in the homogenate only with the Immunodyne membrane. The selectivity in the binding is underlined by the finding that the specific activity of phosphofructokinase decreased in the cell homogenate after incubation with the Immunodyne membrane, whereas with the other two nylon filters it remained equal to the control value.

Therefore, the group used for substitution of the nylon matrix of Immunodyne to preactivate this membrane seems to act as an affinity ligand. The two enzymes for which an interaction with the membrane has been demonstrated (Table I) are both kinases with the substrate ATP in common and both are known to interact with the affinity ligand Cibacron Blue F3G-A[4,6], which has been shown to mimic adenine nucleotides[1,8]. Assuming a binding mechanism due to the presence of the nucleotide binding site on the two enzymes, an affinity elution with several adenine nucleotides (ATP, AMP, NAD, NADP) was performed (Table II, Fig. 3). Together with some other proteins the two kinases are desorbed but the specific activities of the two

TABLE I

TIME-COURSE OF PHOSPHOFRUCTOKINASE BINDING TO DIFFERENT NYLON MEMBRANES

Aliquots of 6 ml of a yeast cell homogenate were incubated with the respective nylon membranes and assayed for phosphofructokinase (PFK) activity at the indicated times. An incubation without a membrane was performed as a control. Values in parentheses are the specific activities at the end of incubation determined in the supernatant from a 25 000 *g* spin.

Time of incubation (min)	*PFK activity (U/ml) in the cell homogenate*			
	Control	*Immunodyne*	*Biodyne A*	*Loprodyne*
0	15.7	–	–	–
60	14.2	7.6	14.7	15.3
120	14.9	4.8	14.2	15.8
	(0.42)	(0.14)	(0.46)	(0.42)

TABLE II

DESORPTION OF PHOSPHOFRUCTOKINASE AND PHOSPHOGLYCERATE KINASE BOUND TO IMMUNODYNE NYLON MEMBRANE BY DIFFERENT ADENINE NUCLEOTIDES

Aliquots of 3 ml of a yeast cell homogenate were incubated with the Immunodyne membranes. Unbound material was removed by washing the membranes with buffer A. Subsequently the membranes were rotated for 20 min with 3 ml of buffer A, containing 10 m*M* of the respective nucleotide. The activities of phosphofructokinase (PFK) and phosphoglycerate kinase (PGK) and the protein content in the eluates were determined.

Enzyme	*Specific activity (U/mg) of PFK and PGK in the elution buffer containing*			
	ATP[a]	*AMP*[b]	*NAD*[b]	*NADP*[b]
PFK	16.5 (±3.5)	19.1 (±2.6)	0.55 (±0.2)	25.7 (±3.9)
PGK	198 (±16)	156 (±12)	117 (±12)	265 (±22)

[a] Values are the means of fifteen experiments.
[b] Values are the means of four experiments.

enzymes after elution and the electrophoretic pattern of the desorbed proteins indicate differences in the action of the nucleotides. All the adenine nucleotides used caused elution of phosphoglycerate kinase, although with different effectiveness. Such differences are more obvious with phosphofructokinase, where almost no elution could be achieved by NAD. With this exception, the other nucleotides (ATP, AMP, NADP) were able to desorb phosphofructokinase from the Immunodyne membrane again, indicating a selective interaction of the two enzymes with the Immunodyne membrane. The electrophoretic patterns of the protein desorbed by these nucleotides are similar. With NADP the two unidentified prominent bands in the low-molecular-weight range are missing, which explains the higher specific activities of the two enzymes in this case (last column in Table II). The adsorption and ATP elution also worked well with the cell homogenate of a different yeast strain (DFY1 in Fig. 3), although differences in the protein distribution are observed. The genetic background and/or different growth conditions might account for these differences.

Using the approach of selective binding and affinity elution with ATP for the partial purification of phosphofructokinase and phosphoglycerate kinase from yeast cell extracts, purification factors of 46.5 and 55.7 were obtained, respectively (Table III). Obviously, there is no difference in the resulting specific activities whether a crude cell homogenate (first column of data in Table II) or the cell extract free of cell debris (Table III) was used in the adsorption step. For purification purposes it might even be advantageous to use the cell homogenate, for the centrifugation step to clear the extract can be omitted.

The capacity of the Immunodyne membrane for the binding of phosphofructokinase and the recovery were determined with purified yeast phosphofructokinase. At a concentration of 1 mg of phosphofructokinase per millilitre of buffer A a maximum of 22 μg of the enzyme was bound per square centimetre of the membrane and was desorbed by a one-step elution with 10 m*M* ATP with a recovery of 72%.

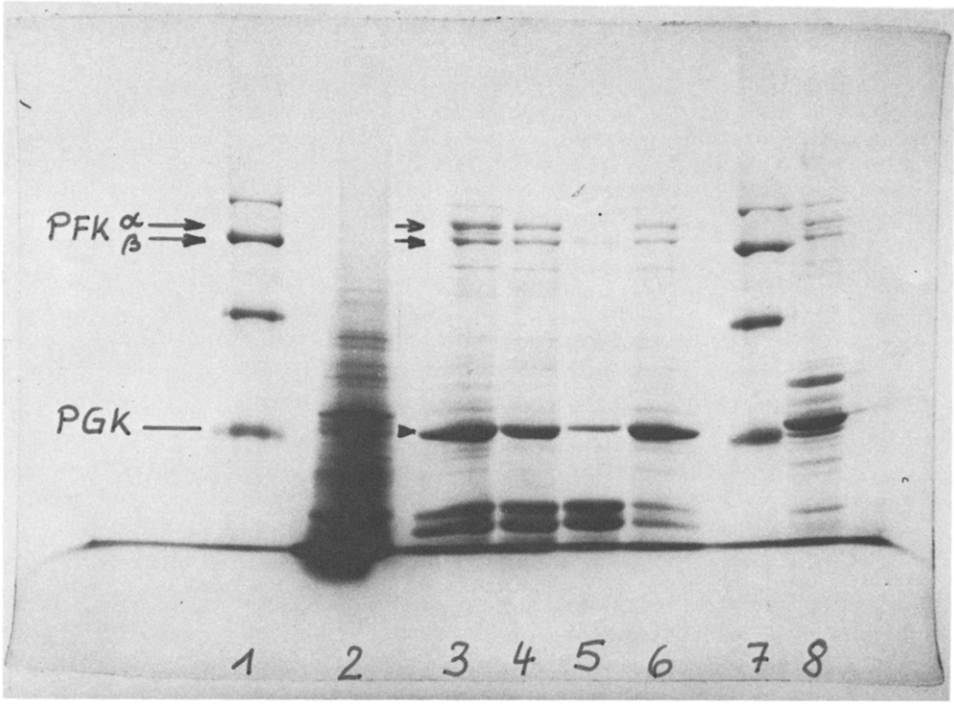

Fig. 3. SDS-PAGE of yeast proteins eluted from the Immunodyne membrane with different adenine nucleotides. Aliquots of 3 ml of yeast cell homogenate were incubated for 60 min with the Immunodyne membranes. Unbound material was removed by washing the membranes with buffer A and the membranes were treated for 20 min with 5 ml of buffer A containing 10 m*M* of the respective adenine nucleotide. Volumes of 50 µl of the eluates and 5 µl of the original cell homogenate were subjected to SDS-PAGE: ATP (lane 3), AMP (lane 4), NAD (lane 5), NADP (lane 6), original cell homogenate (lane 2). The arrows indicate the positions of the two subunits[8] of phosphofructokinase (PFK_β and PFK_γ) and of phosphoglycerate kinase (PGK). They were identified by immunoblotting (not shown). Lanes 1 and 7 are molecular-weight standards: β-galactosidase (116 000), phosphorylase *b* (92 000), bovine serum albumin (69 000) and ovalbumin (45 000). The same experiment (ATP elution) as with the cell homogenate of the commercial yeast in lane 3 was performed with a different strain of *Saccharomyces cerevisiae* (lane 8): DFY1[5] was grown on rich glucose medium to stationary phase and the cell homogenate used for the incubation with the Immunodyne membrane.

TABLE III

PARTIAL PURIFICATION OF PHOSPHOFRUCTOKINASE AND PHOSPHOGLYCERATE KINASE BY SELECTIVE ADSORPTION–DESORPTION WITH IMMUNODYNE NYLON MEMBRANES

A 3-ml volume of yeast cell extract was incubated for 60 min with the Immunodyne nylon membrane. The membranes were washed with buffer A and treated for 20 min with 3 ml of buffer A, containing 10 m*M* ATP.

Sample	*Specific activity (U/mg)*[a]	
	Phosphofructokinase	*Phosphoglycerate kinase*
Cell extract	0.4 ± 0.18	3.52 ± 0.43
Product	18.6 ± 3.2	195 ± 35

[a] Values are the means of six experiments.

The membrane is easily regenerated with sodium hydroxide and its capacity did not decrease after twenty cycles of binding and regeneration. Considering the ease of handling the nylon membrane, the potential use of particle-containing cell homogenates and the overall time to obtain a 50-fold enriched enzyme preparation, affinity sorption on Immunodyne membranes may be superior to other methods as a first step for the extraction of phosphofructokinase and phosphoglycerate kinase from yeast, at least on a small scale of up to a few milligrams.

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