PURIFICATION OF GLYCEROL KINASE BY "DYE-LIGAND" CHROMATOGRAPHY AND HYDROPHOBIC INTERACTION CHROMATOGRAPHY ON BEAD-CELLULOSE DERIVATIVES

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The crude extract of cytosole enzymes was obtained from homogenized cells of *Saccharomyces cerevisiae* by partition. The enzyme was then isolated from the lower aqueous phase displaying higher glycerol kinase activity by dye-ligand chromatography on Cibacron Blue (CB) or Remazol Brilliant Blue R (RB)-derivatized bead-cellulose, ATP being the eluent. The specific activity of glycerol kinase rised more than 10 and 7-times after affinity dye-ligand chromatography and hydrophobic interaction chromatography, respectively. Glycerol kinase obtained by the latter method was purified by CB-bead cellulose. The final preparation maintained its enzymic activity without noticeable losses during a long-term storage at 4 °C in dark.

Glycerol kinase (ATP:glycerol-3-phosphotransferase, E.C. 2.7.1.30) present in the intracellular space of numerous microorganisms is the first enzyme in the enzymic pathway of glycerol utilization as an energetic substrate[1]. Even though the increased appearance of this enzyme was reported in various mezophilic[2 - 4] and termophilic[5] microorganisms, an advantageous source for its preparation can also be the easily accessible yeast *Saccharomyces cerevisiae*, as evidenced by the existence of commercially available preparations of glycerol kinase just from this source. Only few differences in kinetic constants of four commercially available preparations of glycerol kinase of various microbial origin for glycerol analogues differently substituted by nitrogen, sulfur or an alkyl as substrate[6] were found when testing their substrate specificity.

These results prove the very low species difference in glycerol kinase separated from various microbial sources. In addition to chromatographic purification of this enzyme by affinity chromatography on AMP- or ATP-substituted Sepharose[7] also the dye-ligand chromatography[8 - 10] on carriers with immobilized triazine ligands or alternatively by hydrophobic interaction chromatography over Phenyl-Sepharose CL-4B (ref.[11]) could be employed. Carriers for dye-ligand chromatography[12,13] prepared from

bead-cellulose were successfully employed for the zonal chromatography of lactate dehydrogenase[14 - 16]. 3-Phenoxy-2-hydroxypropyl derivatives of bead-cellulose[17,18] substitued the above-mentioned Phenyl-Sepharose CL-4B on hydrophobic interaction chromatography of calmoduline[19].

The aim of this study was to find whether the already described carriers of modified bead-cellulose are suitable for dye-ligand chromatography or hydrophobic interaction chromatography of glycerol kinase of *Saccharomyces cerevisiae*.

EXPERIMENTAL

Materials

Cellulose derivatized with Cibacron Blue 3GA (CB) DS = 31 μmol g^{-1} or Remazol Brilliant Blue R (RB) DS = 56 μmol g^{-1} were obtained by a direct linking of Perloza MT 500 (Secheza, Czechoslovakia) with CB (at 80 °C), or RB (at 25 °C) in alkaline medium[13]. The 3-phenoxyhydroxypropyl derivative of cellulose (PHPC) DS = 25 μmol g^{-1} was prepared by alkylation of Perloza MT 500 with 1,2-epoxy-3-phenoxypropane[18] in alkaline or acid media.

Glycerol 3-phosphate dehydrogenase, NAD, glycerol and ATP are Sigma (U.S.A.) chemicals, the remaining ones of p.a. grade are Serva (Germany) or Lachema (Czechoslovakia) products.

Separation of Proteins by Two-Phase Aqueous Partition

The fresh backer's yeast (100 g) was suspended in 50 mmol l^{-1} phosphate pH 7.5 buffer (100 ml) containing dithiothreitol (5 mmol l^{-1}) and disintegrated (4 × 40 s) in a ballotine homogenizer (ballotine size 0.5 – 0.8 mm) at 2 500 rpm and 4 °C. Sodium citrate (14%, 50 ml) and poly(ethylene glycol) 1 550 (24%, 50 ml) were added to the suspension and the homogenized mixture was centrifuged at 9 000 g for 15 min. Sodium citrate (15.8%, 50 ml) and poly(ethylene glycol) 12 000 (10%, 50 ml) were added to the supernatant, mixed up and allowed to stand at 4 °C till two sharply delimitated phases were formed. The lower citrate phase containing the prevailing part of glycerol kinase was concentrated in an ultrafiltration cell against 5 mmol l^{-1} phosphate pH 7.5 buffer up to a seven-fold concentration of proteins. The concentrate was either purified by chromatography or freeze-dried and stored at 4 °C.

Dye-Ligand Chromatography of Glycerol Kinase on CB-Cellulose and RB-Cellulose

The concentrate was chromatographed on a 5 × 10 cm chromatographic column at a 1 ml min^{-1} flow rate of the mobile phase (20 mmol l^{-1} phosphate buffer) effected by the peristaltic pump Microperpex S (Pharmacia, Sweden) at 4 °C. The absorbance at 280 nm was continually monitored at the outflow by a through-flow detector UVM 4 (Vývojové dílny, Czechoslovak Academy of Sciences). Glycerol kinase was eluted by 20 mmol l^{-1} phosphate buffer containing 5 mmol l^{-1} ATP after removing proteins not integrating with the ligand; chromatographed were 10 ml of the sample (c. 70 mg of proteins), fractions (1 ml) were measured for the proteins content[20] and kinase activity.

Hydrophobic Interaction Chromatography of Glycerol Kinase on PHPC

Conditions: column packing 4.8 × 11 cm, flow rate (10 mmol l^{-1} phosphate buffer pH 7.5 containing 1 mol l^{-1} sodium citrate) as in the preceding experiment, temperature 4 °C. Chromatographed was a 10 ml sample, elution of the glycerol kinase by 10 mmol l^{-1} phosphate buffer containing 0.25 mol l^{-1} sodium citrate.

Purification of Glycerol Kinase after Hydrophobic Interaction Chromatography by Dye-Ligand Chromatography

A sample obtained by hydrophobic interaction chromatography (c. 1.2 mg of proteins in 1 ml; specific activity of glycerol kinase 15 U mg^{-1}) was loaded on a CB-bead-cellulose column (1 × 4.5 cm) and chromatographed at a 0.2 ml min^{-1} flow rate. The sample after elution by 5 mmol l^{-1} ATP was mixed with bovine serum albumin in a 1 : 10 protein to albumin ratio and dialyzed against distilled water and salt, the ATP-free residue was freeze-dried and stored at 4 °C.

Determination of Glycerol Kinase Activity

The activity of glycerol kinase was determined as an amount of glycerol 3-phosphate formed during 1 min (U = 1 μmol min^{-1}). The amount of glycerol 3-phosphate formed was monitored according to the difference of absorbance at 340 nm associated with reduction of NAD to NADH after concerted reaction of glycerol kinase with glycerol 3-phosphate dehydrogenase. This reaction proceeded in 2.8 ml of the reaction medium containing 200 mmol l^{-1} glycine pH 9.8 buffer, 1 mmol l^{-1} hydrazine, 2 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} ATP, 2 mmol l^{-1} NAD, 5 mmol l^{-1} glycerol and 0.2 U glycerol 3-phosphate dehydrogenase and the appropriate sample of glycerol kinase directly in the spectrophotometric cell. Absorbance was recorded in 20-s intervals with a UV-VIS Specord M 40 (Zeiss, Jena) apparatus.

SDS Electrophoresis

The SDS electrophoresis proceeded on a gradient polyacrylamide gel (8 – 20%) with Phast System (Pharmacia, Sweden) instrument. The proteins being separated were visualized with Coomasie Blue by a standard procedure according to the instrument programme.

RESULTS AND DISCUSSION

The average glycerol kinase activity after homogenization was found to be 0.50 ± 0.10 U mg^{-1}. The supernatant after removal of cell walls and cell membranes by centrifugation revealed less than 40% of the original amount of proteins, but more than 80% of the original glycerol kinase activity (Table I). Partition in the two-phase aqueous system led to a further enrichment of glycerol kinase in the citrate phase by approximately 1.2 times.

TABLE I
Preparation of glycerol kinase-enriched intracellular enzymes from *Saccharomyces cerevisiae*

Procedure	Protein content, g	Activity of glycerol kinase	
		total, U . 10^3	specific, U mg^{-1}
Homogenization	3.41	1.768	0.525
Centrifugation (9 000 g)	1.32	1.450	1.152
Phase separation	0.95	1.334	1.407

TABLE II

Chromatographic purification of glycerol kinase (70 mg protein = 98.49 U, spec. act. = 1.41 U mg^{-1}). *A* hydrophobic interaction chromatography on PHPC; *B,C* dye-ligand chromatography on CB- and RB-cellulose, respectively; *D* combination of hydrophobic interaction chromatography on PHPC with dye-ligand chromatography on CB-cellulose

Method	Proteins, mg	Activity	
		total, U	specific, U mg^{-1}
A	3.1	31.43	10.13
B	3.9	86.71	22.23
C	4.4	76.14	17.31
D	0.7	26.60	39.12

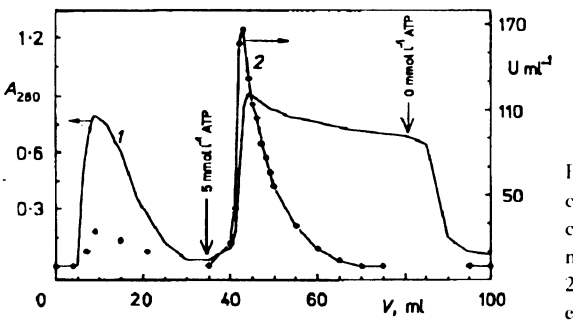

FIG. 1

Elution profile of glycerol kinase on CB-cellulose. Elution with ATP (5 mmol l^{-1}), column packing: 5 × 10 cm, flow rate: 1 ml min^{-1}), temperature: 4 °C. *1* Absorbance at 280 nm, *2* glycerol kinase activity (U ml^{-1} enzyme activity, ml retention volume)

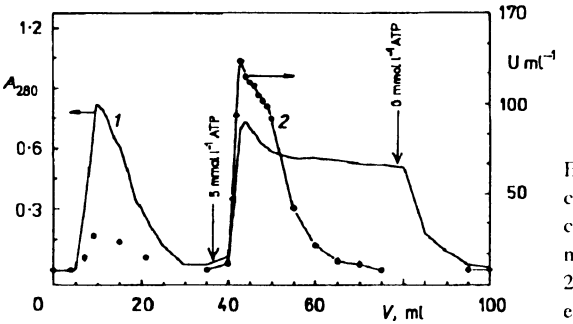

FIG. 2

Elution profile of glycerol kinase on RB-cellulose. Elution with ATP (5 mmol l^{-1}), column packing: 5 × 10 cm, flow rate: 1 ml min^{-1}, temperature 4 °C. *1* Absorbance at 280 nm, *2* glycerol kinase activity (U ml^{-1} enzyme activity, ml retention volume)

The dye-ligand chromatography of the protein samples after enrichment by glycerol kinase (i.e. by partition in two-phase aqueous systems afforded enzyme preparations (Table II) with 22.23 and 17.31 U mg^{-1} glycerol kinase activity on chromatography over CB-cellulose and RB-cellulose, respectively. The enzyme could be eluted with ATP as a mobile ligand in both experiments (Figs 1, 2) what proves competition of the immobilized antraquinone dye with ATP for the same site in the glycerol kinase molecule. The zonal affinity chromatography of lactate dehydrogenase disclosed that the competitive relation between the immobilized ligand and the nucleotide (NADH, ref.[14]) took place during elution only when using CB-cellulose; on the other hand, such a competition was not observed with RB-cellulose. The stoichiochemical biomimetic reaction during adsorption of lactate dehydrogenase on both afore-mentioned celluloses was evidenced only for CB-cellulose[21] and vice versa, RB-cellulose adsorbed unspecifically this enzyme probably due to interaction of hydrophobic nature. Rationalization for this finding offered the fact that CB reacts by an order higher affinity with the nucleotide binding site of lactate dehydrogenase than RB (ref.[22]). Nevertheless, no significant difference in affinity of their interaction with the ATP-binding site of this enzyme was observed on interaction of both anthraquinone dyes with NA^+/K^+-ATPase as an enzyme cleaving ATP. These facts indicated that both dyes are able approximately equally effectively to interact biomimetically with the ATP-binding site in contrast to NADH simulation, where the ability of biomimetic interaction with the NADH-binding site showed mainly CB.

Hydrophobic interaction chromatography on PHPC afforded an enzyme preparation with 10.13 U mg^{-1} glycerol kinase activity (Table II), but approximately 40% of proteins (only c. 10% of the total amount of glycerol kinase) remained on the sorbent even after elution with pure 10 mmol l^{-1} phosphate buffer. A typical elution profile of the chromatography of proteins after two-phase aqueous partition is illustrated in Fig. 3. As follows, the cellulose analogue of Phenyl-Sepharose CL-4B PHPC (ref.[11]) is also suitable for hydrophobic interaction chromatography of glycerol kinase. If the

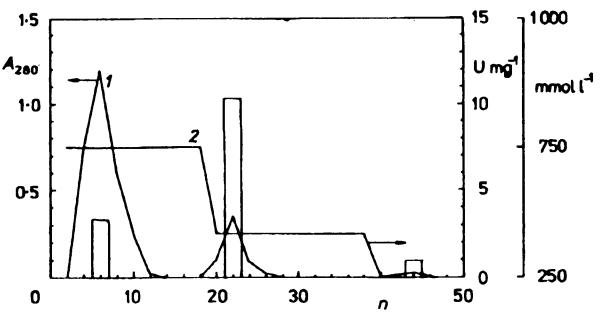

FIG. 3
Elution profile of glycerol kinase on PHPC. Elution by gradient concentration elution of sodium citrate (from 750 to 250 mmol l^{-1}). Column packing: 4.8 × 11 cm, flow rate: 1 ml min^{-1}, temperature: 4 °C. *1* Absorbance at 280 nm, *2* concentration of sodium citrate in mol l^{-1} (columns: glycerol kinase activity)

sample obtained by chromatography on PHPC has been purified by dye-ligand chromatography on CB-cellulose the glycerol kinase preparation activity was approximately 39 U mg^{-1}; after stabilization with bovine serum albumin in the 10 : 1 mg albumin to the protein ratio and freeze-drying, this preparation can be stored at least for 6 months at 4 °C.

Successive purification of glycerol kinase by the above-mentioned chromatographic methods could be also monitored by a more pronounced characteristic band in the SDS electrophoresis with a relative molecular weight (M. w.) within 47 000 and 50 000. Preparation of the enzyme, which was purified after hydrophobic interaction chromatography on PHPC by affinity chromatography on CB-cellulose revealed further three bands of so far not identified proteins (within M. w. 19 000 and 21 000, 67 000 and 70 000 and 98 000 and 102 000) on electrophoretograms.

These procedures afforded enzyme preparations of approximately the same quality as commercial ones currently available, isolated from yeast as a microbial source. Our preparation disclosed specific activity approximately 3 – 5-times lower than glycerol kinase preparations obtained by combination of six to seven chromatographic methods. It is, however, worth noting that this preparation was obtained by combination of only two chromatographic methods.

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