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Fast high-performance liquid chromatographic purification of *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase

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ABSTRACT

A procedure was established for the rapid isolation of *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase (PEPCK) from an overproducing strain. Overexpression was achieved by the transformation of yeast cells with the multicopy plasmid YEp352 harbouring the PEPCK structural gene. The enzyme was purified to homogeneity using first anion-exchange chromatography on Q-Sepharose followed by hydrophobic interaction chromatography on phenyl-Sepharose and gel filtration on Sephacryl S200. The purified phosphoenolpyruvate carboxykinase was further characterized with respect to the molecular mass, displaying an apparent molecular mass corresponding to a tetrameric form.

INTRODUCTION

Phosphoenolpyruvate carboxykinase [ATP; oxaloacetate carboxylyase (transphosphorylating), E.C. 4.1.1.49] from yeast catalyses the decarboxylation of oxaloacetate in the presence of ATP and Mn^{2+} ions to give CO_2, ADP and phosphoenolpyruvate [1]. This reaction is an important step in the formation of glucose from three- and four-carbon precursors. Phosphoenolpyruvate carboxykinase (PEPCK) from yeast differs markedly from vertebrate enzymes, because other enzymes are specific for GTP (or ITP) as a donor of the phosphate group [1]. In addition, the yeast enzyme exists as a tetramer composed of identical subunits [2,3] whereas the PEPCKs from other sources are monomers (relative molecular mass *ca.* 72 000). The primary structures were deduced from cloned DNAs for PEPCK from *Saccharomyces cerevisiae* [4], rat liver [5], chicken kidney [6] and *Drosophila melanogaster* [7]. Comparing the amino acid sequences of the PEPCKs from higher eukaryotes, strong homologies were found [7], and putative GTP-binding regions were suggested to be partially related to a consensus sequence found in different GTP-binding proteins [8]. In the case of the yeast enzyme, no significant similarities to the corresponding en-

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zymes have been obtained [4]. However, a consensus sequence for a putative ATP-binding site [6] can be found in the monomeric PEPCK from yeast at amino acid positions 249–255; further, a conserved sequence interacting with phosphoenolpyruvate [6] may be located at residues 356–382.

Although no detailed investigations of the reaction mechanisms of PEPCK are yet available on a molecular level, the presence of a functional arginyl residue and a reactive lysine residue have been determined [9,10]. Fluorescence spectroscopic measurements support the conclusion that a highly reactive sulphydryl group, located in the nucleotide binding site, is important either for the catalytic mechanism or for the maintenance of the active conformation of yeast PEPCK [11].

Comparing all PEPCKs studied so far, it seems possible that monomeric and tetrameric enzymes may have common features in their secondary or tertiary structures. In order to perform crystallization experiments for subsequent X-ray diffraction analysis, we developed an improved large-scale purification procedure for yeast PEPCK. The purification scheme described here involves the construction of a genetically modified yeast strain that facilitates the isolation of milligram amounts of the enzyme.

EXPERIMENTAL

Enzymes and substrates were purchased from Boehringer (Mannheim, Germany). All chemicals used were commercially available and of analytical-reagent grade.

Yeast strain and overexpression

A DNA fragment harbouring the complete *PCK1* gene encoding PEPCK was ligated into the yeast episomal plasmid YEp352 [12] by standard recombinant DNA techniques [13,14]. The resulting plasmid pPEPCK contained a chromosomal *EcoRI/XhoI* 4-kilobase (kb) DNA fragment obtained from yeast strain WAY. 5-4A ligated into the *EcoRI* and *SalI* restricted YEp352. The plasmid was screened by colony hybridization with an oligonucleotide-derived probe obtained from the *PCK1* sequence [4]. The *PCK1* gene was under the control of the native promoter. Transformation marker genes and sequences necessary for replication in *Escherichia coli* and yeast were derived from the plasmid YEp352. The origin of plasmid replication was originally from the 2-μm circle DNA which results in a high plasmid copy number [15]. Plasmid pPEPCK was transformed in *Saccharomyces cerevisiae* strain WAY. 5-4A according to the lithium acetate method of Ito *et al.* [16]. The transformed yeast was grown under selective conditions on synthetic media lacking uracil to prevent plasmid loss in cultures up to a volume of 200 ml. The preculture so obtained was transferred to 1 l of rich medium. This medium contained 3% glycerol and 3% ethanol as carbon sources. Under this condition the glucose repressible *PCK1* gene was derepressed. Incubation was stopped when the wet mass reached 10–15 g/l. After centrifugation the pellet was resuspended in water. The cells were pelleted again by centrifugation and stored at −20°C.

Enzyme assay

PEPCK activity was assayed spectrophotometrically by coupling the formation of oxaloacetate from phosphoenolpyruvate to the malate dehydrogenase reaction [17]. Standard assays were performed at 25°C in a volume of 1 ml, containing 100 m*M* imidazole–HCl (pH 7.0), 50 m*M* potassium hydrogencarbonate, 1.25 m*M* ADP, 1.0 m*M* $MnCl_2$, 2.0 m*M* glutathione, 0.45 m*M* NADH and 1.5 U/ml malate dehydrogenase (Boehringer). The reaction was started by adding of 100 μl phosphoenolpyruvate (25 m*M*).

Protein determination

The protein peaks of all chromatographic separation steps were recorded using an UV detector at 280 nm (Uvicord SD 2158; LKB). Protein concentrations in PEPCK-containing fractions were determined with the Coomassie protein assay reagent (Pierce). Additionally, pooled fractions and purified samples of PEPCK were assayed with biuret reagent (Sigma).

Chromatographic procedures

Protein purification was performed at 4°C in order to avoid losses of enzyme activity. The columns used were connected to a Model 2249 high-performance liquid chromatographic gradient pump (Pharmacia LKB); sample loading was done using a peristaltic pump.

Starting with 10–35 g of yeast cells, the crude extract was prepared as described in a previous paper [18], except that 100 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.0) containing 1 m*M* dithiothreitol (DTT) and 0.1 m*M* EDTA was used for cell disruption. Immediately after centrifugation the supernatant was applied (1.5 ml/min) to a Q-Sepharose fast-flow column (6 × 5 cm I.D.) previously equilibrated with 100 m*M* HEPES (pH 7.0) containing 1 m*M* DTT, 0.15 m*M* $MnCl_2$ and 0.1 m*M* EDTA (buffer A). The column was washed with the same buffer until the UV absorbance gave a stable baseline and then eluted with a linear sodium chloride gradient (buffer A containing 1 *M* NaCl, gradient volume 700 ml). Fractions containing more than 5 U/ml of PEPCK activity were pooled and adjusted to 30% saturation by addition of solid ammonium sulphate; no precipitation was visible. The sample was loaded onto an equilibrated (buffer A, 30% or 40% ammonium sulphate saturated) phenyl-Sepharose CL-4B column (23 × 2.6 cm I.D.) at a flow-rate of 1 ml/min. PEPCK was eluted with a 750-ml linear gradient of 30–0% (or 40%–0%) ammonium sulphate saturation in buffer A. Pooled fractions containing PEPCK activity were brought to 60% saturation with solid ammonium sulphate. After gentle stirring for about 30 min at 4°C, the suspension was centrifuged at 20 000 *g* for 30 min at 4°C. The protein pellet was dissolved in about 3 ml of buffer A and then applied to a Sephacryl S-200 gel filtration column (53 × 2.6 cm I.D.) that had been equilibrated with buffer A. Buffer A was also needed for elution of PEPCK.

For analytical anion-exchange chromatography, a Mono Q HR 5/5 column (Pharmacia LKB) was used. Crude extracts were prepared from 1–2 h of freshly thawed yeast cells according to the method of Ciriacy [19]. Before sample loading, the solutions were filtered through 0.4-μm filters (Nalgene).

About 300 μg of purified PEPCK were applied (1 ml/min) to a Superformance 75-5 hydroxyapatite column (Merck) that had been equilibrated with 0.005 *M* sodium phosphate buffer (pH 6.6). After washing with equilibration buffer the column was eluted with a linear gradient of 0.005–0.5 *M* sodium phosphate buffer (pH 6.6) in 25 min.

Purified PEPCK (1 mg/ml; 100 μl per run) was injected onto a Superose 6 HR 10/30 column (Pharmacia LKB) equilibrated with buffer A containing 150 m*M* NaCl. Elution was performed with the same buffer at a flow-rate of 0.5 ml/min and fractions of 0.5 ml were collected. The molecular mass of PEPCK was re-examined using standard proteins (low- and high-molecular-mass gel filtration kit, Pharmacia LKB) for calibration. The void volume was determined with blue dextran.

Immobilized metal ion affinity chromatography (IMAC) was carried out using a Chelating Superose HR 10/2 fast protein liquid chromatography (FPLC) column (Pharmacia LKB) loaded with 250 m*M* $CuSO_4$, $CoCl_2$ or $NiCl_2$ dissolved in water. IMAC columns were washed with degassed water (10 ml) in order to remove the unbound metal ions and equilibrated with 0.02 *M* phosphate buffer (pH 7.5) containing 1 *M* KCl and 0.15 m*M* $MnCl_2$. All iminodiacetate (IDA)–M(II) columns were eluted in a falling pH-gradient protocol by using 0.02 *M* phosphate buffer (pH 4.0) containing 1 *M* KCl and $MnCl_2$ as elution buffer (flow-rate 0.6 ml/min; gradient volume 36 ml).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a GE 2/4-LS electrophoresis apparatus (Pharmacia LKB) using the system of Laemmli [20]. Gel electrophoresis under non-denaturating conditions was carried out according to the method of Davis [21] using polyacrylamide gels. Gels were stained with Serva Blue R or with silver nitrate [22].

Crystallization of PEPCK

Before crystallization, samples of purified PEPCK were concentrated by centrifugation in Centricon-30 tubes (Amicon). The crystallization was carried out by the hanging drop vapour diffusion technique [23]. Crystallization experiments were performed at 18°C with 10-μl protein droplets containing 8–16 mg/ml of PEPCK and 1.4 *M* ammonium sulphate (pH 7.0; buffered with 0.2 *M* phosphate) in the presence of 0.001 *M* DTT.

RESULTS

Purification of PEPCK

As the success of protein crystallization is strongly dependent on the total amount of the starting

material, we used the yeast strain WAY. 5-4A harbouring the multicopy plasmid pPEPCK as a source for the isolation of PEPCK. The purification of *Saccharomyces cerevisiae* PEPCK was achieved from this overproducing yeast cells with a yield of about 87%. Crude extracts of the transformed cells showed an enzyme level about ten times higher than wild-type cells. The specific PEPCK activity was about 4.6 U/mg of protein in the crude extract of the recombinant cells.

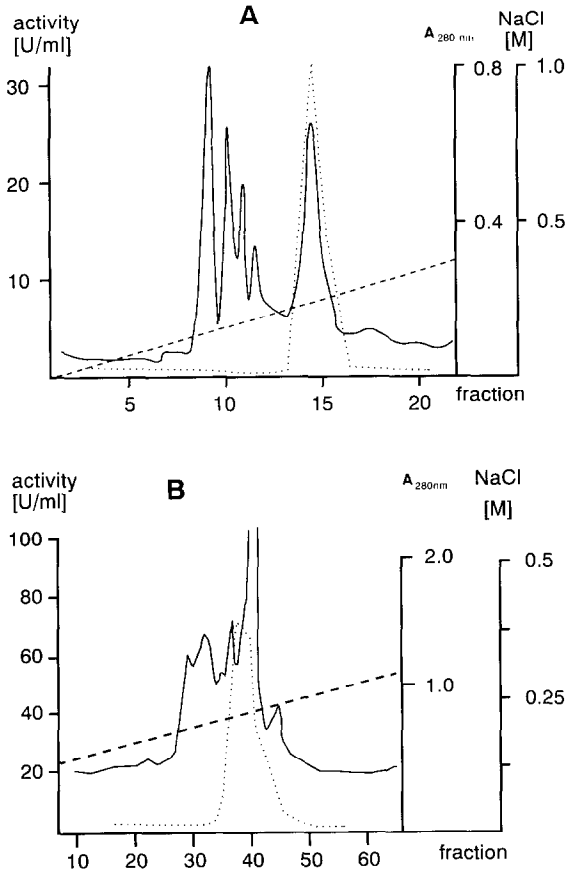

Fig. 1. Scale-up of anion-exchange chromatography of *Saccharomyces cerevisiae* PEPCK form Mono Q to Q-Sepharose. (A) Crude extract (30 U of PEPCK activity) was injected on to a Mono Q HR 5/5 column equilibrated with 100 m*M* HEPES (pH 7.0) containing 1 m*M* DTT, 0.15 m*M* $MnCl_2$ and 0.1 m*M* EDTA (buffer A). Elution was performed using a linear NaCl gradient (0–0.5 *M*) and a gradient volume of 30 ml (flow-rate 1 ml/min). (B) 75 ml of crude extract containing 3670 U of PEPCK were applied to a Q-Sepharose column (bed volume 118 ml) equilibrated with buffer A. Proteins were eluted by using a linear gradient of NaCl (0–1 *M* NaCl in 430 min; flow-rate 1.6 ml/min). Solid lines, protein concentration; dotted lines, enzyme activity; dashed lines, NaCl concentration.

The first step in the purification was anion-exchange chromatography of the supernatant after disrupture of the cells. Fig. 1 illustrates scale-up from a Mono Q HR 5/5 to a K 50/30 column with a 6-cm bed height, packed with Q-Sepharose fast flow. A sharp PEPCK peak was eluted from the Mono Q column at about 250 m*M* NaCl (Fig. 1A). For the preparative run on the Q-Sepharose column, a peak displaying PEPCK activity occurred at about 200 m*M* NaCl (Fig. 1B). This chromatographic step gave an efficient separation of PEPCK from the bulk protein; a recovery reproducibly higher than 100% was observed. The specific activity increased from 4.6 U/mg in the crude extract to 30.9 U/mg in the pooled fractions (Table I).

PEPCK in the combined active fractions was further purified through a phenyl-Sepharose column. All hydrophobic interaction columns resulted in elution profiles comparable to that shown in Fig. 2. The same degree of purification was obtained by using 40% or 30% saturated ammonium sulphate buffer. A relatively large amount of contaminating proteins could be removed during the column washing. A homogeneous peak of PEPCK activity corresponding to a protein peak appeared when the conductivity of the eluting gradient was less than 17 mS (*ca.* 5.5% ammonium sulphate saturation). From the 4600 U that were applied, about 93% could be recovered after hydrophobic interaction chromatography. The specific activity increased slightly from 30.9 U/mg in the pooled Q-Sepharose frac-

TABLE I

PURIFICATION OF YEAST PHOSPHOENOLPYRUVATE CARBOXYKINASE

Data are from a representative purification starting with 11 g of overproducing yeast cells. The yield was calculated as a percentage of the amount of PEPCK present in the crude extract.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	795	3675	4.6	100
Q-Sepharose	150	4640	30.9	126
Phenyl-Sepharose	129	4340	33.6	118
Sephacryl-S 200	80	3218	40.2	87

tions to 33.5 U/mg in the pooled fractions from phenyl-Sepharose. As shown in the inset in Fig. 2, the peak fractions were only poorly contaminated when stained with silver after SDS-PAGE.

The pooled fractions were precipitated and subsequently purified to homogeneity on a Sephacryl S-200 column. Only one nearly symmetric protein peak occurred, which coincided with that of PEPCK activity (Fig. 3). Fractions 17–23 were pooled; the recovery of enzyme activity from Sephacryl S-200 was about 87%. Purified PEPCK showed a specific activity of about 40.4 U/mg.

To illustrate the single purification steps, SDS-PAGE was performed with consecutive samples after anion-exchange, hydrophobic interaction and gel permeation chromatography (Fig. 4A). The results of a representative purification are summarized in Table I. About 80 mg of pure PEPCK were routinely obtained from a purification procedure starting with 11 g of yeast cells. The purified enzyme could be stored at −80°C in buffer A for several months with no detectable decrease in activity.

At room temperature a decrease in of enzyme activity was measured after 3 h. The decay could be considerably reduced when the enzyme was incubated on ice. After 20 h only about 25% of the activity could be detected after storage at room temperature, whereas 80% of the activity was determined after incubation on ice for the same period.

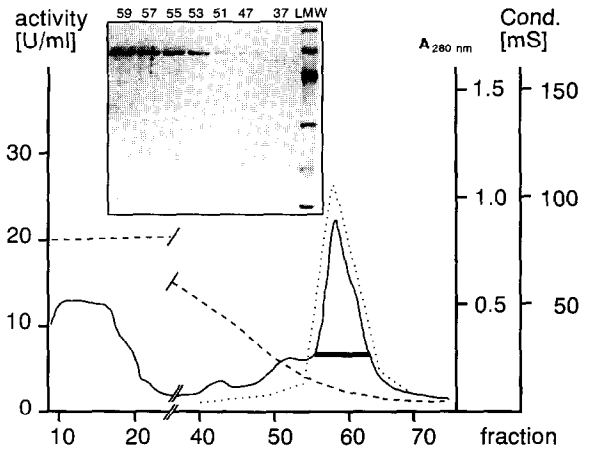

Fig. 2. Hydrophobic interaction chromatography of the pooled fractions from Fig. 1B on phenyl-Sepharose CL-4B. Elution was performed using a linear gradient of decreasing ammonium sulphate saturation (30–0%) in 0.025 *M* piperazine buffer (pH 6.5). PEPCK elution started at about 5.5% saturated ammonium sulphate. Fractions containing more than 5 U/ml (marked with a bar) were pooled. Inset: SDS-PAGE of the peak fractions obtained from phenyl-Sepharose (Coomassie staining). Solid line, protein concentration; dotted line, enzyme activity; dashed line, conductivity. LMW = Low molecular weight standard proteins.

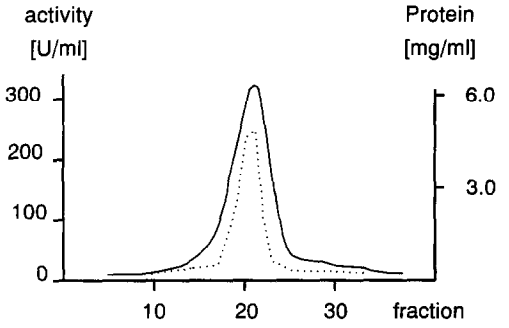

Fig. 3. Gel permeation chromatography of PEPCK on a Sephacryl S-200 column. The active fractions form the phenyl-Sepharose column were precipitated and subjected to gel permeation chromatography on Sephacryl S-200. Solid line, protein concentration; dotted line, enzyme activity.

Criteria of purity

As the quality of an enzyme preparation is an important factor that will influence the crystallization of the protein, the homogeneity of PEPCK was confirmed by different electrophoretic and chromatographic methods. When analysed by SDS-PAGE, the purified PEPCK appeared to be homogeneous, even if as much as 10 μg of enzyme were applied (Fig. 4B; lane 2). A single silver-stained band corresponding to an M_r of about 65 000 was obtained. PEPCK also migrated as a single protein-staining band in a native polyacrylamide gel. After an electrophoresis time of up to 4 h only one protein band was detectable (Fig. 4C). Gel permeation on Superose 6 showed that the PEPCK activity eluted with a single symmetric peak displaying an apparent M_r of about 250 000. The pure enzyme was also injected onto a hydroxyapatite column. The best binding and elution conditions for PEPCK were at pH values in the range 6.6–7.0, where the enzyme eluted in a single symmetric peak. No binding was observed at higher pH values of 7.5 and 8.0. When loading PEPCK onto a column equilibrated with phosphate buffer (pH 6.3), only very poor resolution was achieved.

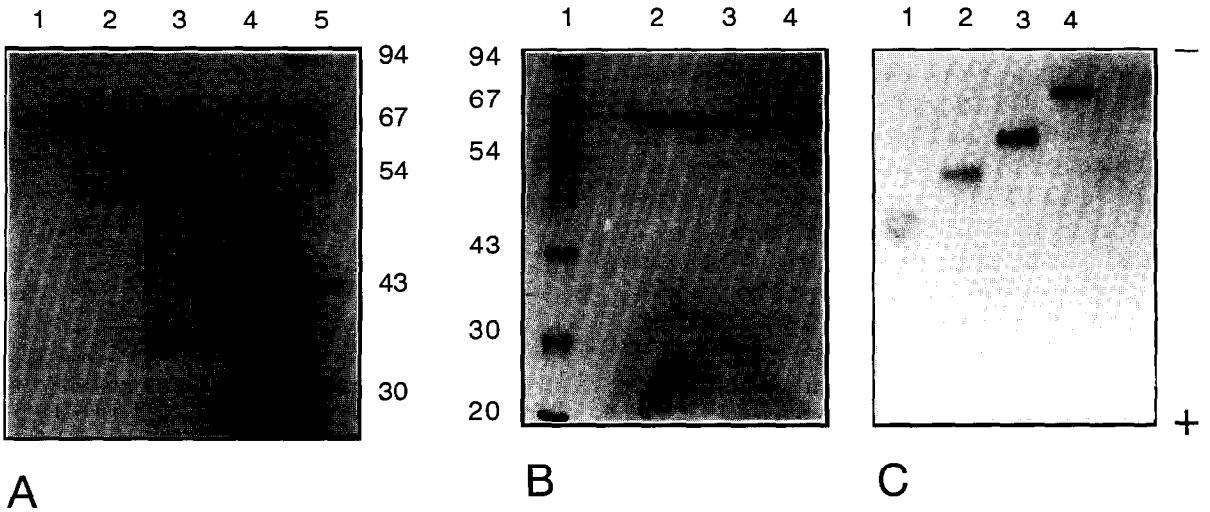

Fig. 4. Electrophoretic analysis of subsequent purification steps of yeast PEPCK and purified PEPCK. (A) SDS-PAGE of pooled fractions from gel permeation (lane 1), hydrophobic interaction (lane 2), anion-exchange chromatography (lane 3) and yeast crude extract (lane 4) (molecular mass markers, $M_r \times 10^3$, lane 5; Coomassie staining). (B) SDS-PAGE of 10 μg (lane 2), 5 μg (lane 3) and 2.5 μg (lane 4) of purified PEPCK (marker proteins, $M_r \times 10^3$, lane 1; silver staining). (C) PAGE of purified PEPCK under non-denaturing conditions electrophoresis time: lane 1, 4 h; lane 2, 3 h; lane 3, 2 h; lane 4, 1 h). The direction of migration is from top to the bottom of the gel.

Crystallization

The protein solution was concentrated by ultrafiltration using Centricon 30 microconcentrators to final concentrations of about 20–30 mg/ml (5000–7000 rpm, 4°C, 1–2 h). Crystals were grown at 18°C in both the presence and absence of ADP at an ammonium sulphate concentration of 1.4 *M*. In spite of the existence of sulphydryl groups in the enzyme [24], the addition of DTT to the protein samples before crystallization had no influence on the size and shape of the PEPCK crystals. Microcrystals were obtained which are too small for X-ray analysis. Further crystallization experiments are in progress.

Affinity of PEPCK for different IDA–M(II) columns

Affinity chromatography on immobilized metal ions was used to investigate the topography of histidine residues on the PEPCK surface [25–27]. The strongest binding was observed when PEPCK was applied to a column loaded with copper. The enzyme could not be eluted from IDA–Cu(II) columns with a decreasing pH gradient. PEPCK displays a weaker affinity for IDA–Ni(II) and IDA–Co(II) columns. Fig. 5 illustrates the chromatography of PEPCK on IDA–Co(II) and IDA–Ni(II) columns. The enzyme was eluted from IDA–Ni(II) columns only after a decrease in the pH to *ca.* 6.3 (Fig. 5A). In chromatography on IDA–Co(II) columns the protein peak occurred at a considerably higher pH value of about 7.0 (Fig. 5B).

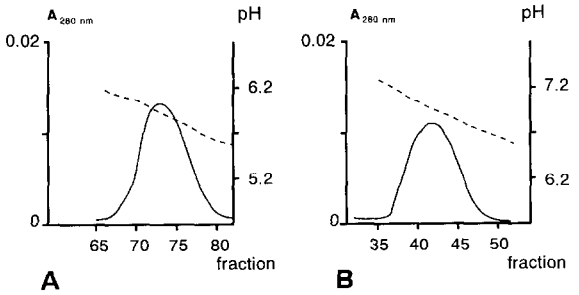

Fig. 5. Chromatography of yeast PEPCK on (A) IDA–Ni(II) and (B) IDA–Co(II) columns. Purified PEPCK was applied to a Chelating Superose column in the equilibration buffer. After washing, the column was developed with a pH gradient using 0.02 *M* phosphate–1 *M* KCl–0.015 *M* $MnCl_2$ (pH 4.0) (0–100% in 60 min; flow-rate 0.6 ml/min). Solid lines, protein concentration; dashed lines, pH gradient.

DISCUSSION

This work involved the overexpression and purification of the *Saccharomyces cerevisiae* PEPCK. The purification was greatly aided by the cloning of the coding region and the overexpression of the inserted gene.

The purification method was adapted to the manipulated yeast strain with the aim of isolating PEPCK in a large amount for subsequent crystallization experiments. The procedure described here indeed resulted in large amounts of highly purified enzyme. The purification of PEPCK using anion-exchange, hydrophobic interaction and gel permeation chromatographies was highly reproducible; results similar to those shown in Table I were obtained from four repeated runs. The conditions required for elution of PEPCK from the analytical Mono Q HR 5/5 column could be applied to fractionation on a large-scale Q-Sepharose column. As shown in Fig. 1, the resolution of the Q-Sepharose column is slightly diminished. Possibly the capacity of the Q-Sepharose column may have been too low, causing the observed decrease in peak resolution. The differences in the separation pattern may be due to the different cell disruption method used for the experiments. However, there is no negative influence on the PEPCK purification efficiency. The peak fractions of both chromatographic steps contained nearly the same specific activity (Mono Q, 25 U/mg; Q-Sepharose, 30 U/mg). Presumably, the removal of adenylate kinase during anion-exchange chromatography caused the apparent increase in PEPCK activity after this purification step [2].

The elution profile of PEPCK on phenyl-Sepharose suggests a relatively hydrophobic character of the enzyme. The average hydropathy of the PEPCK determined according to Kyte and Doolittle [28] was calculated to be −3.1; obviously this value indicates a hydrophobicity that is large enough to be bound to the column material. However, the high ammonium sulphate concentration enhances the binding properties of PEPCK to phenyl-Sepharose. As the purity of the enzyme obtained was the same whether using 40% or 30% ammonium sulphate-saturated buffer, the latter was preferred as the pooled fractions could be adjusted faster.

Gel permeation as the last chromatographic step removed all of the contaminating proteins and the salt that may influence the crystallization experiments. The final specific activity of purified PEPCK after the last step was 40.4 U/mg. This value agrees with the specific activity measured by Encinas *et al.* [11], but is higher than the value reported by Tortora *et al.* [2]. The difference may be due to slightly different assay conditions and different methods of protein determination. The overall recovery of PEPCK was considerably increased (87%) and the yields were at least ten times higher than previously reported [2,29,30].

Although three chromatographic steps were necessary starting from a crude extract, the purification was fast because no time-consuming dialysis was involved. The use of the overexpression yeast strain made it possible to start with a few grams of material. Thus the sample volumes were very small and the columns could be loaded rapidly. Further, the omission of AMP affinity chromatography could lower the cost even in the case of a large-scale purification. We confirmed the results of Tortora *et al.* [2] that the addition of proteinase inhibitors is unnecessary. No significant proteolytic degradation could be detected during the preparations.

The high homogeneity of the purified enzyme was proved by both electrophoretic (native and SDS–PAGE) (Fig. 4) and chromatographic methods using size-exclusion and hydroxyapatite columns. The purified yeast PEPCK exhibited an M_r of *ca.* 65 000 on an SDS-PAGE gel, corresponding to the predicted value calculated by the amino acid sequence (61 500). Although no microheterogeneity of the preparations could be detected, the crystallization of PEPCK resulted in very small crystals not suitable for X-ray analysis.

Chromatography on immobilized metal ions was used in order to investigate the surface topography of histidine residues on the PEPCK molecule. PEPCK showed strong binding affinities for immobilized copper and nickel. PEPCK was also retained on an IDA–Co(II) column. However, the elution from the latter occurred at a considerably higher pH value indicating a weaker binding to cobalt. We interpret the binding of PEPCK to immobilized cobalt as resulting from at least two histidine residues located at the protein surface.

Use of genetic engineering techniques will allow the recovery of large amounts of homogeneous PEPCK, permitting crystallization experiments.

Future work will be aimed at the growth of crystals suitable for X-ray crystallographic studies.

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