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Purification and crystallization of yeast hexokinase isoenzymes

Characterization of different forms by chromatofocusing

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ABSTRACT

The yeast hexokinase isoenzymes PI and PII have been purified in large amounts (20 mg) from overproducing yeast strains. The purification procedures of hexokinase PI and PII include anion-exchange chromatography on DEAE-Sephacel and chromatofocusing on PBE 94, hydrophobic interaction chromatography on phenyl-Sepharose (necessary for the isolation of the isoenzyme PI); in the final step either a Mono Q HR 5/5 or a Fractogel EMD TMAE 650(S) column was used. Hexokinase preparations were characterized before cyrstallization by chromatofocusing on a Mono P HR 5/20 FPLC column, where different forms of hexokinase can be rapidly distinguished by their elution behaviour. From both purified hexokinase PI and PII, large crystals were grown that diffract X-rays to high resolution.

INTRODUCTION

A number of investigations of structure–function relationships of the hexose phosphorylating yeast enzyme hexokinase were based on X-ray diffraction analyses of both the PI and PII isoenzymes. The structure of native hexokinase PII in monomeric form was determined at 2.1 Å resolution [1] and that of the PI isoenzyme with bound glucose at a medium resolution of 3.5 Å [2]. Unfortunately, it was then not possible to crystallize the same isoenzyme with and without bound sugar. Therefore, direct evidence of a conformational change induced by the binding of the substrate could only be derived from results of small-angle X-ray scattering from hexokinase in solution [3]. Further, the correct sequences of the isoenzymes were not known until later [4,5]. A detailed knowledge of the sugar and nucleotide binding sites is of interest for a better understanding of the role of conformational changes in the functioning of hexokinase and of the different specificities of the isoenzymes towards fructose and glucose. It is further of interest for a comparison with other proteins with similar conformations to hexokinase such as actin [6] and the N-terminal ATPase fragment of the 70 000 dalton bovine heat shock cognate protein [7]. In order to provide a basis for detailed crystal structure analyses of the substrate binding sites, we developed an improved scheme for the purification of both yeast hexoki-

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nase isoenzymes on a semi-preparative scale and grew crystals in the native and complexed form which were suitable for X-ray diffraction measurements at high resolution.

Numerous proteolytic degradation products of hexokinase were described in the past, when protein purification methods were less sophisticated [8,9]. Most of the large-scale purification procedures published in the 1960s and 1970s were modifications of the method of Darrow and Colowick [10]. Depending on the elution conditions, different hexose phosphorylating fractions were eluted from DEAE-cellulose columns [8,11–14]. Among these, two predominant proteolysed forms, SI and SII, which are both still active, occur during purification [9]. It has been shown recently by cloning the *Saccharomyces cerevisiae* hexokinase genes that there are two isoenzymes, PI and PII, with overall homologies in their amino acid sequence of only about 76% [4,5]. New attempts to better separate the isoenzymes and eliminate the proteolytic forms have been made successfully by Womack *et al.* [15] using hydroxyapatite column chromatography as the final step and by Kopetzki and Entian [16] using affinity chromatography followed by chromatofocusing. The latter procedure was developed for the small-scale purification of hexokinase.

The preparation described in this paper involves the construction of genetically manipulated yeast strains which facilitate the protein purification of milligram amounts of hexokinase. This work is part of a project aimed at providing information about the kinetics of the hexokinase molecule at a structural level.

EXPERIMENTAL

All chemicals were of pro analysi or puriss grade. Enzymes, substrates and cosubstrates were obtained from Boehringer Mannheim and hexokinase type C301 from Sigma.

Yeast strains and overexpression

A DNA fragment harbouring the complete HXK1 gene of *Saccharomyces cerevisiae* [4] was ligated into the yeast episomal plasmid YEp24 [17]. In a similar approach, the complete HXK2 gene [5] was cloned into the plasmid YEp24. The *in vitro* recombination of DNA was performed by standard techniques [18]. The resulting plasmids pMR22 and pMR47 contained the HXK1 and the HXK2 gene, respectively. Both hexokinase genes were under the control of their native promotors. Transformation marker genes and sequences necessary for replication in *Escherichia coli* and yeast were derived from the plasmid YEp24. The origin of plasmid replication in yeast was derived from the 2-μm circle DNA via YEp24. The use of this origin results in a high plasmid copy number [19]. Plasmids pMR22 or pMR47 were transformed in *Saccharomyces cerevisiae* strain WAY.10-1C according to the method of Ito *et al.* [20]. The chromosomal loci of the HXK1 and HXK2 genes were deleted in this strain [21]. After transformation, hexokinase was only obtained by expression of the plasmid encoded HXK1 and HXK2 genes. The transformed yeasts were grown under selective conditions on synthetic media in order to prevent plasmid loss in cultures up to a volume of 100 ml. The preculture so obtained was transferred to a maximum of 10 l of rich medium (1:20) and incubation was stopped when the wet weight reached 15–20 g/l. After centrifugation, the pellet was resuspended in water. The cells were pelleted again by centrifugation and stored at −20°C.

Enzyme assays

Hexokinase activity was determined spectrophotometrically as described by Bergmeyer [22] by coupling the formation of glucose-6-phosphate from glucose to the reduction of NAD with glucose-6-phosphate dehydrogenase. Unless indicated otherwise, standard assays were performed at 20°C in a total volume of 1 ml containing 50 m*M* triethanolamine–HCl (pH 7.5), 11 m*M* glucose, 1.5 m*M* NAD, 8.9 m*M* magnesium chloride, 0.66 m*M* ATP and 1 U glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Boehringer Mannheim). Isoenzymes were distinguished by their *F*/*G* ratio, *i.e.*, their relative fructose to glucose phosphorylating activities [11]. With fructose as substrate (11 m*M*), 1 unit/ml of phosphoglucose isomerase was added.

Protein determination

The protein peaks of all chromatographic separation steps were recorded at 280 nm (Uvicord SD 2158; LKB). Protein concentrations in hexokinase-

containing fractions were determined with Coomassie protein assay reagent (Pierce). Additionally, pooled fractions and purified samples of hexokinase were assayed with biuret reagent (Sigma).

Preparation of yeast cell extracts

Up to 35 g of freshly thawed cells of the PI-producing strain were suspended in one volume of the above buffer; 2 g of glass beads (0.5 mm) were added to 1 ml of yeast suspension. Purification of the isoenzyme PII started from 10 g of overproducing yeast cells (yielding 15 mg of pure enzyme). An IMA (Zeppelinheim, Germany) disintegrator operating at 4000 rpm for 20 min was used for disruption of yeast cells. Immediately after centrifugation at 20 000 *g* for 30 min at 4°C the supernatant was removed and the cell pellet was resuspended in about 10 ml of piperazine–HCl buffer (0.025 *M*, pH 6.5). After centrifugation, (20 000 *g* for 30 min at 4°C) both supernatants were combined.

Chromatographic procedures

All chromatographic steps were performed in rapid succession at 4°C in order to avoid loss of hexokinase activity. The columns used were connected to a Model 2249 high-performance liquid chromatographic gradient pump (Pharmacia LKB). The cell-free crude extract was rapidly loaded onto a DEAE-Sephacel (Pharmacia LKB) column (5 × 5 cm I.D.) that had been equilibrated with 0.025 *M* piperazine–HCl buffer (pH 6.5). After washing with the equilibration buffer, elution was performed using 0.025 *M* piperazine–HCl buffer (pH 3.5) at a flow-rate of 2 ml/min. The pooled fractions containing hexokinase activity were adjusted to pH 5.5 by the slow addition of 0.025 *M* piperazine (pH 10.5). Large-scale chromatofocusing was performed using Polybuffer Exchanger 94 (Pharmacia LKB) filled into a chromatographic column (46 × 1 cm I.D.). After the equilibration with piperazine buffer (0.025 *M*, pH 5.5), samples which consisted of the pool of active fractions from the DEAE-Sephacel column were loaded at flow-rates of 0.35 ml/min. The columns were washed with equilibration buffer and eluted with decreasing pH gradient developed with 250 ml of Polybuffer 74 (pH 4.0) (diluted 1:10 from stock solution).

The pooled fractions containing hexokinase isoenzyme PI were brought to 40% saturation with solid ammonium sulphate with gentle stirring; no precipitation was visible. The protein solution was applied to a column (20 × 2.6 cm I.D.) filled with 85 ml of phenyl-Sepharose CL-4B (Pharmacia LKB) that had been equilibrated with 40% saturated ammonium sulphate–piperazine buffer (0.025 *M*; pH 6.5) at a flow-rate of 0.5 ml/min. The column was washed with 150 ml of the same buffer and hexokinase was eluted with a 500-ml linear gradient of decreasing ammonium sulphate saturation (40–0%) in combination with an increasing linear gradient of ethylene glycol concentration (0–80%), both in 0.025 *M* piperazine buffer (pH 6.5).

Fractions containing hexokinase PI were desalted using PD 10 columns and then loaded with a 50-ml superloop (1 ml/min) on a Mono Q HR 5/5 FPLC column (Pharmacia LKB) or a Fractogel EMD TMAE 650(S) column (Merck) which had been equilibrated with degassed 0.025 *M* piperazine–HCl buffer (pH 6.5). With the Mono Q column aliquots of 17 mg of protein were applied. The TMAE 650(S) column had a capacity of about 80 mg and the pooled fractions could be loaded in one step. After washing with equilibration buffer, the column was eluted with a linear gradient of 0–1 *M* sodium chloride [in 0.025 *M* piperazine–HCl buffer (pH 6.5)] in 60 min. The protein solution was concentrated by ultrafiltration using Centricon 30 microconcentrators (Amicon) to final concentrations of about 20–30 mg (5000–7000 rpm, 4°C, 1–2 h).

Purified hexokinase (0.1 mg per run) was applied to a Mono P HR 5/20 FPLC column (Pharmacia LKB) that had been equilibrated with 0.025 *M* piperazine–HCl buffer (pH 5.5). The column was developed with Polybuffer 74 (pH 4.0) (1:10 dilution) and fractions of 1 ml were collected. Gel-permeation experiments were done on a Superose 6 HR 10/30 FPLC column (Pharmacia LKB) equilibrated with 0.05 *M* sodium phosphate buffer (pH 7.2) containing 150 m*M* potassium chloride. For each run, 100 µl of purified isoenzymes PI and PII were loaded onto the column. The flow-rate was 0.5 ml/min. The molecular mass of hexokinase PI and PII was re-examined using protein standards (low- and high-molecular-weight gel filtration kit; Pharmacia LKB) for calibration. The void volume was determined with blue dextran.

Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulphate (SDS)-PAGE was performed with the GE 2/4-LS electrophoresis apparatus (Pharmacia LKB) using the system of Laemmli [23]. PAGE under non-denaturing conditions was carried out according to the method of Davis [24] using 7.5% polyacrylamide gels (stacking gel 4.5%).

RESULTS

Purification of hexokinase isoenzyme PI and PII

The purification of *Saccharomyces cerevisiae* hexokinase isoenzymes PI and PII was achieved from overproducing yeast cells with yields of about 23% for the isoenzyme PI and about 42% for hexokinase PII. In order to circumvent co-purification of both isoenzymes, we used a mutant of *Saccharomyces cerevisiae* lacking the HXK2 gene for the purification of isoenzyme PI; the corresponding procedure was employed for PII. We found specific acitivities of about 8.3 U/mg protein in the crude extract of the recombinant PI-producing cells and about 48 U/mg protein for the PII-producing yeast strain.

The first step in the purification of both isoenzymes was anion-exchange chromatography on DEAE-Sephacel using high flow-rates. This chromatographic step resulted in a very good separation of each hexokinase isoenzyme from the bulk protein; a peak of hexokinase PI with a ratio of fructose to glucose phosphorylating acitivity of 2.8 appeared between pH 5.5 and 4.8. Hexokinase PII could be eluted in the same range. The recovery of hexokinase activity from DEAE-Sephacel was about 65% for PI and 70% for PII (Table I). The specific acitivities increased from 8.3 U/mg in the crude extract to 36.7 U/mg in the pooled hexokinase PI fractions. With isoenzyme PII the pooled fractions contained 140 units/mg protein (Table I).

For efficient binding to the PBE 94 polybuffer exchanger column, it was necessary to adjust the pH of the pooled fractions to 5.5. This also prevented a loss of acitvity. All chromatofocusing columns developed with Polybuffer 74 resulted in elution profiles comparable to that shown in Fig. 1. A peak of hexokinase PI was eluted at about pH 4.8 (Fig. 1A). Hexokinase isoenzyme PII was also obtained as a homogenous peak at about pH 4.65 (Fig. 1B). For each isoenzyme, the activities found in the pooled fractions corresponded to 70–80% of the activities applied to the column. Owing to the concentrating effect of chromatofocusing, the volumes of the pooled fractions were about 50 ml, independent of the sample volume (up to 200 ml) that was applied. With hexokinase PII, the peak fractions were nearly free from contaminating proteins. For further purification, therefore, it was sufficient to remove the remaining proteins with a TMAE 650(S) or a Mono Q column. In contrast, the purification of isoenzyme PI required an additional hydrophobic interaction chromatographic step following the chromatofocusing. Hexokinase could be detected at about 25% ammonium sulphate saturation (Fig. 2).

TABLE I

PURIFICATION OF YEAST HEXOKINASE ISOENZYMES

Data are from a representative purification starting with 26 g of hexokinase isoenzyme PI overproducing yeast cells and 7 g PII producing cells. The yield was calculated as a percentage of the amount of hexokinase present in the crude extract.

Purification step	Hexokinase PI				Hexokinase PII			
	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	1140.0	9509	8.3	100	192.3	9176	47.8	100
DEAE-Sephacel	170.0	6208	36.7	65	46.4	6438	140.0	70
Chromatofocusing	76.3	4964	65.0	52	18.8	4465	226.0	49
Phenyl-Sepharose	36.0	3423	94.8	36	–	–	–	–
Mono Q	23.0	2135	95.0	23	12.4	3853	310.2	42

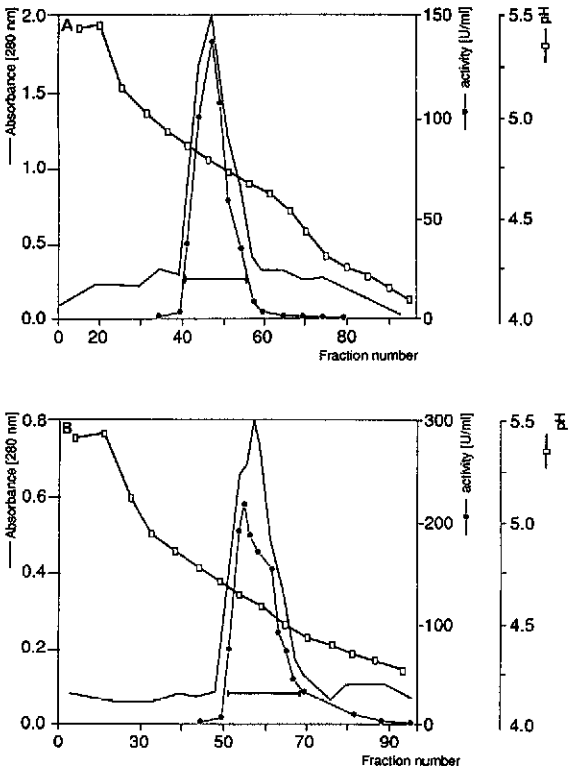

Fig. 1. Chromatofocusing elution profile of the pooled DEAE fractions containing (A) hexokinase PI and (B) isoenzyme PII on a PBE 94 column. Each column was developed using Polybuffer 74 (1:10) (pH 4.0). The pH gradient in the effluent is indicated. and the bars represent the pooled fractions.

About 36% of hexokinase PI could be recovered after the hydrophobic interaction chromatography, and the specific activity increased to 95 U/mg. The last separation to homogeneity was done either on a Fractogel EMD TMAE 650(S) column or on a Mono Q HR 5/5 FPLC column. As a high ionic strength had to be avoided, the remaining ammonium sulphate was removed by rapid desalting on PD 10 columns instead of dialysis. Both anion-exchange chromatographic steps were highly reproducible.

The hexokinase isoenzyme PI was eluted in five fractions (each 1 ml) in the range between 0.28 and 0.35 *M* potassium chloride depending on the total amount of protein that was applied to the column (Fig. 3A). Isoenzyme PII could be recovered under identical conditions (Fig. 3B). As evidenced by protein staining, fractions 14 and 15 contained a single

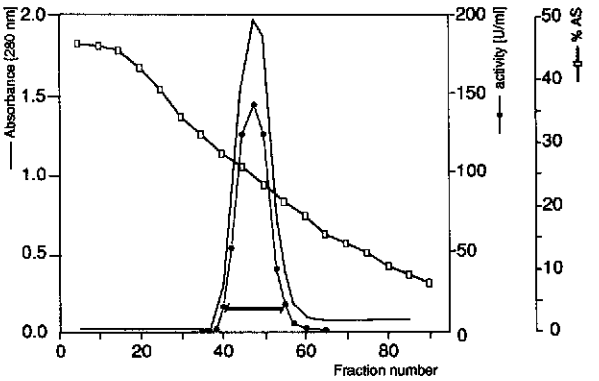

Fig. 2. Hydrophobic interaction chromatography of the pooled fractions from Fig. 1A on phenyl-Sepharose CL-4B. Elution was performed using a linear gradient of decreasing ammonium sulphate saturation (40–0%) in combination with an increasing linear gradient of ethylene glycol concentration (0–80%), both in 0.025 *M* piperazine buffer (pH 6.5). Hexokinase PI eluted at about 25% saturation ammonium sulphate. Fractions containing more than 20 U/ml (marked with a bar) were pooled.

protein in the molecular mass range of hexokinase. In fractions 17 and 18, a smaller protein (40 000 dalton) was visible. Each of the purified isoenzymes could be stored at −20°C in 0.025 *M* piperazine–HCl (pH 6.5) containing 50% glycerol for at least several months with only a minor loss of activity.

Prior to crystallization trials, glycerol was removed by PD 10 columns, and the samples were concentrated to 20–30 mg/ml of protein using Centricon 30 microconcentrators. From this material, it has been possible to obtain for the first time large crystals of hexokinase PI in the presence and absence of glucose, and also crystals of hexokinase PII; the characterization of the crystals by means of X-ray diffraction will be described elsewhere [25].

Criteria of purity

The purities of the different preparations described above and of commercial hexokinase samples which were also used for protein crystallization were determined by a number of electrophoretic and chromatographic methods including SDS-PAGE, non-denaturing PAGE, rechromatography on an anion exchanger, gel permeation and chromatofocusing. Both hexokinase isoenzymes purified from overproducing strains were apparently homogeneous when enzyme preparations were analysed by SDS-PAGE. Both isoenzymes yielded only one

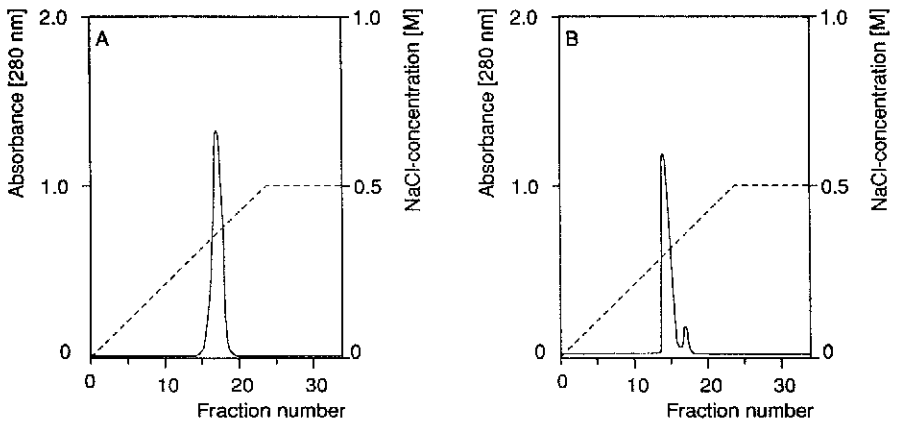

Fig. 3. Anion-exchange chromatography on a Mono Q HR5/5 column of hexokinase isoenzyme (A) PI and (B) PII. With isoenzyme PII a minor peak was separated by a salt gradient. The major peak contains hexokinase PII activity (ratios of fructose to glucose phosphorylating activity 1.4).

protein staining band (10 μg per lane) corresponding to a molecular mass of about 54 000 dalton. On electrophoresis without SDS, isoenzyme PI shows a single band that migrates with nearly the same mobility as the isoenzyme PII (Fig. 4).

In the hexokinase preparation obtained from Sigma, one protein band was visible on the SDS gel, whereas under non-denaturing conditions two major bands could be detected on the gel (Fig. 4). These two proteins could not be separated by anion-exchange chromatography.

During rechromatography of both isoenzymes purified from yeast cells on a Mono Q or TMAE Fractogel column, only one peak was observed which coincided exactly with that of hexokinase activity; this is shown in Fig. 5 for the example of isoenzyme PII. Gel permeation of purified isoenzymes PI and PII on a Superose 6 FPLC column also produced one peak. For each isoenzyme, the hexokinase activity emerged at positions corresponding to a globular protein of molecular weight about 55 000–60 000 dalton.

In order to characterize the protein samples before crystallization, we carried out chromatofocusing using a Mono P HR5/20 column. In our preparation, a distinct species of isoenzyme PI eluted at pH 4.87 (Fig. 6A). With purified hexokinase PII, a peak occurred at pH 4.74 (Fig. 6B). Two species of hexokinase PII could be distinguished (Fig. 6C) for the commercially obtained hexokinase (Type C301) which we also crystallized. The first minor peak occurs at pH 4.53 and corresponds to the slowly migrating band in the native PAGE shown in Fig. 4. A second peak eluted at pH 4.4; it corresponds to the fast band in the non-denaturing polyacrylamide gel. Both proteins separated on the Mono P column had

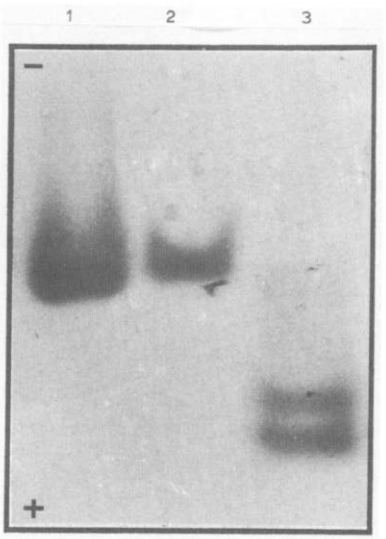

Fig. 4. PAGE at pH 6.8 of hexokinase under non-denaturing conditions. Single band of isoenzyme PI (lane 1), isoenzyme PII (lane 2) and the two bands of commercially obtained hexokinase (lane 3; Sigma Type 301) are shown. The direction of migration is from top to the bottom of the gel.

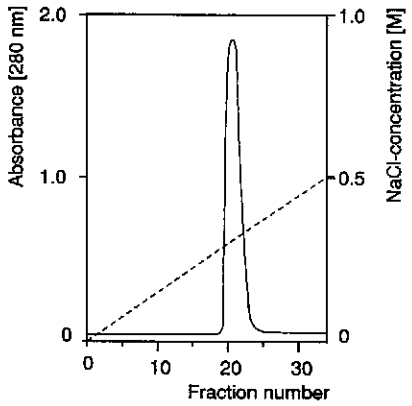

Fig. 5. Anion-exchange chromatography of hexokinase PII on a Fractogel EMD TMAE 650(S) column. Purified isoenzyme PII was applied to the column and eluted as a homogeneous peak. Elution was performed using a linear gradient of increasing sodium chloride concentration.

ratios of fructose to glucose phosphorylating activity of about 1.2, indicating the presence of isoenzyme PII.

DISCUSSION

As shown here, the molecular cloning of each of the *Saccharomyces cerevisiae* hexokinase isoenzymes provides attractive conditions for the purification and subsequent crystallization of the enzyme. From this approach, we obtained crude extracts containing large amounts of either isoenzyme PI or PII at high specific acitivities. Growing sufficiently large crystals for structure determination requires milligram amounts of protein. The procedure described here for the preparation of hexokinase indeed yields large amounts of highly purified isoenzyme PI or PII. As neither the use of protease inhibitors [11] nor rapid cell rupture [13] seems to protect against proteolytic alterations of hexokinase, a combination of rapid cell disruption immediately followed by DEAE-Sephacel chromatography was preferred. During the separation of hexokinase from the bulk protein, most of the proteases were also removed. Chromatofocusing proved to be a convenient method for purifying hexokinase, even if only one isoenzyme is present. The final specific activities were 95 U/mg for isoenzyme PI and 310 U/mg for hexokinase PII. These relatively low values are explained by the assay temperature of 20°C. When the activity of purified isoenzymes was determined at 30°C, specific acitivities of 152 U/mg for hexokinase PI and of 580 U/mg for isoenzyme PII were obtained; these values are comparable to those described by Kopetzki and Entian [16]. The purified isoenzymes were shown to be homogeneous by PAGE both under denaturing and non-denaturing conditions. In contrast to the data reported by Colowick [9], the differences in the mobilities in gel electrophoresis at pH 6.8 are less significant. Both purified yeast hexokinase isoenzymes had a molecular mass of about 54 000 dalton in SDS-PAGE and migrated as a single protein-staining band with the same mobility. For both hexokinase isoenzymes, gel filtration on Superose 6 also showed the same single peak corresponding to the molecular mass of the monomeric form. Similar results for hexokinase PII were described by Furman and Neet [26].

The existence of only one hexokinase isoenzyme in the strains constructed here ensured a rapid isolation, as a distinction between PI and PII during the

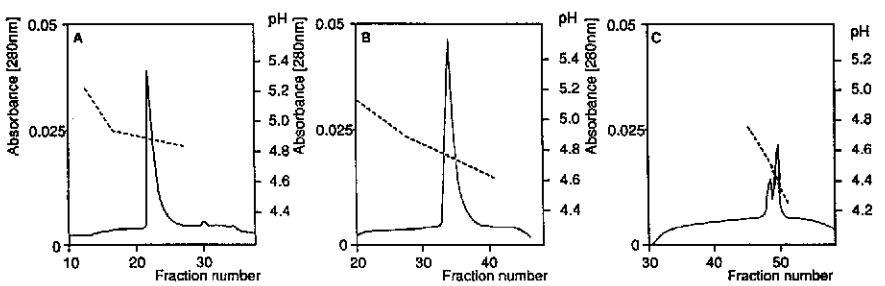

Fig. 6. Chromatofocusing of the purified isoenzyme PI and PII on a Mono P HR5/20 column. Hexokinase PI eluted as a homogenous peak at pH 4.87 (A), whereas a peak containing isoenzyme PII was obtained at pH 4.74 (B). With commercially obtained hexokinase (Sigma, Type C301) two peaks eluting at about pH 4.53 and pH 4.4 could be distinguished (C).

purification was not necessary. The glucose phosphorylating enzyme glucokinase was not detected during the different purification steps. The overall recovery of the hexokinase isoenzymes PI and PII was considerably increased by the cloning and overexpression of the inserted genes. Comparing the final amount of isoenzyme PI and PII obtained here with the results of previous purifications [16], a 10-fold increase for PI and a 40-fold increase for PII was achieved. Hence only small amounts of yeast cells are needed as starting material for the purification. Owing to the small sample volumes, all chromatographic steps could be performed rapidly. The reproducibility and high resolution of the Mono P column make this column particularly suitable for molecular characterization before protein crystallization. As demonstrated in Fig. 5, both purified isoenzymes PI and PII eluted as homogeneous peaks. The pH values at which each isoenzyme eluted differ only minimally, and they are in the same range as those of the chromatofocusing on PBE 94. It should be noted that the precise pH value of elution varied slightly with the total amount of protein that was applied to the columns. In the presence of 50% glycerol, no loss of activity was observed after storage at −20°C for several months; the protein could still be used for crystallization assays.

As the succes of protein crystallization is strongly dependent on the homogeneity and the total amount of the starting material, the combination of genetic engineering techniques with methods of protein crystallization is very useful. Both isoenzymes produced from the cloned genes were suitable for protein crystal growth, and the crystals which were grown diffracted to high resolution [25]. We observed different crystallization behaviours for different commercial preparations which showed homogeneity in SDS-PAGE but exhibited different electrophoretic protein patterns on native gels. Chromatofocusing is a high-resolution method for the rapid characterization of proteins, even in the case of very similar proteins.

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