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Three-step chromatographic purification of Cpr6, a cyclophilin from *Saccharomyces cerevisiae*

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Abstract

Cyclophilins constitute a group of peptidyl-prolyl *cis–trans* isomerases (PPIs), known to be involved in protein folding. Because of their ability to bind the immunosuppresant drug Cyclosporin A (CsA), they are also called immunophilins. Immunophilins, which exhibit a relative molecular mass higher than 40 000, are further found in complex with Hsp90, a major cytosolic molecular chaperone. The present work describes a three-step chromatographic purification of recombinant Cpr6, a cyclophilin from *Saccharomyces cerevisiae*. The cDNA of Cpr6 was cloned into a pRSET A-plasmid with an N-terminal 6 x histidine-tag (his-tag) and transformed into the BL21[DE3]pLysS strain. After collection of the bacterial material and lysis of the cells the cell lysate was centrifuged and loaded onto a metal chelating column. After extensive washing the protein was eluted with a step gradient from 20 to 250 m*M* imidazol. The pooled protein was dialysed against ethylenedinitrilo tetraacetic acid (EDTA)–buffer, and loaded onto a strong anion-exchanger. Cpr6 containing fractions were then, in a last step, loaded onto a gel permeation chromatography column. The purity of the resulting protein was measured by silver stained sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and, additionally, as Cpr6 does not contain tryptophan residues by tryptophan residue titration. Based on a standard curve the content of contaminating tryptophan residues in the purified protein solution was determined. A typical yield of 1 mg pure protein per g of wet cells was achieved with the described procedure.

Keywords: Purification; *Saccharomyces cerevisiae*; Enzymes; Cyclophilins; Cpr6; Peptidyl-prolyl *cis–trans* isomerases

1. Introduction

Two different classes of proteins support the spontaneous process of protein folding in the cell. The first class consists of the heterogenous group of molecular chaperones and the second class is formed by the so-called folding catalysts. The latter are represented by protein disulfide isomerases (PDIs), which accelerate the formation of correct disulfide bonds [1] and the peptidyl-prolyl *cis–trans* isomerases (PPIs), which catalyse the isomerisation of Xaa-Pro peptide bonds [2]. Because of the ability of the cyclophilins and FKBPs to bind the immunosuppressive drugs cyclosporinA (CsA) and FK506, respectively, these two subfamilies of the PPIases are also termed immunophilins. Large immunophilins, like Cpr6 [3,4], with a relative molecular mass between 40 000 and 56 000, are found in association with steroid hormone receptor complexes [5–7]. Binding to these complexes occurs via Hsp90, one of the most abundant cytosolic chaperones [8,9], and is

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promoted by TPR-motifs [10,11]. Similar to Hsp90 the large immunophilins show chaperone activity [12]. Although the large immunophilins have been found to bind to Hsp90-complexes together with several other proteins [13], their function in these complexes is far from being understood.

In this paper, the three step chromatographic purification of Cpr6 is described. The purification consists of an affinity chromatography, an anion-exchange column and as a final step, a gel permeation chromatography. The cDNA of Cpr6 was cloned in a pRSET A-plasmid with an N-terminal 6 x histidine-tag (his-tag) and transformed into the BL21[DE3]pLysS strain. The cell lysate was loaded onto a nickel-agarose, which shows an high affinity to the his-tagged protein [14]. After extensive washing, the protein was eluted by a step gradient of imidazole. Following this affinity chromatography, the protein solution was loaded onto a strong anion-exchanger, the Resource Q. This column was chosen because of the theoretical isoelectric point of 5.84 of Cpr6 and, additionally, because of its small volume (6 ml) the eluted protein is concentrated. The last purification step used was a gel permeation chromatography (Superdex 75 pg). This column was chosen because all remaining contaminating proteins showed different molecular masses to Cpr6 and could easily be removed with this chromatographic technique. The purity of Cpr6 was monitored on a silver stained SDS–PAGE. Additionally, the tryptophan residue content of the protein solution was measured by tryptophan residue titration. The tryptophan residue content of the solution was found to be 0.01 tryptophan residue per molecule Cpr6. Since Cpr6 contains no tryptohpan this stresses the high purity of the preparation. A typical yield of 1 mg pure protein per g of wet cells was achieved.

2. Experimental

2.1. Chemicals

Chemicals were of analytical-reagent grade. All buffer substances, imidazole and guanidium hydrochloride were obtained from ICN (Meckenheim, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany). Protease inhibitors (Complete tablets without EDTA) were obtained from Boehringer (Mannheim, Germany).

2.2. Equipment

As chromatography unit a FPLC from Pharmacia (Uppsala, Sweden) was used. To perform the affinity chromatography the nickel-nitrilo-tri-acetic-acid-superflow (Ni-NTA) from Qiagen (Hilden, Germany) was chosen. The Resource Q (6 ml) and the Superdex 75 prep grade (pg) (120 ml) were prepacked columns from Pharmacia (Uppsala, Sweden). The electrophoresis unit used was a Hoefer Mighty Small from Pharmacia (Uppsala, Sweden). The ultrasonicator B-12 was from Branson (Sonic Power Company, USA). To concentrate the protein solution a ultrafiltration unit from Amicon (Danvers, USA) with a YM3 membrane was used.

2.3. Construction of the Cpr6 expression plasmid

The plasmid containing the cDNA for Cpr6 was a kind gift of Dr. C. Prodromou (Department of Biochemistry and Molecular Biology, University College, London). The Cpr6 gene was cloned into a pRSET A expression vector (Qiagen, Hilden, Germany) via Nhe1 and EcoR1 restriction sites. This plasmid was transformed into a BL21[DE3]pLysS *E. coli* strain (Promega, Madison, USA), which contains the T7 RNA polymerase structural gene under control of the isopropyl-β-D-thiogalactopyranosid (ITPG) inducible *lac*UV5 promotor. The expressed protein carried an N-terminal 6 x his-tag.

2.4. Expression and cell disruption

For expression of Cpr6, a BL21[DE3]pLysS strain containing the pRSET A plasmid was used. This strain contains a lysogen-gene which is activated after freezing and thawing of the harvested cell pellet [15]. An overnight culture with a volume approximately equal to 1/15 of the final culture was inocculated with a single colony or a glycerolculture and grown in Luria Bertani medium supplemented with 100 μg ampicilin per ml (LB_{amp}) at 37°C. Two 5 l flasks, each containing 1 l LB_{amp} were inocculated with these cultures. At a optical density measured at 600 nm of 0.5–0.6 the expression of Cpr6

was induced with a final concentration of 1 m*M* IPTG. After 3 h of growth at 25°C the cells were harvested (8000 *g*, 10 min, 4°C) and the cell pellet was resuspended and washed twice with 50 ml of buffer A (4 m*M* KH_2PO_4, 16 m*M* Na_2HPO_4, 115 m*M* NaCl, pH 7.3). The cell pellet was then frozen in liquid nitrogen and stored at −20°C.

After thawing, the pellets were resuspended with 40 ml of buffer B (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 20 m*M* imidazole, pH 8.0). To complete lysis and to disrupt DNA the protein solution was ultrasonicated (3×15 s on ice). Finally, insoluble and soluble material was separated by centrifugation (40 000 *g*, 30 min, 4°C).

2.5. Chromatographic purification

A XK 16/20 column from Pharmacia (Uppsala, Sweden) was packed with 10 ml of the Ni-NTA-superflow material. Elution of bound Cpr6 was performed by a step gradient from 20 to 250 m*M* imidazole. The Resource Q was equilibrated in buffer C (50 m*M* Tris, 50 m*M* NaCl, pH 8,0). Elution of bound protein was achieved by a linear gradient from 50 to 500 m*M* NaCl. The theoretical isoelectric point of Cpr6 was calculated with the ProtParam tool (http://expasy.hcuge.ch). Finally, a gel permeation chromatography run on a Superdex 75 pg with buffer D (50 m*M* Tris, 50 m*M* NaCl, 5% glycerol, pH 7.5) resulted in pure Cpr6. All runs were carried out at a flow-rate of 0.5 ml/min and were performed at 4°C and all buffers were pre-chilled, filtered and degassed. After every purification step the presence of Cpr6 was confirmed on 12.5% (w/v) SDS–polyacrylamid gels. The gel runs were carried out at a constant current of 15 mA per gel. The gels were either silver stained according to Heukeshoven and Dernick [16] or as described by Fairbanks et al. [17]. The identity of Cpr6 was determined by N-terminal sequencing [18].

2.6. Monitoring the purity by tryptophan residue titration

First, a 10 m*M* tryptophan solution in 40 m*M* Hepes, 6 *M* GdnHCl, pH 7.5 was prepared and filtered. Then, a standard fluorescence curve of increasing amounts of L-tryptopan residues was prepared by exciting at 295 nm and measuring the emission at 350 nm in a 3 ml cuvette. The standard curve was measured with 8 data points from 85 to 345 n*M* L-tryptopan. The standard curve was calculated with the following equation: $y = 1874x + 0.$ 120 n*M* Cpr6 were denatured in the same buffer for 24 h at room temperature. The protein solution yielded a signal corresponding to 1.066 n*M* tryptophan residues, resulting in a calculated value of 0.01 tryptophan residue per molecule Cpr6.

3. Results

3.1. Purification of recombinant Cpr6

Cell were harvested and lysed as described in Section 2.3. To exploit the His-tag of the protein a metal chelating column as first purification step column was chosen. The supernatant of the centrifugation (approx. 40 ml) was loaded onto a 10 ml Ni-NTA agarose which was equilibrated with buffer B. After extensive washing of the column (approx. 15 column volumes) the protein was eluted with an one step gradient, by rising the imidazole concentration of buffer B to 250 m*M*. Cpr6 eluted in a sharp peak and a small volume (approx. 10 ml) from the column (Fig. 1A). The protein solution was then dialysed over night against buffer C, supplemented with 20 m*M* EDTA. The dialysed protein was loaded onto a Resource Q column which was equlibrated in buffer C (10 column volumes). After removing unspecifically bound protein by washing the column with 5 column volumes of the same buffer, bound Cpr6 was eluted with a linear gradient from 50 to 500 m*M* NaCl. At this conditions Cpr6 eluted between 125 and 200 m*M* NaCl (Fig. 1B). The Cpr6 containing fractions were pooled and concentrated to 2 mg/ml. This pool was then loaded onto a Superdex 75 pg which was equlibrated with buffer D. Cpr6 eluted from the column at an elution volume of 60 to 76 ml (Fig. 1C). The course of the three step chromatographic purification of Cpr6 was controlled by SDS–PAGE (Fig. 2). The Cpr6 containing fractions were pooled and dialysed against the same buffer without NaCl, concentrated to 2 mg/ml and stored in 50 μl aliquots at −80°C. The purity of the preparation was, additionally, monitored by a

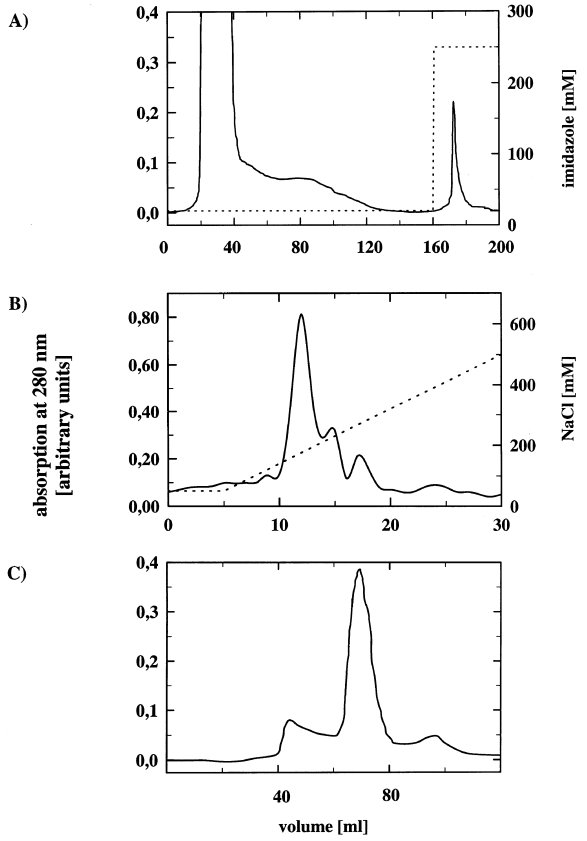

Fig. 1. Elution profiles of the chromatography experiments. Solid lines correspond to eluting protein detected by measuring absorption at 280 nm. The dotted lines show the gradient concentrations. The elution profile of the affinity chromatography (Ni-NTA Superflow, 10 ml) is shown in panel (A). The run of the Resource Q (strong anion-exchanger, 6 ml) is shown in panel (B). Panel (C) depicts the gel permeation chromatography (Superdex 75 pg, 120 ml).

tryptophan residue titration and the identity was controlled by N-terminal sequencing [18].

3.2. Discussion

Of the three purification methods used, the affinity column showed the best purification effect. In this step 70 to 80 percent of the contaminating proteins could be removed. The most important aspect of the affinity chromatography was the extensive washing procedure. Several proteins interacted weakly with the Ni-NTA resin. To avoid this unspecific binding, the column was equlibrated with 20 m*M* imidazole

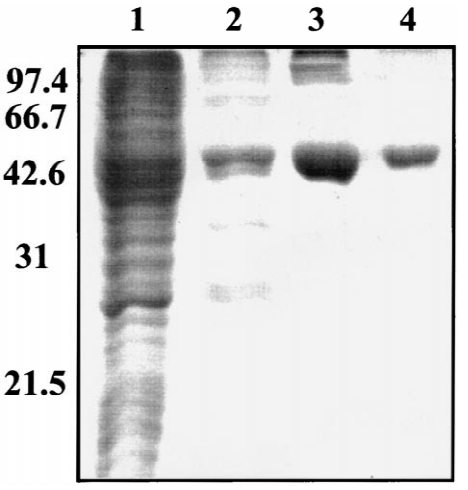

Fig. 2. Purification gel of the three step chromatographic purification of Cpr6 (12.5% SDS–PAGE, Fairbanks staining [17]). Lane (1) shows the crude extract, lane (2) the protein pool of the affinity chromatography, lane (3) the pool after Resource Q. In lane (4) the purified Cpr6 after the gel permeation run is shown. The numbers on the left hand side correspond to molecular mass markers

and 300 m*M* NaCl, and extensively washed. In the case of Cpr6-purification a higher amount of imidazole in the washing buffer (e.g. 50 m*M* imidazole) had no effect on the purity of the eluted protein. The following dialysis in the presence of EDTA was important because His-tagged proteins often tend to aggregate in the presence of bivalent anions. Proteins which precipitate due to this fact can often be resolved with an EDTA dialysis. The next column, the Resource Q, specifically removed one larger and some smaller proteins. This column showed the lowest purification effect but concentrated the protein because of the small column volume. The Resource Q was chosen as second purification step because the protein pool after the first column was not pure enough to perform a gel permeation chromatography immediately. A high amount of contaminating proteins in a gel permeation chromatography will result in an unsatisfactory separation of the target protein from contaminants. After the anion-exchanger column the protein pool showed only some contaminating bands with a higher molecular mass than Cpr6. In accordance with the molecular mass of Cpr6 (40 000) a gel permeation chromatography (Superdex 75 pg) was performed as last purification step.

This column was found to remove the last contaminating protein bands and yielded pure Cpr6. The identity of the protein was confirmed by N-terminal sequencing [18] and the achieved yield was 1 mg pure protein per g of wet cells. Cpr6 can be stored in Tris (as described above) or alternatively in Hepes buffer. Since Cpr6 contains no tryptophan residues, the value of 0.01 tryptophan residues per molecule Cpr6, obtained by tryptophan residue titration proved the high purity of the preparation. The quality of the preparation can also be confirmed by fluorescence spectroscopy because of the characteristic spectra of the different residues (data not shown). Additionally, the functionality of the purified Cpr6 was controlled with a specific PPIase assay [19] (data not shown). Cpr7, a related protein, can be purified with the same method. But, because of its higher instability/protease sensitivity, all puffers were supplemented with 5% glycerol and protease inhibitors were added after every purification step.

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