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# Heterologous overexpression and purification of four common subunits of nuclear RNA polymerases I, II and III of *Schizosaccharomyces pombe*

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**Abstract**

Four subunits of *Schizosaccharomyces pombe* RNA polymerases I–III shared by all three enzymes (Rpb5, Rpb8, Rpb10 and Rpc10 [Rpb12]) have been overexpressed in *Escherichia coli* expression vectors pQE or pET as hexahistidine fusions. The recombinant proteins have been purified to near homogeneity using metal–chelate affinity chromatography and gel filtration. Homogeneity and identity of the purified protein preparations was demonstrated by denaturing polyacrylamide gel electrophoresis and TOF-MALDI mass spectrometry. The proteins were obtained in large amounts, and their preparations are currently in use for monoclonal antibody production and physico-chemical studies of these individual components of eukaryotic transcription enzymes.


*Keywords:* Expression; Purification; Cloning; Nuclear RNA polymerases

## 1. Introduction

Nuclear RNA polymerases I–III are large macromolecular heteromultimeric complexes with a total molecular mass about 0.5–0.6 MDa. At present the most studied, both from biochemical and genetic points of view, are the RNA polymerases I–III of the budding yeast *Saccharomyces cerevisiae*. It is revealed, that RNA polymerase I of *S. cerevisiae* consists of 14 subunits, RNA polymerase II comprises 12 subunits, and RNA polymerase III is composed of 17 different subunits [1,2]. One of the important and still not well-studied components of these enzymes are five so-called common subunits: Rpb5, Rpb6, Rpb8, Rpb10 and Rpc10 [Rpb12] [3–8]. They are shared between all three nuclear RNA polymerases and are essential for cell growth and viability [5,6,9]. All these proteins (with probable exception of Rpb6 [10]) do not have obvious equivalents (homologues) in RNA polymerases of Eubacteria, but are immunologically and structurally conserved in all eukaryotes [11,12]. The majority of common subunits of RNA polymerases I–III have also related subunits in archaeal RNA polymerase in which complexity is comparable with that of eukaryotic transcription enzymes [13–15]. Conservation of main functions of the common subunits in eukaryotic and to some extend in archaeal ([16,17]) lineages was confirmed by heterospecific complementation studies. Indeed, it was demonstrated that four out of five common subunits of *Schizosaccharomyces pombe* and *Homo sapiens* can functionally replace in vivo corresponding counterparts of the budding yeast *S. cerevisiae* [12,18–22].

All five common subunits are proteins with unique structures, for the most part not having clearly recognizable, well-studied structural domains or motifs. This was recently confirmed by established three-dimensional structures of human hRPB6 (hRPABC14.4), *S. cerevisiae* RPB5 and RPB8 subunits [23–25], and spatial structures at high resolution of 10-subunits core of yeast RNA polymerase II [26,27]. Probably the common subunits were specifically selected by evolution to allow nuclear RNA polymerases to transcribe complex chromatin templates.

Despite huge amount of genetic, biochemical and structural data accumulated the exact functions of four common subunits of RNA polymerases I–III, not having homologues in eubacterial enzyme (Rpb5, Rpb8, Rpb10 and Rpc10 [Rpb12]), are largely unclear. Rpb5 subunit contacts downstream DNA duplex in transcribing complex of RNA polymerase II and possibly plays a role in activation of transcription [26]. Apparently, Rpc10 [Rpb12] and Rpb10

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play an indispensable role in assembly and maintenance of integrity of RNA polymerases I–III, forming subcomplexes with subunits Rpb3–Rpb11 (in enzyme II) or Rpc40–Rpc19 (in enzymes I and III) on the early stages of enzymes assembly [8,26]. Still nothing is known about the possible function of Rpb8 subunit.

In order to investigate physico-chemical properties of four common subunits of RNA polymerases I–III of the fission yeast *Sch. pombe*, we have first overexpressed these proteins in bacterial system and purified them to more than 95% homogeneity with use of metal($Ni^{2+}$ or $Co^{2+}$)-affinity and gel filtration chromatographies.

## 2. Experimental

### 2.1. Strains and media

The following *E. coli* strains were used in this study: M15 [pREP4] ($F^-$, $Nal^s$, $Str^s$, $Rif^s$, *lac*$^-$, *ara*$^-$, *gal*$^-$, *mtl*$^-$, *recA*$^+$, *uvr*$^+$, *lon*$^+$) [28] and BL21 (DE3) (lysogene of BL21: $F^-$, *ompT*, $r_B^- m_B^-$) [29]. The bacterial cells were grown on LB-medium with addition of 100 μg/ml of ampicillin in case of BL21 (DE3) and 100 μg/ml of ampicillin and 30 μg/ml of kanamycin in case of M15 [pREP4].

### 2.2. Cloning of cDNAs encoding four common subunits of RNA polymerases I–III of Sch. pombe in prokaryotic expression vectors pQE and pET

The original cloning of cDNAs encoding five common subunits of RNA polymerases I–III of *Sch. pombe* was described by us previously [12,18–21,30]. For the production of recombinant Rpb5, Rpb8, Rpb10 and Rpc10 [Rpb12] proteins, their corresponding cDNAs were recloned in pQE- [28] or pET-based [29] vectors. Three cDNAs (*rpb5*$^+$, *rpb8*$^+$, and *rpc10*$^+$) were recloned from original plasmids pGVS-RPB5(Sp) [19], pYUK9 [30], and pGVS121 [12], respectively, using appropriate restriction sites flanking cDNA inserts or intermediate recloning in pUC19 if necessary. pQE31–Rpb5 was obtained by recloning of *Bam*HI/*Kpn*I fragment (749 bp) containing *rpb5*$^+$ open reading frame (ORF) from pSP53 (pUC-derivative of pGVS–Rpb5(Sp) [19]) into pQE31 vector [28]. For construction of pQE30–Rpb8 *Bam*HI fragment (423 bp) containing *rpb8*$^+$ ORF from pSP81 (pUC-derivative of pYUK9 [30]) was recloned into pQE30 vector [28]. *Sac*I/*Pst*I fragment (342 bp) containing *rpc10*$^+$ ORF from pS10α (pUC-derivative of pGVS121 [12]) was recloned into pQE31 vector [28] generating pQE31–Rpc10.

Polymerase chain reaction (PCR) was used to reclone *Sch. pombe rpb10*$^+$ cDNA from pEL44 [20] into pET-6His/Tb. The following forward and reverse primers were synthesized based on the published sequence of the subunit [20]: 5′-CCGCTAGCATGATCATTCCTATTC-3′ and 5′-CCGGATCCGCTAACCGATTAAAG-3′. The resulting PCR product was cloned into pET-6His/Tb vector, digested with *Nhe*I and *Eco*RV, and sequenced to eliminate the possibility of PCR-induced mutations or other artefacts. The vector pET-6His/Tb is constructed and kindly provided to us by Dr. Marc Vigneron (Ecole Supérieure de Biotechnologie de Strasbourg, France). Compare to original pET vector [29], it contains a hexahistidine tag and encodes a thrombin cleavage site upstream of the polylinker sequence, which allows the recombinant Rpb10 protein to be cleaved from the 6xHis-fusion.

The resulting pQE31–Rpb5, pQE30–Rpb8, pQE31–Rpc10 and pET–Rpb10 plasmids are able to produce the recombinant Rpb5, Rpb8, Rpc10 [Rpb12] and Rpb10 proteins with short 6xHis-tag containing peptides (10–16 aa) fused at their N-termini.

### 2.3. Expression of recombinant fused proteins

For induction of synthesis of the desired proteins cells of *E. coli* strains M15 [pREP4] and BL21 (DE3) were transformed by plasmids pQE31–Rpb5, pQE30–Rpb8, pQE31–Rpc10 and pET–Rpb10, accordingly. The transformants were grown up to $OD_{600} = 0.6$ at 37 °C and then were induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) within 3–4 h at 18 °C or 37 °C. Cells were aliquoted, harvested by centrifugation, and the cell pellets were stored at −70 °C until further use.

### 2.4. Purification of recombinant proteins

Recombinant proteins were purified from a soluble fraction of bacterial lysates with the help of a metal-affinity chromatography on $Ni^{2+}$–NTA agarose (QIAGEN, Germany) or TALON ($Co^{2+}$) Metal Affinity Resin (Clontech, USA) using the same procedure for both sorbents. Bacterial cells were precipitated by centrifugation, suspended in 4 ml of a buffer A1 (50 mM $NaH_2PO_4$, pH 8.0; 200 mM NaCl; 1 mM DTT; 10 mM of imidazole for Rpb5), buffer A2 (20 mM Tris–HCl, pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM DTT, 10 mM imidazole for Rpb8) or buffer A3 (50 mM sodium acetate, 300 mM NaCl, 50 μM $ZnCl_2$, 10% glycerol, 1 mM DTT, 10 mM imidazole for Rpb10 and Rpc10 [Rpb12]), then were sonicated on ice (22 kHz; four times till 10 s) and again centrifuged. Decanted supernatant containing soluble fraction of proteins was filtrated through the 0.45 μm filter (NALGENE, Denmark).

For purification of proteins by metal-affinity chromatography, 4 ml of the clarified lysate were mixed with 1 ml of 50% suspension of $Ni^{2+}$–NTA agarose (in case of Rpb8 and Rpc10 [Rpb12] subunits) or TALON ($Co^{2+}$) Metal Affinity Resin (in case of Rpb5 and Rpb10 subunits) equilibrated with buffers A1, A2 or A3. The mixture was incubated at 4 °C with shaking during 60 min. The unbound proteins were removed by centrifugation, metal-affinity agarose was rinsed five times by 4 ml of a buffer B1 (50 mM $NaH_2PO_4$, pH 8.0; 1 M NaCl; 1 mM DTT; 20 mM of imidazole for

Rpb5), buffer B2 (20 mM Tris–HCl, pH 7.5, 1 M NaCl, 10% glycerol, 1 mM DTT, 20 mM imidazole for Rpb8) or buffer B3 (50 mM sodium acetate, 1 M NaCl, 50 μM $ZnCl_2$, 10% glycerol, 1 mM DTT, 20 mM imidazole for Rpb10 and Rpc10 [Rpb12]). Then target protein was eluted in a buffer C1 (50 mM $NaH_2PO_4$, pH 8.0; 200 mM NaCl; 1 mM DTT; 250 mM imidazole for Rpb5), buffer C2 (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM DTT, 250 mM imidazole for Rpb8) or buffer C3 (50 mM sodium acetate, 100 mM NaCl, 50 μM $ZnCl_2$, 10% glycerol, 1 mM DTT, 250 mM imidazole for Rpb10 and Rpc10 [Rpb12]). Both resins used ($Ni^{2+}$–NTA agarose and TALON ($Co^{2+}$)) show very similar efficiency in our hands.

It is worth to mention that in contrast to other three subunits, subunit Rpb10 was mostly found in insoluble protein fraction. For this reason for obtaining the large amount of this subunit renaturation procedure was used. Cells were lysed in buffer D1: 6 M guanidine–HCl, 20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol, 10 mM imidazole. Cleared lysate was applied on TALON ($Co^{2+}$) Metal Affinity Resin, the protein contained beads were washed with 10 volumes of buffer D2: 8 M urea, 20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 50 μM $ZnCl_2$, 10% glycerol, 1 mM β-mercaptoethanol, 10 mM imidazole. Immobilized Rpb10 subunit was refolded by washing the beads with 10 volumes of buffer R1: 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 50 μM $ZnCl_2$, 10% glycerol, 1 mM β-mercaptoethanol, 10 mM imidazole. Refolded protein was eluted with buffer R2: 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 50 μM $ZnCl_2$, 10% glycerol, 1 mM β-mercaptoethanol, 250 mM imidazole.

Further purification of subunits was achieved by gel-filtration (size-exclusion) chromatography. The following sorbents were used: Sephadex G-50 Superfine (Pharmacia, Sweden), Sephacryl G-200 Superfine (Pharmacia, Sweden) or TSK G-2000 (TOSO-H, Japan). In a typical procedure, 0.5 ml of protein solution at a concentration of 2 mg/ml was applied on a Sephadex G-50 Superfine column ($V_o \sim 5$ ml), using 10 mM Tris–HCl, pH 7.6, 100 mM NaCl, 1 mM DTT. The purified recombinant proteins were concentrated with a Centricon-10 microconcentrator (Amicon, USA). Protein concentrations were determined by the Bradford colometric Protein Assay (Bio-Rad, USA) using BSA as standard. The identity of purified recombinant subunits was confirmed by TOF-MALDI mass spectroscopy.

## 3. Results and discussion

We have used recombinant techniques to express four common subunits of *Sch. pombe* RNA polymerases I–III in quantities sufficient for subsequent studies of physicochemical properties of these individual proteins. To produce recombinant common subunits of *Sch. pombe* RNA polymerases I–III with a hexahistidine tag at their N-termini, we cloned correspondings cDNAs (ORFs) in bacterial expression vectors pQE30, pQE31 [28] or pET-6His/Tb, derivative of pET [29]. In case of vector pET-6His/Tb transcription of a target gene, placed under control of promoter of T7 phage, is driven by a potent RNA polymerase of this phage. Synthesis of this enzyme in bacterial strain *E. coli* BL21 (DE3) is directed by a lambdoid prophage and is induced by IPTG [29]. In case of vectors of pQE series, the expression of a target gene is under control of promoter of T5 phage and two *lac*-operators [28]. The transcription of a cloned gene from pQE-plasmids in *E. coli* strain M15 [pREP4] is performed by bacterial RNA polymerase after inactivation of lac-repressor with IPTG. Polylinkers of both types of vectors contain sequences encoding six consecutive histidine residues placed in the same frame as the coding sequences of cloned RNA polymerase subunits and fused with them at their N-terminal. This allows rapid purification of the fused polypeptide in one step using chromatography on metal-affinity resin.

Recombinant full-length Rpb5, Rpb8 and Rpc10 (Rpb12) were overexpressed as 6xHis-fusion proteins in *E. coli* M15 [pREP4] cells (Fig. 1A, B, D). The complete open reading frames of Rpb5, Rpb8 and Rpc10 (Rpb12) were recloned from previously described plasmids pGVS-Rpb5(Sp) [19], pYUK9 [30], and pGVS121 [12] containing native sequences of the coresponding cDNAs of *Sch. pombe*. In case of Rpb10, the corresponding cDNA was amplified by PCR and cloned into pET-6His/Tb vector. The expression plasmid pET-Rpb10 was transformed into competent *E. coli* BL21 (DE3) cells (Fig. 1C).

After transformation into competent *E. coli* M15 [pREP4] or BL21 (DE3) cells, production of recombinant 6xHis-Rpb fusion proteins by several independent transformants was monitored by purification of analytical amounts of recombinant RNA polymerases I–III subunits from 2 ml of bacterial culture and their analysis by SDS-PAGE in the following sample buffer: 25 mM Tris–HCl (pH 7.5), 4% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.005% Bromphenol Blue (Fig. 1). The cells were first frozen overnight, then thawed on ice and boiled before loading samples on the gel. The first step in this analytical procedure of recombinant protein purification from bacterial cultures without cells lysis was partially adopted from publication, first describing use of repeated cycles of freezing and thawing for isolation of recombinant protein [31].

Representative clones producing the best yield of desired proteins were selected for large-scale recombinant protein purification. In a typical preparative-scale experiment, exemplified here for subunit Rpb8 (Fig. 2), 200 ml of bacteria harbouring the expression plasmid were grown in LB-medium with 100 μg/ml ampicillin and 30 μg/ml of kanamycin at 37 °C. At midlog phase ($A_{600} \sim 0.5$–$0.7$), the incubation temperature in cases of Rpb5, Rpb10, Rpc10 [Rpb12] was lowered to 18 °C [32] and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The lower temperature considerably reduced the extent of inclusion-body formation in case of

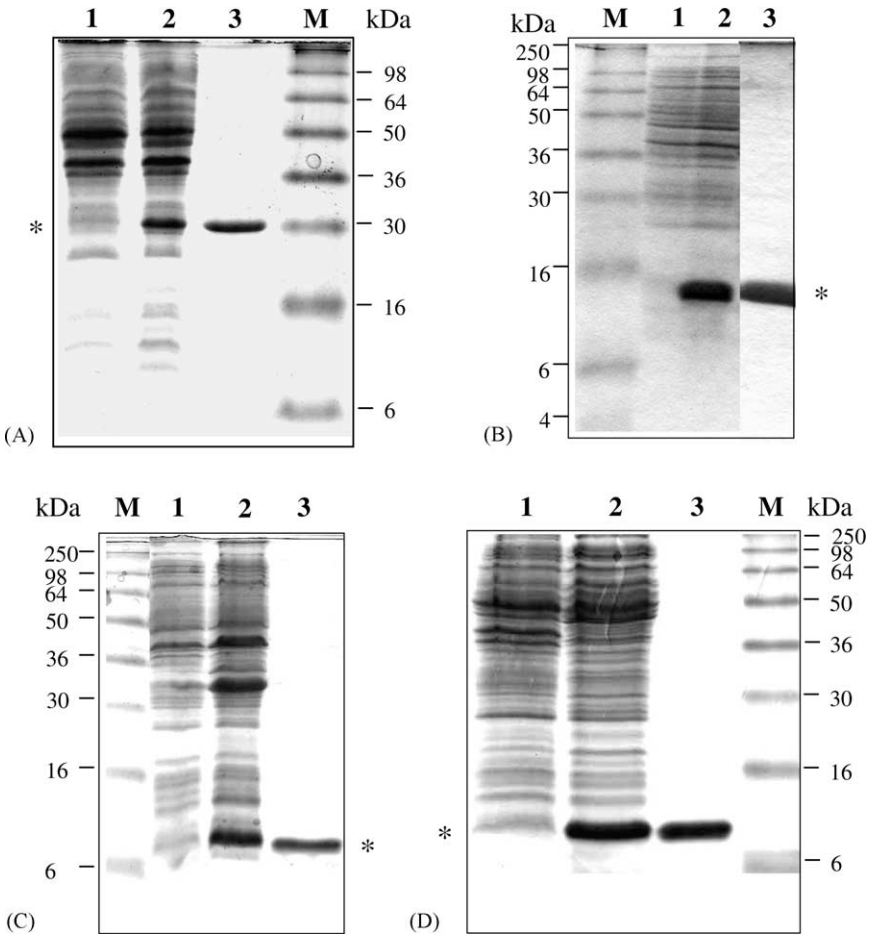


Fig. 1. Overexpression and purification of recombinant Rpb5 (A), Rpb8 (B), Rpb10 (C), and Rpc10 [Rpb12] (D) proteins. Cellular proteins were separated by 17% SDS-PAGE and visualized by Coomassie blue staining. Lanes: 1—uninduced cell extract of *E. coli* strains M15 [pREP4] (A, B and D) or BL21 (DE3) (C); 2—lysates of the same bacterial cells after induction with IPTG; 3—purified subunits of RNA polymerases I–III; M—molecular weight markers of proteins (SeeBlue$^{TM}$ Pre-Stained Standards, Novex, USA). Recombinant proteins are indicated by asterisks.

not-completely soluble fusion proteins and increased the yields of soluble Rpb5 and Rpc10 [Rpb12]-fusion proteins. This is not necessary for Rpb8 which is produced in bacterial cells as completely soluble protein. After induction for 3.5 h, the cells were harvested by centrifugation and stored at −70 °C.

Cells were lysed by sonication, filtered and cleared lysate with the released recombinant proteins was directly mixed with a batch of the pre-equilibrated $Ni^{2+}$ or $Co^{2+}$ resin for the metal–chelate affinity chromatography (Fig. 2A). To reduce nonspecific interactions of the proteins with the resin, 10 mM imidazole was included in the equilibration buffer. To remove imidazole and for further purification of proteins an additional gel filtration step on Sephadex G-50 Superfine (for purification of Rpb8), Sephacryl S-200 Superfine (for Rpb5) or TSK G-2000 columns (for Rpc10 [Rpb12] and Rpb10) usually was necessary (Fig. 2B).

In case of the subunit Rpb10, which was always found predominantly in insoluble fraction, we found it very helpful to use immobilization of recombinant protein during renaturation instead of dialysis procedure (see also [33]). The presence of Zn-ions in the refolding buffer is also important in this protocol, because of stabilizing role of zinc in the maintaining of Rpb10 native structure.

After assessing the purity of recombinant subunits in various fractions by SDS-PAGE, the fractions containing electrophoretically pure protein were concentrated with a Centricon-10 microconcentrator (Amicon, USA) to a final protein concentration of 0.5–1 mg/ml and stored at 4 °C or at −20 °C with addition of glycerol to final 50% concentration (for a long storage). Using these procedures, 5–20 mg of recombinant proteins per liter of culture could be purified to more than 98% homogeneity as judged from SDS-PAGE Coomassie blue-stained gel analysis (Fig. 1) and/or by TOF-MALDI mass spectrometry data (Fig. 2C).

In conclusion, we have performed the bacterial overexpression and purification of four hexahistidine-taged subunits shared by all nuclear RNA polymerases of *Sch. pombe*. Obtaining of these purified proteins in large quantities constitutes a prerequisite step toward characterization of their physico-chemical and functional properties. They will be also useful for monoclonal antibody production

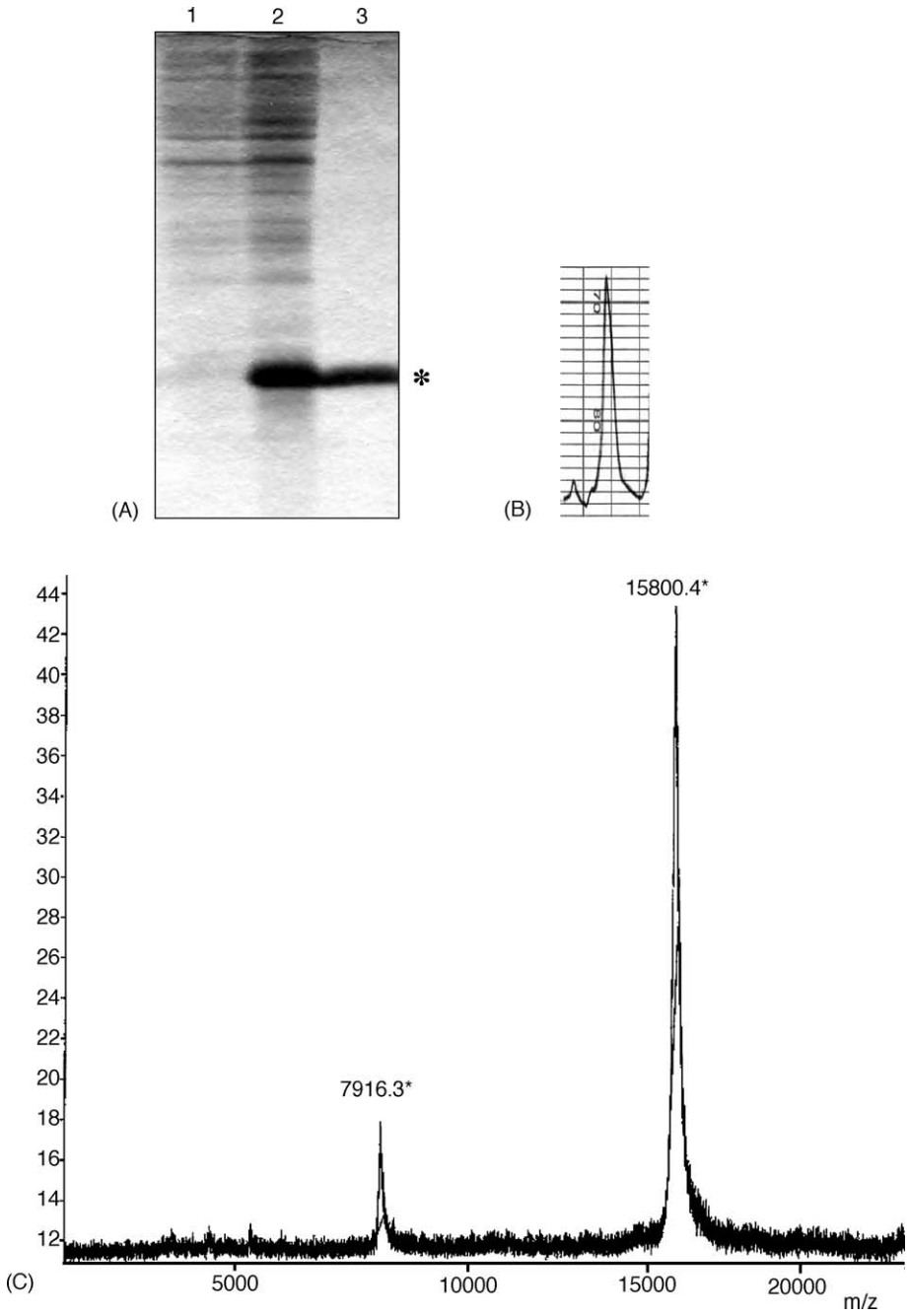


Fig. 2. Large scale purification and identification of recombinant Rpb8 subunit of the fission yeast *Sch. pombe*: (A) 17% SDS-PAGE analysis of the following fractions is shown: 1—uninduced lysate of *E. coli* strains M15 [pREP4] bacterial cells; 2—lysate of bacterial cells after induction with IPTG; 3—fraction of Rpb8 protein eluted after metal affinity and size-exclusion chromatographies. (B) representative elution profile obtained by gel filtration on Sephadex G-50 Superfine column of a Rpb8 fraction followed metal-ion-affinity chromatography. (C) TOF-MALDI mass spectrum of the main peak of recombinant protein obtained after gel filtration. The smaller peak corresponds to recombinant Rpb8 molecules bearing two charges.

and study interactions with other macromolecules, such as nucleic acids (DNA and RNA) in a first place. Presence of hexahistidine tags on recombinant proteins allows their direct immobilization and simplifies interaction studies. The procedure used in this work could be applied for purification of other subunits of eukaryotic RNA polymerases including the specific ones, such as Rpb11 (our preliminary data, not shown).

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