2.16 Purification of RNAP α subunits

2.16.1 Day 1

Competent BL21(λDE3) cells were transformed with pHTT7f1NHα and plated on lactose MacConkey agar plates containing ampicillin.

2.16.2 Day 2

5 ml of LB, containing ampicillin, was inoculated with a single transformant and left shaking at 37 °C overnight.

2.16.3 Day 3

200 ml of fresh LB, containing 200 μg/ml ampicillin, was inoculated with 0.2 ml of overnight culture. Cultures were grown to an OD_{600} of approximately 0.6 before the addition of IPTG (200 μM final concentration). Following IPTG addition, cells were grown for a further three hours before being harvested in a 500 ml pot at 7,000 rpm for 15 minutes at 4 °C.

2.16.4 Day 4

The cell pellet was resuspended in 10 ml of Buffer A and cells were lysed by sonication. Lysis was checked by measuring the OD_{650} before and after sonication, at a 1/500 dilution 'before' and 1/100 dilution 'after'. Complete lysis of the culture resulted in a 10-fold drop in OD_{650}. After lysis, the lysate was transferred to an Oakridge tube and centrifuged at 17,000 rpm for 30 minutes at 4 °C. The pellet was discarded and the supernatant was filtered through a 2 μm syringe filter before being loaded onto a Ni^{2+} agarose affinity column (Amersham Pharmacia).

Prior to use, the column was washed with five column volumes of Buffer A at a flow rate of 1 ml/min. The entire supernatant was then loaded onto the column using a flow rate of 1 ml/min. When proteins that did not bind to the Ni^{2+} agarose matrix had passed through the column, the matrix was washed with 7 % Buffer B, removing proteins loosely associated with the Ni^{2+} agarose. Finally, a gradient from 7 % Buffer B to 100 % Buffer B, over a period of 35 minutes and a flow rate of 1 ml/min, was used to elute RNAP α subunits from the column. Fractions containing α subunits were analysed by SDS PAGE and the appropriate fractions were pooled

2.1.5 Purification of MelR 173

Lysis buffer: 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM EDTA

Inclusion body wash buffer 1: 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM EDTA, 4 M Urea

Inclusion body wash buffer 2: 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM EDTA, 4 M Guanidine-HCl

Inclusion body wash buffer 3: 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM EDTA, 6 M Guanidine-HCl

Refolding buffer: 50 mM Sodium phosphate (pH 8.0), 1 M NaCl, 10 % (v/v) Glycerol, 2 M Guanidine-HCl

2.1.6 Purification of MelR 303

Lysis buffer: 20 mM Sodium phosphate (pH 7.5), 20 % (v/v) Glycerol, 300 mM NaCl

Inclusion body wash buffer 1: 20 mM Sodium phosphate (pH 7.5), 20 % (v/v) Glycerol, 1 M NaCl

Inclusion body wash buffer 2: 20 mM Sodium phosphate (pH 7.5), 20 % (v/v) Glycerol, 1 M NaCl, 4 M Urea

Inclusion body wash buffer 3: 20 mM Sodium phosphate (pH 7.5), 1 M NaCl, 6 M Guanidine-HCl

Refolding buffer: 20 mM Sodium phosphate (pH 7.5), 20 % (v/v) Glycerol, 1 M NaCl

2.1.7 Purification of RNAP α subunits

Buffer A: 20 mM Tris (pH 8), 500 mM NaCl, 10 % (v/v) Glycerol

Buffer B: 20 mM Tris (pH 8), 500 mM NaCl, 10 % (v/v) Glycerol, 200 mM Imidazole

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

Acrylamide monomer (resolving gel): 30 % (w/v) acrylamide, 0.8 % (w/v) N, N'-methylene bis-acrylamide. Filtered through Whatman 47 mm diameter glass fibre filter

Acrylamide monomer (stacking gel): 30 % (w/v) acrylamide, 0.45 % (w/v) N, N'-methylene bis-acrylamide. Filtered through Whatman 47 mm diameter glass fibre filter