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Affinity chromatography and capillary electrophoresis for analysis of the yeast ribosomal proteins

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We present a top down separation platform for yeast ribosomal proteins using affinity chromatography and capillary electrophoresis which is designed to allow deposition of proteins onto a substrate. FLAG tagged ribosomes were affinity purified, and rRNA acid precipitation was performed on the ribosomes followed by capillary electrophoresis to separate the ribosomal proteins. Over 26 peaks were detected with excellent reproducibility (<0.5% RSD migration time). This is the first reported separation of eukaryotic ribosomal proteins using capillary electrophoresis. The two stages in this workflow, affinity chromatography and capillary electrophoresis, share the advantages that they are fast, flexible and have small sample requirements in comparison to more commonly used techniques. This method is a remarkably quick route from cell to separation that has the potential to be coupled to high throughput readout platforms for studies of the ribosomal proteome. [BMB reports 2012; 45(4): 233-238]

INTRODUCTION

Two dimenstional gel electrophoresis in combination with MALDI-TOF has proven to be a powerful proteomic platform (1). We are developing a gel-free miniaturised analogue of this known as the Proteome-On-A-Chip where we seek to deposit a high density of protein spots with sizes of 100 μm or less on a substrate. Such a format allows the proteins to be analysed in a variety of ways including using electron-vibration-vibration two dimensional infrared spectroscopy (EVV 2DIR) and antibody blotting. EVV 2DIR is a top-down, label free technique which has been shown to identify and quantify proteins (2, 3). Capillary electrophoresis (CE) is an ideal final separation stage for the Proteome-On-A-Chip as it can retain protein functionality whilst allowing the deposition of small spots of protein with sufficiently high throughput to be useful. An early objective of this platform is to analyse eukaryotic ribosomes. In this paper, we demonstrate the affinity purification of yeast ribosomes and separation of the ribosomal proteins with CE. This paves the way for subsequent deposition and analysis. The high degree of reproducibility achieved demonstrates the potential utility of the approach.

The ribosome is a large ribonucleoprotein complex that, together with many interacting factors, is the site of protein synthesis in the cell. In eukaryotes the 4 strands of ribosomal RNA give the complex its main structure. They form a ribozyme centre at the interface between the subunits, where nascent peptide bond formation occurs (4-6). Eukaryotic ribosomes contain approximately 80 proteins, with many serving to maintain ribosome structural stability, whilst others regulate ribosomal activity or cotranslationally modify the emerging nascent peptide (7).

Traditional ribosome knowledge classesd the function of ribosomal proteins as a mainly structural one. Today it is known that they play a more complex role, with questions surrounding extraribosomal functions and substoichiometries of ribosomal proteins. In 1996, Wool first described the extraribosomal functions of a list of just over 30 ribosomal proteins across species (8). These extraribosomal functions include roles in transcription, translation, DNA repair, mRNA processing, apoptosis and developmental regulation. In the last decade, the list has grown and, not surprisingly, much research is ongoing to confirm these as exhibiting bonafide extraribosomal activity and to find new candidates (9).

Substoichiometries arise from several scenarios including ribosomal proteins exhibiting extraribosomal functions which result in them being absent in the ribosome for periods of time (10), ribosomal proteins present at more than one copy, and the presence of ribosome interacting proteins (11). Some ribosomal proteins play roles within complex signalling networks. An example is the Receptor for Activated C-Kinase, RACK1 (known as Asc1p in *Saccharomyces cerevisiae*), which has been confirmed as a core eukaryotic 40S ribosomal protein

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but is also a scaffold protein, recruiting a range of kinases and membrane-bound receptors to the ribosome (12).

To briefly summarize, the ribosome, though in itself a large and complex protein-RNA machine, to be understood properly must be considered in its wider cellular context. This means studying the extraribosomal functions of ribosomal proteins and ribosomal functions of extraribosomal proteins. The demand for high throughput quantitative proteomic approaches is therefore pressing.

Many of the proteomic strategies used to study the ribosome have included various forms of gel electrophoresis with MS (13-15). Although some great successes have been had using these methods, for many ribosomal studies the demand is for automated methods with a higher degree of reproducibility. Accordingly, liquid chromatography methods with MS have become increasingly popular. However, only a limited number of separations on intact ribosomal proteins have been reported (16, 17), most likely because MS instrumentation for top-down proteomic analyses are not yet common-place and are expensive to purchase and maintain. CE has been infrequently used to study ribosomal proteins. Moini separated out intact proteins from a bacterial ribosome with an ESI-MS interface enabling 55 out of the 56 ribosomal proteins to be resolved in two runs (18). Moini also analysed a tryptic digest of ribosomal proteins using a novel technique to improve sequence coverage, $(CE\text{-}MS/MS)^n$, whereby multiple injections and anlyses were performed in one run (19).

Perhaps the paucity of studies of ribosomal proteins using CE is because of predicted difficulties in both separation and sample preparation. The separation of ribosomal proteins using CE is challenging because of the relatively large number of proteins that exist within a small range of pI values, and the fact that separation in free solution is based on the size to charge ratio. However, CE is an excellent separation technique for resolving complex protein mixtures (18, 20). The fact that capillary zone electrophoresis can be performed without surfactants or organic solvents make it an ideal technique for the study of intact proteins. With careful optimisation of sample preparation and separation conditions, the advantages of small sample consumption, short separation times and exceptional efficiency can be exploited for the yeast ribosome.

Most ribosome studies commence with a sucrose density gradient to isolate the ribosomes. Unfortunately, this process is labour intensive, requires large quantities of starting material, can be difficult to reproduce and yields final fractions that contain other co-purifying proteins and sucrose, which can cause difficulties in further analyses. Ribosome affinity chromatography techniques have been developed most widely in *Escherichia coli* (21-23) and *Saccharomyces cerevisaie* (24, 25). These are based on the use of either a tagged ribosomal protein or rRNA strand, which is used to capture the attached subunit or even the whole ribosome (often with associating proteins and mRNAs) onto a matrix. These can provide one-step methods achieving a highly purified sample with vastly improved yields and can be easily scaled down but despite this, their use is not common place.

Accordingly, in this paper, we describe a usefully high throughput separation technique that utilises affinity purification and CE to achieve a highly reproducible separation of ribosomal proteins. Using affinity purification as the first dimension of separation, as opposed to a sucrose gradient, offers increased flexibility, facilitating prospects such as the analysis of co-purifying proteins. The potential to scale down purification will be essential in future studies where, for example, growth conditions may be changed in several samples and the results compared.

RESULTS

The overall analysis procedure consists of two stages: ribosome affinity purification and CE. A critical feature of the approach is that both techniques are quick, efficient and have small sample requirements in comparison to more traditional alternatives.

Ribosome affinity purification (RAP)

RAP was performed using the Inada method (25). The mutant strain, YIT613, expressed a C-terminally FlagHis6 epitope tagged ribosomal protein Rpl25. Purification was achieved using anti-FLAG affinity resin and competitive elution with a FLAG

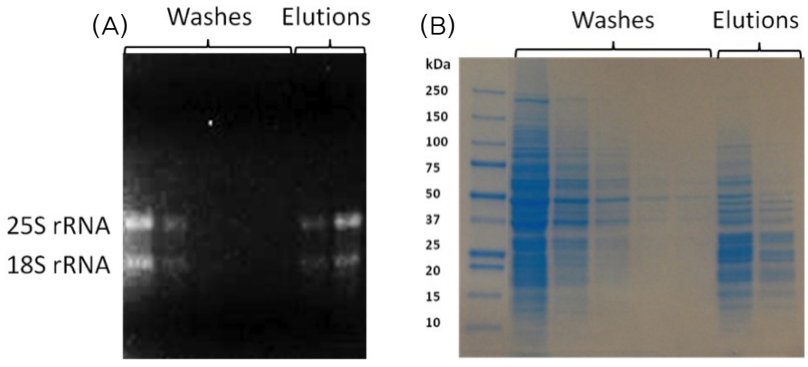

Fig. 1. (A) RNA gel of affinity purification. 25S and 18S rRNA bands can be seen to be disappearing through successive washes (lanes 1-5) and reappearing in elutions (lanes 6-7). (B) SDS-PAGE of successive washes (lanes 2-6) and elutions (lanes 7-8).

peptide. Although the tagged protein is on the 60S subunit, the purification method resulted in purification of 80S ribosomes, as is demonstrated by the RNA gel (Fig. 1A), which shows both the 25S and 18S rRNA strands from the 60S and 40S subunits respectively. This is in agreement with the work of Inada *et al*. (25).

Ribosomal protein capillary electrophoresis

To the best of our knowledge, this is the first time that proteins from the eukaryotic ribosome have been separated using CE. The separation is challenging because of the number of proteins, the relatively small range in size, pI and other similarities which will arise from the proteins all originating in similar cellular molecular environment (almost all are nucleic acid binding proteins, for example).

Using the CE set-up described and with optimisation of buffer conditions, ribosome purification and sample preparation conditions, over 26 peaks were resolved (Fig. 2). Based on a series of 7 consecutive runs, high reproducibility of the separation was observed, with RSD migration times $<0.5\%$. With this degree of reproducibility it is evident that the method is not only high throughput, but also gives quantifiable data that can be compared across experiments. We have found the profile to be reproducible throughout our affinity purified ribosomes, providing a 'fingerprint' for ribosomal proteins prepared under these conditions. This paves the way for future studies with modified cell growth conditions (such as dietary restriction, which has been observed to correspond with a reduction in 60S subunits (26)) and probing of the attached ribosome on the affinity matrix before elution to see alterations in the CE profile.

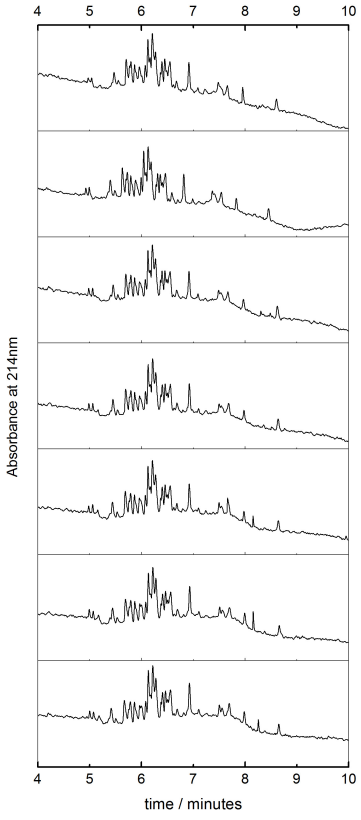

Fig. 2. Separation of ribosomal proteins from affinity purified ribosomes using CE, 7 consecutive repeats. Sample was separated in a 50 μm i.d. capillary with an effective length of 33 cm. A 1.1 cm sample plug was introduced hydrodynamically and separated under an applied voltage of 20 kV.

It should be noted that some single peaks in the electropherograms shown in Fig. 2, may contain more than one protein. The use of laser induced fluorescence detection, which can achieve unrivalled detection limits, would enable injection of smaller sample plugs and is likely to result in the resolution of a greater number of peaks. MS identification with reconstruction of the electropherogram would also enable a greater number of peaks to be resolved (27, 28). To demonstrate the potential for CE-ESI-MS of eukaryotic ribosomal proteins, the separation has also been performed with a volatile BGE, 50 mM ammonium acetate, pH 4.0 (see supplementary information†). Due to differences in the separation conditions, the electropherogram is not identical to Fig. 2.

A crucial stage in this protocol is the preparation of ribosome sample prior to CE to ensure that ribosomes are reproducibly dissociated into ribosomal proteins. The intention of the first stage of the ribosome sample preparation method is to use RNase to cleave the rRNA which maintains the structure of the ribosome. The acetic acid RNA precipitation step is essential to then remove rRNA from the sample. Experiments without this step gave irreproducible profiles. Dialysis into a 10 times dilution of the BGE was found to be crucial in achieving high efficiencies.

MS performed on the ribosomal protein sample prior to CE confirms that ribosomal proteins were injected into the capillary (see supplementary information†). Simply, following CE runs the sample as used for CE experiments was analysed by LC-MS/MS. Out of 54 hits above 20%, 39 were ribosomal proteins. With a stringent cutoff of 100%, 19 out of 22 were ribosomal proteins. Hence it is reasonable to assume that the separation is indeed a separation of almost entirely ribosomal proteins. A small number of non-ribosomal proteins were identified in the MS data and could account for peaks seen, many of which were highly abundant glycolytic enzymes. However, the molecular weights and pI values (as predicted with ExPasy) of these proteins are such that, with the exception of phosphoglycerate mutase 1 (MW 27.5 kDa, pI 8.86), it is likely that these proteins would migrate much later than the bulk of the ribosomal proteins. Interestingly, regulatory components of the ribosome are also present, notably guanine nucleotide-binding protein subunit beta-like protein, also known as Asc1p, with a protein identification probability of 100%.

It is important to note that MS did not identify the presence of all of the yeast ribosomal proteins. The missing may be due

to co-precipitation with RNA, although two acidic proteins were identified; it is more likely that they were simply not identified due to the relatively low concentration of the final sample used for MS which was 1-5 μg/ml per protein. However, the purpose of incorporating MS analysis at this stage in the methodology was not to assess the affinity purification method, but rather to verify that ribosomal proteins were being injected into the capillary. The same MALDI-MS/MS analysis performed on eluate after affinity purification (but prior to preparation for CE) identifies 61 ribosomal proteins out of 74 hits above 20% probability, and, 41 ribosomal proteins out of 47 hits above a cutoff of 100%, including Asc1p. This may be due to the higher concentration of the sample.

DISCUSSION

CE has been successfully used for top-down separation of yeast ribosomal proteins, resolving over 26 peaks with outstanding reproducibility within a run time of 10 minutes. Ribosomes used were purified using affinity chromatography, which is both simple and efficient. We believe the readout provides a ribosomal protein fingerprint, from which a wide variety of applications are possible. The high level of reproducibility inherent in this method in terms of migration time and peak height suggests application in studies of proteins exhibiting extraribosomal functions and thus absent from the ribosome, substoichiometries, posttranslational modifications resulting in shifting migration times and fluctuations resulting from changes in growth conditions. To study these, the use of laser induced fluorescence detection would be required to enable the injection and detection of reduced sample quantities. The affinity purification method results in the co purification of proteins interacting with the ribosome. As the entire method is of high throughput and sample requirements are low, this would provide an ideal platform to scan for ribosome interacting proteins. Output to an identification technique will then create a novel platform for fast analyses of the ribosomal proteome. We intend to apply this ribosomal separation strategy to CE-2DIR. For studies into ribosomal substoichiometries and extraribosomal activities, the quantitative nature of this technique will be a distinct advantage. There is no reason why the method described cannot be applied also to miniaturised CE systems.

MATERIALS AND METHODS

Affinity purification of Ribosomes

Cell Growth and Lysis: Ribosome samples were prepared using a FLAG affinity purification method. Strain YIT613 was a kind gift of Toshi Inada at Nagoya University, Japan. YIT613 (CB012 *rpl25::LEU2*) contains the plasmid BIT 700 [pRPL25-FH-URA3CEN] and so expresses a c-terminally FLAG tagged version of Rpl25. Cells were grown to an optical density at 600nm of 0.8. Cells were pelletted and resuspended in 400μl of lysis buffer (20 mM HEPES pH 7.4, 2 mM $Mg(OAc)_2$, 100 mM KOAc, 100 μg/ml cycloheximide, 0.5 mM dithiothreitol). Cell culture was then transferred to 2 μl tubes and the cells pelletted and resuspended in an equal volume of lysis buffer + protease inhibitor (1 × Roche EDTA-free complete protease inhibitor cocktail). Cell lysis was performed using the FastPrep system (Qbiogene, Inc., Carlsbad, CA). The cells were transferred to 2 ml FastProtein Red Matrix™ tubes and lysed by 6 repetitions of 20 second runs followed by 1 minute on ice. The tubes were then spun at 10,000 rpm for 1 minute to pellet the beads, and the lysate transferred to 2 ml eppendorfs and cleared by spinning at 10,000 rpm for 1 minute. The cleared lysate was transferred to 2 ml Eppendorfs, frozen in liquid nitrogen and stored at −80°C.

Ribosome affinity purification (RAP): Anti-FLAG M2-agarose affinity resin (Sigma-Aldrich, Dorset, UK) was used for capture of FLAG-tagged ribosomes from YIT613 lysate. 200 μl of resin (100 μl bead volume) was washed twice in 4 ml TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and then washed three times in 4 ml 2×binding buffer (100 mM Tris-HCl pH 7.5, 24 mM $Mg(OAc)_2$, 50 U/ml RNAsin (Promega, Southampton, UK), 1 mM PMSF).

Following this, 125 μl of lysate (approximately 4 A260 units) was added to an equal volume of ice cold 2×binding buffer and 100 μl of resin (bead volume) in a 2ml Eppendorf tube. Binding proceeded for 2½ hours at 4°C with gentle rocking.

The resin was washed five times with 0.2 ml of ice-cold IXA-100 buffer (50 mM Tris-HCl pH 7.5, 12 mM $Mg(OAc)_2$, 100 mM KCl, 1 mM PMSF). Two consecutive elutions were performed, each time, by incubating the resin for 20 minutes at 4°C with gentle rocking in IXA-100 buffer containing FLAG peptide (Sigma-Aldrich) at a concentration of 100 μg/ml. Washes and elutions were carried out by adding buffer to the resin, centrifuging at 3,000 rpm in a Centra CL3R Thermo centrifuge at 4°C for 2 minutes and removing and retaining the supernatant. The absorbance at 260 nm of flow-through, washes and elutions was measured. Extracts were then frozen in liquid nitrogen and stored at −80°C until required for gels or for CE analysis.

Capillary electrophoresis of ribosomal proteins

Electrophoretic preparation of ribosomal protein samples: 25 μl of affinity purification eluate was incubated for 15 minutes at 37°C with 2.4 μg/ml RNase A (Sigma-Aldrich, molecular biology grade). Following this, RNA precipitation was performed as a modification of work by Hardy *et al.* (29), by adding 40 μl of ice cold glacial acetic acid and centrifuging at 12,000×g for 6 minutes at 4°C. The liquid phase was then transferred to a 500 μl tube and diluted with 60 μl ice cold MilliQ water. This was then dialysed with 2 mM sodium citrate (10 times dilution of the background electrolyte, see below) for 3 ½ hours with 4 changes of buffer at 4°C in Pierce Mini Dialysis Units with Microtubes to give the final sample.

Separations: Experiments were performed using a homebuilt CE system fitted with a UV/Vis absorption detector (Scientific Systems Inc model 500 variable UV/Vis absorption). Capilla-

ries were 50 μm i.d. × 375 μm o.d. fused silica with a neutral polyvinyl alcohol (PVA) inner coating (Agilent Technologies, Cheshire, UK) to eliminate electroosmotic flow (EOF) and prevent protein adsorption. Capillaries were trimmed to 44 cm total length and 33 cm effective length. A 20 mM sodium citrate, pH 4.0 background electrolyte (BGE) was used. 20 kV was applied in the positive polarity mode. Sample was injected hydrodynamically by applying a pressure at the inlet and introducing a plug 1.1 cm in length. 20 mM sodium citrate, pH 4.0 was prepared from monobasic sodium citrate anhydrous ($C_6H_7NaO_7$) and dibasic sodium citrate sesquihydrate ($C_6H_6Na_2O_7.1.5H_2O$).

MALDI-MS/MS of ribosomal protein sample

Following CE, the sample was analysed by liquid chromatography MALDI-TOF/TOF-MS in the Mass Spectrometry Centre for Integrative Systems Biology at Imperial College London. Briefly, the fully prepared sample was desalted and trypsin digested. Fragments were then separated by reversed phase-HPLC and robotically spotted onto a metal MALDI target using an Ultimate 3000 nanoLC system (Dionex, Sunnyvale CA) coupled to a Probot automated spotting device (LC Packings, Sunnyvale CA). MALDI-MS/MS was performed using an Applied Biosystems 4,800 TOF/TOF. All MS/MS data were analysed using the Mascot search engine (Matrix Science, London, UK). Mascot was set up to search the SwissProt S*accharomyces Cerevisiae* database (UnitProt release 2010_09) for the digestion enzyme Trypsin. The database was searched with a fragment ion mass tolerance of 0.30 Da and a parent ion tolerance of 100 ppm. Oxidation of methionine residues and carboxymethylation of cysteine residues were specified as variable modifications. Scaffold (version 3.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS based protein identifications. Protein identifications stated in the supplementary information have greater than 20.0% probability and contain at least one identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (30).

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Appendix A Supplementary Data

†Electronic Supplementary Information (ESI) contains MS information of the ribosome sample prior to injection into the capillary and electropherograms from CE performed on the sample using an ammonium acetate BGE.

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