Video Article

Chromatographic Purification of Highly Active Yeast Ribosomes

Arturas Meskauskas[1,2], Jonathan A. Leshin[1], Jonathan D. Dinman[1]

[1]Department of Cell Biology and Molecular Genetics, University of Maryland
[2]Department of Biotechnology and Microbiology, Vilnius University

Correspondence to: Jonathan D. Dinman at dinman@umd.edu

URL: http://www.jove.com/details.php?id=3214
DOI: 10.3791/3214

Keywords: Cell Biology, Issue 56, Ribosome, purification, DNA, yeast, chromatography, Saccharomyces cerevisiae,

Date Published: 10/24/2011

Citation: Meskauskas, A., Leshin, J.A., Dinman, J.D. Chromatographic Purification of Highly Active Yeast Ribosomes. J. Vis. Exp. (56), e3214, DOI : 10.3791/3214 (2011).

Abstract

Eukaryotic ribosomes are much more labile as compared to their eubacterial and archael counterparts, thus posing a significant challenge to researchers. Particularly troublesome is the fact that lysis of cells releases a large number of proteases and nucleases which can degrade ribosomes. Thus, it is important to separate ribosomes from these enzymes as quickly as possible. Unfortunately, conventional differential ultracentrifugation methods leaves ribosomes exposed to these enzymes for unacceptably long periods of time, impacting their structural integrity and functionality. To address this problem, we utilize a chromatographic method using a cysteine charged Sulfolink resin. This simple and rapid application significantly reduces co-purifying proteolytic and nucleolytic activities, producing high yields of intact, highly biochemically active yeast ribosomes. We suggest that this method should also be applicable to mammalian ribosomes. The simplicity of the method, and the enhanced purity and activity of chromatographically purified ribosome represents a significant technical advancement for the study of eukaryotic ribosomes.

Video Link

The video component of this article can be found at http://www.jove.com/details.php?id=3214

Protocol

1. Preparation of a cysteine charged Sulfolink resin

1. Prepare Sulfolink resin (Pierce) at room temperature.
2. Place a total of 10 ml of a 50% slurry of Sulfolink coupling gel as supplied by the manufacturer in two 10 ml plastic vials, with 5 ml distributed into each vial.
3. Briefly centrifuge vials at 850 x g and carefully remove supernatants.
4. Wash the gel three times in coupling buffer (50 mM Tris, pH 8.5, 5 mM EDTA) by resuspending the beads in 5 ml, centrifuging briefly at 850 x g and removing the supernatant by pipette.
5. Add five ml of a 50 mM solution of L-cysteine in coupling buffer to each tube and mix the slurry for 1 hour at 25°C.
6. Remove the residual L-cysteine by washing as described in step 1.4 above.
7. Resuspend the gel in 10 ml of binding buffer [20 mM HEPES, pH 7.6, 5 mM $Mg(OAc)_2$, 60 mM NH_4Cl, 2 mM DTT], and equilibrate the resin by washing in 10 ml of binding buffer 3 times as described in step 1.4. Decant resin into a 10 ml centrifuge column, cap and stored at 4°C. The resin capacity for RNA binding is ~20 A_{260} units per ml.

2. Chromatographic purification of ribosomes using a cysteine charged Sulfolink resin

Note: If working with a strain where protease activity proves to be an issue, protease inhibition cocktails can be used in all subsequent buffers.

1. Maintain all components on ice and prepare all solutions using RNase-free water and sterile filtration.
2. Grow yeast cells overnight to mid-log phase ($O.D._{595}$ = 0.6 - 1.2) in appropriate media. Pellet cells by centrifugation, and wash 1 gram cells in binding buffer and centrifuge at 3700 x g for 5 minutes at 4°C.
3. Resuspend cells in 1 ml of binding buffer to an approximate total volume of 2 ml, and disrupt using an equal volume of 0.5 mm glass beads chilled to 4°C using a Biospec Mini-bead beater (Bartlesville, OK). Samples can be split into multiple tubes as needed.
4. Remove unbroken cells, organelles, and cellular debris by centrifugation at 30,000 x g for 30 minutes in a Beckman-Coulter Optima Max E ultracentrifuge (Fullerton, CA).
5. Immediately before use, pellet the resin for one minute at 1,000 x g to remove the storage solution. Two ml of resin is used for every one gram of cells.
6. Remove cell lysate supernatants from the 30,000 x g spin (S30), taking care to minimize contamination from either the lipid fraction at the very top or the cell debris at the bottom of the tubes, and place directly into the charged Sulfolink slurry. The lipid layer can be avoided either by removal or by pushing out from the pipet after the tip has crossed into the supernatant.
7. Incubate the S30/Sulfolink mixture on ice without mixing for 15 minutes.
8. Place the columns into 15 ml conical tubes and centrifuge at 1,000 x g for one minute.
9. Place the flowthrough fractions back into the columns, mix by hand, and incubate for a further 15 minutes on ice.
10. Place the columns into 15 ml conical tubes and centrifuge at 1,000 x g for one minute. Discard the flowthrough.

11. Cap columns and add 5 ml of binding buffer. Mix columns by hand until resuspended, remove the caps, and centrifuge the columns centrifuge at 1,000 x g for one minute. Repeat this washing protocol two more times.
12. Add 1.5 ml of elution buffer (20 mM HEPES-KOH, pH 7.6, 10 mM $Mg(OAc)_2$, 500 mM KCl, 2mM DTT, 0.5 mg/ml heparin) to each column. Mix slurries by hand, and incubate on ice for 2 minutes
13. Place columns into new 15 ml conical tubes and centrifuge at 1,000 x g for 1 minute. Collect eluate. Repeat the elution step once more so that the final combined sample volumes are ˜ 3 ml. Used resin can be washed with elution buffer without heparin and followed by equilibration with binding buffer, and stored in 10 ml volumes of binding buffer at 4°C for re-use later.

3. Puromycin treatment

Note: An alternative method to remove contaminating tRNA species is to switch from a glucose rich to glucose depleted media to promote ribosome runoff. However, this does change the metabolic status of the yeast cells, which could affect ribosome function and concentration.

In order to strip the ribosomes from endogenous peptidyl-tRNA, a treatment with puromycin is performed.

1. Neutralize to pH 7.5 100 mM puromycin solution by adding 1M KOH dropwise at room temperature.
2. Add neutralized puromycin solution to ˜ 1mM final concentration in eluate.
3. Add 100 mM GTP to a final concentration of 1 mM.
4. Incubate for 30 minutes at 30°C.

4. Purification of ribosomes by sedimentation through glycerol cushions

1. Place one ml of cushion buffer (20 mM HEPES, pH 7.6, 10 mM $Mg(OAc)_2$, 500 mM KCl, 2 mM DTT, 25% glycerol) into a 4 ml volume polycarbonate ultracentrifuge tube.
2. Gently layer puromycin treated elution fractions on top of the cushion, and centrifuge samples at 100,000 x g overnight at 4°C.
3. Remove tubes from the centrifuge rotor, and aspirate supernatants.
4. Wash pellets containing purified ribosomes twice with 1 ml of cushion buffer.
5. Resuspend ribosomes in 100 µl of cushion buffer by gentle disruption using a glass rod.
6. After disruption, cover tubes with parafilm and shake at a moderate speed in a cold room vortex for one hour.
7. Transfer the contents to a microcentrifuge tube, centrifuge for 5 minutes at maximum speed at 4°C, and remove supernatants to fresh tubes.
8. Repeat steps 4.1 to 4.7 using 2 ml of cushion buffer and, except that 100 µl of storage buffer (50 mM HEPES -KOH pH 7.6, 50 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 1 mM DTT, 25% glycerol) is used in steps 4.4 and 4.5 instead of cushion buffer.
9. Quantify the purified ribosomes spectrophotometrically (1 A_{260} = 20 pmoles of yeast ribosomes), and store at -80°C. Typical results from 1 gram of yeast are 300-400 pmoles of ribosomes.

5. Representative results:

An example of RNA species extracted from each of the three major steps of the protocol is shown in Figure 2. While rRNAs are the major species present in total cell lysates (T) these also contain a large number of other RNA species. Ribosomes purified from the Sulfolink column (SL) also contain a large amount of tRNAs due to the high affinity of the column bed for these species as well. Treatment of this fraction with puromycin results in hydrolysis of peptides from peptidyl-tRNAs, and promotes dissociation of these species from ribosomes. The puromycin treated samples (Pm) lack co-purifying tRNA species, and thus represent completely pure ribosomes. As previously reported[8], these ribosomes are highly intact and biochemically active, making them ideal substrates for detailed functional and structural analyses.

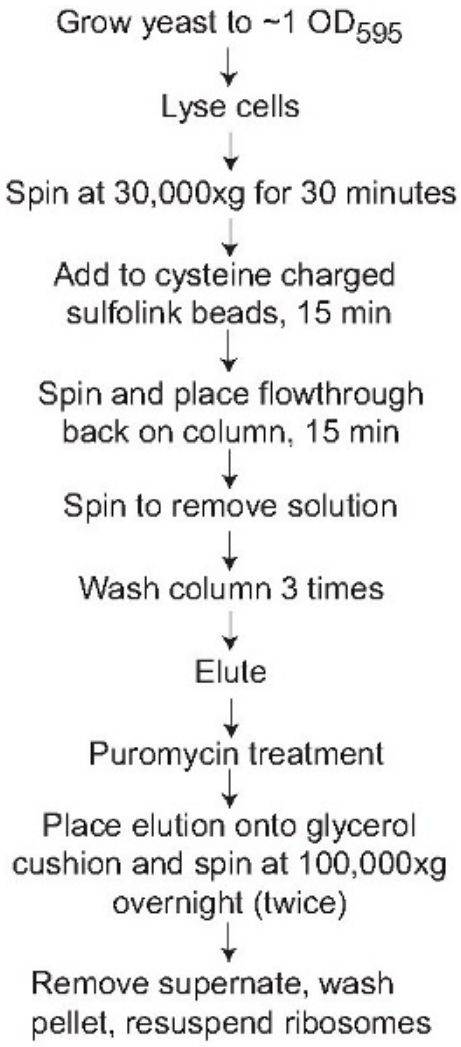

Figure 1. Method Flowchart. Chromatographic purification of ribosomes using the cysteine linked sulfolink resin is depicted.

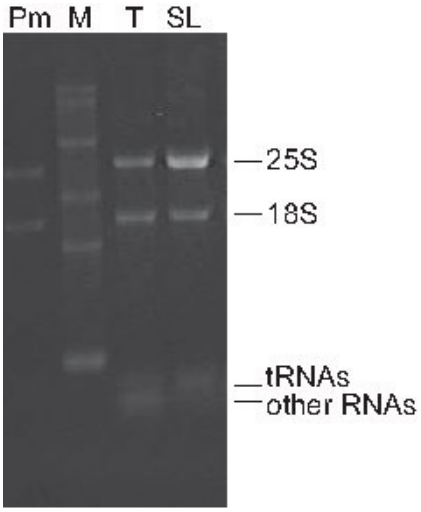

Figure 2. Representative analysis of ribosome preparations. Total RNA species were extracted from total cell lysates (T), Sulfolink purified ribosomes (SL) and Sulfolink purified ribosomes subsequently treated with puromycin and sedimented through a glycerol cushion (Pm). Lane M represents RNA size markers. Bands representing 25S and 18S rRNAs, as well as tRNAs and other RNA species are indicated. All lanes were loaded with 2 µg of RNA.

Discussion

Ribosome purification protocols basically involve lysing cells, harvesting a cytosolic fraction from a low speed spin, and then pelleting ribosomes by high speed centrifugation [1]. While a few novel methods have been used for purifying bacterial ribosomes, the same had not been true for eukaryotes [2-4]. Although additional steps have been added along the years, e.g. salt washes, and glycerol cushions (e.g. see [5]), biochemical and

structural studies of yeast ribosomes have been hampered by their tendency to become degraded by endogenous degrading enzymes during the purification process, most likely due to the long periods of time during the ultracentrifugation steps during which ribosomes are exposed to these classes of enzymes [6].

The major problem associated with traditional protocols is the co-purification of proteases and nucleases with ribosomes. This results in their degradation during the purification process, resulting in lower yields of biochemically active ribosomes. The high levels of proteases and nucleases present in clinical isolates of pathogenic bacteria led to the development of a chromatographic method for ribosome purification using a cysteine charged Sulfolink resin [7]. The rRNAs and proteins derived from bacterial ribosomes isolated using this method showed much lower levels of degradation, and the ribosomes so purified were significantly more able to bind erythromycin and to synthesize proteins. The specific chemistry of ribosome binding to the Sulfolink resin is unknown, however it has been speculated that it involves hydrophobic interactions [7]. These observations suggested that the cysteine charged Sulfolink resin chromatography method may also be applicable to yeast ribosomes, and if so, that it could solve many of the problems described above. Thus we adapted this protocol for isolation of intact, highly active yeast ribosomes. The column chromatographic method rapidly and efficiently results in separation of a significant fraction of contaminating nucleases and proteases from ribosomes resulting in purer, more biochemically active ribosome preparations with enhanced biochemical and structural properties, which scales well to higher quantities of ribosomes [8]. No significant differences were seen on a denaturing protein gel between traditionally purified ribosomes and those purified via column chromatography, indicating that these ribosomes contain all the same ribosomal elements as traditionally purified ribosomes.

There are some steps in protocol that require special attention. With regard to disruption of yeast cells, a precise 1:1 ratio of cell suspension to glass beads (vol/vol) is critical. Typically, ˜80% of cells are disrupted in 2 min. Importantly, over-disruption should be avoided to prevent the release of degrading enzymes from cell organelles. Although ribosomes obtained directly from the Sulfolink column were shown to be nearly completely free of protease and nuclease contamination, the observation of high levels of tRNA in this fraction indicated that the resin binds tRNA as well [7,8]. We have found that the presence of tRNA species interferes with downstream biochemical assays e.g. ribosome/ligand binding, assays of peptidyltransferase activity and rRNA structural analyses. Puromycin treatment [9] of chromatographically isolated ribosomes results in production of peptidyl-puromycin, which is released from ribosomes, along with deacylated tRNAs [10-14]. The final overnight high speed centrifugation of samples through a glycerol cushion is critical for removing the remaining free tRNAs from the ribosome samples. If separate subunits are required, they may be obtained by sedimentation through a high salt sucrose gradient[15]

Disclosures

No conflicts of interest declared.

Acknowledgements

We wish to thank all of the members of the Dinman laboratory, including Ashton Belew, Karen Jack, Sharmishtha Musalga, Sergey Sulima, Shivani Reddy, and Michael Rhodin for their help and input on this project. This work was supported by grants to AM from the American Heart Association (AHA 0630163N) and to JDD from the National Institutes of Health (5R01 GM058859-12). JAL was supported by an American Reinvestment and Recovery Act of 2009 supplement to the parent grant (3R01GM058859-11S1).

References

1. Palade, G.E. & Siekevitz,P. Liver microsomes; an integrated morphological and biochemical study. *J. Biophys. Biochem. Cytol.* **2**, 171-200 (1956).
2. Fabry, M., Kalvoda, L., & Rychlik, I. Hydrophobic interactions of *Escherichia coli* ribosomes. *Biochim. Biophys. Acta* **652**, 139-150 (1981).
3. Jelenc, P.C. Rapid purification of highly active ribosomes from *Escherichia coli*. *Anal. Biochem.* **105**, 369-374 (1980).
4. Le goffic, F., Moreau, N., Chevelot, L., Langrene, S., & Siegrist, S. Purification of bacterial ribosomes using chloramphenicol and erythromycin columns. *Biochimie.* **62**, 69-77 (1980).
5. Meskauskas, A., Petrov, A.N., & Dinman, J.D. Identification of functionally important amino acids of ribosomal protein L3 by saturation mutagenesis. *Mol. Cell Biol.* **25**, 10863-10874 (2005).
6. Algire, M.A., *et al.* Development and characterization of a reconstituted yeast translation initiation system. *RNA.* **8**, 382-397 (2002).
7. Maguire, B.A., Wondrack, L.M., Contillo, L.G. & Xu, Z. A novel chromatography system to isolate active ribosomes from pathogenic bacteria. *RNA.* **14**, 188-195 (2008).
8. Leshin, J.A., Rakauskaite, R., Dinman, J.D., & Meskauskas, A. Enhanced purity, activity and structural integrity of yeast ribosomes purified using a general chromatographic method. *RNA. Biol.* **7**, 354-360 (2010).
9. Triana, F., Nierhaus, K.H., & Chakraburtty, K. Transfer RNA binding to 80S ribosomes from yeast: evidence for three sites. *Biochem. Mol. Biol. Int.* **33**, 909-915 (1994).
10. Azzam, M.E. & Algranati, I.D. Mechanism of puromycin action: fate of ribosomes after release of nascent protein chains from polysomes. *Proc. Natl. Acad. Sci. U. S. A.* **70**, 3866-3869 (1973).
11. Nathans, D. Puromuycin inhibition of protein synthesis: incorporation of puromycin into peptide chains. *Proc. Natl. Acad. Sci. U. S. A.* **51**, 585-592 (1964).
12. Rabinovitz, M. & Fisher, J.M. A dissociative effect of puromycin on the pathway of protein synthesis by Ehrlich ascites tumor cells. *J. Biol. Chem.* **237**, 477-481 (1962).
13. Allen, D.W. & Zamecnik, P.C. The effect of puromycin on rabbit reticulocyte ribosomes. *Biochim. Biophys. Acta.* **55**, 865-874 (1962).
14. Yarmolinsky, M.B. & Haba, G.L. Inhibition by puromycin of amino acid incorporation into protein. *Proc. Natl. Acad. Sci. U. S. A.* **45**, 1721-1729 (1959).
15. Becker-Ursic, D. & Davies, J. *In vivo* and *in vitro* phosphorylation of ribosomal proteins by protein kinases from *Saccharomyces cerevisiae*. *Biochemistry.* **15**, 2289-2296 (1976).

