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Investigation of methods suitable for the matrix-assisted laser desorption/ionization mass spectrometric analysis of proteins from ribonucleoprotein complexes

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A variety of protein isolation and purification techniques for ribonucleoprotein (RNP) complexes were investigated for their compatibility with downstream analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Ribosomal proteins from *Escherichia coli* 70S ribosomes were obtained using methods such as phenol extraction and precipitation by organic solvents or acids. Under optimal conditions, more than 90% of the expected ribosomal proteins were detected in a single MALDI-MS experiment. The most effective approach combined ribosome denaturation by buffer exchange with acid precipitation of the ribosomal ribonucleic acids. An improved acid precipitation approach, involving the sequential additions of acetic and trifluoroacetic acid, yielded more complete protein coverage while minimizing loss of ion signal from lower molecular weight proteins. With phenol extraction, substantial gains in ion abundance of higher molecular weight proteins are noted, although some of the lower molecular weight proteins were not efficiently extracted. These results illustrate several effective approaches for protein isolation from protein complexes such as RNPs that are MALDI-MS compatible, and these approaches should extend the use of MALDI-MS for proteomics-based analyses of other protein–nucleic acid complexes.

Keywords: MALDI-MS, ribosomes, ribosomal proteins, extraction, precipitation, RNP complex

Introduction

Mass spectrometry is now established as one of the key workhorse technologies for proteomics.^{1–3} Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been a popular choice for proteomic analyses. Typically, MALDI-MS is used for protein identification with prior sample separation by electrophoresis or chromatography.^{4–6} MALDI-MS has also been used to obtain health-specific protein fingerprints during screening of biological fluids or tissues.^{7–10}

In addition to the now ubiquitous use of MALDI-MS for protein identification and protein profiling, this technique remains a powerful approach for characterizing complex mixtures of proteins. Indeed, a recent focus has been to use proteomics-based approaches for the characterization of protein complexes^{11–14} including ribonucleoprotein (RNP) complexes.^{15,16} However, many of these previous applications utilized enzymatic digestion followed by MALDI peptide mass fingerprinting for protein identification.

An alternative use of MALDI-MS is for the direct screening of intact proteins present in isolated protein complexes. Arnold and Reilly demonstrated this use of MALDI-MS during the complex-wide analysis of proteins present in the ribosome.¹⁷ Ribosomes are RNP complexes composed of several (3–4) ribonucleic acids (RNAs) and more than 50 proteins. Bacterial ribosomes are ideal RNP complexes for method development because they are abundant, readily isolated and well-characterized by conventional biochemical approaches.¹⁸ In addition, high quality protein analysis would be useful in elucidating the mechanism of ribosome function, identifying and characterizing the role of post-translational modifications in protein structure/function studies¹⁹ and identifying biomarkers for microorganism screenings.²⁰

In their previous work, Arnold and Reilly used MALDI-MS to characterize the ribosomal proteins and their post-translational modifications from *Escherichia coli* ribosomes.¹⁷ They used 1% TFA to precipitate rRNAs and to enhance the signal from the ribosomal proteins. In addition,

molecular weight cut-off (MWCO) filters were used during sample preparation to improve the data obtained from higher molecular weight proteins present in this complex. They also showed that optimization of various MALDI instrumental parameters allowed for the identification of all ribosomal proteins, albeit with the trade-off of requiring multiple MALDI-MS experiments. Subsequent to that work, Pearson and co-workers used MALDI-MS to identify the mutations present in ribosomal proteins relating to antibiotic resistance.²¹ Ribosomes and ribosomal proteins have also been characterized by electrospray ionization-based approaches.^{22–24}

A key to the effective use of mass spectrometry, including MALDI-MS, for the characterization of proteins from complexes such as RNPs is the sufficient purification of the protein mixture. A variety of approaches for obtaining and concentrating biomolecules from complexes including ribosomes exist.²⁵ Phenol extraction is commonly used for isolating proteins or nucleic acids from complex particles.^{26,27} Organic solvents such as acetone and ethanol have been used for concentrating proteins and ribosomal subunits from dilute solution²⁸ and are popular for the purification of nucleic acids.²⁹ Additionally, ribosomal proteins have been precipitated by acetic acid with subsequent analysis by 2-dimensional gel electrophoresis.³⁰

To determine which sample preparation approach is most effective for use in MALDI-MS-based analyses of RNP complexes, we have investigated the applicability of several common protein isolation procedures. Using *E. coli* 70S ribosomes as the model complex system, a combination of electrophoretic and mass spectrometric analyses was done to characterize sample yields and MALDI compatibility. We show that an initial sample-denaturing step improves subsequent protein recovery by precipitation. All of the methods investigated were found to be compatible with downstream MALDI-MS and to provide the opportunity for the analysis of protein complexes in a single mass spectrometric experiment.

Experimental

Materials

Tryptone and yeast extract were obtained from Difco Laboratories (Detroit, MI, USA). Buffer reagents and peptide calibration kit were obtained from Sigma (St Louis, MO, USA). Trizol reagent, a monophasic solution of phenol and guanidine isothiocyanate, was from Invitrogen (Carlsbad, CA, USA). Sinapinic acid (SA) and α -cyano-4-hydroxycinnamic acid (CHCA) were obtained from Fluka (Milwaukee, WI, USA). Acids and organic solvents were HPLC grade or better. Endoproteinase LysC was purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). DNase was purchased from Promega (Madison, MI, USA). Molecular weight cut-off membrane tubing (3.5 kDa) was obtained from Spectrum (Laguna Hills, CA, USA).

Methods

Preparation of 70S ribosomes

Ribosomes were cultured in-house, following previously published procedures.²⁵ For ribosome extractions, the method of Spedding²⁵ was used with minor modifications. Briefly, ~15 g of frozen *Escherichia coli* MRE 600 cell paste was thawed, suspended in 40 mL buffer A (20 mM Tris-HCl, 10.5 mM magnesium acetate, 100 mM NH₄Cl, 0.5 mM EDTA, 3 mM β -mercaptoethanol) and disrupted in a French press. DNase was added to the homogenate up to 0.5 μ L mL⁻¹ and the mixture was incubated at 4°C for 20 min. Cell debris was removed by centrifugation for 1 h at 30,000 \times g, the mixture was resuspended in buffer A and ribosomes were purified by centrifugation through a sucrose cushion containing buffer B (20 mM Tris-HCl, 10.5 mM magnesium acetate, 500 mM NH₄Cl, 0.5 mM EDTA, 3 mM β -mercaptoethanol). Centrifugation was carried out for 15 h at 143,000 \times g. The pellets were resuspended in buffer C (10 mM Tris-HCl, 5.25 mM magnesium acetate, 60 mM NH₄Cl, 0.25 mM EDTA, 3 mM β -mercaptoethanol) and dialyzed against the same buffer using 3.5 kDa MWCO membrane tubing to remove the excess sucrose. All procedures were carried out at 4°C. Aliquots at a concentration of 30 mg mL⁻¹ of ribosomes in buffer C were stored at -80°C. Ribosome suspensions were diluted 2- to 10-fold prior to MALDI-MS analysis. The final protein concentrations were determined by using a modified Bradford protein assay with bovine serum albumin (BSA) as a standard.

Acid extraction

Trifluoroacetic acid (TFA) extraction of ribosomal proteins was done as described by Reilly.¹⁷ About 1 μ L of TFA of different concentrations was added to 9 μ L of buffered 70S ribosomes. The resulting supernatant was mixed with a MALDI matrix for MALDI-TOF MS analysis. Acetic acid extraction of ribosomal proteins was done according to Hardy.³⁰ A solution of 67% acetic acid and 0.1 M MgCl₂ was added to the 70S ribosome suspension and then stirred in an ice bath for 1 h. After removal of the rRNA precipitate, the supernatant was lyophilized and reconstituted in deionized water prior to further analysis.

Acetone and ethanol precipitation

Acetone and ethanol extraction were performed with minor modifications to the methods described by Hames³¹ and Hamel,³² respectively. One volume of ice-cold organic solvent was added to ribosomes prepared at a concentration of 20 absorbance units ($\lambda = 260$ nm : A₂₆₀) mL⁻¹ in buffer C. After incubation for two hours at -80°C, the precipitated ribosomes were collected by centrifugation and resuspended in deionized water. Before MALDI analysis, a 2.5% TFA solution was added to the resuspended ribosomal solution. The resulting supernatant was combined with matrix and analyzed by MALDI-MS.

Phenol extraction

A complete description of the phenol extraction is given elsewhere.³⁶ Briefly, in order to extract ribosomal proteins from ribosomes, 100 μ L of Trizol reagent was added to 30 μ L of buffered 70S ribosomes followed by the addition of 20 μ L of chloroform. After phase separation, proteins in the organic layer were obtained by precipitating with 2-propanol, dried under vacuum and resuspended in deionized water. Precipitation by 2-propanol was used to recover low molecular weight proteins remaining in the aqueous layer.

Sodium dodecylsulfate polyacrylamide gel electrophoresis and in-gel digestion

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10% or 12.5% polyacrylamide gels of 1 mm thickness, run at 300 V for six to ten hours. The SDS-PAGE gels were visualized by staining with Coomassie Brilliant Blue R250. To confirm the identity of ribosomal protein S1, the gel band visualized at 66 kDa was excised, cut into pieces and transferred into a siliconized microcentrifuge tube. The gel pieces were destained and washed with a solution of 30% methanol and 10% acetic acid. The pieces were reduced in gel by 10 mM dithiothreitol and alkylated by 55 mM iodoacetamide.³³ After washing, the gel pieces were dehydrated by acetonitrile and dried by a SpeedVac. The dried gel pieces were re-swollen in a buffer solution (5 mM Tris-HCl, pH 8.0) containing LysC followed by overnight digestion at 37°C. The digested products were vortexed for 5 min and extracted twice by sonication for 5 min in 50 μ L of extraction solution (50% acetonitrile, 5% formic acid). The supernatants containing the extracted peptides were pooled prior to MALDI-MS analysis.

MALDI analysis

All MALDI-MS experiments were done on a Bruker Reflex IV reflectron MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a nitrogen laser. Protein mass spectra were obtained in the positive-ion mode at an acceleration voltage of 20 kV by accumulating 200 laser shots. All samples were analyzed under identical parameters. These parameters were determined by optimizing the instrument for both signal intensity and resolution using a set of standard proteins and sinapinic acid.

For all protein analyses, saturated sinapinic acid in 33% aqueous acetonitrile + 0.1% TFA was used as the matrix. Samples were prepared by mixing 1 μ L of sample solution (~0.5–2 μ g total protein) with 9 μ L of matrix. For peptide experiments, 100 mM CHCA in 50% aqueous acetonitrile + 0.1% TFA was used as the matrix. Calibration of protein mass spectra was done using a mixture of insulin, ubiquitin, cytochrome c, myoglobin and BSA and then recalibrated internally with well-resolved ribosomal proteins. Calibration of peptide mass spectra was done using the Sigma

peptide calibration kit composed of angiotensin II, ACTH (fragment 18–39) and insulin chain B.

Results and discussion

The analysis of complex mixtures of proteins such as ribosomal proteins, requires optimization of a variety of experimental parameters such as type of matrix, solvent and sample preparation conditions.^{34,35} For the analysis of ribosomal proteins, various matrices including 2,5-dihydroxybenzoic acid, CHCA and sinapinic acid were investigated. Sinapinic acid was found to be the most effective matrix for analyzing the wide range of molecular masses present in this sample and was used for all subsequent investigations. Once the matrix was chosen, the primary focus of the remaining optimization studies involved examination of various sample preparation conditions.

Ribosomal proteins are unique in that they originally exist in combination with ribosomal RNAs. Thus, ribonucleoprotein complexes such as the ribosome present unique challenges in the optimization of protein isolation/sample preparation steps. As ribosomal proteins are intimately associated with rRNAs within the ribosome, the primary goal of this work was to examine various approaches for isolating ribosomal proteins that would allow their analysis by MALDI-MS in a single experiment. There are two experimental concerns associated with such isolations. First, because some ribosomal proteins are known to be tightly associated within the interior of the ribosome,^{36–41} an effective protein isolation approach should result in the release and recovery of all ribosomal proteins present. Second, to limit the potential suppression of protein signals by nucleic acids, an effective protein isolation approach should completely separate rRNAs from ribosomal proteins. Thus, three approaches previously used for the isolation of ribosomal proteins were investigated to determine their effectiveness at recovering all ribosomal proteins in the absence of any rRNA as well as their downstream compatibility with MALDI-MS. Ribosomal proteins are denoted using standard nomenclature wherein ribosomal proteins from the large subunit are identified by an L and ribosomal proteins from the small subunit are identified by an S.

Acid extraction

Arnold and Reilly previously showed that the addition of trifluoroacetic acid (TFA) is effective at precipitating rRNA from intact ribosomes and is compatible with downstream MALDI-MS analysis.¹⁷ Therefore, TFA was chosen as a reference with which other protein isolation protocols could be compared. Here, several TFA solutions of increasing concentration were used to establish an optimum TFA concentration and generate a suitable reference mass spectrum.

As seen in Figure 1, the relative abundances of the various ribosomal proteins increased with increasing concentration of TFA up to 5% TFA. For TFA concentrations between 1 and 5%, the spot-to-spot signal was found to be very reproducible. The reproducibility appeared to correlate with sample morphology (data not shown) as expected.⁴² Those concentrations of TFA yielding the highest reproducibility also resulted in the most homogeneous crystals. Above 5% TFA, crystal morphology becomes less homogeneous and the ion abundances decrease overall with minimal protein signal detected above ~ 14 kDa. Similar studies done using the calibration standards also resulted in the loss of ion signal at acid concentrations above 5% (data not shown).

As discussed in part previously,^{17,21} the observed effect of TFA on the quality of the mass spectra in Figure 1 is likely due to several effects. The addition of TFA leads to the precipitation of rRNAs and limits their concentration in the final sample solution, thereby reducing sample suppression effects. Another assumption is that TFA serves as a proton source, thereby enhancing the signals from ribosomal proteins. However, when the concentration of TFA is too high, the loss of ion signal could be attributed to the lack of crystal homogeneity, the loss of proteins by precipitation,

signal suppression due to ion pairing or some combination of these possibilities.

To examine whether the loss of signal at higher concentrations of acid is due to co-precipitation of ribosomal proteins with the rRNAs, additional experiments were done. Supernatants of ribosomal samples treated with TFA were run by SDS-PAGE to visualize the amount of proteins remaining in the supernatant. It was observed that the molecular ion abundance of the higher molecular weight proteins decreased with increasing concentrations of TFA (data not shown). In addition, after TFA precipitation, the precipitate was resuspended in deionized water and analyzed by MALDI-MS. Several ribosomal proteins were detected in the precipitate (data not shown). These observations were consistent with the MALDI data in Figure 1 and suggest that the lower pH leads to protein co-precipitation with rRNA.

Although the various ribosomal proteins, except for L7/L12,⁴³ should be equally represented in the original sample, significant variation in ion abundances among the ribosomal proteins was consistently seen. Such variation is expected due to the differing basicities and hydrophobicities of the constituent proteins. When using TFA, the ribosomal proteins L30, L29, S16, S19, L16, S5 and L6 were consis-

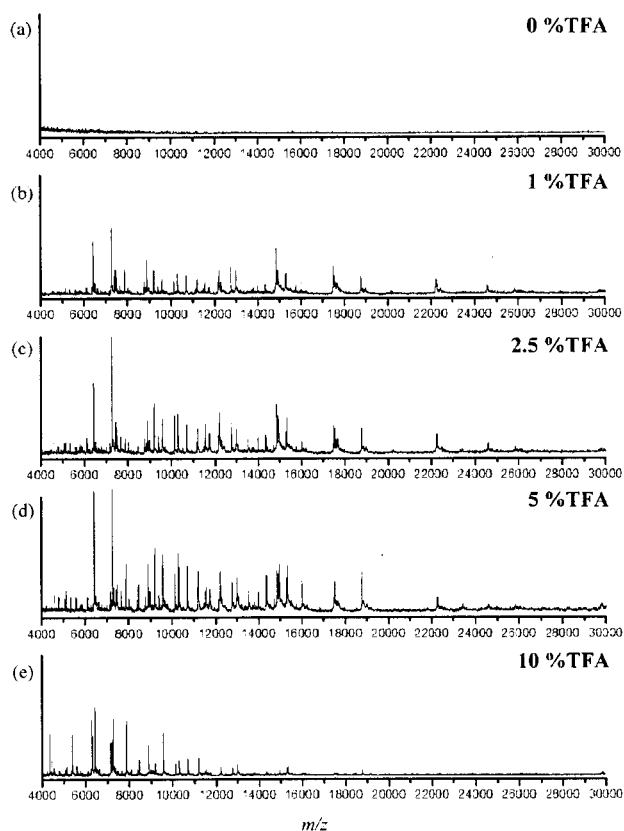


Figure 1. Effect of TFA precipitation on the MALDI mass spectral analysis of 70S ribosomal proteins. (a) No TFA, (b) 1% TFA, (c) 2.5% TFA, (d) 5% TFA and (e) 10% TFA added to 70S ribosome sample with supernatant analyzed by MALDI-MS. The information obtained from the addition of 2.5% TFA is used as a comparison against other sample preparation methods investigated.

tently detected at relatively high abundance, whereas the signals from L36, S22, L34, L33, L32, L35, S21 and L27 depend on the amount of TFA present. Arnold and Reilly found that L36, S22, L34, L33 and L32 could be observed when CHCA was used as a matrix, presumably because these low molecular weight proteins are better suited for this matrix.¹⁷

From the MALDI-MS and SDS-PAGE results, it was determined that the optimum TFA concentration was 2.5% based on the number and abundance of the proteins detected. For all subsequent comparisons, the MALDI-MS data obtained from this concentration was chosen as the reference mass spectrum [Figure 1(c)].

In the TFA approach first demonstrated by Reilly,¹⁷ the supernatant is analyzed directly without any additional steps. By way of comparison with that approach, precipitation of ribosomal RNAs by acetic acid was done as described by Agafonov.⁴⁴ In this approach, acetic acid is used to precipitate the rRNA and the resulting supernatant is first lyophilized and then reconstituted in deionized water before MALDI-MS analysis. As seen by the data in Figure 2(a), this approach results in a greater number of higher molecular weight proteins of high ion abundance than does the TFA approach. However, unlike the TFA approach, lower molecular weight proteins such as L36, S22, L34, L33 and L32 are not observed when using this approach. However, if 2.5% TFA is added to the supernatant resulting from acetic acid treatment, these lower molecular weight proteins are detected with the trade-off of reduced ion abundance for the higher molecular weight proteins [Figure 2(b)]. It was also found that if comparable amounts of TFA (> 50%) are used

instead of acetic acid no precipitate forms. Because TFA is a stronger acid than acetic acid, the large amount of TFA may lead to RNA degradation or protein co-precipitation thereby limiting its effectiveness as a precipitating agent.

Acetone or ethanol precipitation

It is known that RNA folding is effected by and stabilized in the presence of magnesium ions.^{45,46} Within the ribosome, ribosomal proteins assist in ribosome assembly. Because of these effects, the exchange of higher ionic strength buffers with deionized water was examined to determine if an initial destabilization of the ribosome structure would increase the number and abundance of ribosomal proteins detected by MALDI-MS.

To exchange out the high ionic strength buffer with deionized water, ribosomes were precipitated by the addition of acetone or ethanol, and the precipitates were resuspended in deionized water after centrifugation. MALDI-MS analysis of the resuspended solution or supernatant did not yield any significant ion signals (data not shown). However, after treating the resuspended solution with 2.5% TFA, protein mass spectra were obtained (Figure 3).

Several distinct differences were noted in this approach. For both acetone and ethanol precipitation, a greater number of proteins were detected at higher ion abundances than found by the addition of TFA alone. Both precipitations resulted in an increase in ion signal from lower molecular weight proteins such as L36, S22, L34, L33 and L32 with ethanol yielding higher ion signals from these proteins than acetone. Interestingly, acetone precipitation resulted in the detection of L7/L12 whereas ethanol precipitation did not.

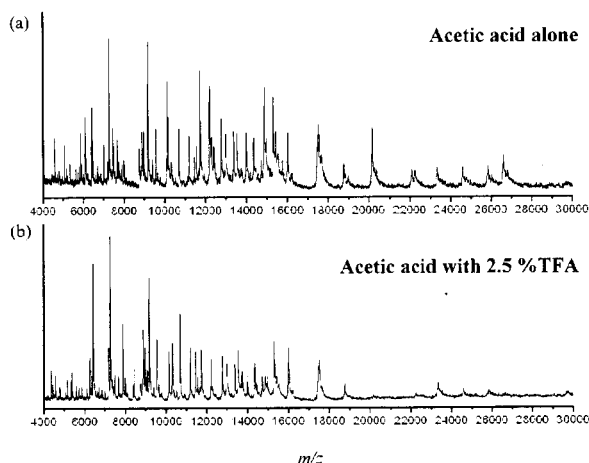


Figure 2. MALDI mass spectral data obtained after 70S ribosomes were precipitated with acetic acid. The supernatant was lyophilized and resuspended in (a) deionized water and (b) 2.5% TFA prior to MALDI-MS analysis. Acetic acid precipitation is comparable with TFA precipitation, although the subsequent addition of TFA before MALDI-MS analysis leads to the loss of ion signal for higher molecular weight proteins.

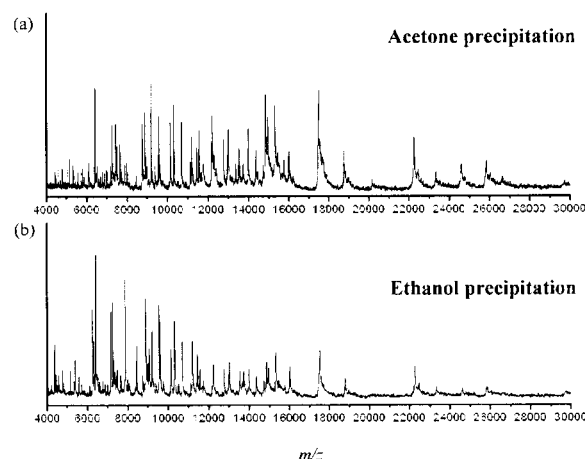


Figure 3. MALDI mass spectral data obtained after 70S ribosomes were precipitated with (a) acetone and (b) ethanol. Precipitates were resuspended in deionized water to denature ribosomes and then treated with 2.5% TFA to precipitate ribosomal RNA prior to MALDI-MS analysis. Initial acetone precipitation leads to a higher yield of ribosomal proteins.

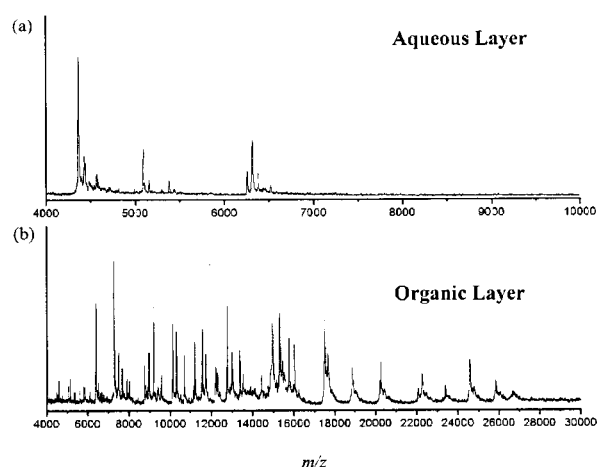


Figure 4. MALDI mass spectra obtained after phenol extraction of 70S ribosomes; (a) aqueous phase and (b) organic phase. The lower molecular weight proteins L36, S22, L34, L33 and L32 are found in the aqueous phase. The proteins analyzed in the organic phase yielded higher ion abundances than those found by TFA precipitation alone.

These two proteins are only weakly associated with the ribosome complex, and they are typically lost during many sample preparation treatments.⁴⁷ The likely explanation for this result is the reduced solubility of these two proteins in acetone as compared with ethanol. Thus, they likely do not precipitate during ethanol treatment and would, therefore, be lost in this sample preparation approach.

Phenol extraction

As an alternative to either of the precipitation-based approaches previously discussed, organic extraction of ribosomal proteins was examined. Phenol extraction is a method commonly used for isolating nucleic acids from cell lysates.²⁶ The extraction efficiency of phenol was first verified using the protein calibration standards. After phenol extraction, the aqueous layer, which should not contain proteins, was analyzed by MALDI-MS and no protein signals were observed (data not shown). The organic layer resulting from phenol extraction was treated with 2-propanol to precipitate any proteins. The precipitate was re-dissolved in deionized water and then analyzed by MALDI-MS. Each of the proteins from the calibration standards was detected, suggesting the viability of this approach (data not shown).

This same protocol was then performed on intact 70S ribosomes. As seen in Figure 4, ribosomal proteins were observed after phenol extraction. Although all of the expected ribosomal proteins were not detected, the quality of the mass spectral results was improved as compared with that obtained when using 2.5% TFA. In particular, improved ion abundance for the higher molecular weight proteins was observed [Figure 4(b)]. Analysis of the aqueous layer result-

ing from phenol extraction yielded the lower molecular weight proteins L36, S22, L34, L33 and L32 [Figure 4(a)].

A significant difference in the phenol extraction approach is the absence of any suppression effects that can arise when higher amounts of acid are added in the precipitation-based approaches. Although some of the lower molecular weight ribosomal proteins were found to remain in the aqueous layer, optimization of the phenol extraction solvents/conditions may allow the complete isolation of ribosomal proteins. Such work is currently underway in our laboratory.

Comparison of methods

The mass spectral results from ribosomal proteins isolated by the approaches described above are summarized in Table 1. In general, the lower molecular weight proteins are the most sensitive to the sample preparation conditions. For example, ribosomal proteins such as L36, S22, L34, L33, L32, L35, L31, S21 and L27 were only observed at reasonable ion abundances after precipitation of RNA with high amounts of acid or after acetone precipitation of ribosomes. SDS-PAGE analysis of either precipitates or supernatants from various treatments was done to determine if the absence of abundant ion signals from these lower molecular weight proteins is due to the loss of these proteins during isolation or ion suppression effects (Figure 5). As seen in Figure 5, weak bands arising from lower molecular weight proteins are present in the ethanol and acetone supernatant. However, both the ethanol and acetone precipitates still contain bands representative of these lower molecular weight proteins suggesting that, while some sample loss is found, the lack of lower molecular weight protein signals in the MALDI data arises through ion suppression.

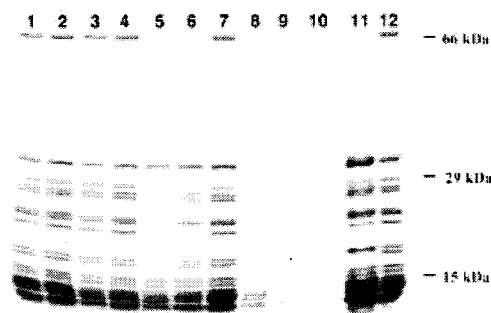


Figure 5. 12.5% SDS PAGE gel of ribosomes treated by the approaches described in text. Lanes 1, 2, 7 and 12: intact ribosomes; Lane 3: pellet obtained after ethanol precipitation; Lane 4: pellet obtained after acetone precipitation; Lanes 5 and 6: supernatant obtained after acetic acid precipitation; Lane 8: supernatant obtained after ethanol precipitation; Lane 9: supernatant obtained after acetone precipitation; Lane 10: pellet obtained after acetic acid precipitation; Lane 11: supernatant obtained after 2.5% TFA precipitation. Lanes 1–7 and 12: 1.0 μ L sample loaded; Lanes 8–11: 5.0 μ L sample loaded. See text for discussion.

Table 1. Masses and relative abundances of *E. coli* ribosomal proteins obtained by different sample preparation procedures.

Protein	Expected mass (Da)	Observed mass (Da)	2.5% TFA	AA/ 0% TFA	AA/2.5% TFA	Acetone	EtOH	Phenol	Modification ^a
L36	4364.4	4364.0			M	W	M	H°	
S22	5095.8	5095.8			W		W	M°	
L34	5380.4	5380.3			M		M	W°	
L33	6240.4	6255.7			M	W	H	M°	Methylated
L32	6315.2	6316.5			M	W	M	M°	
L30*	6410.6	6411.2	H	M	H	H	H	H	
L35	7157.8	7158.1			M	W	M		
L29*	7273.5	7273.1	H	H	H	H	H	H	
L31	7871.1	7870.6	W		M	M	H	M	
S21	8368.8	8369.2	W		M	M	M		
S18	8855.3	8896.1	W	M	M	M	M		Acetylated
L28	8875.3	8875.1	M	M	M	H	H		
L27	8993.3	8993.2			M	W	M		
S16*	9190.6	9191.0	M	H	H	H	H	H	
S20/L26	9553.2	9553.5	W	W	M	M	H	W	
S17	9573.3	9573.7	M	M	M	M	H	M	
S15	10137.6	10138.2	M	H	M	M	M	H	
S19	10299.1	10299.6	M	M	M	H	H	H	
L25*	10693.5	10693.4	M	M	M	M	M	M	
L24	11185.0	11184.9	M	M	M	M	M	M	
L23	11199.1	11198.7	M	M	M	M	M	M	
S14	11449.3	11449.3	W	M	M	M	M		
L21	11564.4	11564.8	M	M	M	M	M	H	
S10	11735.6	11736.6	M	H	M	M	M	M	
L7	12164.0	12164.6	M	M		M	W	W	Acetylated
L7	12207.3	12206.1	M	M	W	M	M	M	
L12	12164.0	12176.8	M	W	W	W	W	W	Methylated
L22	12226.3	12225.8	M	M	M	M	M	M	
L18*	12769.6	12769.8	M	M	M	M	M	H	
S13	12968.2	12969.8	W	M	M	M	M	W	
L19	13002.1	13002.9	M	M	M	M	M	M	
L20	13365.8	13365.0	W	M	M	W		M	
L14	13541.1	13541.5	M	M	M	M	M	M	
S12	13605.9	13654.5			W		M		β-Methylthiolated

Table 1. Masses and relative abundances of *E. coli* ribosomal proteins obtained by different sample preparation procedures (continued).

Protein	Expected mass (Da)	Observed mass (Da)	2.5% TFA	AA/ 0% TFA	AA/2.5% TFA	Acetone	EtOH	Phenol	Modification ^a
S11	13713.8	13727.5	W	W	M	M	M		Methylated
S8*	13995.4	13995.2	M	M	M	M	M		
L17	14364.6	14364.9	M	M	M	M	M	M	
S9	14725.0	14724.0	W	M	M	M	M	M	
L11	14744.2	14871.0	M	H	M	H	M	H	Methylated (9 times)
L15	14980.4	14966.0	M	M	M	M	M	M	Unknown
S6	15703.5	15446.3	W	M	W	M	M	M	Loss of EEEE
L16	15281.2	15325.3	M	H	H	H	M	H	Unknown
L9	15769.1	15769.5	W	M		M	W	M	
L13*	16018.6	16019.3	M	M	M	M	M	M	
S5	17472.2	17515.6	M	M	M	H	M	H	Acetylated
L10	17580.4	17579.4	M	M	M	M	M	M	
L6*	18772.6	18772.8	M	M	M	M	M	M	
S7	19887.9	17474.1	M	M	M	M	M	M	Loss of residue 156–178
L5	20170.4	20170.2	W	M		W		M	
L4	22086.6	22084.9		M				W	
L3	22243.6	22258.9	M	M	W	M	M	M	Methylated
S4	23338.0	23337.9	W	M	M	M	W	M	
L1*	24598.5	24597.5	M	M	W	M	W	M	
S3	25852.1	25851.8	W	M	W	M	W	M	
S2	26612.5	26615.2		M		W		W	
L2	29729.3	29727.9	W	W	W	W	W		
S1	61158.2	—							
S6			45	45	51	51	51	45	

W = relative intensity below 15%, M = relative intensity between 15–60%, H = relative intensity above 60%. Sample treatment: 2.5% TFA added; acetic acid (AA) precipitation with no added TFA; acetic acid precipitation followed by addition of 2.5% TFA; acetone precipitation; ethanol precipitation; phenol extraction. *Proteins marked by asterisks are used for internal calibration. ° Those proteins were identified from aqueous layer after phenol extraction (see text). ^aAll post-translational modifications observed here have been detected previously by other techniques.

Surprisingly, whereas acetic acid precipitation of rRNA appears to lead to losses in ribosomal proteins present in the remaining supernatant, no proteins are observed in the precipitated pellet (Figure 5, lane 10). Moreover, the combination of acetic acid and TFA yielded the highest quality mass spectral coverage of ribosomal proteins at lower mass. Acetic acid alone yielded high quality MALDI-MS data for

higher molecular weight proteins, again suggesting that the addition of TFA can lead to suppression effects for the higher molecular weight components of the sample.

In general, with the exception of ribosomal protein S1 as noted below, the approaches investigated here provide a ready means of isolating proteins from RNP complexes in a manner compatible with MALDI-MS analysis. Except for

the variations noted for the lower molecular weight proteins, no other general trends relating to the absence of proteins in the final mass spectra were noted. Based on these results, if a global picture of proteins present in RNP complexes is desired, the use of acetic acid for RNA precipitation is recommended. Addition of TFA should be limited to the minimal amount required to improve results from the lower molecular weight components of the sample. For ribosomes in particular, an additional step of ribosome denaturation (using ethanol) also proves effective in improving the subsequent mass spectral results.

Ribosomal protein S1

The highest molecular weight ribosomal protein, S1, was not observed in the MALDI-MS data obtained after performing any of the protocols mentioned above. However, SDS-PAGE analysis of ribosomal proteins showed a band around 66 kDa that should correspond to this ribosomal protein during all treatments except TFA precipitation (Figure 5). To confirm that this band was, in fact, ribosomal protein S1, in-gel digestion of the peak circled in Figure 6(a) was done. Figure 6(b) is the mass spectrum resulting from the in-gel digestion of S1 with LysC. The peaks in Figure 6(b) that can be assigned to the S1 protein are summarized in Table 2, and these yield 38% sequence coverage of the

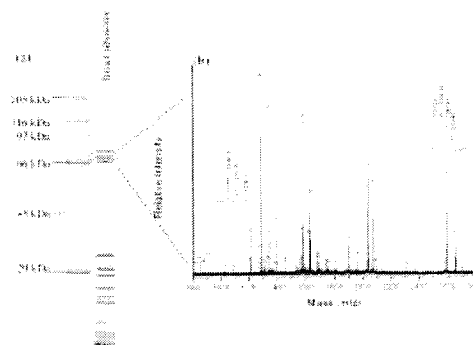


Figure 6. (a) 10% SDS PAGE gel of protein markers and intact ribosomes; (b) MALDI mass spectrum of peptide products obtained after LysC digestion of band circled in Figure 6(a). The insets show the resolution of ~ 11000 FWHH at m/z 1214.76 and 7000 FWHH at 2595.35. All assigned masses marked by asterisks correspond to LysC digested peptides within 25 ppm of database values. The peaks marked by + are from modification by carbamidomethylation.

protein. Thus, based upon the SDS-PAGE analyses of various sample preparations (Figure 5), S1 is present in the original sample mixture even though it is not detected during MALDI-MS. It appears that, whenever TFA is added, S1 is lost to precipitation. For those analyses done in the absence

Table 2. MALDI-TOF MS analysis of in-gel LysC-digested putative S1 protein band (Figure 6).

Measured mass	Calculated mass ^a	ΔM (ppm) ^b	Modification	Amino acid residues	Sequence
822.45	822.4474	3.2		505–511	(K)FTGVDRK(N)
850.45	850.4423	9.1		273–279	(K)RYPEGTK(L)
1028.60	1028.5992	0.8		34–43	(K)DVVLVDAGLK(S)
1206.59	1206.6006	–8.8		44–54	(K)SESAIPAEQFK(N)
1214.75	1214.7333	14		512–522	(K)NRAISLSVR(K)(D)
1282.69	1282.6908	–0.6		91–100	(K)RHEAWITLEK(A)
1309.68	1309.6792	0.6	PyroGlu	261–272	(K)QLGEDPWVAIAK(R)
1326.69	1326.7058	–12		261–272	(K)QLGEDPWVAIAK(R)
1570.73	1570.7403	–6.5		351–363	(K)ANPWQQFAETHNK(G)
1576.88	1576.8923	–7.8		248–260	(K)FDRERTRVSLGLK(Q)
1675.78	1675.7889	–5.3	1Met-ox	1–14	(–)MTESFAQLFEESLK(E)
1741.83	1741.8550	–14	PyroGlu	435–449	(K)QLAEDPFNNWVALNK(K)
1758.84	1758.8815	–24		435–449	(K)QLAEDPFNNWVALNK(K)
1802.76	1802.7656	–2.5	1Met-ox	537–552	(K)QEDANFSNNAMAEAFK(A)
1902.01	1902.0045	13		512–528	(K)NRAISLSVRKDEADEK(D)
2020.10	2020.1191	–9.5		227–244	(K)RVKHPSEIVNVGDEITVK(V)
2038.18	2038.1661	6.8		15–33	(K)EIETRPGSIVRGVVVAIDK(D)
2312.10	2312.1172	–7.4		351–370	(K)ANPWQQFAETHNKGDRVEGK(I)
2595.44	2595.4470	–2.7		411–434	(K)KGDEIAAVVLQVDAERERISLGVK(Q)

^athe monoisotopic singly-protonated mass of the peptides is shown

^bmeasured minus calculated mass from the MALDI spectrum in Figure 6. The peptide sequence for identified peptide is indicated

of TFA, S1 could be suppressed by the other sample components or requires MALDI sample conditions more conducive to high molecular weight protein analysis.

Conclusions

Here we have investigated the applicability of several ribosomal protein isolation approaches to downstream MALDI-MS analysis. Because ribosomal proteins associate with rRNAs in this ribonucleoprotein complex, methods allowing the isolation of all ribosomal proteins and the absence of any rRNA were sought. All of the methods investigated were generally successful at isolating ribosomal proteins and allowed their subsequent analysis and identification by MALDI-MS. The approach yielding the most reproducible and significant ion abundances from the majority of ribosomal proteins was the use of acetic acid for rRNA precipitation. Alternatively, buffer exchange by acetone or ethanol precipitation of ribosomes followed by acid precipitation of ribosomal proteins also provides high protein coverage. Phenol extraction appears potentially attractive due to the lack of suppression effects, although optimization of the solvent conditions would be necessary to effectively isolate the lower molecular weight proteins currently retained in the aqueous phase.

Most of the approaches investigated here were also found to be at least as effective as the TFA approach previously described.^{17,21} Of particular note, these methods allow for the simultaneous analysis of nearly all of the *E. coli* 70S ribosomal proteins in a single MALDI-MS experiment. Whereas separate mass spectral conditions for low and high molecular weight proteins reduce suppression effects, the strategies discussed here should improve our ability to rapidly screen proteins present in a ribonucleoprotein complex such as the ribosome. In addition, these methods should also be useful for isolation and MALDI-based analysis of proteins from other RNP complexes.

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