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# Maximizing recovery of water-soluble proteins through acetone precipitation

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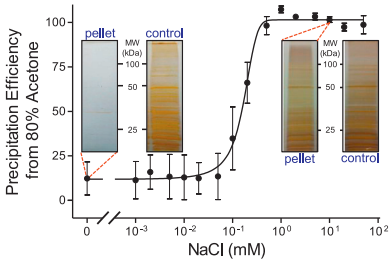
## HIGHLIGHTS

- For most proteins, ionic species are required for precipitation in organic solvent.
- The amount of salt correlates with the protein and organic solvent concentrations.
- Ionic species are also required to precipitate complex proteome mixtures.
- A model of ion pairing is proposed to explain this synergistic precipitation.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Solvent precipitation is commonly used to purify protein samples, as seen with the removal of sodium dodecyl sulfate through acetone precipitation. However, in its current practice, protein loss is believed to be an inevitable consequence of acetone precipitation. We herein provide an in depth characterization of protein recovery through acetone precipitation. In 80% acetone, the precipitation efficiency for six of 10 protein standards was poor (ca. ≤15%). Poor recovery was also observed for proteome extracts, including bacterial and mammalian cells. As shown in this work, increasing the ionic strength of the solution dramatically improves the precipitation efficiency of individual proteins, and proteome mixtures (ca. 80–100% yield). This is obtained by including 1–30 mM NaCl, together with acetone (50–80%) which maximizes protein precipitation efficiency. The amount of salt required to restore the recovery correlates with the amount of protein in the sample, as well as the intrinsic protein charge, and the dielectric strength of the solution. This synergistic approach to protein precipitation in acetone with salt is consistent with a model of ion pairing in organic solvent, and establishes an improved method to recover proteins and proteome mixtures in high yield.



## 1. Introduction

Precipitation is a classic approach to purify proteins, being first described through the application of high salt concentrations (salting out) [1] and later through the addition of organic solvents [2]. Protein precipitation in acetone remains an active approach for sample purification ahead of mass spectrometry (MS). For example, the method is applicable to the effective elimination of sodium dodecyl sulfate (SDS), a notoriously persistent sample additive which interferences with protein analysis through HPLC and MS. SDS is of considerable benefit to the proteomics workflow, both as an effective solubilizing additive and to impart mass-based protein separation [3–5]. To permit HPLC and LC/MS analysis, the concentration of SDS in the sample must be reduced to below 0.01% [6]. Acetone precipitation has previously been shown effective to purify SDS-containing protein samples ahead of MS analysis [6,7]. While analyte purity is an important consideration of any sample clean-up approach, one cannot neglect the importance

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of maintaining high recovery of the analytes themselves. In this regard, conventional strategies for protein precipitation have inevitably contributed to analyte loss, and are often overlooked in favor of other cleanup approaches (e.g. solid phase extraction).

The degree of protein loss through acetone-precipitation has been widely reported, and appears to be dependent on several conditions of the sample. For example, Thongboonkerd et al. compared acetone precipitation to ultracentrifugation for recovery of urinary proteins, showing a preference toward the loss of basic and hydrophobic proteins in acetone [8]. In this study, the concentration of acetone was 50%, which may not be optimal for recovery of all proteins. Srivastava et al. employed 50% acetone to enrich gamma-crystallin from human eye lenses, noted that this 20 kDa protein remains soluble in the solvent system [9]. Puchades et al. quantified the precipitation efficiency in a 4:1, acetone:water solvent system, reporting 80% protein yield. Their recovery value is based on visual assessment of protein band intensity within SDS PAGE, and further represents only two standard proteins (myoglobin and cytochrome c) and under specific sample conditions [10]. Through $^{35}S$ radiolabelling, Barritault et al. quantified the recovery of ribosomal proteins in five volumes cold acetone as a function of the protein concentration (between 0.2 and 20 g $L^{-1}$) [11]. For samples prepared in 0.5% SDS, protein recovery was significantly less than that obtained in water, with ~80% yield reported. As a front-end purification technique, the seemingly variable recovery of protein places doubt on the utility of solvent precipitation, particularly when characterization of all sample components is desired. For optimal recovery of complex mixtures through protein precipitation, it is generally accepted to employ three or four parts cold acetone (i.e. 75–80% acetone, overnight incubation of the sample on ice or in the freezer) [6,7,12]. Other solution conditions, including the ionic strength, presence of surfactants or buffer pH, may interfere with protein recovery. A detailed investigation of the possible influence of solution conditions on protein precipitation efficiency in acetone has not been reported.

To optimize protein recovery through solvent precipitation, it is critical to first understand the factors controlling protein solubility in non-aqueous solvents. In aqueous solution, proteins adopt a structure which exposes hydrophilic regions to the surrounding aqueous, allowing formation of a hydration layer that shields protein–protein interactions. Disruption of this hydration layer generally causes protein precipitation [13]. The current model which explains solvent-induced precipitation can be explained by Coulomb's law, which relates the magnitude of the electrostatic force to the dielectric constant of the medium. Organic solvents, having reduced dielectric strength, increase the attractive force between opposingly charged ions [14]. For a heterogeneously charged protein surface, the positive charges of one protein can combine with the negative charges of another, leading to aggregation of the sample in organic solvent [15]. However, by a similar logic, repulsive forces between like charges on opposing proteins would also increase in organic solvents. Thus, this simple model of precipitation does not fully describe the cause of protein aggregation in acetone.

In the current investigation, the variables influencing protein recovery through acetone precipitation are explored. The solubility of a given protein in 80% acetone is highly dependent on the pH of the solution, which in turn relates to the charge density of the protein. To improve protein recovery, an ion pairing reagent is added, which effectively neutralizes the charge of the protein. In high organic solvent, ion pairing can occur between protein and simple salts such as NaCl. The combined influence of salt and organic solvent results in consistent and quantitative recovery for all water soluble proteins including complex proteome mixtures.

## 2. Experimental

### 2.1. Reagents

Individual protein standards as well as lyophilized *Saccharomyces cerevisiae*, type II (YSC2), was purchased from Sigma (Oakville, Canada). Tris, SDS, iodoacetamide and dithiothreitol (DTT), along with materials for preparation and running polyacrylamide gels were purchased from BioRad (Hercules, CA). Milli-Q grade water was purified to 18.2 MΩcm. Sodium chloride and HPLC grade acetone were from Fisher Scientific (Ottawa, Canada). *Escherichia coli* was grown and harvested according to established protocols (Qiagen Manual for Good Microbiological Practices). The rat proximal tubule cell line NRK-52E was a gift from Dr. Dawn MacLellan (IWK Health Centre, Halifax, Canada) and was grown according to manufacturer's instructions (American Type Culture Collection, Burlington, Canada).

### 2.2. Yeast proteome extraction

Ten grams of lyophilized yeast were washed three times in $dH_2O$, and suspended in $dH_2O$ at a final volume ratio of 1:1 (wet pellet:water). The slurry was added drop-wise to liquid nitrogen, and ground with a mortar and pestle for 10 min under liquid nitrogen. The frozen powder was quantitatively transferred to a vial and $dH_2O$ was added at a volume to weight ratio of 5 mL $g^{-1}$. Proteins were extracted on ice with gentle shaking for 10 min; the insoluble material was removed by centrifugation at 16,000 × g for 10 min. The protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). The sample was frozen at −25 °C until further use.

### 2.3. Escherichia coli proteome extraction

Bacterial cells were suspended in $dH_2O$ and heated to 95 °C for 5 min. The cooled suspension was then subjected to multiple passed through a 26 gauge syringe in order to lyse the cells. Solid materials were separated by centrifugation at 16,000 × g for 10 min. The concentration of the resulting *E. coli* proteome extract was 0.5 g $L^{-1}$ (as determined by Bradford assay). The sample was frozen at −25 °C until further use.

### 2.4. NRK-52E osmotic lysis

Cells were pelleted at 300 rpm for 5 min in a benchtop centrifuge, and then twice washed in PBS buffer. Cells were suspended in 1 mL of ice cold water and the sample was held on an ice bath for 10 min to complete osmotic lyses. Cellular debris was separated through centrifugation at 16,000 × g for 10 min. The resulting concentration of the cellular protein extract was 0.2 g $L^{-1}$.

### 2.5. Acetone precipitation

Acetone precipitation was carried out as described [6]. Protein samples were prepared in water along with a specified concentration of NaCl, described in the results. The total volume of sample plus acetone was maintained at 500 μL. For example, precipitation in 80% acetone involves addition of 400 μL organic solvent to 100 μL protein solution. Samples were incubated overnight at −20 °C, and then centrifuged at 16,000 × g for 20 min. The supernatant was carefully removed with a pipette, leaving behind less than 20 μL of solution. An additional washing step was performed by adding 400 μL of cold acetone to the pellet, and removing the bulk of the supernatant following centrifugation. Residual acetone was removed from the pellet by air drying in a fume hood. The