

Protein Extraction From Frozen Brain Samples

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Introduction

Proteins from frozen tissue samples need to be extracted efficiently and without degradation to make the best use of a limited resource and to ensure, as much as possible, that an accurate representation of the proteins in the living tissue is obtained. The emergence of new and powerful proteomic techniques that make possible studies of many proteins simultaneously and the possibility of automated dispensations from biobank facilities have refocused attention on optimized and standardized tissue extraction protocols.

Two broad categories of complementary analytical uses of extracted proteins can be defined, which also place different demands on the extraction procedure: 1/ identification and quantitation studies and 2/ functional studies, including enzymatic assays and binding studies.

Identity, including identification of posttranslational modifications, and quantity determination is based on protein sequencing or antibody, or other ligand, binding and does not require preservation of secondary, tertiary or quaternary structure. Efficient extraction is in fact, in part, dependent on breaking protein interactions to release proteins bound in macromolecular assemblies. Disruptive methods of solubilization can, and should, therefore be applied, short of hydrolyzing the protein amino-acid chain or posttranslational modifications. In contrast, native protein extraction for functional studies inevitably compromises extraction efficiency for preservation of protein function. Native protein extraction is not the purpose of this current manual, and will not be dealt with further here. It is, of course an important issue for optimal use of biobanked tissue samples and should be dealt with systematically. Those studies will be performed separately.

Perhaps the most common method in biomedicine to solubilize proteins for identity and quantity determination is by solubilization with the ionic detergent sodium dodecyl sulfate (SDS). While not suitable for functional studies, and indeed for many other kinds of studies, SDS extraction still probably constitutes the most universally applicable protein extraction methodology, and the one most suitable for standardization. SDS-extracts can be used for SDS electrophoresis, Western blotting, including identification of post-translational modifications, and, after dilution (to reduce the SDS concentration), for two-dimensional electrophoresis and enzyme-linked immunosorbent assay (ELISA) (Harlow and Lane 1999) and mass spectrometry. We have chosen not to study solubilization in two-dimensional gel electrophoresis sample buffer since we think this solubilization system may be more in need of individual optimization, and be less suited for a standardized approach. It should be noted, however, that SDS extracts can be subjected to 2-D PAGE and that such extracts might, in fact, be close to optimal (Harder, Wildgruber et al. 1999).

No systematic study of the parameters of frozen tissue solubilization with SDS appears to exist in the scientific literature (Laemmli 1970; Laemmli and Favre 1973; Dignam 1990; Bollag and Edelstein 1991; Harlow and Lane 1999; Simpson 2003). We have therefore

initially focused our attention on establishing standardized conditions for optimal frozen brain tissue extraction using SDS solubilization.

Yield of extracted protein

It is currently not possible to determine the total amount of protein in a tissue prior to solubilization. The yield of extracted proteins will therefore, by necessity, be an indirect measure. The best measures are percent solubilization of total wet or dry weight of the tissue and/or the total amount of solubilized protein per weight unit of tissue, which both can readily be determined.

Avoidance of tissue degradation

Solubilization of tissue in SDS denatures and inactivates most enzymes, including proteases. We have adopted a solubilization scheme that includes disintegration of the frozen tissue, without thawing, to minimize or eliminate proteolysis in this step. The disintegrated tissue is then immediately solubilized in SDS-containing buffer. To avoid proteolytic and morphological degradation of the devascularized tissue prior to solubilization it should be stored cold but not frozen for an as short a time as possible. Setting the outside limits of temperature and time that are compatible with intact proteins and morphology requires analysis of these parameters. We suggest using Western blotting as a means to assess the amount of specific intact protein, and to investigate the existence of any degradation intermediates. Please see the accompanying Western blotting protocol for a detailed description of the technology, for current recommendations and for a summary of the current status of knowledge.

Parameters for efficient extraction

Optimal solubilization in our protocol, presented below, results in more than 97% brain tissue solubilization yield, with minimal aggregation and undetectable proteolytic degradation. Suboptimal extraction results in less than 40% extraction yield, proteolytic degradation or in aggregation. These results will be published separately. What follows is a summary of our current results and a suggestion for a standardized SDS extraction protocol. The extract should be suitable for automated dispensation.

Methods for Disintegration of Tissue Samples

- Intact tissue (no disintegration)
- Manual homogenization
- Glass Bead Vortexing
- Grinding in liquid nitrogen
- Sonication
- Ball mill grinding

Sonication and ball mill grinding give considerably higher extraction yield than other means of tissue disintegration. The aerosol that forms during sonication results in a loss of about 7.5% of the material as well as constituting a potential infection hazard. Sonication is also difficult to perform without risk of contamination of the sample. Ball mill grinding can be performed in a sealed cryogenic tube, obviating all risk of losses, infection and contamination.

A ball mill is a device that can disintegrate frozen tissue contained in a tube together with a steel ball, by vigorous shaking. A picture of a commercial ball mill is shown as figure 1.



Figure 1. The B. Braun Mikrodismembrator S ball mill

We recommend ball mill grinding.

Contamination

Human tissue should be regarded as potentially contagious, and treated accordingly. In the context of protein extraction this means that methods should be favored that contain the tissue so that the risk of infection is eliminated. Likewise, it is desirable that the tissue is contained so that the risk of molecular contamination of the sample is eliminated.

In weighing the various methods of tissue disintegration, we favor the ball mill method since it combines the highest levels of protein yield (together with sonication) with containment within a cryogenic tube during the disintegration process, thus eliminating both exogenous contamination and risk of infection.

Solvent

The concentration of SDS in the solubilization buffer affects protein solubilization. 2% SDS (1x SDS) gives about half as much maximal protein solubilization as 4% (2x SDS). The potential need to dilute the SDS concentration for 2-D PAGE or ELISA would favor the use of 2% (1x SDS) and instead using a compensatory larger volume.

We recommend 1x SDS solubilization buffer (2% SDS).

Time of extraction

There is no effect of time on total protein extraction efficiency between 2 minutes and 60 minutes of extraction time.

We recommend 10 minutes of extraction time.

Temperature of extraction

There is no effect of temperature on total protein extraction efficiency between 50°C and 99°C. The recovery of specific proteins, however, decreases with increasing temperature, possibly due to aggregation. In some cases solubilization at 60°C results in partial proteolytic degradation.

We recommend a 70°C extraction temperature.

Volume of extraction

Any volume of 1x SDS extraction buffer (2% SDS) larger than 10 times the wet weight of normal brain tissue (or larger than 5x the wet weight if using 2x SDS sample buffer) will give maximal solubilization.

We recommend a 1x SDS extraction buffer volume 10 times the wet weight of the tissue.

Protein Concentration Determination

Several different protein concentration assays exist, including the following common assays: Absorbance at 280nm, Lowry Assay, Bradford Assay, Bicinchoninic (BCA) Assay, Pierce Assay, Dot Filter Binding Assay, BioRad RC DC Assay. Measuring the protein concentration in an SDS extract requires that the assay is compatible with the detergent and reducing agent in the solution.

We recommend the BioRad RC DC protein assay.

Suggested standard protocol for protein extraction from frozen tissue samples

- The work should be organized so that any contagion in the sample cannot infect the personnel.
- The work should be organized so that the sample is not contaminated.
- Relevant safety precautions for working with liquid nitrogen should be taken.

Label and preweigh a sufficient number of “Eppendorf” 1.5 ml capped polypropylene tubes (Treff# 96.7246.9.01). Make notes of the tube weight.

Remove the desired number of tissue pieces from the -70°C freezer. The size should be about 100 mg (about 5x5x4 mm). Keep them on liquid nitrogen so that they do not thaw.

Weigh the tissue pieces on weigh-paper on an analytical scale. Be quick so the samples do not thaw. Make notes of the tissue weight. Place the tissue pieces in a frozen (on liquid nitrogen) sterile cryogenic tubes (Corning #2012, 1.2 ml) each with a sterile steel ball (Sartorius BBI-8546703).

Place the frozen tissue in cryogenic tubes in the frozen ball mill tube holder (B. Braun International #8531960). Shake the samples for 1 minute at 2000 Hertz (B. Braun Mikrodismembrator S). Remove the frozen tubes. Inspect the samples to ensure that the tissue has been disintegrated and that the tube is intact. Store the tubes on liquid nitrogen until processed.

Take out one tube at the time and start to thaw at room temperature. While the sample is still frozen, add a volume of 1x SDS-buffer (0.125 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 0.001% bromophenol blue and 5% 2-mercaptoethanol (Laemmli 1970; Laemmli and Favre 1973)) corresponding to 10x the wet weight of the sample. Vortex the sample for 1 minute at room temperature (Scientific Industries, Vortex Genie®2). Check that the disintegrated tissue is macroscopically solubilized. Transfer the solubilized contents

quantitatively to the labeled and preweighed Eppendorf tube. Incubate the sample for 10 minutes at 70°C with shaking at 1400 Hertz (Eppendorf Thermomixer Compact).

(If the contents is viscous due to DNA and this is a concern, the DNA can be fragmented by sonication (Sonics & Materials, Vibra cell™) according to the manufacturers instructions, with precautions taken to avoid contamination (it may be impossible to avoid contamination with this technique). The sample should be cooled on ice during sonication.)

Sediment any residual undissolved tissue by centrifugation at 16,100xg (Eppendorf Centrifuge 5415 D or equivalent) for 10 minutes at room temperature. Decant the supernatant to a fresh Eppendorf tube. Note the presence of any sediment. Weigh the empty tube to determine the weight of the sediment.

Set aside a 25 µl aliquot for measuring the protein concentration. Freeze the rest of the sample at -20°C. We expect the solubilized and denatured samples to be stable at -20°C for months, if not longer. Use the BioRad RC DC Protein Assay #500-0119, 500-0120, 500-0121, 500-0122 or equivalent. Follow the manufacturers instructions.

References

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