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**ORIGINAL ARTICLE**

# Optimized protein extraction from cryopreserved brain tissue samples

CHRISTER ERICSSON[1], INTI PEREDO[2] & MONICA NISTÉR[1]

[1]*Department of Oncology-Pathology, Karolinska Institute, Cancer Center Karolinska, R8:05, Karolinska University Hospital-Solna, 171 76 Stockholm, Sweden, and* [2]*Department of Clinical Neuroscience, Karolinska Institute, Karolinska University Hospital – Solna, 171 76 Stockholm, Sweden*

**Abstract**
Optimal standard conditions for protein extraction and solubilization from frozen tissue samples have been examined. Quantitative differences in specific protein amounts or post-translational modifications underlie many, if not all, disease states. Maximal and standardized extraction and solubilization of protein from diseased or healthy tissue is important to make the whole protein complement available for proteomic analysis, and to make the best use of a precious resource. Minimal degradation of the protein amino acid backbone, or of phosphorylated amino acid side chains, during sample preparation is essential to preserve the analytical utility of the extract. We have investigated parameters of brain tissue disintegration, and of extraction/solubilization temperature, time and volume and have reached 98% extraction of brain tissue, corresponding to about 100 μg protein per mg tissue wet weight, by an SDS-based method: Tissue disintegration in the frozen state, by ball mill grinding followed by extraction and solubilization in 2% SDS for 10 min, at 70°C, in a volume corresponding to ten times the tissue wet weight, with shaking. The treatment with SDS sample buffer can inhibit protease and phosphatase activity. Moreover, endogenous enzymes can be inhibited by incubation at high pH. The resulting protein extracts can be used for both one-dimensional SDS gel-electrophoresis and for two-dimensional isoelectric focusing/SDS electrophoresis. The proposed standard protocol has the potential to find wide application where protein extraction, solubilization, identification and quantitation from cryopreserved clinical samples are desirable.

The medical value of stored tissue samples has increasingly come into focus. The reasons are at least twofold: 1. identification and quantitation of proteins from human tissue samples have become more rapid and more sensitive with the availability of the human genome sequence, the recent developments in mass spectrometry, other protein analysis technologies [1], and the increasing number of specific antibodies; and 2. with an increasing understanding of fundamental biological processes stemming from research using model organisms and *in vitro* systems, it has become increasingly urgent to test specific hypotheses on patient material, in order to determine its validity for the human condition. Human tissue can now increasingly be investigated directly for alterations from disease by the improved proteomic techniques. Formaldehyde-fixed historical histo-pathological specimens from repositories have been, and continue to be, invaluable in this latter respect. Formaldehyde cross-links the proteins in place, reducing artefactual diffusion or extraction. It allows the investigator to study the tissue and sub cellular distribution of specific proteins in health and disease, by the use of antibodies, most notably by immunohistochemical techniques including, recently, on samples mounted as tissue arrays [2].

Many powerful types of protein analysis, however, such as SDS-PAGE, Western blotting, 2-D PAGE and MALDI mass spectrometry or liquid chromatography and ESI mass spectrometry, for the most part require that the proteins first be extracted and solubilized. These techniques do not always work well with formaldehyde cross-linked material that was not designed to release proteins. To apply these proteomic techniques optimally requires preserving the tissue sample in a native state, free of chemical fixative. Currently the best technique for native tissue preservation seems to be snap freezing, e.g. in liquid nitrogen, followed by cold storage at −70°C, or below. Such samples can be extracted by a variety of techniques, and the solubilized proteins subjected to analysis [3]. New and existing biobanks

Correspondence: Christer Ericsson, Department of Oncology-Pathology, Karolinska Institute, Cancer Center Karolinska, R8:05, Karolinska University Hospital-Solna, 171 76 Stockholm, Sweden.

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are organized to snap freeze as well as formaldehyde fix tissue samples to make the best use of the material.

Sample preparation is widely recognized as being an essential component of any protein analysis [4]. Cryopreserved samples stored in biorepositories are no exception. Since extraction efficiency may vary with different techniques, it is also widely recognized that a reproducible and standardized protocol needs to be adopted to obtain samples that are comparable. As high an extraction rate as possible is desirable to avoid selective extraction, and to use the material with maximal efficiency. There exists an important dividing line between protein solubilization for proteomic techniques designed to identify and quantitate proteins on the one hand, and those designed to study functions, such as enzymatic activities or other binding activities, on the other. Study of identity and quantity can, and should, use much more disruptive, and so more efficient, solubilization schemes. Study of function requires that the extraction be sufficiently non-disruptive that the function under study is preserved, and so inherently is less complete.

Our aim with this work was to, as much as possible, design a tissue extraction method that would be universally applicable for protein identification and quantitation. While in defined cases it is essential to adapt the extraction conditions to the goal at hand [1], we offer a technique that should be useful in the many cases when a specialized extraction technique is not necessary or desirable. We chose to use the well-established ionic detergent sodium dodecyl sulfate in aqueous buffer [5], currently perhaps the most effective general protein extraction and solubilizing agent to complete the protein extraction as much as possible. It has been shown that the SDS extraction method is compatible not only with SDS-PAGE, but also, if SDS concentration is sufficiently low, with 2-D PAGE [6], trypsin digestion followed by liquid chromatography and ESI mass spectrometry [7], ELISA and antibody array [8]. In order to have a well-defined and experimentally relevant source of biological material for testing, we chose mouse brain as a model. Selected results were subsequently validated using patient material. Variables of tissue disintegration and of extraction temperature, volume, and time were studied and optimized. Conditions of protein degradation and degradation of phospho-groups, and inhibition of these processes, were studied in order to minimize these problems in an informed way. Finally, several common and powerful protein analysis techniques were applied to test the utility of the proposed standard extraction protocol.

## Experimental Procedures

### *Patient samples*

Glioblastoma tissue was removed as part of routine diagnosis and therapy. All human samples were from the Department of Neurosurgery, Karolinska University Hospital, Solna, Sweden. The appropriate ethical permission was obtained.

### *Mouse strains and handling*

Wild type C57 Bl 6 mice were used throughout. The mice were kept at the animal facility at the Karolinska University Hospital, Solna, Sweden. The appropriate ethical permission was obtained.

Mice were sacrificed with $CO_2$ and cerebrum was divided into six equally sized pieces.

### *Brain tissue preparation*

A pathologist inspected human samples, and removed representative parts for diagnosis. Surplus material was used for this work. Mouse or human brain samples were placed in sealed plastic bags and were subsequently snap frozen by immersion in liquid nitrogen ($N_2$ (l)) and stored at $-70^{\circ}C$ until used.

### *Frozen sample disintegration*

Sample disintegration was performed by 1. solubilizing intact pieces of tissue by shaking with SDS sample buffer in an Eppendorf tube, 2. grinding the frozen tissue in a mortar, held in $N_2$ (l) followed by shaking with SDS sample buffer in an Eppendorf tube, 3. sonicating on ice in SDS sample buffer in an Eppendorf tube, and 4. using frozen ball mill grinding (Mikro-dismembrator S, B. Braun International) in a cryogenic tube (Corning Incorporated, #2028) with a steel ball, followed by shaking in SDS sample buffer in an Eppendorf tube. The samples in "4/" were cooled in $N_2$ (l) prior to, and after, the sample disintegration, and remained frozen during the entire disintegration procedure.

### *Sample solubilization*

Solubilization of the protein content was done in Eppendorf tubes (Treff Lab AG, #96.8668.9.01) by addition of $1 \times$SDS buffer (2% SDS, pH 6.8 [5]), ten times the weight of the frozen tissue and subsequent incubation at $70^{\circ}C$ and shaking at 1400rpm (Eppendorf, Thermomixer Compact, #5350 000.013) for 10 min. Any remaining solid residue was sedimented at $13.2 \times 10^3$ xg (Eppendorf, Centrifuge 5415 D, #5425 000.219) for 5 min at room temperature.

*Purified proteins*

Ovalbumin (OVA) was from Sigma (A5503). Protein molecular weight standards were from Amersham Biosciences (RPN 5800). MagicMark™XP Western Standard (Invitrogen) standards were used for the Western blots.

*Protease digestion*

Disintegrated brain samples were incubated with trypsin (Sigma T6567) at 1:20 w/w in 100 mM Tris-HCl, pH 8.5.

*Phosphatase digestion*

Disintegrated brain samples were incubated with alkaline phosphatase (Sigma P3681) at 0.2 unit/ml in 1.0 M diethanolamine, 0.50 mM $MgCl_2$, pH 9.8.

*Protein concentration determination*

The protein concentration was determined, using a commercial assay (BIO-RAD, RC DC Protein Assay #500-0119) and using bovine serum albumin (BIO-RAD, Protein Standard II bovine serum albumin #500-0007) as the standard.

*Soluble sample storage*

The solubilized protein samples were frozen and stored at $-20^{\circ}$C until used.

*Sample preparation*

Protein samples were in some cases purified by precipitation with the 2-D Clean-Up Kit (Amersham Biosciences), followed by resolubilization.

*One dimensional electrophoresis*

The protein expression was analyzed by SDS-PAGE electrophoresis using either manually prepared gels (10%) [5] or a commercial system (Invitrogen, Nu Page System and Nu PAGE 10% Novex Bis-Tris Gels, NP0301BOX). Equal amounts of proteins were loaded into each lane. Whole protein amount was verified using a total protein stain (see below). Parallel gels were in some cases blotted onto nitrocellulose paper as described below.

*Two dimensional electrophoresis*

2-D Clean-Up Kit (Amersham Biosciences) treated samples were solubilized in 7M urea, 2M thiourea 2-D PAGE sample buffer and loaded onto 7 cm pI 3–11 NL isoelectric focusing strips (Amersham Biosciences). The samples were focused; the strips equilibrated in SDS sample buffer, and the second dimension SDS-PAGE performed [9].

*Protein detection*

The gels were immersed in 10% methanol/ 7% acetic acid to fix the separated proteins. The proteins were detected using either SYPRO Orange or SYPRO Ruby (Molecular Probes) [10]. The gels were scanned on a BioRad FX laser scanner, and the resulting images analyzed using the PDQuest 7.2 software package. Images were exported as TIFF files.

*Phosphoprotein detection*

The protein samples were either delipidated and desalted by extracting with methanol and chloroform, or by precipitation with the 2-D Clean-Up Kit (Amersham Biosciences) and subsequently dissolved in 1 × SDS buffer (2% SDS). SDS-PAGE electrophoresis was performed and the gels immersed in 50% methanol/ 10% acetic acid to fix the separated proteins. The phosphoproteins were detected using the Pro-Q Diamond Phosphoprotein Gel Stain (#MP-33300, Molecular Probes) [11], followed by total protein detection.

*Western blot*

The gel-separated proteins were transferred [12] to a nitrocellulose paper (Schleicher & Schuell, Protran BA 83 Cellulosenitrate #AT0894-1). The following antibodies were used: anti ERK (p44/42 MAP Kinase) (Cell Signaling Technology, #9102), anti pERK mouse monoclonal antibody (Santa Cruz Biotechnology Inc, #sc-7383), anti alpha tubulin (Abcam, ab7750), anti-SAPK/JNK (Cell Signalling, #9252), and anti Na/K ATPase (Abcam, ab7671). An HRP conjugated anti rabbit secondary antibody was used, diluted 1:1000 (Amersham Biosciences, #NA934V) or an HRP anti mouse antibody diluted 1:1000 (Amersham Biosciences, #NA931V). The papers were then developed, using chemiluminescence (Amersham Biosciences, ECL #1059250) and imaged on a Fuji Film LAS 1000 cooled CCD camera.

## Results

*Tissue disintegration*

A variety of common disintegration methods were tried, followed by SDS sample buffer extraction, for their extraction efficiency, measured as percent of tissue wet weight that becomes non-sedimentable by centrifugation. The data are summarized in

Figure 1. It is clear that sonication and frozen ball-mill grinding were the most efficient.

*Protein extraction and solubilization*

We extracted and solubilized brain tissue, following ball mill grinding, under different conditions of temperature, time and volume to determine the optimal parameters. Non-sedimentable wet weight after solubilization relative the original frozen wet weight (extraction), as well as total amount of soluble protein relative original frozen wet weight (solubilization), were used as criteria. Figure 2 shows the effect of temperatures 70°C to 95°C on amount extracted. The different temperatures gave essentially equal extraction efficiency. When comparing the amount of three specific proteins available for a Western blot assay, we found that 70°C solubilization gave a higher amount of available Na/K ATPase and SAPK/JNK proteins measured as Western blot signal, than either 80°C or 95°C solubilization, even though equal amount of protein was loaded in each lane. The p44/42 MAPK signal appeared less sensitive, if at all, to variation in the extraction temperature.

Figure 3 shows the effect of increasing the extraction/solubilization volume from 2.5 times the tissue wet weight to 40 times the wet weight. A plateau of maximal extraction is reached at a volume of ten times the tissue wet weight.

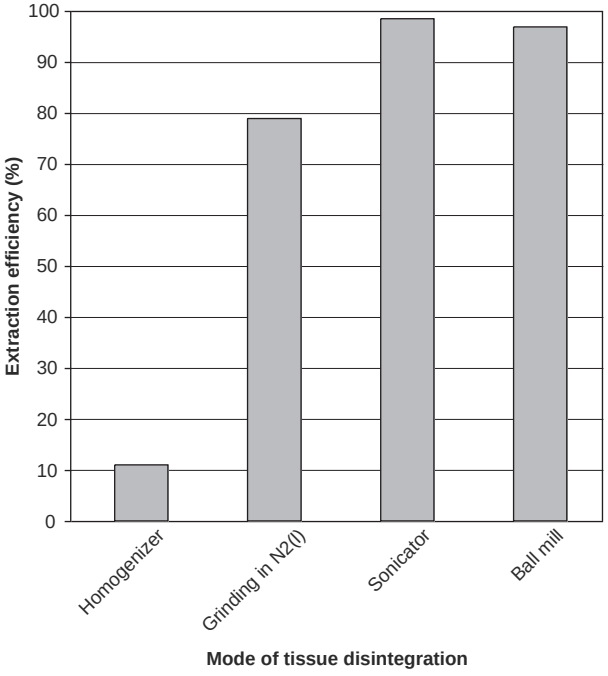


Figure 1. Comparison of the relative amount of tissue extraction following various disintegration methods. 1. Manual disintegration of tissue in Eppendorf tube with miniature pestle, 2. Grinding of the frozen tissue in a mortar cooled on liquid nitrogen, 3. Sonication on ice, 4. Ball mill grinding of frozen samples in equipment cooled in liquid nitrogen. The solid residue following extraction was determined and related to the initial wet weight. The fraction of initial wet weight that was extracted is plotted.

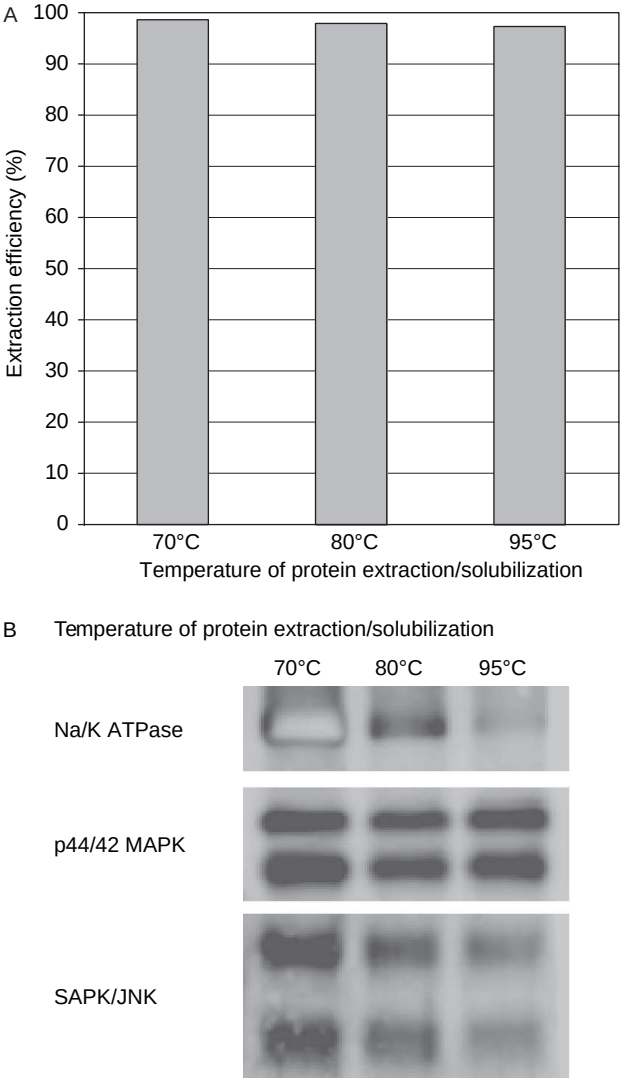


Figure 2. Effect of temperature on tissue extraction. Ball mill disintegrated samples were suspended in 10 ×v/w sample buffer and incubated for 10 min at the indicated temperatures. The solid residue following extraction was determined and related to the initial wet weight. The samples were then subjected to a protein content determination and subsequent Western blot immunoassay. A. The fraction of initial wet weight that was extracted was plotted. B. Western blot assayed with antibodies to Na/K ATPase, P44/42 MAPK and SAP/JNK. 50 μg of protein was loaded in each lane.

Figure 4 shows the effect of extraction/solubilization time tested from 2 min to one hour and measured as relative amount of tissue extracted. There is no observable difference in extracted tissue amount between 2 min and one hour. We did not try shorter extractions than 2 min.

In summary, about 98% of the initial wet weight of tissue becomes resistant to centrifugation (extracted) under the optimized conditions. This corresponds to

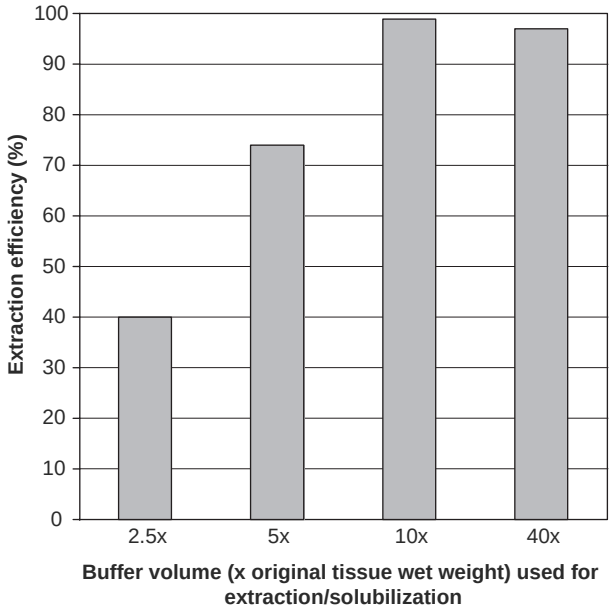


Figure 3. Effect of buffer volume on tissue extraction. Ball mill disintegrated samples were suspended at the indicated v/w in sample buffer, and incubated at 70°C for 10 min. The solid residue following extraction was determined and related to the initial wet weight. The fraction of initial wet weight that was extracted was plotted.

about 100 μg solubilized protein per mg wet weight of brain tissue.

*Protein degradation*

We have chosen to study two parameters of protein degradation that are frequently of concern: amino acid backbone degradation and degradation of the amount of posttranslational protein phosphorylation. The mouse total brain protein extracts and OVA were sensitive to proteolysis by added trypsin (Figure 5A, compare lanes 2, 3 and 4, and lanes 7, 10 and 11). Arrows indicate the degradation fragments of OVA in lane 10. The sensitivity to proteolysis, from endogenous proteases, increased with lower pH of the solvent in the interval 8.5 to 4 (compare lanes 3, 5 and 6). While there was no observable degradation at pH 8.5, there was considerable degradation at pH 7. The most extensive degradation in the experiments was at pH 4.0. From a practical standpoint, solubilization of protein extracts in pH 8.5 buffer seems protective against proteolysis. These observations are confirmed when studying the amount of tubulin in the same extracts by Western blot (Figure 5B, compare lanes 1, 2, 4 and 5). The result presented in Figure 5A (compare lanes 7, 8, 9, 10 and 11) shows that SDS can be a powerful protease inhibitor.

The total mouse brain protein extract and OVA were noticeably reduced in detectable phosphate groups by treatment with alkaline phosphatase (Figure 6A and B, compare lanes 2, 3, 4 and lanes 7, 9, 8). The total brain protein pattern was not noticeably altered by the phosphatase treatment. The phosphoprotein ovalbumin (OVA) was used as a control. Treatment with alkaline phosphatase removed the phosphate groups efficiently from OVA as evidenced by a shift to lower apparent molecular weight (Figure 6A) and by a loss of ProQ Diamond reactivity (Figure 6B) (compare lanes 7–11).

The sensitivity of protein phosphorylation to endogenous phosphatases was investigated by incubating mouse brain protein extracts in three different pH buffers ranging from pH4 to pH 9.8 (Figure 6A and B, compare lanes 2, 3, 5 and 6). Incubation of mouse brain protein extracts at pH 4 caused a marked reduction in detectable protein phosphorylation. The effect was smaller at pH 7 (lane 6) and at pH 9.8 (lane 3). The experiment where the alkaline phosphatase was treated with SDS (lanes A10 and B10, compared to lanes 7, 9 and 11) shows that SDS effectively inhibits alkaline phosphatase. Moreover, phospho ERK was markedly sensitive to incubation at pH 7.0 and pH 4.0 (Figure 6C). Therefore treatments at high pH or with SDS are effective means to reduce phosphatase activity as well as protease activity.

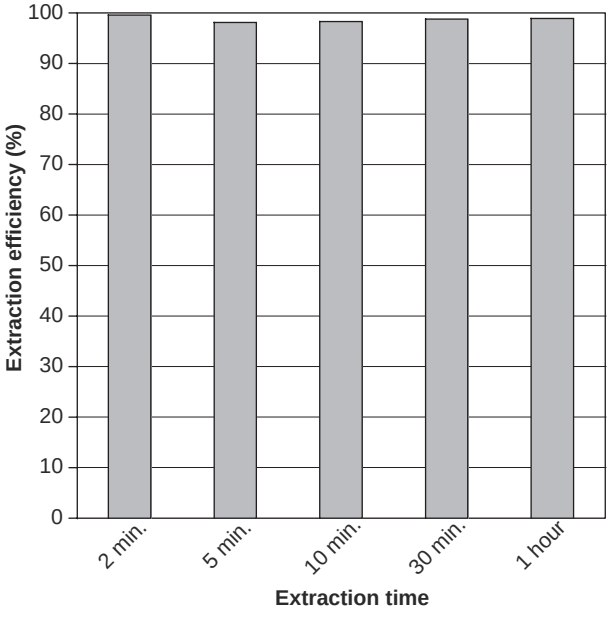


Figure 4. Effect of time on tissue extraction. Ball mill disintegrated samples were suspended in 10 × v/w sample buffer and incubated for the indicated times at 70°C. The solid residue following extraction was determined and related to the initial wet weight. The fraction of initial wet weight that was extracted was plotted.

*Effect of freezing and thawing*

A sample of solubilized tissue proteins was subjected to 20 cycles of freeze (liquid nitrogen) and thaw (37°C water bath) and compared to an untreated control. We could observe no major effect on total solubilized protein amount and only a slight decrease in the amount of a specific protein, Na/K ATPase (Figure 7).

*Reduction of SDS concentration for proteomic analysis*

The efficiency of the SDS extraction would be especially beneficial for analysis of precious human samples. In order to use this benefit maximally it will be important to validate the extracts with a high-resolution proteomic technique such as 2-D PAGE. 2-D PAGE requires SDS concentration to be 0.25%, or less. We have explored dilution or precipitation/resolubilization to lower the amount of SDS in 2% extracts sufficiently to perform 2-D PAGE experiments. Samples with high protein concentration worked well with both approaches (data not shown). Samples with low concentration of protein, however cannot achieve optimal protein concentration when diluted to the required low SDS concentration. In order to have a method that would accommodate both high and low protein concentrations, we used a commercial protein precipitation/resolubilization method: 2-D Clean-Up Kit (Amersham Biosciences). A frozen sample of human glioblastoma tissue was extracted in SDS, precipitated by 2-D Clean-Up Kit treatment, and completely resolubilized in 2-D PAGE sample buffer, and then subjected to 2-D PAGE followed by SYPRO Ruby staining (Figure 8). There was a sufficiently low conductivity to avoid overheating and little, or no, horizontal streaking, indicating that the SDS-level was brought down to levels that are compatible with 2-D PAGE.

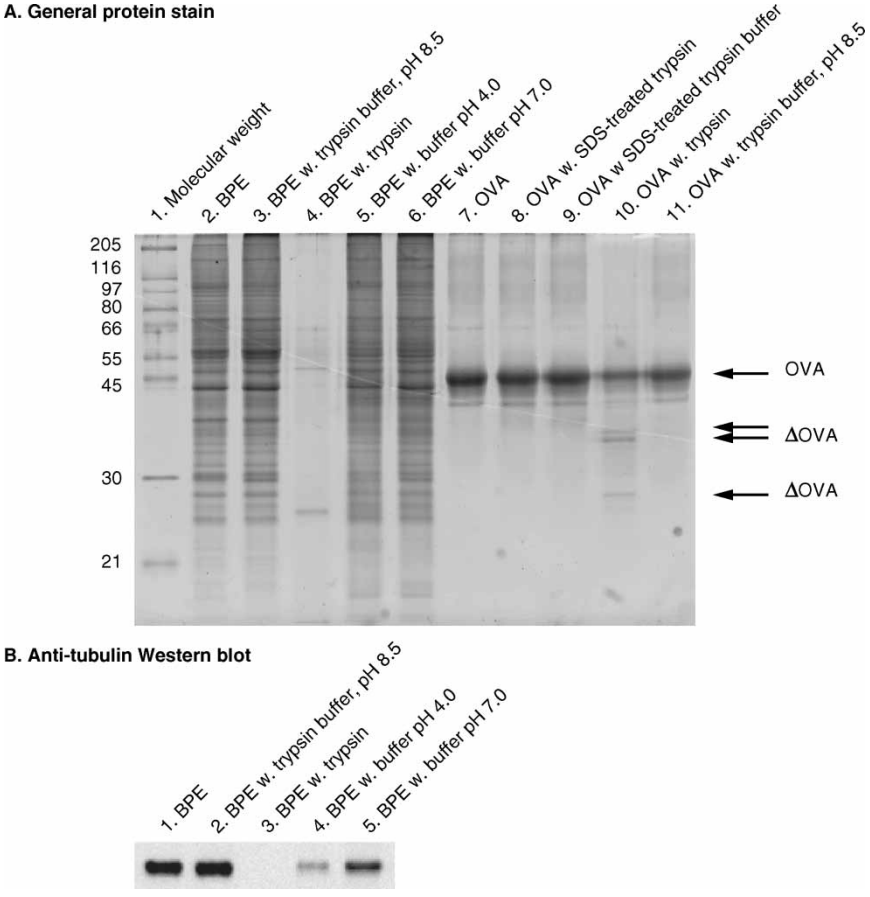


Figure 5. Proteolytic activity in the tissue sample. Mouse brain protein samples corresponding to 50 μg *before treatment* were loaded in the indicated lanes. Ovalbumin (OVA) samples corresponding to 1 μg *before treatment* were loaded in the indicated lanes. Disintegrated samples were either solubilized directly in SDS sample buffer or were suspended in the indicated buffer and incubated at 37°C, and then solubilized in SDS. A. Total protein detected by SYPRO Ruby and B. Western blot of the same protein extracts as in A, detected with an antibody to tubulin, lane 1-Molecular weight markers, lane 2-brain protein extract (BPE), lane 3-BPE in trypsin digestion buffer without enzyme, lane 4-BPE in trypsin digestion buffer with enzyme, lane 5-BPE in phosphate buffer, pH 4.0, lane 6-BPE in phosphate buffer pH 7.0, lane 7-ovalbumin (OVA), lane 8-OVA in trypsin digestion buffer *with* enzyme, pretreated with SDS, lane 9-OVA in trypsin digestion buffer *without* enzyme, pretreated with SDS, lane 10-OVA in trypsin digestion buffer *with* enzyme, Lane 11-OVA in trypsin digestion buffer *without* enzyme.

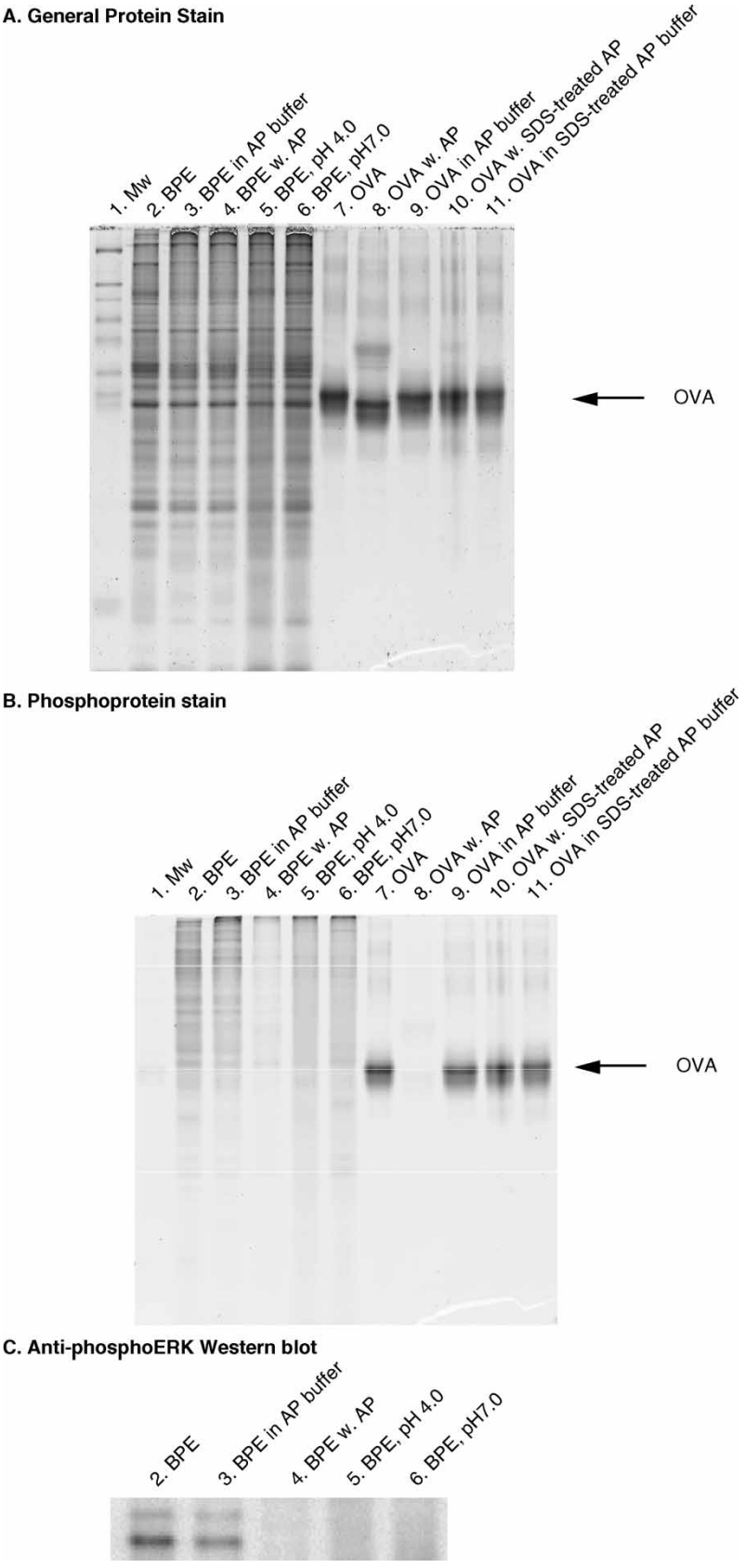


Figure 6 (*Continued*)

## Discussion

Diseased tissue is expected to contain many differences in its protein complement from healthy tissue. An accurate representation of the samples requires that tissue-handling and protein-extraction does not introduce alterations in the protein complement.

There exists a need for standardized and optimized protein extraction protocols that minimize sample loss, or other degradation, and are compatible with contemporary proteomic technologies. The chemical diversity of proteins makes this challenging. While it may not be possible to devise a single extraction protocol for all proteomic applications, we would like to suggest that for many applications a simple and straightforward disintegration, extraction and solubilizing scheme with the powerful solubilizing agent SDS might fulfill the requirements, especially when combined with methods to subsequently reduce the SDS concentration to levels that do not interfere with downstream analysis.

### *Tissue disintegration*

It would be expected that disintegration methods that yield the smallest tissue fragments would be the most effective in terms of achieving a high extraction yield, due to the high surface to volume ratio. Our results generally confirm this supposition, in that sonication and frozen ball mill grinding allowed for the most efficient extraction of the tissue. A hand-held homogenizer was much less efficient. Also tissue that was ground by mortar and pestle had a suboptimal yield. We attribute this to the generation of variably sized tissue fragments. For research on human specimens there are several additional parameters to consider: 1. Since patient tissue is a limiting resource it is essential to minimize losses in handling. Having the entire disintegration and solubilization procedure in a contained space, in a cryotube, such as those used for ball mill grinding best prevents losses. Sonication results in the loss of material due to aerosol formation. These aerosols are also a potential source of contamination and infection and so are less desirable; 2. Methods that allow the tissue specimen to be contained, such as ball mill grinding, are also preferable due to the reduced risk of exposing personnel to known or unknown contagions. In addition, 3. current sonicators require a probe to be inserted in the sample. These probes provide a potential source of contamination of the sample, since they are reused. The cryotubes and steel balls used for ball mill grinding are disposable; 4. Methods that can be performed in the frozen state, such as ball mill grinding, are preferable to those that require thawing due to the reduced risk of artefactual protein degradation. In all these respects ball mill grinding is superior to sonication, while being similar or equal in terms of amount of protein extracted. We have therefore used

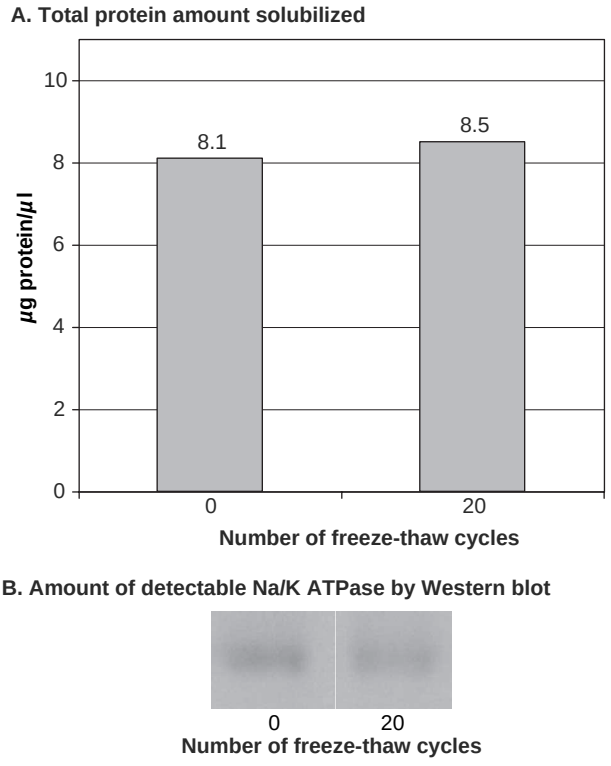


Figure 7. Effect of freeze-thaw cycles on sample usability. A. Total protein determination, B. Tubulin detected by Western blot analysis.

Figure 6. Protein phosphatase activity in the tissue sample. Brain protein samples corresponding to 50 µg were loaded in the indicated lanes. Purified protein samples corresponding to 1 µg were loaded in the indicated lanes. Disintegrated samples were either solubilized directly in SDS sample buffer or were suspended in the indicated buffer and incubated at 37°C, and then solubilized in SDS. A. General protein staining by SYPRO Ruby (Molecular Probes, Inc), B. Phosphoprotein staining by ProQ Diamond reagent, and C. Anti-phosphoERK Western Blot. Lane 1, molecular weight markers, including the phosphoprotein ovalbumin, lane 2-brain protein extract (BPE), lane 3-BPE incubated in alkaline phosphatase digestion buffer, pH 9.8, *without* enzyme, lane 4-BPE incubated in alkaline phosphatase digestion buffer, pH 9.8, *with* enzyme, lane 5-BPE incubated in phosphate buffer, pH 4.0, without enzyme, lane 6-BPE incubated in phosphate buffer, pH 7.0, without enzyme, lane 7-OVA, a phosphoprotein, lane 8-OVA incubated with alkaline phosphatase in digestion buffer, lane 9-OVA incubated in alkaline phosphatase in digestion buffer *without* enzyme, lane 10-OVA incubated with alkaline phosphatase, *pretreated with SDS*, in digestion buffer, lane 11-OVA incubated with alkaline phosphatase digestion buffer without enzyme, but pretreated with SDS. The specificity of the ProQ Diamond detection of phosphoproteins is demonstrated by its reactivity only with the OVA molecular weight marker (lane A1 compared to lane B1) and not with the other non-phosphoprotein molecular weight markers, as well as by the loss of ProQ reactivity upon alkaline phosphatase treatment of OVA.

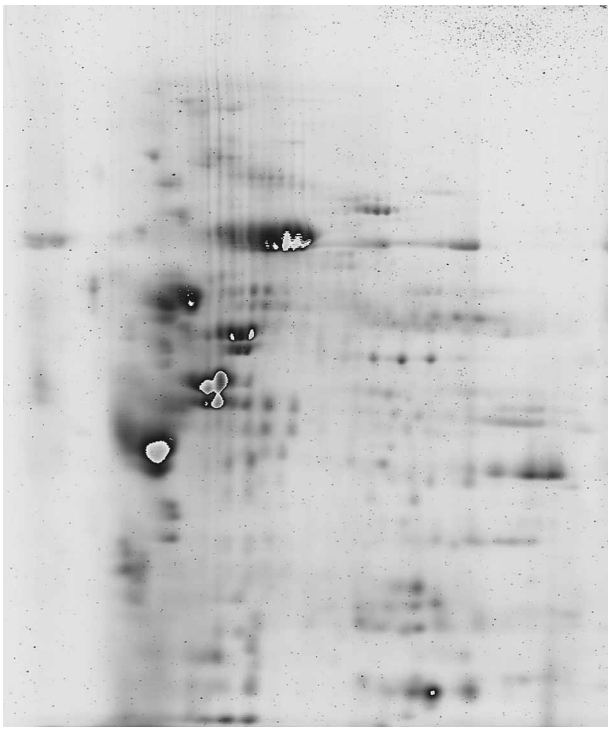

Figure 8. 2-D PAGE of human glioblastoma solubilized in SDS. The 2% SDS extracted sample was precipitated using the 2-D Clean-Up kit, resolubilized in 2-D PAGE sample buffer, resolved by 2-D PAGE followed by protein detection by SYPRO Ruby.

the ball mill grinding in all further work and recommend this disintegration method for routine purposes.

*Protein solubilization*

The proteins in the disintegrated tissue need to be extracted and solubilized as efficiently as possible. We tested several parameters. As expected the solvent becomes saturated by high concentration of protein. The plateau is reached as the volume of solvent corresponds to about ten times the wet weight of the tissue (10 ×v/w). We therefore suggest 10 ×v/w as a safe volume to use for maximal solubilization. The time of solubilization is optimal already at the shortest time we measured, 2 min. The most remarkable observation was that although the total amount of extractable and solubilized protein was about the same, the amount of available protein in a Western blot assay decreased with increasing temperature in the same interval. We hypothesize that at the higher temperatures some proteins form low speed centrifugation non-sedimentable aggregates. These aggregates may be soluble as assayed by low speed centrifugation, but may still not enter an electrophoretic gel. The observation that total protein extracted at the various temperatures remains about the same, while the availability of some specific proteins goes down, would suggest that the phenomenon is restricted to only certain proteins. This conclusion is supported by the observation that p42/44 MAPK availability in a Western blot assay stays about the same. This finding is also similar to the decrease in detectable titin from muscle when heated above 73°C [13]. In weighing the effects on total tissue solubilized and amount of protein actually detected, it would seem that 70°C is the optimal temperature for solubilization, among those tried.

In distributing samples from a frozen biorepository, it may sometimes be advantageous to distribute samples of optimized extracts, rather than the frozen tissue itself. Researches requesting samples from the biorepository would be given the option to request an aliquot of such a standard extract in amounts required for his or hers application. In this way each and every scientist would not have to request an entire piece of tissue. A simple calculation, assuming a 100 mg piece of tissue, indicates that about 10 mg of protein would be extracted. A typical experiment may require about 50 μg of total tissue protein (e.g. one lane in a Western blot). If so, about 200 experiments could be performed with the material from one piece of tissue. If each investigator requested enough material for 20 experiments, then a piece of tissue would be enough for ten scientists, rather than perhaps only one, if each scientist requested one piece of tissue each. An additional advantage would be that the solubilization would be performed under quality-controlled conditions, adding a level of reproducibility as well as saving on laboratory time. The extraction and solubilization method for clinical samples is summarized in Figure 9.

*Protein degradation*

It is highly desirable that the proteases be inhibited during protein disintegration, when subcellular compartments that are kept separate *in vivo* are mixed, exposing the cellular proteins to secreted, lysosomal and other digestive enzymes they are not normally exposed in this way. We consider it another advantage of ball mill grinding, that it is performed in the

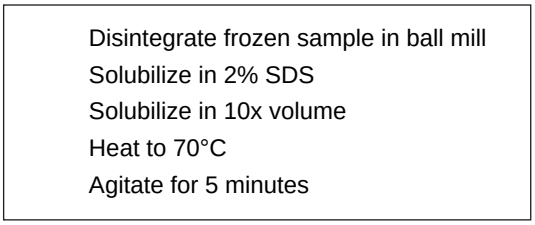


Figure 9. Recommended procedure for tissue protein extraction by SDS.

frozen state, thereby all but guaranteeing that the tissue proteases and phosphatases will not have an opportunity to degrade the cellular proteins, during disintegration.

Our experiments with incubating disintegrated tissue at various pH, in the absence of SDS, show that there does indeed exist endogenous enzymes that could degrade the proteins. The observation that the degradation occurs most efficiently at pH 4 suggests that lysosomal enzymes may predominate under these conditions. It would therefore seem advisable to avoid any lengthy incubation of the thawed protein homogenate, in the absence of SDS, especially at low pH. It is interesting to compare this result with those of Castellanos-Serra and Paz-Lago who found degradation of marker proteins by added protease, even at pH 11.0 [14]. As pointed out by the authors, the added enzymes had alkaline pH optima. In our experiments, the proteases under study were the endogenous brain proteases. We therefore feel that there is no contradiction between the results. It would therefore seem that whenever incubation of brain protein extract is required, it would be advisable to perform those incubations at an elevated pH, whenever possible, in order to minimize proteolysis.

SDS was shown to be a powerful inhibitor of both added trypsin and of added alkaline phosphatase. It is interesting to compare also this result with those of Castellanos-Serra and Paz-Lago [14] who found degradation of a marker protein by trypsin even after boiling in 1% SDS. The present experiments were designed slightly differently from those of Castellanos-Serra and Paz-Lago, in that in the present experiments the trypsin and alkaline phosphatase were heated with SDS prior to adding to the protein substrate, while in their experiments the enzyme was added, followed by heating. We think this small difference in procedure explains the slightly different results. We suspect that the enzymes degraded their substrate in the short period between addition and heating. In our experiments no such possibility existed, since the enzyme was pretreated with hot SDS. We think this comparison underscores the importance of a quick denaturation of enzymes to avoid degradation.

It is shown by our results that SDS can cause an efficient inhibition of enzymatic activity. If this were true for proteases and phosphatases generally, there would be no need to add specific inhibitors for the purpose of the present type of experiment. While we have no indication from the current experiments that there exists any residual protease activity following treatment with hot SDS, it deserves pointing out that these results do not guarantee the integrity of protein extracts when incubated for longer times than those used in the experiments presented here. Given that some protease inhibitors are peptide-like and that downstream analysis may involve peptide analysis, it is clear that it is sometimes a disadvantage to add this class of inhibitors. The samples stored in a biorepository may in some cases be stored for many years. It is therefore, in principle, desirable not to make any additions to the samples that are not absolutely necessary, no matter how innocuous they may seem today. New and unforeseen analytical methods may have been developed in the future, and may be sensitive to additions that are considered harmless today.

### *Effect of freezing and thawing*

Our study of the effect of freezing and thawing an SDS solubilized tissue extract indicates that by the criteria used the extracted protein could safely be frozen and thawed 20 times without observable loss in total protein or major loss in the membrane protein Na/K ATPase. While this is an encouraging observation, additional studies are required to see whether this is true generally. If this was found to be the case it would obviate the need to aliquot samples and so would be space-efficient for, for example, a protein extraction core facility associated with a biorepository.

### *Reduction of SDS concentration for proteomic analysis*

Maximal protein extraction and solubilization in SDS containing buffer requires an initial excess of SDS, frequently standardized to be 2% SDS. However, some powerful analytical applications, such as 2-D PAGE, immunoassays or trypsin digestion/ESI-MS, are sensitive to concentrations of SDS above about 0.2%. To combine the advantages of a high SDS concentration during extraction and solubilization, with the requirements for a low SDS concentration for downstream applications, requires lowering the SDS concentration post-solubilization. Only those protein samples that contain a relatively high original protein concentration can be diluted the required eight-fold or more and still have an optimal protein amount in the maximal volume for loading on an isoelectric focusing strip for 2-D PAGE. For this reason it may be convenient to use a precipitation/resolubilization method, as a standard procedure to lower SDS concentration. The precipitated proteins can then be solubilized in a suitable solvent and at a suitable concentration. In order to demonstrate the potential for use of a high resolution proteomic technique that would require low SDS concentration and that would accommodate both high and low initial protein

concentrations, we treated a 2% SDS extract of a human glioblastoma with a protein precipitation/resolubilization method: 2-D Clean-Up Kit, followed by 2-D PAGE. We found that the precipitated sample could be completely resolubilized in 2-D PAGE sample buffer, with no detectable sedimentable residue, and that a high-resolution separation was achieved. Refinement of this procedure and testing of other applications are challenges for the future.

## Conflict of interest

The authors have no personal or financial relationships that might bias their work. The funding sources had no involvement in the design, analysis or interpretation of the report, or in the decision to submit the report for publication.

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