Total Protein Extraction from Cultured Cells for Use in Electrophoresis and Western Blotting


ABSTRACT

Experimentation with cultured cells often requires analyzing cellular protein extract by gel electrophoresis and immunoblotting. Traditional methods for extracting cellular proteins by homogenization or detergent solubilization usually produce protein samples that are viscous (due to the presence of DNA) and prone to degradation due to the presence of endogenous protease activity. We have developed a method that involves solubilization of cells with sodium dodecyl sulfate (SDS), precipitation of proteins with trichloroacetic acid (TCA) with special physical excitation of DNA aggregate and reconstitution of precipitated protein with Tris base. These protein samples prepared by this method contain little DNA, making them ideal for long-term storage. The solubilized total protein extract is fully compatible with protein assay, gel electrophoresis and Western blotting. When compared to protein extract from a homogenization method, those from the TCA method showed an identical total protein staining pattern on SDS polyacrylamide gel electrophoresis and contained distinct cellular proteins recognized by many monoclonal and polyclonal antibodies (including anti-actin, spectrin, protein kinase C (4λ), tubulin and spectrin) on Western blots.

INTRODUCTION

For experimentation with cultured cells that investigate protein degradation or modification such as changes in protein expression, degradation and phosphorylation state, analysis of total cellular protein extract by polyacrylamide gel electrophoresis (PAGE) and immunoblotting are often utilized (8–10,13–15,18). Existing methods for protein extraction usually call for certain mechanical disruption of the cells by homogenization, sonication or shearing (2,6,8,15). Also, protein degradation and other modifications during both the protein extraction procedure and long-term storage are potential problems, unless effort is made to arrest endogenous protease/enzyme activity permanently (11). Another issue is the effectiveness of the physical disruption since cells may not be fully lysed by methods such as homogenization. An alternative sometimes used is direct lysis of cells with Tris-glucose buffer containing sodium dodecyl sulfate (SDS) (9). However, the SDS in these samples interferes severely with either the Bradford's or the Lowry protein assay (3,7,14). Our attention was then turned to developing a novel method of total protein extraction for cultured cells. Several criteria to be satisfied by a new method were simplicity, rapid arrest of most endogenous enzymatic activities, cellular DNA exclusion, compatibility with protein assays, compatibility with PAGE, Western blotting and antibody binding, and long-term stability of samples.

MATERIALS AND METHODS

Materials

The DC protein assay kit was purchased from Bio-Rad (Hercules, CA, USA), Calcium Iodophore A23187 (A23187), dibutylribofuranoside (DTF) and EGTA were from Sigma Chemical (St. Louis, MO, USA), MEM medium, RPMI medium and serum were from Life Technologies (Gaithersburg, MD, USA). Anti-spectrin monoclonal antibody was from Affiniti Research Products (Nottingham, England, UK) and anti-talin monoclonal antibody and anti-actin polyclonal antibody were from Chemicon International (Temecula, CA, USA). Anti-protein kinase C (α isoform) was from Transduction Laboratories (Lexington, KY, USA).

MOLT-4 Cultures

MOLT-4, a leukemia cell line (ATCC, Rockville, MD, USA), was maintained in suspension in RPMI medium with 10% (vol/vol) fetal bovine serum, penicillin-streptomycin and fungizone. Before use, the cells were washed three times with serum-free RPMI medium and resuspended to 1.5 million cells/ml with medium and transferred to a 12-well plate (0.5 ml/well). To activate endogenous calpain, 20 μM A23187 were then added, and the cells were further incubated for 90 min in a 37°C incubator (17). The cell suspension was then subjected to protein extraction as described below.

Fetal Rat Cerebrocortical Cultures

Fetal rat cortical cells were harvested and cultured in a 12-well plate format as described previously (15). On the 17th day post-plating, the cultures were used for protein extraction.

Homogenization Protein Extraction Method

Cerebrocortical cultures in a 12-well plate format at 1 milliliter/cell/ml were washed with 20 mM Tris-HCl (pH 7.4) at 25°C, 150 mM NaCl, 1 mM EDTA (TBS-EDTA). Cells were then collected by scraping in 300 μl of homogenization buffer containing 20 mM Tris-HCl (pH 7.4 at 4°C), 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 5 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml N-acetyl-L-cysteine (N-ac-L-cys) (TPCK) and 10 μg/ml N-acetyl-L-phenylalanine chloromethyl ketone (TPCK) and transferred to a mini-homogenization tube (size 18; 1.5 ml capacity; Kontes, Vineland, NJ, USA). For MOLT-4 cultures in suspension, 3–6 million cells were washed with 10 ml of TBS-EDTA and centrifuged for 4 min (2000g; H1000B rotor; SORVALL, R18000D Centrifuge; Du Pont, Wilmington, DE, USA) three times. Cell pellets were resuspended to 30 μl with homogenization buffer and transferred to a mini-homogenization tube. Cells were homogenized by 10 passes at room temperature. For rat brain samples, portions of fresh brain (ca. 3 g) were chopped into pieces and homogenized in three volumes of homogenization buffer with 10–15 passes at 4°C. All samples were aliquoted and quick-frozen with isopropyl alcohol and stored at −70°C until use.

TCA Protein Extraction Method

For attached cerebrocortical cells in a 12-well plate format, medium was removed from wells, and cells were washed with 1 ml TBS-EDTA (20 mM Tris-HCl at pH 7.4, 155 mM NaCl, 1 mM EDTA) twice at room temperature.

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Lysis buffer (300 µL) containing 2% (w/vol) SDS, 5 mM EGTA, 5 mM EDTA, 0.5 mM PMSE, 10 µg/mL AEBSF, 5 µg/mL leupeptin, 10 µg/mL pepstatin, 10 µg/mL TLCK, 10 µg/mL TPCK, 20 mM Tris-HCl (pH 7.4) at room temperature was added. The cells were allowed to lyse for 10–15 min. One hundred microliters of 100% (v/vol) trichloroacetic acid (TCA) were added to the lysate. DNA aggregate was removed while protein precipitate suspension from three wells was collected into microcentrifuge tubes. After centrifugation (3600 × g for 5 min), pellets were washed with 1 mL of 2.5% (v/vol) TCA. The final pellets were neutralized with 25 µL of 3 M Tris base for 30–60 min and then diluted with 25 µL of water. For cells in suspension (MOLT-4), 3–6 million cells in 0.5 mL were added to one well, and 50 µL of 100 mM EDTA were added before 500 µL of lysis buffer were added. The rest of the procedure was the same as above. For rat brain samples, portions of fresh rat brain (3–5 g) were cut into pieces and then homogenized using a mini-homogenizer with 10 passes in a homogenization buffer (300 µL) (see above). The homogenate was then transferred to a 12-well plate and 50 µL of 100 mM EDTA and 500 µL of lysis buffer were added. The rest of the procedure was identical as described above.

**Electrophoresis and Western Blot**

The solubilized protein samples were analyzed for protein concentration using a modified Lowry assay (Bio-Rad (14)). Whole cell protein samples (50 µg) were run on SDS-PAGE (120 V, 2.3 h) in a mini gel unit with the Tris-glycine running buffer system (25 mM Tris base, 192 mM glycine, 0.1% [w/vol] SDS [pH 8.3]) and transferred onto a PVDF membrane (Novex, San Diego, CA, USA) at 0.55 A for 2 h in a Tris-glycine buffer system. The blots were probed with the first antibody overnight at 4°C, a biotinylated second antibody (1:500) for 1 h with streptavidin-alkaline phosphatase conjugate (1:5000) for 30 min (18). The blots were developed with substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indoly phosphate.

**RESULTS AND DISCUSSION**

**The TCA Protein Extraction Method**

During the development of this novel method for cellular protein extraction, we took advantage of the facts that SDS is not precipitated by TCA at room temperature, and cellular proteins and DNA are precipitated by TCA in a different manner; DNA tends to produce a filamentous aggregate, while protein precipitate exacts as a suspen-
tion of fine powder. Slightly different methods were derived for attached and suspension cells.

Cells in suspension (e.g., leukemic MOLT-4 cells) were washed and resuspended to 6 million/ml with medium without serum, and 0.5 mL was added to each well. Cell treatment was initiated at this point (e.g., by adding stimu-

lus to evoke a response). After the ex-

periment, we chose not to remove the

incubation media, as any such attempts would potentially result in cell loss and

introduction of variability. Instead, 10 mm EDTA were added to chelate calci-

um and other metal ions in the media, preventing activation of proteases in-

cluding the calcium-activated protease once cells were lysed. A lysis buffer that

contains 2% (v/v) and a protease inhibitor cocktail (see Materials

and Methods) were then added to achieve a final SDS (30% SDS). It general-

ly took about 5–15 min to achieve com-

pletely lysis as evident by the formation of a clear but viscous solution. TCA

was added to achieve a final concentra-

tion of 10% (w/v). This caused preci-
sipation of both cellular proteins and

DNA in 10–20 s.

For adherent cell cultures (such as cerebrocortical cultures), cells were

washed with and maintained in 1 mL of

medium without serum. Cell treatment

was initiated if desired at this point.

After cell treatment, medium was

removed by aspiration, and attached

cells were washed three times with an

isotonic buffer containing 1 mM EDTA

(TBS-EDTA). This step removes

media, medium, and other constituents in the me-

dia. Cells were then lysed with the ad-

dition of a lysis buffer as described

above. Upon complete cell lysis, a TCA

solution was then added to precipitate proteins.

At this point, the procedure for fur-

ther protein extraction is identical with

either method. We removed the DNA

aggregate with a pipet tip using a slow

circular motion. The protein precipitate

was transferred to micro-

centrifuge tubes within 5 min. A longer

delay sometimes leads to the attach-

ment of protein precipitate to the plas-

tic plate; this results in decreased pro-

tein yield. It is also important to stress

that this procedure must be done at

room temperature, since at 4°C, SDS

precipitates out of solution, which com-

plicates the protein extraction and sub-

sequent protein assay. The collected

protein samples were then centrifuged

and the protein pellets washed once or

twice with 2.5% TCA. The final protein

pellets were dissolved in 3 M Tris base.

It takes up to 20–30 min for full protein

resolution. Samples were stored at

-20°C until use.

Protein Extract Characterization

It appeared that the protein samples

generated had very little residual

SDS left, probably because of the

washing of the protein prints with

TCA. As a result, we encountered little

problem in directly assaying the

samples for protein concentration

using either the Bradford method or the

Lowry method (data not shown) (3,14).

For comparison, we carried out a tradi-

tional protein extraction method in

which MOLT-4 cells were homo-

geinized (see Materials and Methods for
details). We examined the protein

pattern on PAGE for samples devided

from the two methods by analyzing an

equal amount of protein. The protein

pattern of these samples was virtually

identical (Figure 1A). Western blotting

for similar samples was also per-

formed. The blot was then probed with

a monoclonal antibody against a

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cytoskeletal protein, talin. Samples from both methods showed a major band at 250 kDa, corresponding to the molecular weight of talin (Figure 1B). In terms of protein recovery, the homogenization method yielded 39.5 ± 1.2 μg/million cells (n = 3), while the TCA method yielded 12.7 μg/million cells (n = 3). The lower yield in the TCA method could be due to adhesion of protein precipitate to the plastic plates. However, the yield of about 12 μg/million cells using the TCA method was still reasonable.

For adherent cells (such as cerebrocortical cultures), an advantage of the TCA method over the homogenization method is that there is no need for scraping cells from the wells for processing. The cell solubilization and protein precipitation are done in the wells directly. It can potentially save time when a large number of samples are processed. Also, because endogenous enzymatic activities are mostly arrested by SDS and TCA used in the procedure, minimal protein alteration during the protein extraction procedure is expected. We also applied the TCA method to pre-homogenized brain samples. We analyzed these samples immunologically for the presence of several cytosolic proteins (protein kinase C, calmodulin-dependent protein kinase II) as well as membrane/cytoskeletal proteins (actin, talin, spectrin) with monoclonal and polyclonal antibodies. So far, all antibodies, including a monoclonal anti-protein kinase C and a polyclonal anti-actin antibody, reacted equally well with samples from either method (Figure 2). The brain samples also worked well.

We further characterized the material produced from the MOLT-4 and rat brain samples that had been processed using the TCA extraction method by loading different amounts of protein for immunoblotting. Anti-actin antibodies showed that the samples reacted with the antibody concentration dependently and reproducibly (Figure 3). As low as 1.0–2.5 μg of total protein loaded, actin was easily detected. These data also suggest that quantification of a particular protein is possible with these samples, especially if an internal standard can be included to generate a standard curve.

Finally, we looked at the utility of the TCA extraction method to detect protein changes (such as degradation, expression alteration or phosphorylation). Previously, it has been demonstrated that when MOLT-4 cells are treated with A23187, endogenous calpain is activated, which results in the degradation of cytoskeletal protein, spectrin (17). We examined the effect of incubation of MOLT-4 cells with 20 μM of A23187 on the integrity of cellular spectrin. Upon completion of cell treatment (1 h), total protein was extracted using the TCA method. Equal amounts of protein (3 μg) from both control and A23187-treated cells were loaded onto a gel and subjected to electrophoresis. The blot was then probed with a monoclonal anti-α-spectrin antibody. It was evident that treatment of cells with A23187 indeed resulted in spectrin breakdown.

![Figure 3. Anti-actin immunoblot of different amounts of MOLT-4 and rat brain protein extracts from the TCA method.](image)

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converting the intact 280 kDa form to antigenic fragments of 150 and 145 kDa (Figure 4).
It is also worth noting that since SDS lyses nuclei readily, nuclear pro-
teins can also be extracted using the TCA method. Previously, DNA re-
moval was a major issue for studying DNA-binding proteins (1). This new
method may provide a solution to that.
However, while most cytoplasmic pro-
tein complexes are acidic, several highly basic proteins (e.g., histones) are reported to
be TCA-soluble (7). These proteins may not be extracted efficiently using the
TCA method. Also, small peptides are generally not TCA-precipitable.
While we do not know the exact lower molecular weight limit of our TCA ex-
traction, we did observe protein bands just below the smallest molecular
weight standard used (14.5 kDa) (Fig-
ure 1). This is consistent with a report
that protein as low as 12 kDa is TCA-
precipitable (10). As we focused on to-
total protein extraction from cultured
mammalian cells, the TCA method is
indeed applicable to tissue samples (see
Figure 2), as well as non-mammalian
(e.g., insect cells) and bacterial cul-
tures too. Another potential applica-
tion of the TCA extraction method is
in preparing samples for enzyme-linked
immunosorbent assay (ELISA) for ei-
ther research or clinical purposes (16).
In summary, we have developed an
improved method of cellular protein ex-
traction ideal for cell cultures. We have
also showed that the method is
applicable to small-scale tissue sam-
ple. The TCA extraction method is
simple, fast and robust and thus espe-
cially useful when a large number of
samples are to be processed. Resulting
protein samples are fully compatible
with protein assay, PAGE and Western
blotting.

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Figure 4. Visualization of spectrin breakdown
in A23187-treated MOLT 4 cells. MOLT 4 cells
were either untreated or treated with 20 pg
A23187, total protein was then extracted using
the TCA method. Protein samples of 15 pg per
tube were analyzed using SDS-PAGE and West-
ern blotting with anti-spectrin antibody. The
arrow shows the position of intact spectrin (280
kDa), whereas the spectrin breakdown products
(ODBP) (190, 140 kDa) are indicated by the
open triangles. Results show are representative
of at least 5 experiments.

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