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High-yield skeletal muscle protein recovery from TRIzol after RNA and DNA extraction

Yuan Wen*[,1,2,3], Ivan J Vechetti Jr[1,2], Taylor R Valentino[1,2] & John J McCarthy[1,2]

*[1]Department of Physiology, College of Medicine, University of Kentucky, Lexington, KY, USA; [2]Center for Muscle Biology, University of Kentucky, Lexington, KY, USA; [3]MD/PhD Program, College of Medicine, University of Kentucky, Lexington, KY, USA; *Author for correspondence: ywen2@g.uky.edu*

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ABSTRACT

Extraction of DNA, RNA and protein from the same sample would allow for direct comparison of genomic, transcriptomic and proteomic information. Commercially available kits exhibit poor protein yield and the TRIzol® reagent produces a protein pellet that is extremely difficult to solubilize. In response to these limitations, this study presents an optimized method for the extraction of protein from the organic phase of TRIzol that allows for higher yield recovery of skeletal muscle protein compared with direct homogenization in a common protein lysis buffer. The presented method is inexpensive, simple and fast, requires no additional treatment of the protein pellet for dissolution, and is compatible with downstream western blot applications.

LAY ABSTRACT

Scientists analyze DNA, RNA and protein using separate kits and techniques that do not allow for effective analysis of all three macromolecules from the same sample. Simultaneous extraction kits and techniques are limited by poor protein yield after nucleic acid isolation. We present a fast, effective, inexpensive and high-yield method of recovering protein (including large proteins such as titin) from tissue using the TRIzol reagent after RNA and DNA recovery.

METHOD SUMMARY

The method of high-yield protein extraction from TRIzol after RNA and DNA isolation involves replacing chloroform with bromochloropropane. Instead of producing a tightly packed protein pellet using isopropanol, the protein in the organic phase is precipitated using ethanol and water. Complete dissolution of the resulting protein pellet is achieved using a sodium dodecyl sulfate-urea buffer that allows solubilization of large protein species.

KEYWORDS:

ethanol–bromochloropropane–water method • guanidinium thiocyanate–phenol–chloroform extraction • modified TRIzol protein isolation • skeletal muscle

Skeletal muscle tissue is routinely analyzed for DNA, RNA and protein content during functional adaptation. Although commercially available kits allow for the simultaneous extraction of all three macromolecules from the same sample, their protein yields are poor [1]. TRIzol®, Tri Reagent™ and TriFast™ are commercially available formulations based on the guanidinium thiocyanate–phenol–chloroform extraction method first developed by Chomczynski and Sacchi in 1987 [2]. The underlying principle of that extraction method relies on the differential solubility of the three macromolecules. After centrifugation, RNA is retained in the aqueous phase while DNA and protein remain in the interphase and organic phase, allowing for selective sequential precipitation of each macromolecule [3]. TRIzol is already commonly used to isolate total cellular RNA in skeletal muscle samples; however, each sample is usually cut into three smaller pieces in order for DNA, RNA and protein to be isolated separately. One of the reasons for separating the extractions is that the standard TRIzol protocol produces a protein pellet that is difficult to dissolve. Methods involving special treatments of the pellet have been reported to improve solubility and protein recovery [4–7]. In addition to being time-consuming, such special treatments may require materials and equipment not commonly available in all laboratories. Excessive agitation may contribute to significant protein degradation, especially for large proteins like titin that are sensitive to mechanical shearing and heat [8]. An alternative and much simpler method using ethanol, bromochloropropane and water (EBW), adapted from an earlier methanol–chloroform–water protein isolation method, has produced good protein yields from TRIzol for various tissues [9,10]. However, skeletal muscle was not included in the tissues studied, and the yield for large sarcomeric proteins is unknown.

Initial experience in our laboratory using the published EBW method on mouse skeletal muscle resulted in tightly packed protein pellets that were resistant to solubilization by a 4% sodium dodecyl sulfate (SDS) buffer. We hypothesized that this difficulty is due to the presence of substantial amounts of large striated muscle proteins (e.g., myosin heavy chain, dystrophin, nebulin and titin) per unit mass

of tissue. As such, we attempted to formulate a buffer to improve solubilization of these large proteins. Here we present a modified EBW method specifically enhanced for isolating protein from skeletal muscle samples homogenized in TRIzol. We report complete dissolution of the protein pellet using a modified SDS-urea buffer without any heating or mechanical agitation such as sonication. Using this method, we demonstrate effective solubilization of large striated muscle proteins such as myosin heavy chain, nebulin and titin. Efficient protein recovery after RNA isolation using TRIzol allows for rapid analysis of preciously small samples.

Materials & methods

Protocol modification

The original EBW method included the addition of chloroform for RNA phase separation, but this can be replaced entirely with bromochloropropane (BCP) [9,11]. In our lab, we routinely replace chloroform with BCP because the latter is less toxic and results in a tighter interphase. Adding an additional volume of BCP (as described in the EBW method) during protein extraction results in a tightly packed protein pellet which is difficult to solubilize. This is likely because BCP is a stronger, more hydrophobic organic solvent than chloroform. Indeed, replacing chloroform with BCP and omitting the second addition of BCP produced a less densely packed protein pellet; this enhanced solubilization of the pellet, eliminated a toxic component (chloroform) and reduced the total amount of reagent used.

Buffer formulation

Titin, being the largest protein, necessitates some special considerations in terms of resolubilization [12]. The presence of urea in SDS buffers has been reported to improve the solubility of titin whereas β-mercaptoethanol was shown to prevent titin aggregating by reducing disulfide bonds [13–16]. Titin is also sensitive to residual protease activity in SDS buffers, and therefore protease inhibitors are necessary [14,17–21]. Finally, heating damages titin as well; the amount of intact titin decreases in proportion to heating temperature and duration [8,14,22]. The SDS-urea buffer recipe described here incorporates all of the above considerations.

Mechanical overload & muscle tissue preparation

All animal procedures were conducted in accordance with institutional guidelines for the care and use of laboratory animals as approved by the Animal Care and Use Committee of the university. C57BL6/J mice were housed in a humidity- and temperature-controlled facility, maintained on a 14:10 hour light-dark cycle with food and water *ad libitum*. Mice were subjected to either sham or synergist ablation surgery to induce hypertrophy of the plantaris muscle as previously described [23]. Mice were euthanized 24 h after surgery and the plantaris muscles excised, flash-frozen in liquid nitrogen, cut into two smaller pieces along the mid-belly on dry ice and weighed before homogenizing in RIPA or TRIzol using a Bullet Blender (Next Advance, Inc., NY, USA) with one scoop (100 μl) of 1-mm diameter zirconium oxide beads at speed 10 for 3 min ×2. Insoluble materials and beads were removed after centrifugation at 10,000×*g* for 10 min at 4°C. RIPA samples were quantified using the DC protein assay (Bio-Rad, CA, USA) and prepared for SDS-PAGE by boiling for 5 min in sample buffer containing 2% SDS, 1% β-mercaptoethanol, 6% glycerol, 50 mM Tris-HCl (pH 6.8 at room temperature), and 0.004% bromophenol blue. TRIzol samples were subjected to the optimized protocol as described in detail above. Protein bands were visualized after SDS-PAGE using Coomassie blue stain (Bio-Rad).

Cell culture

C2C12 myoblasts (ATCC®) were cultured in growth medium (Dulbecco's modified Eagle medium with 10% fetal bovine serum and 1% penicillin/streptomycin) at low, medium and high densities. When 100% confluent, myoblasts were switched into low-serum differentiation medium (Dulbecco's modified Eagle medium with 2% Horse Serum and 1% Penicillin/Streptomycin). Myotubes were cultured in differentiation medium for 3 days before protein collection.

Western blot analysis

A total of 10 μg of protein was loaded into each lane for SDS-PAGE, and protein was transferred to Bio-Rad Immun-Blot® PVDF membranes and blocked with 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBST). Primary antibodies against pan-myosin heavy chain (mouse monoclonal A4.1025; EMD Millipore, 05-716) and α-tubulin (rabbit monoclonal EP1332Y; Abcam, ab52866) were diluted 1:1000 in 5% bovine serum albumin in TBST. Secondary antibodies coupled to Alexa Fluor 680 nm fluorophores against mouse IgG and rabbit IgG were diluted 1:10,000 in 5% bovine serum albumin in TBST. Band intensities were analyzed using the gel analysis tool in ImageJ [24].

Statistics

Reported values represent mean ± SEM. Statistical analyses were performed using GraphPad Prism version 7.00 for Windows, (GraphPad, CA, USA) with $p < 0.05$ considered to be statistically significant. A paired, two-tailed Student's *t*-test was performed for the protein yields, which passed the Shapiro–Wilk test for normality; a two-way analysis of variance, followed by Sidak's post-hoc test comparing 'overload' with 'sham' groups, was used to analyze protein phosphorylation following mechanical overload of the plantaris muscle.

Results & discussion

Figure 1A shows that using the optimized TRIzol method, protein can be recovered from skeletal muscle tissue with a significantly higher

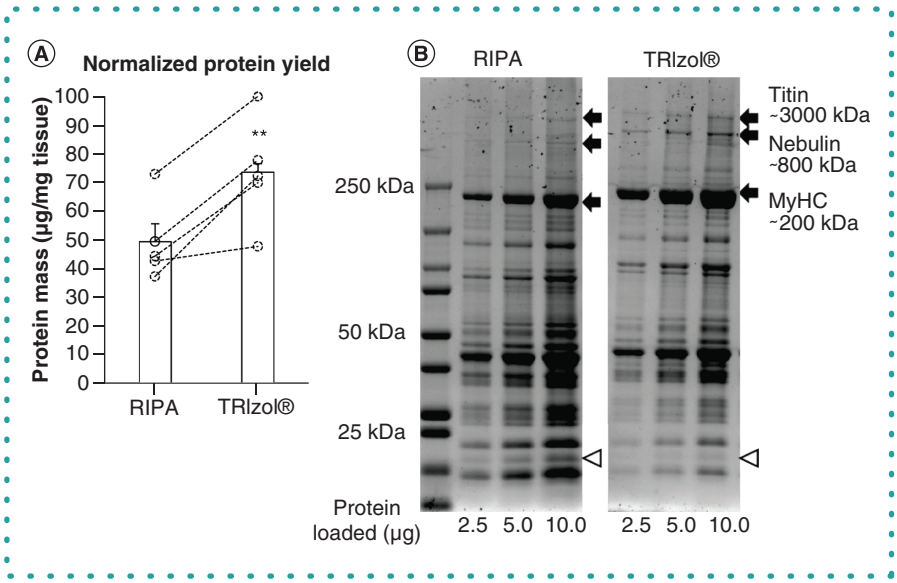

Figure 1. Protein yield from TRIzol is higher compared with RIPA buffer, likely due to increased solubilization of high molecular weight sarcomeric proteins. (A) Protein quantification comparing RIPA and TRIzol® protein isolation methods using mouse plantaris muscles. The open bar represents the mean of the five measurements shown as open circles and the error bar shows standard error of the mean. A dotted line connects measurements of muscle samples from the same animal. **(B)** Coomassie-stained SDS-PAGE for total protein per lane showing increased intensity of high molecular weight bands in the TRIzol-isolated protein samples compared with RIPA. Increasing amounts of protein (2.5, 5 and 10 µg) were loaded for each method. All samples were run on the same gel at the same time; two sections of the gel image are cropped to allow for side-by-side comparison.
**$p < 0.01$.
RIPA: Radioimmunoprecipitation assay.

yield than with direct lysis in RIPA buffer ($p < 0.01$). Total protein from mouse plantaris muscle, as seen on Coomassie-stained SDS-PAGE, reveals that the TRIzol method contains prominent high molecular weight bands (>200 kDa) while the RIPA isolation method shows more prominent lower molecular weight bands given the same total amount of protein loaded (Figure 1B). The superior protein yield from our modified TRIzol method is likely secondary to improved solubilization of high molecular weight components of skeletal muscle sarcomere. There is a notable decrease in the band intensities of lower molecular weight proteins, especially those less than 25 kDa, in the TRIzol-isolated samples relative to RIPA samples. We believe this is not due to loss of the small molecular weight proteins, but is secondary to the increased abundance of high molecular weight proteins, which causes a decreased relative proportion of the lower molecular weight proteins given the same total protein mass loaded per well. The lower molecular weight protein band (Figure 1B, arrowhead) that is not present at 2.5 µg of total protein becomes more visible with increases in the amount loaded, indicating that the band is not lost.

We used C2C12 cells in culture to assess whether proteins isolated using our TRIzol method and buffer are compatible with downstream western blot analyses. Under conditions of proliferation, myoblasts do not express myosin heavy chain, which is only expressed upon differentiation into myotubes. Using our isolation method, we were able to detect myosin heavy chain in C2C12 myotube lysates but not in undifferentiated myoblasts, even though α-tubulin was detectable in all samples (Figure 2A). After demonstrating compatibility with western blot analysis, we wanted to test whether protein phosphorylation is preserved using our protocol, because detecting phosphorylation status is a common method of assessing cell signal transduction. For example, phosphorylation of residues on key proteins, such as mTOR, AKT and RPS6, is vital for increased protein synthesis during skeletal muscle growth [25]. C57BL6/J mice were subjected to sham or synergist ablation surgery and after 24 h of mechanical overload, the plantaris muscle was collected for analysis. Protein was extracted using RIPA buffer or TRIzol as described, with the addition of phosphatase inhibitors in the RIPA and the SDS-urea buffers. The phosphorylation status of Ser2448 on mTOR, Thr308 on AKT and Ser240/244 on RPS6 were analyzed for the same muscle after 1 day of mechanical overload using both protein extraction methods (Figure 2B). Quantification of western blot bands following TRIzol protein isolation demonstrated significant increases in RPS6 and AKT phosphorylation in response to 24 h of mechanical overload of the plantaris muscle (Figure 2C). Phosphorylation of AKT was increased in the RIPA samples with overload but failed to reach statistical significance with a sample size of three. mTOR phosphorylation was not significantly different at 24 h of overload, regardless of the protein isolation method used. Two-way analysis of variance indicates significant main effect of hypertrophy on RPS6 and AKT phosphorylation without significant interaction. No significant main effect or interaction was detected for mTOR phosphorylation.

Our method for extracting protein from tissue using TRIzol reagent after the isolation of RNA and DNA is fast, efficient and inexpensive. We obtained complete dissolution of the protein pellet without special treatment or equipment and achieved higher yield relative to

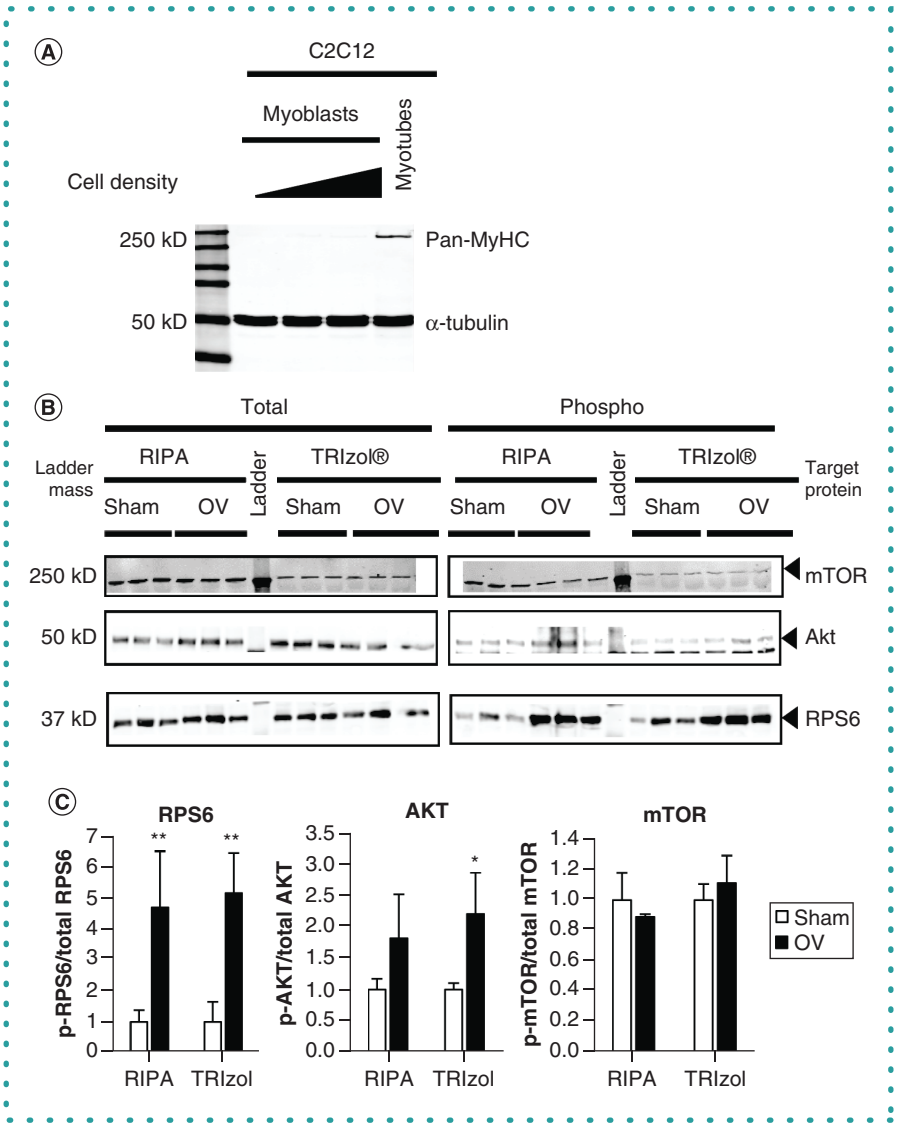

Figure 2. TRIzol protein isolation preserves phosphorylation and is compatible with downstream western blot analysis. (A) TRIzol-isolated protein lysates from C2C12 myotubes *in vitro* were successfully detected using anti-pan-myosin heavy chain (MyHC) and α-tubulin antibodies in western blot analyses. MyHC is only expressed after myoblasts differentiate into myotubes, but α-tubulin is expressed in all stages of myogenesis. **(B)** Western blot analysis of total and phosphorylated mTOR, AKT and RPS6 (Ser2448, Thr308 and Ser240/244, respectively). **(C)** Quantification of bands in image **(B)** expressed as a ratio of arbitrary band intensities of phosphoprotein relative to total protein, normalized to the average of the Sham group (n = 3 for both sham and OV groups).
***p < 0.05; **p < 0.01. Error bar indicates standard deviation.**

direct homogenization using RIPA lysis buffer. Despite significant interest in simultaneous isolation of RNA, DNA and protein, most other previous methods only recover a small fraction of the precipitated proteins [1,5–7,26]. The higher yield even compared with direct homogenization in RIPA buffer may be explained by improved solubility of large sarcomeric proteins (e.g., myosin, nebulin and titin) while still recovering all of the smaller protein species. The entire procedure can be performed in less than 30 min with no specialized equipment or heating, in stark contrast to the multiple hours of heating or overnight treatments that are necessary for other methods [4,27]. Our presented method decreases the time as well as the cost of extracting DNA, RNA and protein simultaneously from the same biological sample.

It is important to note that, despite obtaining higher protein mass per unit of input tissue by our method compared with using RIPA buffer, this may represent only a fraction of the protein that can be recovered through direct homogenization in the optimized buffer. In 2017 Kopec *et al.* described an optimized buffer for brain tissue that effectively solubilizes protein from TRIzol after boiling the protein pellet at 50°C for 2–18 h [27]. They showed that compared with direct homogenization in the optimized buffer, the yield for direct homogenization in RIPA was only about 70%; after using their optimized buffer to solubilize protein from TRIzol after RNA and DNA

removal, they obtained a protein yield of roughly 70% compared with direct homogenization in their optimized buffer. Our own data show that direct homogenization in RIPA gives a yield of about 70% compared with using our buffer and modified method after TRIzol homogenization. This raises the possibility that our proposed method and buffer produce yields on a par with direct homogenization in the optimized buffer. An alternative interpretation of the data is that there may be tissue-specific differences; RIPA buffer may be less effective in solubilizing skeletal muscle protein compared with brain tissue. This alternative interpretation is supported by the finding of Kopec *et al.* that at least one of the high molecular weight (~200 kDa) proteins was solubilized by their optimized buffer much more than by RIPA buffer. Our data also highlight the inadequacies of RIPA buffer in solubilizing the high molecular weight proteins. However, for researchers currently using RIPA buffer to homogenize tissue, our proposed method may achieve higher quantities of recovered protein.

We confirmed that the currently presented isolation protocol is compatible with subsequent western blot analysis of total and phosphorylated proteins, which is consistent with previous reports showing that protein from TRIzol samples retains good quality for downstream analyses [6,26–28]. Our method can help laboratories routinely study changes in protein species from TRIzol samples that would otherwise be thrown away. Furthermore, studying genomic, transcriptomic and proteomic changes within the same sample can help to minimize biological and technical variability that may confound study results. Finally, this method can decrease the number of animals needed to complete a study and is especially useful in situations where sample availability and quantity are limiting factors.

Future perspective

More than three decades have come and gone since the single-step RNA isolation method that formed the basis of the TRIzol reagent was described. Although isolation of nucleic acids from the same sample has become mainstream, protein extraction has not been a trivial undertaking; however, many studies have demonstrated that good quality protein can be extracted from TRIzol following RNA and DNA isolation. With every study, improvements have been made to permit more efficient and higher yield recovery. It can be expected that within the next few years, more standardized protocols and commercially available kits would allow for RNA, DNA and protein to be extracted with improved quality and yield at lower costs in terms of both time and money.

Author contributions

Y Wen developed, tested and optimized the protocols and recipes, performed the overload surgeries, generated the figures and drafted the manuscript. I Vechetti tested and optimized the protocols and recipes, performed experiments, participated in the surgeries and tissue collections, analyzed the data and critically revised the manuscript. T Valentino tested the protocol, participated in data collection, analysis and interpretation, and critically revised the manuscript. J McCarthy participated in the study design, coordinated the data collection and analysis and oversaw the drafting and revision of the manuscript.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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