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Protein from the fraction remaining after RNA extraction is useful for proteomics but care must be exercised in its application

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ABSTRACT

Simultaneous isolation of mRNA and proteins from a single small biopsy specimen can be useful for integrated omics studies. Here, we have improved the method for extracting protein from the fraction remaining after RNA isolation by TRIzol reagent, for application in protein and proteome analyses. Protein yield was reduced by half, but the patterns developed on 2D gels were equivalent to conventional urea extractions. Thus, although quantitative profiles of individual proteins were different from conventionally-isolated samples, overall profiles were similar. Therefore, this particular protein source is useful for proteomics but care must be exercised in its application.

Introduction

Disease omics studies have markedly developed in the post-genomic decade. In addition to transcriptomics, proteomics has been useful for investigating the pathogenesis of different liver diseases such as viral hepatitis and hepatocellular carcinoma (Niu et al., 2010; Perdomo et al., 2012). mRNA levels are not always informative for the amount of protein present, which itself is not always paralleled by the functional activity of a given gene. Post-translational modification such as processing and phosphorylation is occasionally related to the function of a given gene. Thus, liver proteomics including assessment of post-modified proteins is valuable in disease omics in addition to investigations at the transcriptional level. Although clinical specimens provide a great deal of such pathobiological information, they are commonly too small to thoroughly examine both the transcriptome and the proteome. For this, it would be necessary to simultaneously isolate these macromolecules from a single specimen. This would also be useful for integrating transcriptomic and proteomic data for a more complete view of the regulatory mechanisms controlling gene expression. Chomczynski (1993) described an RNA extraction reagent which allows simultaneous extraction of DNA and protein from a single sample. Such protein extracts could be subjected to Western blotting without any problems (Chomczynski, 1993). Nonetheless, it was an issue that the efficiency of protein extraction from the remaining lysate was so poor that the original method required essential modifications (Hummon et al., 2007; Likhite and Warawdekar, 2011; Man et al., 2006; Nolan and Teller, 2006). It is still not optimized for application to protein analysis. In the present study, we attempted to improve protein extraction from the lysate remaining after RNA extraction. We also determined whether such protein extracts could be used for quantitative proteomics using two dimensional fluorescence difference gel electrophoresis (2D-DIGE).

Materials and methods

Samples

Non-cancerous liver tissues were obtained from 4 cases of hepatocellular carcinoma positive for hepatitis C virus by surgical resection. All cases had solitary tumors less than 5 cm in diameter, and no metastasis to lymph nodes and other organs. Histopathology of non-cancerous liver tissues was chronic hepatitis without liver cirrhosis, with fibrosis scores corresponding to F2 or F3 (Ichida et al., 1996). Our study protocol was approved by the Ethics Committee of our School, and informed consent was obtained from the patients.

Protein extraction

Total RNA was extracted from liver tissues with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and the remaining fractions, middle and lower phases of the first phase separation were stored at −80 °C for longer than 1 year. Protein was extracted from the stored fraction by modifying the manufacturer's protocol. Briefly, after DNA precipitation, protein was precipitated with isopropanol from the supernatant (phenol–ethanol layer).

Abbreviations: 2D-DIGE, two dimensional fluorescence difference gel electrophoresis; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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The protein precipitate was then washed, dried and dissolved in 7 M urea, 2 M thiourea, 4% (w/v) 3-(3-cholamidepropyl) dimethylammonio-1-propanesulphonate, 1 mM phenylmethanesulfonyl fluoride, and 30 mM Tris–HCl, pH 8.5 (Urea-buffer). After sonication, the extracted protein was re-precipitated with acetone, and the pellet was washed with acetone, dried and re-dissolved in Urea-buffer.

Protein was also extracted directly from the same liver tissues by homogenization in the Urea-buffer described above, followed by incubation at room temperature for 30 min. After sonication and centrifugation, the supernatant was used for analysis. The protein concentration was determined using the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA) (Bradford, 1976). Bovine serum albumin was used as a standard.

Western blotting

Twenty micrograms of protein was electrophoresed on sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gels (Laemmli, 1970), which was then stained with SYPRO Ruby (Life Technologies) according to the manufacturer's instructions. Gel images were acquired using an FLA-5100 Fluorescent Image Analyzer (GE Healthcare, Little Chalfont, UK). Acquired gel images were analyzed using Multi Gauge version 3.0 (GE Healthcare).

For Western blotting, the separated proteins were transferred to a nitrocellulose membrane using the iBlot Dry Blotting System (Life Technologies). After blocking, membranes were incubated with 0.1 μg/ml of anti-β-actin antibody ab8226 (Abcam, Cambridge, MA, USA), with 0.2 μg/ml of anti-calreticulin antibody SPA-600 (Enzo Life Sciences, Inc., Farmingdale, NY, USA) and 1 μg/ml of anti-vimentin antibody MAB3400 (Chemicon, Temecula, CA, USA), and then with horseradish peroxidase-conjugated secondary antibodies, anti-mouse IgG (Dako, Glostrup, Denmark) or anti-rabbit IgG (Dako). Secondary antibodies bound to the membranes were detected by ECL Plus or ECL Advance Western Blotting Detection System (GE Healthcare) and the chemiluminescence was acquired by Light Capture (ATTO, Tokyo, Japan) and analyzed by CS Analyzer (ATTO) and Multi Gauge version 3.0 (GE Healthcare). Serial dilutions of mixed samples were also subjected to Western blotting to establish a standard curve for quantification of target proteins. The signal intensity of each protein band was expressed as a value relative to the standard.

2D-DIGE

Fifty micrograms of each sample was labeled with Cy5 (Minimal Labelling Dye, GE Healthcare), and an internal standard (a pool of an equal amount of all samples) was labeled with Cy3 (Minimal Labelling Dye, GE Healthcare) according to the manufacturer's instructions. 2D gel electrophoresis was carried out according to the method of Toda and Kimura (1997) and a web protocol (http://www.proteome.jp/2D/2DE_method.html). Isoelectric focusing (IEF) was performed with CoolPhoreStar IPG-IEF Type-P (Anatech, Tokyo, Japan) after loading a mixture of 50 μg each of a Cy3-labeled internal standard and the Cy5-labeled sample on an Immobiline DryStrip (pH 4–7, 18 cm length, GE Healthcare). After reducing and alkylating the strip, the second dimension SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on a 12.5% polyacrylamide gel (190 × 173 × 1 mm) using CoolPhoreStar SDS-PAGE Dual-200 (Anatech). Gel images were acquired using an FLA-5100 Fluorescent Image Analyzer (GE Healthcare). Spot-pattern and spot-abundance were analyzed by Progenesis SameSpots (NonLinear Dynamics, Newcastle upon Tyne, UK). To determine the abundance of each spot, the fluorescence intensity was calculated as a percent value per gel. To normalize inter-gel differences, Cy5 percent value was normalized to Cy3 in each spot and the normalized value was used for statistical analysis. Cluster analysis was performed by GeneSpring ver. 12.5 (Agilent Technologies, Santa Clara, CA, USA) using normalized values.

Results

Protein yield

From 14 to 44 mg of liver tissues, 2.7 to 6.1 mg of proteins were directly extracted (10 to 20% w/w) with Urea-buffer, designated Urea-protein (U). In contrast, only 0.5 to 0.9 mg of proteins were extracted from 14 to 28 mg of tissues after RNA extraction with TRIzol (3 to 5% w/w), designated TRIzol-protein (T). The yield from the remaining fraction following our solubilizing procedure was approximately 50%.

1D pattern of proteins by SDS-PAGE

Fig. 1A shows gel images stained with SYPRO Ruby after SDS-PAGE of extracted proteins. Total intensity of proteins in an individual lane was determined and the ratio of each molecular weight fraction to the total was calculated (Fig. 1B). The pattern of TRIzol-protein was similar to that of Urea-protein, but the content of high molecular weight proteins (>75 kDa) was significantly decreased ($p = 0.029$ by Mann–Whitney U test) and that of low molecular weight proteins tended to be increased in the TRIzol-protein ($p = 0.057$).

Western blotting

Three representative proteins with similar molecular weights (40 to 60 kDa) were analyzed by Western blotting (vimentin, calreticulin and β-actin, Fig. 2). Different proteins showed different variations between Urea- and TRIzol-proteins, occasionally depending on sample variation. β-Actin was relatively constant in Urea- and TRIzol-proteins of all samples, while the content of 57 kDa vimentin was high in TRIzol-proteins of all samples (Fig. 2B). Three minor bands of lower-molecular weight vimentin cleaved by caspases (Byun et al., 2001) were less well represented in the TRIzol-protein. The calreticulin signal was also constant, but occasionally low in the TRIzol proteins (Fig. 2B). Thus, particular proteins, but not the majority, showed variable content in the same manner as sample variation.

2D-DIGE

To validate that TRIzol-protein was suitable for proteomics investigations, we performed 2D-DIGE using 4 samples of non-cancerous liver tissues with chronic hepatitis C. An additional 4 samples of Urea-protein

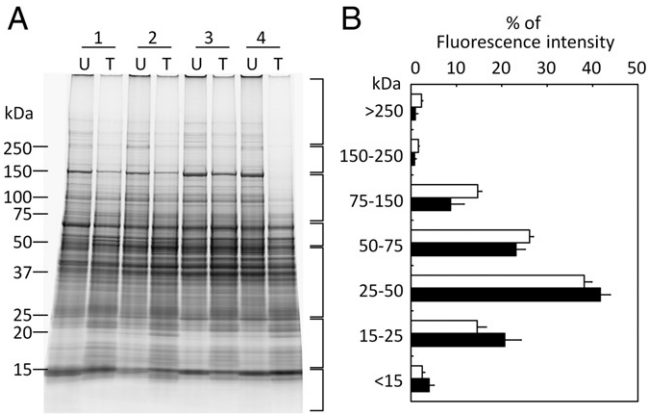

Fig. 1. SDS-PAGE of proteins extracted from 4 non-cancerous liver tissues by two different methods. (A) SYPRO Ruby staining of the gel. Twenty micrograms of Urea-protein (U) and 20 μg of TRIzol-protein (T) were loaded per lane and separated on an SDS-12.5% polyacrylamide gel. (B) Relative amounts of protein in the molecular weight fractions are indicated. Fluorescence intensity of each fraction was measured and its percentage relative to total intensity was calculated. Open bars, Urea-protein; closed bars, TRIzol-protein.