A Modified Gelatin Zymography Technique Incorporating Total Protein Normalization

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ABSTRACT

Gelatinase zymography is a commonly used laboratory procedure; however, variability in sample loading and concentration reduce the accuracy of quantitative results obtained from this technique. To facilitate normalization of gelatinase activity by loaded protein amount, we developed a protocol using the trihalocompound 2,2,2-trichloroethanol to allow for gelatin zymography and total protein labeling within the same gel. We showed that detected protein levels increased linearly with loading, and describe a loading concentration range over which normalized gelatinase activity was constant. We conclude that in-gel total protein detection is feasible in gelatin zymography and greatly improves comparison of gelatinase activity between samples.

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Gelatinases are involved in tissue homeostasis as well as pathologic conditions, such as cancer [1], neurodegenerative diseases [2], and cardiovascular diseases [3], and are thus a major focus of current research. Gelatin zymography is commonly used to quantify gelatinase activity. This technique uses SDS-PAGE to separate proteins and assesses enzymatic activity by incorporating a gelatin substrate within the polyacrylamide gel [4–7]. However, current gelatin zymography protocols do not use a standard normalization method to account for variability in sample loading. It has been demonstrated that normalizing the densitometric signal by protein loading greatly improves data reliability in Western blotting, a similar SDS-PAGE based technique [8,9]. To date, the only method for zymography normalization is to run a parallel gel for analyzing total protein by Coomassie staining [10] or for immunoblotting against β -actin or similar loading controls [11]. However, these approaches require additional time and resources and, more importantly, cannot account for the variability in the quantity of loaded sample.

Recently, trihalocompound activation [12] has been used for total protein quantification and subsequent band normalization in Western blotting. When tryptophan residues are excited by ultraviolet (UV) radiation, they react with a trihalocompound to form a compound that emits visible light [13]. Thus, when trihalocompounds are added within a polyacrylamide gel, the tryptophan residues within the loaded proteins can be visually detected following UV exposure. This allows total protein in the loaded sample to be quantified, and has been shown to be a reliable method of normalization [14].

Since collagen contains few tryptophan residues [15,16], trihalocompounds would be expected to induce minimal background signal by binding to collagen in zymography

gels following UV exposure. We therefore reasoned that trihalocompounds could be used to quantify total protein loading in gelatin zymography. Among tested trihalocompounds, 2,2,2-trichloroethanol (TCE) has been shown to provide superior fluorescent intensity when compared to trichloroacetic acid and chloroform [17]. Therefore, TCE was chosen to test this approach.

HT1080 fibrosarcoma cells (CCL-121; American Type Culture Collection, Manassas, VA) were cultured at 25,000 cells/cm² in phenol-free media (without serum) with phorbol 12-myristate 13-acetate (PMA, 25 ng/mL) overnight to upregulate MMP expression [18]. Cells were lysed with 25 mM Tris-HCl, pH 7.5; 100 mM NaCl; and 1% (v/v) Nonidet P-40 (NP-40). Protease inhibitors (10 µg/mL aprotinin, 2 µg/mL leupeptin, and 4 mM benzamidine) were added immediately prior to lysis. The samples were centrifuged at 14,000g for 10 minutes to remove debris. Protein concentration in the lysates was measured via micro bicinchoninic acid assay (BCA, Thermo Scientific, Pierce Protein Research Products, Rockford) per the manufacturer's protocol. 4x non-reducing sample dye (250 mM Tris-HCl, pH 6.8; 40% (v/v) glycerol; 8% (w/v) SDS; and 0.01% (w/v) bromophenol blue) was added to the samples. Additional lysis buffer was added to create a protein loading range of 2.5-30 µg per lane in equal volumes.

Gelatin zymography was carried out using 10% SDS-polyacrylamide gels with 0.2% (w/v) gelatin. SDS-PAGE was run at 125 V for 90 minutes at room temperature in running buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.3, BioRad, Hercules, CA). Following electrophoresis, the gels were removed and placed in 2.5% (v/v) Triton X-100 to renature enzymes for 40 minutes. Then, gels were incubated in 50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl₂, pH 7.8 overnight to allow sufficient time for

genatinolytic activity to occur. Following overnight incubation at 37°C, gels were incubated in 10% (v/v) TCE in a 1:1 methanol-water mixture for 10 minutes. Note that since TCE binds to tryptophan residues and subsequently alters protein structure, TCE cannot be directly incorporated into the gel as Western blotting methods have used [17] and should be added after enzymatic activation to avoid inhibition of gelatinase activity (data not shown). Following incubation with TCE, the gels were exposed to 302 nm, 48W UV light for 2.5 minutes to activate fluorescent modification of tryptophan residues in the presence of TCE [17], then imaged with a ChemiDocTM MP gel imaging system (Fig. 1a, BioRad, Hercules, CA). Next, the gels were incubated with Coomassie stain (0.05% (w/v) Coomassie Brilliant Blue G-250, 25% (v/v) methanol, 10% (v/v) acetic acid) for 1 hour, followed by destaining (4% (v/v) methanol, 8% (v/v) acetic acid) until clear bands were visible, at which time the gels were imaged (Fig. 1b). Note that Coomassie makes total fluorescent protein signal undetectable and therefore must be added after total protein determination. Total protein and gelatinase activity were quantified using ImageQuant TL 8.1 (GE Healthcare Life Sciences, Marlborough, MA). For a more detailed protocol, please see supplementary material.

We first show that gelatinase activity and total protein can be obtained independently on the same gel. Total protein densitometric value was measured with no detectable gelatin background signal (Fig. 1a). TCE fluorescent signal increased linearly with total loaded protein (Fig 1c), similar to previous results in Western blotting [14]. MMP-2 activity also increased linearly until saturation was reached at approximately 15 µg of loaded protein per lane (Fig. 1d).

Figure 1: (a) Total protein visualized with TCE in a polyacrylamide gel with a gelatin substrate. Values at the top of each lane indicate the nominally loaded mass of protein. (b) Zymogram indicating gelatinase activity in the same gel. Plots showing (c) the TCE fluorescence intensity and (d) the total gelatinase activity versus loaded protein concentration. In both panels, the plotted values were normalized to the relevant value for the smallest amount of loaded protein (2.5 µg).

Due to concerns about gelatinase activity saturation, we next determined the working range of sample protein loading that gave adequate normalization for HT1080 cell lysates. Gelatinase activity (Fig. 1c) was normalized by the corresponding total protein densitometric values (Fig. 1d). A working range was defined as the region in which the normalized gelatinase activity values varied only minimally (Fig. 2a). For HT1080 lysates, this range was found to be 5 - 15 µg of loaded protein per lane. At higher sample loadings, the MMP activity signal became saturated, leading to a drop in

normalized values. Similarly, at lower protein loadings, insufficient loading or lack of either gelatinase or total protein values led to poor normalization.

Therefore, we carried out further testing using 8 protein loading levels in the 5-15 μg range, with diluted preparations of a single master sample being loaded in each well to create different protein loading levels. This was repeated for three gels, and gelatinase activity for each loaded protein amount was averaged over these three gels, both with and without total protein normalization (Fig. 2b). Our results showed that, when normalized by total protein, gelatinase activity was nearly constant between different loading conditions. However, as expected, the non-normalized gelatinase activity rose with the total amount of loaded protein.

Figure 2: (a) Gelatinase activity (Fig. 1b, loaded from 2.5-30 μg) normalized by total protein (Fig. 1a). The yellow-shaded region is the range where normalized gelatinase activity was approximately constant. (b) Total gelatinase activity within yellow-shaded concentration range from panel (a), with and without total protein normalization ($n=3$, error bars denote standard deviation).

Due to known issues with protein concentration assays and possible gel loading variations, normalizing gelatinase activity to total loaded protein is essential in zymography. To our knowledge, this is the first method that enables total protein measurement and gelatinase activity within the same gel, facilitating normalization of gelatinase activity.

There remain a few potential limitations of this new method. First, only HT1080 lysates have been tested; we recommend that other types of samples be tested before use. For example, conditioned media has not been extensively tested and may work less well, due to having a lower total protein TCE fluorescent signal, making normalization noisier. Further, whole tissue samples were not investigated; due to the high collagen content in most tissues it is unclear if this technique can be applied. In addition, due to the variability in tryptophan composition of various tissue and cell protein samples, the working range for adequate normalization should be assessed for each sample type prior to the use of TCE total protein normalization. Despite these limitations, we expect that the use of this normalization technique will improve sample-to-sample comparison within the same polyacrylamide gel and enable more reliable comparison of gelatinase activity across multiple samples and experiments.

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REFERENCES

- [1] C. Gialeli, A.D. Theocharis, N.K. Karamanos, Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting., *FEBS J.* 278 (2011) 16–27. doi:10.1111/j.1742-4658.2010.07919.x.
- [2] M. Brkic, S. Balusu, C. Libert, R.E. Vandenbroucke, Friends or Foes: Matrix Metalloproteinases and Their Multifaceted Roles in Neurodegenerative Diseases., *Mediators Inflamm.* 2015 (2015) 620581. doi:10.1155/2015/620581.
- [3] P. Liu, M. Sun, S. Sader, Matrix metalloproteinases in cardiovascular disease., *Can J Cardiol.* 22 Suppl B (2006) 25B–30B.
- [4] P.A.M. Snoek-van Beurden, J.W. Von den Hoff, Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors., *BioTechniques.* 38 (2005) 73–83.
- [5] M. Toth, R. Fridman, Assessment of Gelatinases (MMP-2 and MMP-9 by Gelatin Zymography., *Methods Mol Med.* 57 (2001) 163–174. doi:10.1385/1-59259-136-1:163.
- [6] M.O. Platt, Y. Xing, H. Jo, A.P. Yoganathan, Cyclic pressure and shear stress regulate matrix metalloproteinases and cathepsin activity in porcine aortic valves., *J Heart Valve Dis.* 15 (2006) 622–629.
- [7] H. Frankowski, Y.-H. Gu, J.H. Heo, R. Milner, G.J. Del Zoppo, Use of gel zymography to examine matrix metalloproteinase (gelatinase) expression in brain tissue or in primary glial cultures., *Methods Mol Biol.* 814 (2012) 221–233. doi:10.1007/978-1-61779-452-0_15.
- [8] G.M. Aldridge, D.M. Podrebarac, W.T. Greenough, I.J. Weiler, The use of total protein stains as loading controls: an alternative to high-abundance single-protein controls in semi-quantitative immunoblotting., *J Neurosci Methods.* 172 (2008) 250–254. doi:10.1016/j.jneumeth.2008.05.003.
- [9] S.C. Taylor, A. Posch, The design of a quantitative western blot experiment., *BioMed Research International.* 2014 (2014) 361590. doi:10.1155/2014/361590.
- [10] A.E. Awad, V. Kandam, S. Chakrabarti, X. Wang, J.M. Penninger, S.T. Davidge, et al., Tumor necrosis factor induces matrix metalloproteinases in cardiomyocytes and cardiofibroblasts differentially via superoxide production in a PI3Kgamma-dependent manner., *Am J Physiol, Cell Physiol.* 298 (2010) C679–92. doi:10.1152/ajpcell.00351.2009.

- [11] W. Xiong, T. Meisinger, R. Knispel, J.M. Worth, B.T. Baxter, MMP-2 regulates Erk1/2 phosphorylation and aortic dilatation in Marfan syndrome., *Circ Res.* 110 (2012) e92–e101. doi:10.1161/CIRCRESAHA.112.268268.
- [12] D. Kazmin, R.A. Edwards, R.J. Turner, E. Larson, J. Starkey, Visualization of proteins in acrylamide gels using ultraviolet illumination., *Anal Biochem.* 301 (2002) 91–96. doi:10.1006/abio.2001.5488.
- [13] R.A. Edwards, G. Jickling, R.J. Turner, The light-induced reactions of tryptophan with halocompounds., *Photochem Photobiol.* 75 (2002) 362–368.
- [14] J.E. Gilda, A.V. Gomes, Western blotting using in-gel protein labeling as a normalization control: stain-free technology., *Methods Mol Biol.* 1295 (2015) 381–391. doi:10.1007/978-1-4939-2550-6_27.
- [15] G.P. Talwar, S.E. Hasnain. *Textbook of Biochemistry, Biotechnology, Allied and Molecular Medicine*, PHI Learning Pvt. Ltd., n.d. 2015.
- [16] O. del Barco, J.M. Bueno, Second harmonic generation signal in collagen fibers: role of polarization, numerical aperture, and wavelength., *J Biomed Opt.* 17 (2012) 045005. doi:10.1117/1.JBO.17.4.045005.
- [17] C.L. Ladner, J. Yang, R.J. Turner, R.A. Edwards, Visible fluorescent detection of proteins in polyacrylamide gels without staining., *Anal Biochem.* 326 (2004) 13–20. doi:10.1016/j.ab.2003.10.047.
- [18] M.W. Roomi, T. Kalinovsky, J. Monterrey, M. Rath, A. Niedzwiecki, In vitro modulation of MMP-2 and MMP-9 in adult human sarcoma cell lines by cytokines, inducers and inhibitors., *Int J Oncol.* 43 (2013) 1787–1798. doi:10.3892/ijo.2013.2113.