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The scaled volume as an image analysis variable for detecting changes in protein expression levels by silver stain

A new variable for measuring the relative intensities of silver stained protein spots on two-dimensional gels is described. The scaled volume (SV) more accurately measures the intensity of protein spots and accounts for differences frequently encountered when trying to compare two gels than other variables such as relative volume ratio, optical density, or relative optical density. The SV scales the signal of interest by the noise (gel background) with secondary signals removed (spots not of interest, e.g., technical artifacts). The SV of spot intensities offers a better dynamic response to protein amount for the model proteins studied here. Depending on the quantity of protein loaded onto gels, we have observed a coefficient of variation range of 0.2 to 1.3. We also observe that the SV silver stain response follows a characteristic exponential profile for different proteins.

Keywords: Two-dimensional electrophoresis / Proteomics / Silver stain

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1 Introduction

Two-dimensional gel electrophoresis (2-DE) is capable of resolving thousands of peptides and proteins from biological samples. Some common protein detection methods for 2-DE include radiolabel, Coomassie blue stain, and silver stain. In addition, fluorescent tags, which require specialized image acquisition devices, have demonstrated good sensitivity and quantitation [1]. While new technology for quantifying protein expression changes using a mass spectrometer [2] holds great promise for proteomic analyses, this technology is not yet widely accessible. Metabolic labeling of proteins offers excellent quantitation, but this approach is not well suited for a wide variety of biological specimens. Attempts to use 2-DE gels stained with either Coomassie blue or silver for the comparison of proteomic changes based on protein spots have largely been limited to the identification of qualitative or semiquantitative differences between gels.

Coomassie blue stain is known to be related linearly to protein concentration over a 20-fold range in concentration, but is not as sensitive as other stains such as silver stain [3]. Silver stain and protein concentration have been reported to be related linearly over a 40-fold range in concentration, but Merrill *et al.* [4] have shown that the rela-

tionship of stain intensity to protein concentration and the range of concentrations are different for each protein. These studies were completed without the use of sophisticated image acquisition equipment and image analysis software.

Melanie 3 (GeneBio, Geneva, Switzerland) is a commercially available third generation gel analysis software package that features automated analysis and quantitation of multiple gel images obtained by an image acquisition device such as a laser densitometer. Melanie automatically defines five variables that can be used to measure the characteristics of each spot: AREA, optical density (OD), relative OD (%OD), integrated optical density (VOL), and relative integrated optical density (%VOL). The %VOL and %OD of a spot have been used to relate stain intensity to protein amount [5], although %VOL has recently been shown to provide a more accurate reflection of the protein amount [6]. Neither of these variables consider background stain levels and have a limited range of linearity. Attempts to account for background stain levels have focused on scaling factors based on the spot volumes of ubiquitous proteins [7]. An alternate option is to scale each gel by the total volume (VOL) of the gel background. It is desirable to remove the influence of spots not of interest (proteins or technical artifacts) by subtracting their VOL from the gel background. Also, the AREA of the background could be a significant factor when comparing gels of disparate loadings. Therefore, a normalization of the AREA of the gel background is desirable.

To investigate how silver stain intensity relates to protein concentration, a set of three protein standards were separated by 2-DE. These proteins were run both indivi-

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Abbreviations: **DI**, deionized water; **OD**, optical density; **SV**, scaled volume; **VOL**, integrated optical density

dually and as a mixture. The proteins run individually were used to evaluate several methods for measuring stain intensity. The variable that offered the best correlation to protein amount was the scaled volume (SV). The SV is a composite of two Melanie variables, VOL and AREA, for different components of the gel. A calculation of the SV requires manual editing of Melanie detected gel images, so it is not a variable that can easily be calculated in an automated fashion. We use the SV to measure the reproducibility of silver staining among duplicate gels and to estimate the minimum differential SV needed to conclude statistically that two proteins are differentially expressed.

2 Materials and methods

2.1 Protein samples

Trypsinogen (bovine), trypsin inhibitor (soybean), and bovine serum albumin (BSA) were obtained from Sigma Chemicals (St. Louis, MO, USA). These proteins were selected for their disparate *pI* and molecular mass (*M_r*) values. The *pI* values for trypsinogen, trypsin inhibitor, and BSA are 8.7, 4.5, and 5.6, respectively. The *M_r* values for trypsinogen, trypsin inhibitor, and BSA are 25 kDa, 22 kDa, and 66 kDa, respectively. Each pure lyophilized protein was reconstituted in 40 mM Tris (Bio-Rad, Hercules, CA, USA), pH 8.5, to a stock concentration of 10 mg/mL. Serial dilutions of 1 mg/mL, 100 µg/mL, 10 µg/mL, 1 µg/mL, 100 ng/mL, and 10 ng/mL were made from the stock solutions using 40 mM Tris. The volume of dilutions loaded onto 2-DE gels was 2–20 µL, the range of accurate volume measurement for the pipette used.

Each of the three protein samples was loaded individually at 10², 10³, and 10⁶ ng per gel. In addition, the proteins were loaded as a mixture. For the mixture gels, each individual protein ranged from 10⁰ to 10⁵ ng per gel. The protein loadings for the mixture gels are shown in Table 1.

Table 1. Protein loadings used in this study

	Protein (ng)		
	Trypsinogen	Trypsin inhibitor	BSA
A	0.6	0.8	1.0
B	8.0	10.0	15.0
C	50.0	100.0	120.0
D	300.0	400.0	500.0
E	1500.0	2500.0	3500.0
F	7000.0	8000.0	10000.0

The gels for D, E, and F are shown in Fig. 1.

2.2 Electrophoresis

2-DE was performed as described [8]. IEF was performed in pH 3–10 nonlinear 18 cm IPG strips (Pharmacia, Piscataway, NJ, USA). The IPG strips were rehydrated overnight in a solution of the protein samples supplemented with rehydration solution containing 8 M urea (Bio-Rad), 2% CHAPS (Sigma Chemicals), 1.33% BioLyte pH 3–10 carrier ampholytes (Bio-Rad), 0.67% BioLyte pH 5–7 carrier ampholytes (Bio-Rad), and 0.3% DTT (Bio-Rad) such that the total rehydration volume was 400 µL. The IPG strips were run for a total of 64 250 Vh at 20°C on a Multiphor II (Pharmacia). Subsequently, the gel strips were equilibrated in a solution of 6 M urea, 30% glycerol (Mallinckrodt Baker, Phillipsburg, NJ, USA), 2% SDS (Bio-Rad), 2% DTT, and 11.75 mL 0.05 M Tris, pH 6.8. The gel strips were then equilibrated with a solution of 6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide (Fluka, Milwaukee, WI, USA) and 11.75 mL 0.05 M Tris, pH 6.8. Second-dimensional 12% T gels were prepared by mixing appropriate amounts of a stock solution of 30% acrylamide monomer (Bio-Rad) and 0.8% piperazinediacrylamide (Bio-Rad) with 1.5 M Tris, pH 8.6, and Milli-Q purified deionized water (dl). Polymerization was initiated by the addition of a fresh solution of 10% ammonium persulfate (Bio-Rad) and TEMED (Bio-Rad). These gels were overlaid with the IPG gel strip and run at a constant 40 mA at 12°C until the dye front reached the gel label.

2.3 Detection

After SDS-PAGE, proteins were visualized with an ammoniacal silver stain [8]. All steps were performed on an orbital shaker in 250 mL of liquid in glass staining trays with two gels per tray. Following SDS-PAGE, the gels were washed for 5 min in dl and fixed for 1 h in a solution of 40% ethanol and 10% acetic acid. The gels were rehydrated overnight in a solution of 5% ethanol and 5% acetic acid. Gels were incubated in a 10% glutaraldehyde solution for 30 min and excess glutaraldehyde was washed by four short dl washings and three 30-min dl washings. Fresh ammoniacal silver solution was prepared by dissolving 6 g of silver nitrate in 30 mL dl. This solution was slowly added to a solution of NaOH and ammonium hydroxide to a final concentration of 0.8% silver. Gels were incubated in this silver solution for 20 min before being washed by three times in dl. The gels were developed in 0.01% w/v citric acid and 0.037% v/v formaldehyde. The gels with single protein species were developed optimally by visual inspection. For the gels with a mixture of proteins, four replicates were performed. Two of the four replicates were developed optimally based on visual inspection. The third replicate gel was

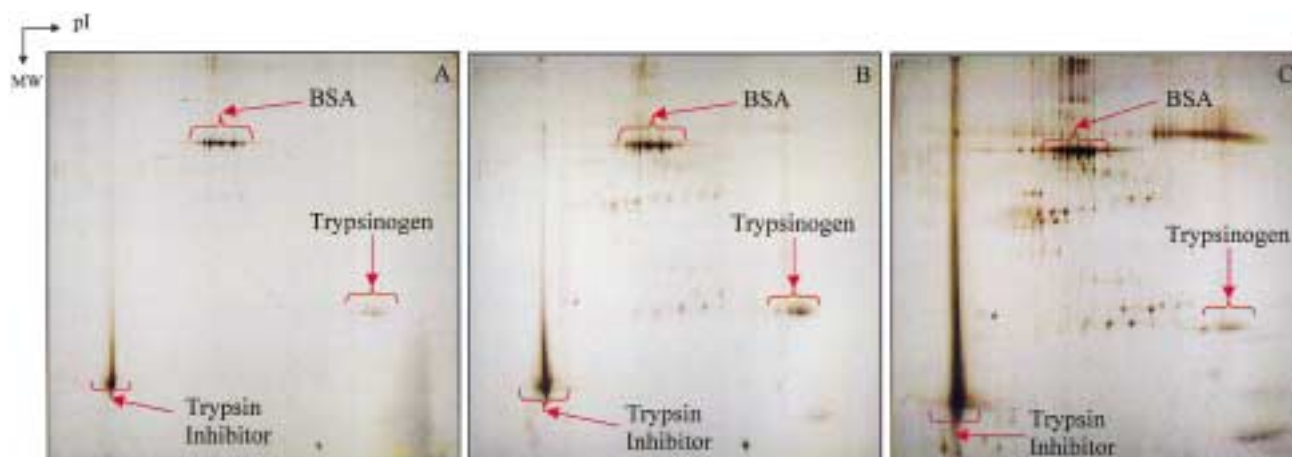


Figure 1. (A) Silver-stained 2-DE gel corresponding to D from Table 1. The three model proteins are indicated with arrows. (B) Silver-stained 2-DE gel corresponding to E from Table 1. The three model proteins are indicated with arrows. (C) Silver-stained 2-DE gel corresponding to F from Table 1. The three model proteins are indicated with arrows. The appearance of other spots in (A)–(C) were considered to be contamination of the lyophilized protein and therefore proteins not of interest.

purposefully overdeveloped and the fourth gel was underdeveloped. Development was stopped by incubation in a 30% w/v citric acid solution before scanning with a Molecular Dynamics Personal Densitometer SI (Sunnyvale, CA, USA) at 50 μm resolution and 12 bits per pixel.

2.4 Gel analysis

Melanie 3 (GeneBio) was used for gel image analysis. Spot boundaries were determined with the default automatic detection parameters and were subsequently edited by visual inspection. The gray levels (contrast) for each gel were set to the same values. Subsequently, individual pixels that were a minimum of 25% darker than the background were selected as part of a spot. One user completed spot editing for the entire set of gels. To measure the staining variability of the gel background, a copy of each gel image was made and the entire image above the dye front minus the edges was manually selected as one spot. All spot data were exported from Melanie to Microsoft Excel (Microsoft, Redmond, Washington, USA). Excel was used to calculate the SV for spots of interest and to plot spot data for each gel.

3 Results

Representative mixture gels are shown in Fig. 1. The data from the set of nine gels with individually loaded proteins was used to compare several variables for stain intensity with respect to protein quantity. Three variables defined by Melanie were considered. First, %VOL was used to quantify the protein amounts. In addition, OD and %OD were considered. A fourth stain variable was constructed

by a combination of two standard Melanie variables: VOL and AREA. The SV is calculated to account for differences in stain intensity for the protein of interest, background staining differences, and to remove differences caused by spots that are not of interest, e.g., technical artifacts. The defining equation for the SV is given in Eq. (1).

$$SV = \frac{VOL_{\text{spots of interest}}}{\frac{(VOL_{\text{gel background}} - VOL_{\text{spots not of interest}})}{(AREA_{\text{gel background}} - AREA_{\text{spots not of interest}})}} \quad (1)$$

The numerator measures the signal response – the quantity of the protein of interest. The normalization factor scales the signal response by the noise (gel background) with secondary signals removed (spots not of interest). Moreover, to account for variable gel sizes and quantities of spots not of interest, the normalization factor is scaled by AREA. Therefore, the SV is a variable that represents the stain intensity of a spot of interest normalized with respect to variations in background staining with disparities caused by spots not of interest removed. Another description is that the SV represents the signal of interest, normalized by the noise, and without secondary signals. Figure 2 shows how the four stain intensity variables relate to protein quantity for the individual protein gels over four orders of magnitude. No obvious relationship for OD, %OD, and %VOL is observed on linear or log-linear plots. We also observe no obvious trend for VOL or AREA (data not shown).

The gels loaded with a mixture of the proteins were used to estimate the experimental error inherent in our silver staining protocol, as measured by SV. An important con-

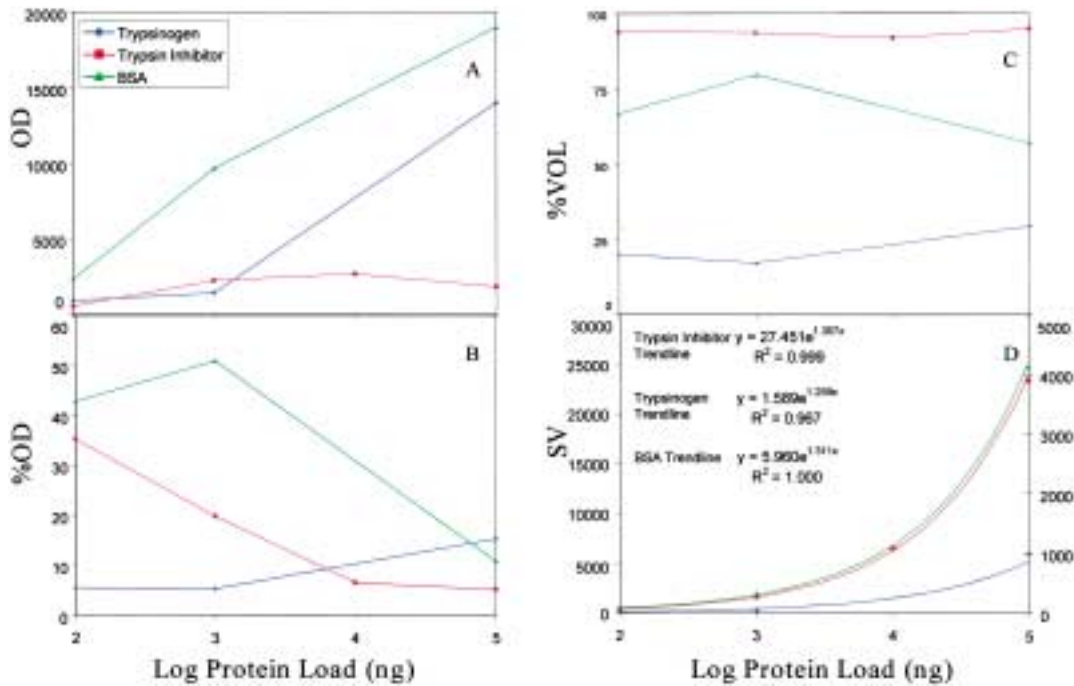


Figure 2. (A) The relationship between OD and protein amount over four orders of magnitude for trypsinogen, trypsin inhibitor, and BSA as determined by Melanie. (B) Relationship between %OD and protein amount. (C) Relationship between %VOL and protein. (D) Relationship between SV and protein amount. Trypsin inhibitor is plotted on the primary ordinate axis on the left-hand side of the graph. Trypsinogen and BSA are plotted on the secondary ordinate axis on the right-hand side of the graph. The exponential fit for each protein is shown, as well as the coefficient of determination (generated by Excel). The similarity of the trendlines for each protein indicates a common mechanism of stain development.

sideration is the relationship between the amount of error and the quantity of protein considered. A plot of the logarithm of SD against logarithm protein load shows that the SD increases linearly with increasing protein loads (Fig. 3). Therefore, we express the error as CV [9]. The CV is the SD divided by the mean. Four data points for each protein load (two normally stained gels, one overstained gel, one understained gel) were used to calculate the average, SD, and CV for that load. The maximum CV value for one order of magnitude of protein concentrations is shown in Fig. 4. There was not a significant difference in the SV for each protein for understained or overstained gels as compared to the normally stained gels.

4 Discussion

4.1 Reproducibility

Figure 4 shows that the maximum variation in silver stain intensity as measured by the CV for SV is 1.3 over the range 1–10 000 ng of protein. Anderson *et al.* [9] reported that the CV of integrated spot densities (VOL) for 106 out of 137 proteins (77%) detected with Coomassie blue from a mouse

liver sample are less than 0.15. In a similar study, it was shown that 41% of human leukocyte proteins had a VOL CV of less than 0.15 when ammoniacal silver stain was the detection method used [7]. Our results indicate that 50% of the protein loadings for the three proteins studied have SV CV values less than 0.35. An important consideration is that previous studies used proteins from tissue samples, not purified proteins. The range of protein load for the tissue samples was based on the number of source cells per gel. However, purified proteins enable us to better define the range of protein load where the SV is representative of protein amount. In addition, we used different computer-assisted gel analysis software than both of these previous studies. Proteins loaded at < 1 ng were not considered in this analysis because we observe that this is the reliable limit for silver staining according to our protocol. This is in agreement with limits observed by others [4].

4.2 Staining characteristics

Figure 2 indicates that the SV is a variable that correlates silver stain intensity and protein amount somewhat better than OD, %OD, or %VOL for the three proteins studied. In

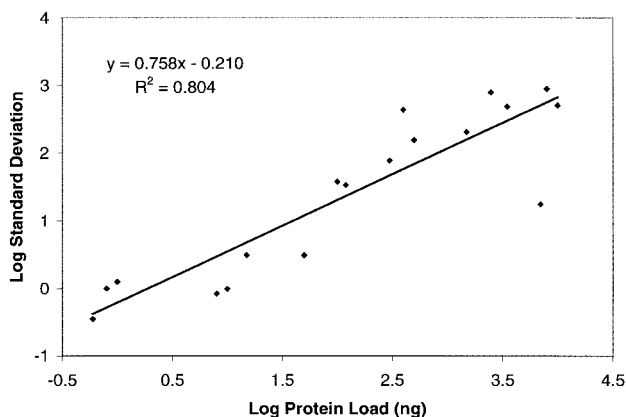


Figure 3. A plot of the log standard deviation versus log protein amount for the gels with one protein loaded per gel. The data are consistent with the assumption of a linear relationship between abundance and standard deviation.

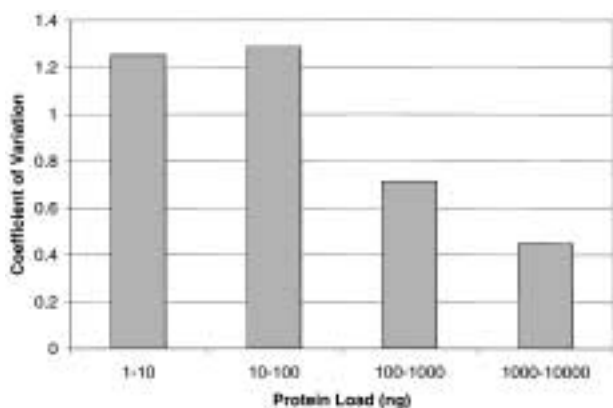


Figure 4. CV of variation for discrete quantities of protein. The data suggest that the silver stain is more reproducible at higher protein loads.

addition, the shape of the SV plot for all three proteins is very similar. They all fit an equation of the form $y = a \cdot \exp(bx)$ where y is SV, x is the log of the protein quantity, and a and b are parameters which have similar values for all three proteins studied. The values of a and b suggest that although different proteins respond to silver stain differently, there is an inherent similarity in the stain response for all proteins. Indeed our results of similar experiments with other proteins also yield values for a and b which are similar to these (data not shown).

Although the exact mechanism of silver staining is unknown, some probable mechanisms have been described [10]. Our data suggest that many proteins respond to silver stain in a similar manner, *i.e.*, the shape

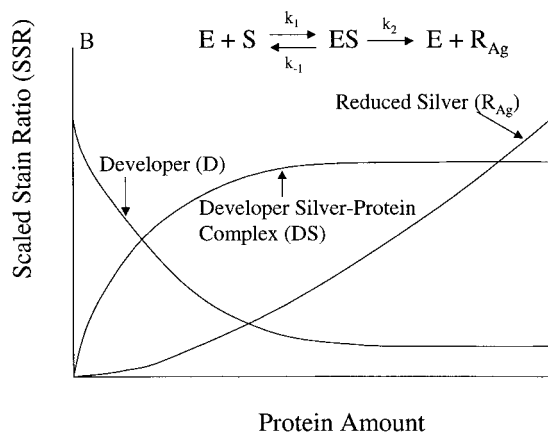
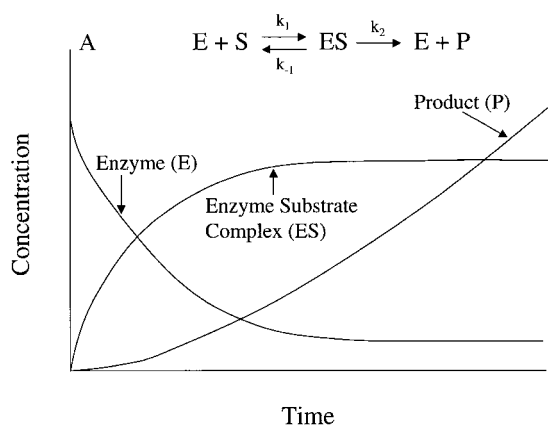


Figure 5. (A) The initial time evolution for species in Michaelis-Menten type kinetics. (B) Michaelis-Menten type kinetics used as a model for the development of silver stain. The essential feature of the graph is that the curve for reduced silver is similar to the protein curves in Fig. 2D.

of the curves for all three proteins on Fig. 2D are similar. We also note that the shape of these curves is similar to the initial time course of product formation for Michaelis-Menten type kinetics [11], shown in Fig. 5A. To make an analogy to silver stain, the variables of Fig. 5A are changed; time is replaced by protein amount, concentration is replaced by SV, the enzyme is the developing agent (formaldehyde here), the substrate is the silver ion-protein complex, and the product is reduced silver (in the areas where it was complexed to a protein). These changes are shown in Fig. 5B. Limitations to this model are that the SV does not plateau at high protein amounts and that the developer is not reused during the reaction. However, the essential features of this model suggests a possible reason for the similarity of the curves for multiple proteins – all three proteins were developed with formaldehyde as the developing agent. This should result in the same rate

constant (k_1) for the initial step in development. The differences in the curves could be caused by differences in the second rate constant (k_2); k_2 could be a function of the affinity of a given protein (trypsinogen, trypsin inhibitor or BSA here) for silver ions.

In general, silver stain response, as measured by the SV, increases exponentially as protein amount increases. Our results indicate that the most important variables that control final stain intensity, are protein amount, protein composition (sequence), and development time. However, the SV is able to correct for differences in development time as evidenced by the similarity of overstained, understained, and optimally stained gels. This illustrates that the SV is potentially useful in correcting for more subtle variations in staining intensity that might be caused during normal staining procedures. Further studies using real mixtures of proteins from biological samples are planned to explore the full utility of the SV.

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