Solid-Phase Metal Chelate Assay for Quantifying Total Protein: Resistance to Chemical Interference

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ABSTRACT

Recently, we developed reversible metal chelate stains that are fully compatible with immunoblotting and protein sequencing. Membrane supports are incubated in Ferrozine®/ferrous complex followed by ferrocyanide/ferric complex (double-metal chelate [DMC] stain). Proteins are quantified by computerized densitometry. In this study, the metal chelate stains are used for routine protein quantitation. Manually applying samples to membranes leads to variable spot spreading. Better results are achieved using a slot-blot apparatus to maintain a constant application area. The Ferrozine/ferrous and DMC assays are compared to colloidal gold and bicinchoninic acid (BCA) assays with respect to chemical interference, protein-to-protein variation, dynamic linear range and sensitivity. The DMC assay provides a superior linear range (100-fold) when compared with colloidal gold (10-fold range) and BCA assays (47-fold). Though the colloidal gold assay is more sensitive, it suffers from poor reproducibility, high protein-to-protein variation and lower tolerance to interfering agents. The BCA assay has the least protein-to-protein variation but is also least sensitive and most susceptible to interfering agents.

INTRODUCTION

Proteins are quantified by a variety of methods based upon different physical principles. UV spectrophotometry utilizes the intrinsic absorptive properties of aromatic amino acids for quantitation (2,16). Though proteins are not chemically altered, the UV method is insensitive and inherently exhibits protein-to-protein variation since the frequency of tryptophan and tyrosine residues varies among different proteins. Turbidimetric methods rely upon protein precipitation and subsequent spectrophotometric analysis (2,16). Trichloroacetic acid is a popular precipitating agent, but nucleic acids can also precipitate and interfere with analysis (2). Also, turbidimetric assays display poor sensitivity and accuracy, high protein-to-protein variation and limited dynamic range (16). Other solution-based techniques, such as the biuret and Folin-Ciocalteu (Lowry) assays, rely upon chemical reactions with proteins to generate a colored product (2,16). These techniques are susceptible to interference from some commonly used reagents that also produce colored product (2). In the solution-based bicinchoninic acid (BCA) protein assay, reduced copper is chelated to two molecules of BCA, yielding a purple product with strong absorbance at 562 nm (23). The BCA assay is more tolerant to interfering agents, but remains sensitive to reducing agents such as β -mercaptoethanol (BME) and dithiothreitol (DTT), as well as chelating agents such as ethylenediaminetetraacetic acid (EDTA). Finally, solution-

based methods such as the Coomassie Brilliant Blue and pyrogallol red-molybdate assays measure shifts in the wavelength maximum of dye absorbance upon formation of a dye-protein complex (1,16,26). Dye-binding methods are highly tolerant to chemical agents in the sample (2). Most depend upon electrostatic interaction of anionic dyes with α - and ϵ -amino side-chain moieties at acidic pH and thus are more subject to protein-to-protein variation than the biuret and Folin-Ciocalteu assays (5,18,25,27).

Many solution-based total protein assays and electrophoresis stains (amido black, Ponceau red, Fast green, BCA, Coomassie Brilliant Blue, Folin-Ciocalteu, Benzoxanthine yellow, colloidal gold, colloidal iron and India ink) are adaptable to solid-phase assays (4-9,11-15,21,22,24). Solid-phase protein assays are less susceptible to interfering agents commonly found in samples. Since protein is specifically bound to membranes, contaminants are readily washed away. Total protein quantitation is achieved directly on the membrane by densitometry using instrumentation for quantitation of protein bands in polyacrylamide gels. Alternatively, the stains can often be eluted from membranes and quantified by spectrophotometry.

Recently, we developed a sensitive metal chelate stain for proteins electroblotted to nitrocellulose and polyvinylidene difluoride (PVDF) membranes (18). The stain is fully reversible and compatible with protein sequencing and immunoblotting (18). It is based upon binding of Ferrozine®/ferrous

complexes to electroblotted proteins, followed by binding of ferrocyanide/ferric complexes for enhanced sensitivity. The double-metal chelate (DMC) stain is evaluated in this study for its use in a solid-phase total protein assay. The DMC stain is compared to the Ferrozine/ferrous, BCA and colloidal gold assays with respect to sensitivity, dynamic range, protein-to-protein variation and tolerance to interfering agents.

MATERIALS AND METHODS

Materials

Potassium ferrocyanide, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine disodium salt (Ferrozine), polyvinyl pyrrolidone-40 (PVP-40), thioglycolic acid, light white mineral oil, chicken ovalbumin, bovine serum albumin (BSA), bovine gelatin and bovine hemoglobin were obtained from Sigma Chemical (St. Louis, MO, USA). Nitrocellulose membranes, 0.45- μ m pore size, were obtained from Bio-Rad (Hercules, CA, USA). Nytran membranes, 0.45- μ m pore size, were obtained from Schleicher & Schuell (Keene, NH, USA). Ferric chloride was obtained from Matheson Coleman & Bell (Norwood, OH, USA). Gold chloride was obtained from Polysciences (Warrington, PA, USA).

Dot Blotting

For analysis of the linear response of the protein assays, dilutions of BSA ranging from 1.5 ng/ μ L to 10 μ g/ μ L were prepared from a 10 μ g/ μ L stock in distilled and de-ionized water (dd-H₂O). All BSA stock solutions were prepared gravimetrically. Interfering agents were prepared as 2 $\times$ stocks and mixed with equal volumes of 0.2 μ g/ μ L BSA. One microliter of each sample was applied onto nitrocellulose strips ($n = 2$). After allowing the membranes to air dry, they were incubated in 0.1% PVP-40 in phosphate-buffered saline (PBS). The Ferrozine/ferrous complex was prepared as a solution of 0.75 mM Ferrozine, 30 mM FeCl₃, 15 mM thioglycolic acid in 2% acetic acid. The ferrocyanide/ferric complex was prepared as a solution of 100 mM potassium ferrocyanide (K₄Fe(CN)₆), 60 mM FeCl₃, in 100 mM sodium acetate, pH 4.0. The

colloidal gold was prepared with modifications as previously described (10). Membranes were incubated in staining solutions for 10–15 min, until colored spots appear. DMC staining was performed by sequential incubation in the Ferrozine/ferrous and ferrocyanide/ferric complexes (18). After staining the membranes, unbound dye was washed away with a 1–2-min incubation in dd-H₂O.

Slot Blotting

Slot blotting was performed using a Bio-Dot[®] SF vacuum apparatus (Bio-Rad) according to the manufacturer's instructions. Membranes were rehydrated with 100 μ L/well of dd-H₂O, samples were loaded and the wells rinsed. In all slot blot procedures, equal volumes of sample and wash buffers were used. Washes were always with dd-H₂O for colloidal gold stain and 100 mM sodium acetate, pH 4.0, for metal chelate stains.

For analysis of dynamic range and protein-to-protein variation, stock solutions of proteins were first prepared in dd-H₂O at concentrations ranging from 0.02 to 450 ng/ μ L and subsequently diluted with 4 volumes of the appropriate wash buffer. Five hundred microliters per well of sample were applied to yield total protein amounts ranging from 2.1 ng to 45 μ g. BSA was used to determine the dynamic linear range of each stain. For analysis of protein-to-protein variation, we used a five-point standard curve corresponding to the midpoint of the linear range for each stain. The proteins evaluated were BSA, gelatin, hemoglobin and ovalbumin. For both the Ferrozine/ferrous and DMC stains, the five standard curve points were 0.23, 0.34, 0.49, 0.73 and 1.1 μ g. For colloidal gold, the points were 16, 21, 37, 53 and 68 ng.

For analysis of interfering agents, a quantity of BSA was chosen that roughly corresponded to the midpoint of the linear range for each stain. This ensured the detection of both false-positive and false-negative responses to the tested reagents (Table 1). BSA (0.70 μ g) was used for the Ferrozine/ferrous chelate stain, 0.25 μ g for the DMC stain and 53 ng for the colloidal gold. Proteins and interfering agents were

prepared separately in dd-H₂O as 2 $\times$ stocks. Equal volumes of each were combined and mixed on a vortex mixer. Solutions of the interfering agents were combined with equal volumes of dd-H₂O. The proteins were also prepared in dd-H₂O in the absence of interfering agents to serve as controls. Samples were diluted 4-fold in the appropriate wash buffer and applied to the slot blot apparatus (500 μ L/well). Following sample application to the slot blotter, membranes were blocked for 10–15 min with 0.1% PVP-40 in 100 mM sodium acetate pH 4.0 for the metal chelate stains and in PBS for colloidal gold. The staining and rinsing steps were identical to those of the dot blots described above.

Densitometry

Membranes were air-dried prior to analysis. Reflective imaging was employed for opaque nitrocellulose membranes using a document scanner (UC-630 MaxColor[®]; UMAX Data System, Hsinchu, Taiwan) interfaced with a Macintosh[®] Power PC 7100/66 (Apple Computer, Cupertino, CA, USA). A yellow cellophane photographic filter (Smith-Victor, Griffith, IN, USA) was used to enhance detection of the colored stains (3). A 3 $\times$ 3 pixel median filter was applied to the images to reduce noise (19). Quantitation was performed with NIH Image Version 1.57 public domain software developed by Dr. W. Rasband, Research Services Branch, NIMH (wayne@helix.nih.gov). Background subtraction was applied using the 2-D rolling ball algorithm within NIH Image; however, it had no effect on any of the assays (data not shown). NIH Image was also used to measure spot area for dot blots. Briefly, the scanned images were calibrated with a standard metric ruler, and the spot areas were obtained using the circular selection tool in NIH Image. Similar results were obtained utilizing the charge-coupled device (CCD)-based Bio Image system running on a Sun Microsystems SPARCstation[®] (Bio Image, Ann Arbor, MI, USA) (17).

To extend the dynamic range of the metal chelate stain, membranes were rendered clear for transmission imaging. Dry membranes were immersed in a small quantity of light white mineral

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oil and stored between two sheets of overhead transparency film. Transmission imaging was performed with a Videk® CCD camera (Eastman Kodak, Rochester, NY, USA) and Bio Image's Whole Band Analyzer software (17). Neutral density (0.3 optical density [OD]) and 600 ± 70 nm band-pass filters were used to block excessive transmitted light and enhance the signal-to-noise ratio, respectively. A 3×3 pixel median filter was also applied to these images for noise reduction (18).

BCA Assay

The BCA assay was performed according to the method of Smith et al. using the Pierce Chemical (Rockford, IL, USA) 60°C enhanced protocol (23). For analysis of dynamic linear range and protein-to-protein variation, proteins were prepared at concentrations ranging from 100 ng/100 μ L to 300 μ g/100 μ L in dd-H₂O. BSA was used for analysis of the dynamic linear range ($n = 4$), while BSA, hemoglobin, ovalbumin and gelatin were used to evaluate protein-to-protein variation ($n = 2$). The five-point mid-range standard curve used to measure protein-to-protein variation was 5.7 μ g/100 μ L, 6.7 μ g/100 μ L, 10.0 μ g/100 μ L, 15 μ g/100 μ L and 23 μ g/100 μ L. Interfering agents ($n = 2$) were prepared as 2 $\times$ stocks and mixed with equal volumes of 20 μ g/100 μ L BSA (both in dd-H₂O). Interfering agents were also evaluated without addition of protein. Control samples were prepared with and without 10 μ g/100 μ L of BSA (final concentration) in dd-H₂O in the absence of interfering agents.

RESULTS AND DISCUSSION

Dot Blot Assay

When integrated optical density is plotted against protein amount, a biphasic response is observed in dot-blot assays visualized with the Ferrozine/ferrous, DMC and colloidal gold stains (Figure 1B). Plots are characterized by a steep initial slope, followed by a shallower slope at higher protein concentrations. The phenomenon is observed with all proteins evaluated in this study and is illustrated with gelatin visualized with the DMC stain (Figure 1A). The

initial slope, ranging from 5 to 340 ng, appears to be due to changes in stain intensity (Figure 1A, row 1). Stain intensity saturates at about 0.5 μ g. The shallower slope, generated with protein amounts ranging from 0.5 to 10 μ g, appears to result from progressive increases in spot area (Figure 1A, row 2). Plotting spot area as a function of the mean pixel density (average grey-scale intensity) verifies the cause of the two curves (data not shown). With 5 to 400 ng of applied protein, spot area remains constant at 1.2 mm², but mean pixel density increases from 1 to 8 arbitrary units (AU). For protein amounts of 400 ng to 10 μ g, the mean pixel density increases only slightly (from 8 to 9.5 AU), while spot area increases from 1.2 to 6 mm². Tris-HCl, sodium dodecyl sulfate (SDS) and Triton® X-100 increase spot area, while MgCl₂ and CaCl₂ decrease spot area in both colloidal gold- and DMC-stained dot blots (data not shown). It is expected that us-

ing membranes with different protein binding and wetting capacities would alter the shape of solid-phase dot blot standard curves. Metal chelate stains can be used with a variety of membranes including nitrocellulose, PVDF and nylon (Reference 18, and unpublished observations).

Linear Range

Variation in spot spreading is effectively eliminated when a vacuum slot-blot device is used, which restricts the protein application region to a defined area that cannot be altered by buffer composition or protein concentration. The device used in this study maintains a constant membrane surface area of 5.25 mm² for protein binding. In the remainder of this paper, all comparisons of dynamic linear range, sensitivity, protein-to-protein variation and susceptibility to interfering agents utilize the device. Sensitivity is expressed as total protein per sample for all assays.

Figure 1. Evaluation of linearity of the solid-phase dot-blot assay using serially diluted bovine gelatin. Panel A: 5–340 ng (row 1) and 0.5–10 μ g (row 2) of total protein were applied to nitrocellulose strips and visualized with the DMC stain. Panel B: Stained spots were quantified using computer-assisted image analysis, which revealed two distinct linear regions.

Limits of linearity are determined by the following criterion: Standard deviations (error bars) of data points at the high and low end of standard curves are required to overlap the best fit line to be considered part of the range.

Evaluation of nitrocellulose membranes by reflectance imaging indicates that the dynamic linear range for protein detection is 0.15 to 1.5 μg (10-fold range) for both the Ferrozine/ferrous (Figure 2A) and the DMC stains (data not shown). The dynamic linear range of the colloidal gold assay is 10.5 to 100.0 ng (10-fold) (Figure 2C). The linearity of the colloidal gold assay ($r = 0.94$) is poorer than the other solid-phase and solution-phase assays ($r = 0.99$ for the Ferrozine/ferrous chelate assay and $r = 1.0$ for the BCA and DMC assays). The dynamic linear range for the BCA assay is 1.1 μg to 49 μg (47-fold) (Figure 2D).

When high amounts of protein ($>1.6 \mu\text{g}$) are applied to nitrocellulose membranes, staining is often observed on the membrane surface opposite the protein application. Though the protein on the reverse side of the membrane is

stained by the solid-phase procedures, it is not detected by reflectance imaging, leading to low estimates of total protein amount. This problem can be circumvented by rendering membranes transparent and subsequently quantifying the stain by transmission imaging. The dynamic linear range of the DMC stain increases to 100-fold when nitrocellulose membranes are rendered transparent with mineral oil and imaged transmissively (0.10–10.0 μg) (Figure 2B). Because of significant fading of the stain when membranes are rendered transparent, the dynamic linear range of the Ferrozine/ferrous chelate stain does not increase. Because of extensive stain smearing and flaking with higher amounts of protein, the linear dynamic range of the colloidal gold stain does not improve.

Protein-to-Protein Variation

Protein quantitation requires predictable relationships between the amount of protein and integrated OD (20). Ideally, all proteins should produce identical slopes and threshold responses when integrated OD is plotted as a

Figure 2. Determination of the dynamic linear range of various solid-phase and solution-based total protein assays using serially diluted BSA. Panel A: Ferrozine/ferrous chelate stain. Panel B: DMC stain. Panel C: Colloidal gold stain. Panel D: BCA assay. “b” is the Y-intercept, “m” is the slope and “r” is the linear correlation coefficient of the best fit line through the data points. Error bars correspond to the standard deviation of each determination. Integrated OD is determined by transmission imaging in Panel B; and reflectance imaging, in panels A and C.

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Table 1. Evaluation of Interference from Commonly Used Chemical Agents

Agents		% of Control $\pm$ SD			
		Ferrozine	DMC	Gold	BCA
Buffers					
Tris-HCl	1 M	91 $\pm$ 12	91 $\pm$ 15	233 $\pm$ 47	28 $\pm$ 4
	0.1 M	92 $\pm$ 4	96 $\pm$ 8	243 $\pm$ 35	102 $\pm$ 3
	0.01 M	111 $\pm$ 6	91 $\pm$ 8	174 $\pm$ 5	105 $\pm$ 2
Glycine	1 M	91 $\pm$ 12	110 $\pm$ 11	275 $\pm$ 415	44 $\pm$ 0.2
	0.5 M	92 $\pm$ 6	124 $\pm$ 13	161 $\pm$ 178	76 $\pm$ 0.2
	0.1 M	91 $\pm$ 6	104 $\pm$ 7	202 $\pm$ 131	117 $\pm$ 1
Salts					
(NH ₄) ₂ SO ₄	20%	117 $\pm$ 15	103 $\pm$ 13	220 $\pm$ 64	50 $\pm$ 2
	10%	113 $\pm$ 5	111 $\pm$ 6	249 $\pm$ 62	80 $\pm$ 4
	0.2%	105 $\pm$ 14	101 $\pm$ 10	233 $\pm$ 14	110 $\pm$ 0.1
KCl	100 mM	92 $\pm$ 13	98 $\pm$ 15	194 $\pm$ 38	105 $\pm$ 5
	10 mM	110 $\pm$ 13	98 $\pm$ 25	252 $\pm$ 68	102 $\pm$ 7
	1 mM	120 $\pm$ 5	100 $\pm$ 20	180 $\pm$ 48	107 $\pm$ 4
MgCl ₂	100 mM	117 $\pm$ 13	116 $\pm$ 26	178 $\pm$ 61	88 $\pm$ 4
	10 mM	119 $\pm$ 6	115 $\pm$ 4	227 $\pm$ 27	102 $\pm$ 6
	1 mM	109 $\pm$ 8	100 $\pm$ 22	229 $\pm$ 20	103 $\pm$ 6
Detergents					
Triton X-100	10%	19 $\pm$ 9	18 $\pm$ 8	153 $\pm$ 9	188 $\pm$ 5
	1%	10 $\pm$ 8	27 $\pm$ 12	164 $\pm$ 36	121 $\pm$ 5
	0.1%	16 $\pm$ 1	46 $\pm$ 14	112 $\pm$ 8	118 $\pm$ 1
SDS	10%	57 $\pm$ 6	154 $\pm$ 10	8 $\pm$ 4	117 $\pm$ 2
	1%	81 $\pm$ 4	129 $\pm$ 8	22 $\pm$ 11	115 $\pm$ 0.2
	0.1%	107 $\pm$ 4	144 $\pm$ 8	22 $\pm$ 11	108 $\pm$ 7
Tween® 20	10 %	17 $\pm$ 6	28 $\pm$ 6	233 $\pm$ 36	195 $\pm$ 2
	1%	17 $\pm$ 15	21 $\pm$ 12	282 $\pm$ 77	125 $\pm$ 1
	0.1%	4 $\pm$ 1	34 $\pm$ 21	240 $\pm$ 108	115 $\pm$ 2
Reductants					
DTT	10 mM	107 $\pm$ 5	116 $\pm$ 15	107 $\pm$ 131	685 $\pm$ 16
	1 mM	111 $\pm$ 15	104 $\pm$ 19	175 $\pm$ 93	385 $\pm$ 10
	0.1 mM	101 $\pm$ 14	104 $\pm$ 11	183 $\pm$ 113	114 $\pm$ 3
BME	10%	91 $\pm$ 13	111 $\pm$ 14	20 $\pm$ 7	0.0 $\pm$ 0.0
	1%	108 $\pm$ 13	97 $\pm$ 13	43 $\pm$ 10	653 $\pm$ 9
	0.1%	112 $\pm$ 9	108 $\pm$ 26	141 $\pm$ 21	653 $\pm$ 12
Miscellaneous					
EDTA	100 mM	100 $\pm$ 9	108 $\pm$ 11	162 $\pm$ 32	0.0 $\pm$ 0.0
	10 mM	110 $\pm$ 5	96 $\pm$ 11	101 $\pm$ 19	99 $\pm$ 2
	1 mM	103 $\pm$ 6	117 $\pm$ 14	37 $\pm$ 4	103 $\pm$ 1
Sucrose	40%	100 $\pm$ 5	107 $\pm$ 4	77 $\pm$ 8	209 $\pm$ 0.2
	20%	96 $\pm$ 8	108 $\pm$ 15	92 $\pm$ 12	163 $\pm$ 3
	0.4%	98 $\pm$ 5	101 $\pm$ 13	80 $\pm$ 22	116 $\pm$ 2
Glycerol	20%	118 $\pm$ 9	136 $\pm$ 16	292 $\pm$ 97	637 $\pm$ 4
	10%	102 $\pm$ 11	117 $\pm$ 7	232 $\pm$ 75	513 $\pm$ 17
	0.1%	94 $\pm$ 10	117 $\pm$ 16	216 $\pm$ 35	144 $\pm$ 2
PVP-40	0.1%	15 $\pm$ 14	48 $\pm$ 9	132 $\pm$ 61	104 $\pm$ 3
	0.01%	22 $\pm$ 5	67 $\pm$ 15	128 $\pm$ 61	104 $\pm$ 3
	0.001%	68 $\pm$ 12	86 $\pm$ 16	92 $\pm$ 16	105 $\pm$ 4
Ampholyte	1%	95 $\pm$ 12	120 $\pm$ 5	149 $\pm$ 26	113 $\pm$ 7
	0.1%	99 $\pm$ 3	122 $\pm$ 13	79 $\pm$ 25	100 $\pm$ 5
	0.01%	110 $\pm$ 7	122 $\pm$ 13	62 $\pm$ 12	92 $\pm$ 4

100 μ L of each interfering agent were used. Samples also contained 0.70 μ g/well (Ferrozine assay), 0.25 μ g/well (double-metal chelate assay), 53 ng/well (colloidal gold assay) or 10 μ g/100 μ L (BCA assay) of BSA. Control samples were prepared in dd-H₂O containing the appropriate quantity of BSA. Integrated intensities (Ferrozine, double-metal chelate and colloidal gold) and absorbance (BCA) values were obtained in the presence of the interfering agents and calculated as a percentage of the corresponding control values.

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function of protein amount. In reality, slope and threshold values for different proteins often vary, making it difficult to assign absolute mass to a given protein based upon measurable integrated OD. Numerous dye-binding protein assays are characterized by individual proteins producing linear standard curves, but with slopes and threshold detection levels that differ for each protein. This is due to differences in the mole percentage of basic amino acids in each protein (5,18,25,27). For truly quantitative estimates of protein amount, a standard curve must be produced for each protein assayed.

Protein-to-protein variation for the solid-phase and solution-phase assays are assessed using standard curves generated for four known proteins (Figure 3). The curves are evaluated by comparing the mean integrated OD values (solid-phase assays) or absorbance values (solution-based assay) obtained for

ovalbumin, hemoglobin and gelatin with the values obtained from equal amounts of BSA. The colloidal gold stain significantly underestimates the amount of ovalbumin, yielding a mean ratio of 0.3 ± 0.2 relative to BSA. The DMC stain also slightly underestimates (0.7 ± 0.10), while the Ferrozine/ferrous chelate and the BCA methods accurately estimate the amount of ovalbumin (1.0 ± 0.1). The Ferrozine/ferrous chelate, DMC and colloidal gold stains overestimate hemoglobin (1.5 ± 0.3 , 1.2 ± 0.2 and 1.7 ± 0.6 , respectively) and gelatin (1.5 ± 0.3 , 1.5 ± 0.2 and 2.0 ± 0.3 , respectively). The BCA assay accurately estimates the amount of hemoglobin (1.0 ± 0.1), but underestimates the amount of gelatin (0.7 ± 0.0).

Next, the slopes from the calculated best line fits for each standard curve are compared to the slope of the BSA curve. The colloidal gold and Ferro-

zine/ferrous chelate stains show the highest protein-to-protein variation with respect to this parameter. Relative to BSA, the colloidal gold yields standardized slope values of -0.1, 0.6 and 1.3 for ovalbumin, hemoglobin and gelatin, respectively. The Ferrozine/ferrous chelate produces a steeper slope with hemoglobin (1.5), but ovalbumin and gelatin are similar to the BSA reference (0.8 and 0.9, respectively). The DMC and BCA methods demonstrate the lowest protein-to-protein variation. The DMC stain produces slope values of 0.9, 0.9 and 0.8 for ovalbumin, hemoglobin and gelatin, respectively, whereas the BCA assay yields values of 0.8 for all three proteins when compared to the BSA reference. Evaluation of the standard deviations obtained from measuring the four test proteins indicates that the BCA assay has the lowest standard deviation value (6%), followed by the DMC stain (9%),

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Ferrozine stain (12%) and colloidal gold stain (23%).

Thus, the DMC assay displays relatively little protein-to-protein variation with respect to slope of the standard curve, but does exhibit moderate variation in threshold detection sensitivity. The BCA assay also exhibits low variation in slope; and, among the five proteins evaluated, threshold detection sensitivity differs substantially only for gelatin. With respect to both threshold detection sensitivity and slope, the Ferrozine/ferrous chelate stain exhibits moderate, while the colloidal gold stain exhibits high, protein-to-protein variation. Ovalbumin, in particular, is very poorly stained using the colloidal gold method.

Although four model proteins are evaluated in this paper, the DMC protocol effectively stains 800–1000 proteins in complex cell lysates separated by two-dimensional gel electrophoresis and electroblotted to nitrocellulose membrane, with a sensitivity similar to silver staining (18). The DMC stain is more sensitive and exhibits lower protein-to-protein variation than other commonly used dye-binding stains (Ponceau red, Fast green and amido black) (18). In addition, the stain is suitable for quantifying small peptides (leucine enkephalin and apidaecin) (18).

Interfering Agents

In solid-phase protein assays, dilute protein samples are easily concentrated onto the membrane, since multiple sample applications can be performed to accommodate large volumes. Solution-based assays detect only a finite protein concentration. If the detection limit for a solution-based assay is reached, the sample-to-assay reagent ratio must be increased or the sample must be concentrated. Increasing sample volume is likely to increase chemical interference from buffer components (23). Solution-based assays, however, generally involve fewer handling steps where protein losses may occur.

The solid-phase and solution-based assays were examined with respect to susceptibility to interfering agents (Table 1). The number of agents tested was extensive, but easily interpreted by defining significant interference as a 2-fold increase or decrease in integrated

OD. The Ferrozine/ferrous and DMC assays are significantly inhibited by 0.1%–10% Triton X-100 and Tween 20. PVP-40 also inhibits the Ferrozine/ferrous chelate stain at concentrations of 0.01%–0.1% (wt/vol), whereas the DMC stain is affected only at a concentration of 0.1%. Neither of the metal chelate stains generates significant false-positive signals with any of the tested agents. Experiments performed in the absence of protein further substantiate the lack of false-positive signals produced by the agents (data not shown). The colloidal gold stain is sensitive to interference from most agents at the concentrations listed in Table 1. Interference is primarily manifested as increased signal, though SDS inhibits staining at all concentrations tested (0.1%–10% wt/vol) and BME inhibits staining at 1%–10% (vol/vol). False-positive staining is observed, in the absence of protein, with 10% SDS, 1–10 mM dithiothreitol (DTT) and 1%–10% BME (data not shown). DTT and BME cause the colloidal gold to change from red to black and result in excessive band smearing in the presence or absence of protein. Color development in

the BCA assay is inhibited by 1 M Tris-HCl, 1 M glycine, 20% ammonium sulfate (wt/vol), 10% BME and 100 mM EDTA. False-positive signals are produced with 1–10 mM DTT, 0.1%–1% BME, 40% sucrose and 10%–20% glycerol. Thus, of the assays evaluated in this study, the Ferrozine/ferrous and DMC assays display the lowest susceptibility to chemical interference, with only non-ionic detergents and PVP-40 inhibiting staining. This is reasonable since binding to nitrocellulose membranes is thought to occur largely through hydrophobic interactions (11). The inability of PVP-40 to be stained by the DMC procedure and its effectiveness as a membrane-blocking agent are key to protein sequencing and immunoblotting applications (18). A related stain, copper phthalocyanine 3,4',4'',4'''-tetrasulfonic acid, shares many properties with our stain but binds avidly to PVP (10).

CONCLUSION

The DMC stain is resistant to interference from common chemical reagents, has low protein-to-protein

Figure 3. Response curves for different proteins using various solid-phase and solution-based total protein assays. Panel A: Ferrozine/ferrous chelate stain. Panel B: DMC stain. Panel C: Colloidal gold stain. Panel D: BCA assay. The proteins evaluated are bovine serum albumin (squares), ovalbumin (circles), gelatin (diamonds) and hemoglobin (triangles). Error bars correspond to the standard deviation of each determination. Integrated OD is determined by reflectance imaging in Panels A, B and C.

variation, is sensitive and reproducible, and displays a 100-fold dynamic linear range. The DMC stain is readily quantified using an inexpensive document scanner interfaced with a personal computer. In addition, a standard yellow cellophane photographic filter and a slot-blotting apparatus are required. Using the slot-blot apparatus, 48 samples can be applied to the nitrocellulose in less than 30 min, and staining requires another 30 min. Our laboratory routinely utilizes the staining procedure to standardize samples in immunoblotting. Cell lysates or subcellular fractions are conveniently stained for total protein, quantified, destained and re-probed with a specific antibody detection system on a single nitrocellulose membrane.

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