

# The Staining of Acidic Proteins on Polyacrylamide Gels: Enhanced Sensitivity and Stability of "Stains-All" Staining in Combination with Silver Nitrate

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Received January 27, 1997

**A number of acidic proteins, such as those found in bone and dentin, are poorly resolved on acrylamide gels using Coomassie blue or silver nitrate staining. The cationic dye Stains-all allows visualization and identification of these proteins due to their differential staining: highly acidic proteins stain blue and intact proteoglycans stain purple, whereas less acidic proteins stain pink. However, the use of Stains-all is limited due to relatively poor staining sensitivity and lack of stability to light. A procedure which addresses these deficiencies has been developed utilizing established protocols for Stains-all staining followed by silver nitrate incubation and development. In this way, phosphoproteins such as osteopontin, bone sialoprotein, dentin phosphophoryn, and other acidic glycoproteins are visualized at higher sensitivity (greater than five-fold) and staining stability than normally achieved with just Stains-all. The protocol stains a greater variety of proteins than a combined alcian blue/silver staining procedure previously described. Utilizing the Stains-all/silver protocol, porcine bone osteopontin, a protein not visualized by standard silver staining, can be observed in amounts as little as 0.25 ng on polyacrylamide gels. Furthermore, densitometric scans demonstrate that the staining intensity is proportional to osteopontin amount and can be used for quantification over a range from 0.25 to 50 ng.** © 1997 Academic Press

Methods for detection of proteins on polyacrylamide gels after electrophoretic separation have been extensively studied (1, 2). The use of Coomassie brilliant

blue and the more sensitive silver nitrate stains allows for detection of most proteins on PAGE.<sup>2</sup> Silver staining techniques detect amounts as little as 50 pg per band on SDS-PAGE (3). However, several classes of highly negatively charged proteins are detected poorly, if at all, with these stains. These include proteoglycans and mucins, which contain high levels of sulfated sugar residues, and the acidic proteins and phosphoproteins, such as those found in mineralized tissues. This latter group includes osteopontin (4), bone sialoprotein (BSP; 5), and dentin phosphophoryn (6), proteins which contain high levels of acidic amino acids, phosphate, and, in some cases, sialic acid and sulfate residues. Due to the poor interaction of Coomassie blue and silver nitrate with these highly acidic proteins, other dyes, specifically those of a cationic nature, have been used to stain these proteins on polyacrylamide gels.

The cationic carbocyanine dye, Stains-all, stains sialoglycoproteins and phosphoproteins blue, while proteins of other classes stain red (7). Furthermore calcium-binding proteins, such as calsequestrin, calmodulin, and troponin C stain blue with Stains-all, presumably through an interaction within the calcium-binding domains (7). Stains-all has proven to be useful in the staining of mineral-associated acidic sialoproteins such as those found in bone (8). However, problems with the use of Stains-all for labeling acidic proteins include limited sensitivity and photobleaching or lack of stability toward light. Red-stained bands fade within 1 min, and blue-stained bands fade within 1 h.

Previous studies on mucins (9) and proteoglycans (10) have shown that staining with alcian blue, a cationic dye, followed by silver nitrate enhances the inten-

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<sup>2</sup> Abbreviations used: BSA, bovine serum albumin; BSP, bone sialoprotein; MW, molecular weight; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.

sity of staining of these acidic glycoproteins. Similar intensification of stained mucin bands was achieved with the use of a combined periodic acid–Schiff reagent as the first dye, followed by silver nitrate (9). These studies demonstrated that staining with dyes which interact with anionic groups on proteins could be intensified using a stain that interacts with the cationic groups of the first dye. Whereas these dyes have been used successfully for the study of proteoglycans and mucins, acidic proteins, including phosphoproteins with lower carbohydrate contents, stain poorly with these dyes. Osteopontin, a protein that has attracted a great deal of research interest, is one such phosphoprotein. This protein which has a distinct tissue distribution has been implicated in pathological and physiological processes involved in cancer (11), atherosclerosis (12) kidney stone formation (13), as well as bone formation and inhibition of mineral formation (5, 14, 15).

In this investigation we demonstrate that silver nitrate incubation after staining with Stains-all enhances and stabilizes bands corresponding to acidic proteins, including osteopontin, BSP, dentin phosphophoryn and osteoglycan.<sup>3</sup> This stabilization eliminates the need to handle and process gels in the dark prior to photography and analysis. Furthermore, the increase in sensitivity (at least fivefold) allows for the detection and quantification of subnanogram amounts of acidic proteins.

## MATERIALS AND METHODS

### Materials

Bovine serum albumin (BSA) and Stains-all<sup>4</sup> were purchased from ICN Biochemicals (Cleveland, OH). Electrophoretic reagents for the PhastSystem, glutaraldehyde, and molecular weight (MW) markers were obtained from Pharmacia (Montreal, Quebec). Other electrophoretic chemicals were from Bio-Rad (Toronto, Ontario). Silver nitrate (AnalaR) was from BDH. Alcian blue 8GX was obtained from Allied Chemical (New Jersey). All other reagents were from BDH and all were of AnalaR quality. Reverse-osmosis-treated water was further purified by passing through a Millipore Q Plus system.

### Proteins

Purified preparations of BSP (16), osteopontin (17), and osteoglycan (18) were prepared from adult porcine calvariae using protocols previously described. Amino acid analysis for BSP and osteopontin was consistent with the composition deduced from the cDNA of the

respective proteins (19, 20) and indicated purity of greater than 95%. Osteoglycan composition was similar to that previously described (18). The protein core of the osteoglycan was released from the attached glycosaminoglycan by treatment with chondroitinase ABC at a ratio of 0.01 unit/ $\mu\text{g}$  protein as described previously (18). Phosphophoryn was purified from adult porcine dentin as described (21). Amino acid analysis was consistent with that published for bovine dentin phosphophoryn (6), with a serine, aspartic acid, and asparagine content of approximately 80% (with no adjustment made for loss of serine during hydrolysis).

### Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

The electrophoresis of proteins followed standard protocols described in the Pharmacia manual for the Phast-System, using precast gels (12.5% acrylamide) and SDS-containing buffer strips. The polyacrylamide gels (0.45 mm thick) contained 0.112 M acetate, 0.112 M acetate, 0.112 M Tris, pH 6.4, and the buffer strips contained 0.2 M tricine, 0.2 M Tris, 0.55% SDS, pH 8.1. The protein samples were dissolved or diluted into buffer containing (final concentration) 2 M urea, 2% (w/v) SDS, 0.2 M dithiothreitol, and 0.001% (w/v) bromophenol blue. Samples were heated to 95°C for 4 min or 56°C for 30 min and cooled to room temperature. To avoid variability in loading, 1- $\mu\text{l}$  aliquots of sample were applied by pipettor to the gel surface. Electrophoresis conditions were as specified by the manufacturer: 250 V, 10 mA, for 70 Vh at 15°C.

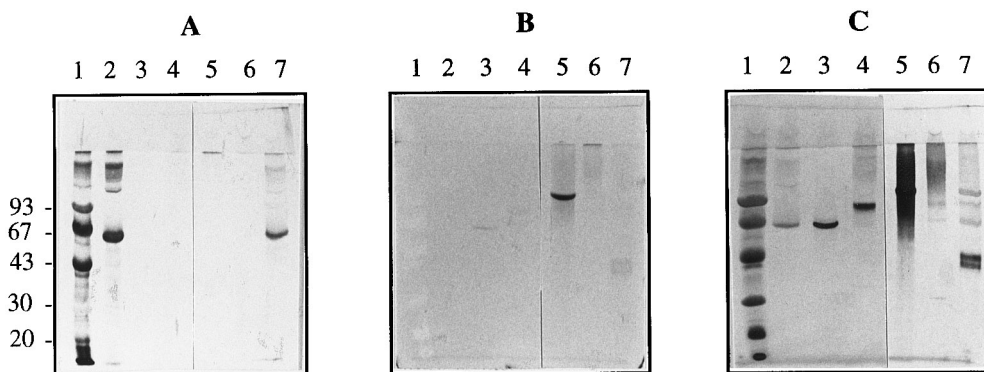
### Silver Nitrate Staining

The protocol for silver staining was adapted from that described (22). In brief, after electrophoresis, the gels were fixed in 40% (v/v) methanol and 10% (v/v) acetic acid for 10 min, and then washed three times in 10% (v/v) ethanol and 5% (v/v) acetic acid for 10 min each. Gels were then incubated in 3.4 mM potassium dichromate containing 3.2 mM nitric acid for 5 min. Shaking in H<sub>2</sub>O with several changes (5 min) was followed by incubation in 12 mM silver nitrate for 20 min. Following a rinse with water (0.5 min) with changes and rapid rinses with developer solution, the gels were developed in a solution containing 0.28 M sodium carbonate and 0.15% (v/v) formaldehyde until protein bands were developed. Reaction was terminated with 10% (v/v) acetic acid and the gels stored in 5% (v/v) glycerol, 10% (v/v) acetic acid.

An alternate silver staining protocol utilizing glutaraldehyde instead of potassium dichromate as the sensitizer (10) was used to compare the alcian blue/silver method (see below) to the Stains-all/silver nitrate protocol.

<sup>3</sup> In a previous study, decorin was described as HAPG1, whereas osteoglycan, a chondroitin-sulfate-containing proteoglycan, was originally described as HAPG2 and HAPG3 (18).

<sup>4</sup> Registry number for Stains-all, 7423-31-6.



**FIG. 1.** Comparative staining of acidic proteins. Identical samples ( $1 \mu\text{l}$ ) were pipetted directly onto the gels and electrophoresed on 12.5% acrylamide gels and stained with (A) the standard silver nitrate protocol with (potassium dichromate as the sensitizer), (B) Stains-all, and (C) the combined Stains-all/silver protocol. Lane 1, MW markers consisting of phosphorylase B (93 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and  $\alpha$ -lactalbumin (14 kDa, not labeled); lane 2, BSA (20 ng); lane 3, osteopontin (20 ng); lane 4, BSP (20 ng); lane 5, dentin phosphophoryn (100 ng); lane 6, osteoglycan (100 ng); lane 7, chondroitinase ABC-treated osteoglycan (100 ng), which contains albumin added to stabilize the enzyme preparations.

### Stains-all Staining

The protocol for Stains-all was adapted from that described previously (7). After electrophoresis, the gels were rinsed three times with 25% (v/v) isopropanol followed by washing in 30–50 ml of the same solution on a shaker for 10 min. The cycle of rinsing (three times) and washing was repeated three times. This procedure ensures the removal of all SDS, which, if present, would cause the precipitation of the Stains-all dye. Isopropanol was then replaced by 30 ml of Stains-all solution (30 mM Tris, 7.5% (v/v) formamide, 25% (v/v) isopropanol, adjusted to pH 8.8 with HCl, followed by addition of 0.025% (w/v) Stains-all). Due to the photosensitivity of Stains-all, gels were incubated in light-tight containers on an orbital shaker at room temperature normally for at least 2 h.

### Combined Stains-all/Silver Staining

After Stains-all staining, the location and color of the bands were noted and then washed with 30 ml of 25% (v/v) isopropanol with a change every 5 min until background became clear (approx 20 min). The gels were rinsed with  $\text{H}_2\text{O}$  three times and then incubated in 30 ml of 12 mM silver nitrate on an orbital shaker for 20 min. The gels were rinsed in water three times, rapidly followed by the incubation with developer (as above). The reaction was terminated, usually at 5 min, by the removal of developer and the addition of 10% (v/v) acetic acid. The gels were stored in a solution containing 5% glycerol and 10% acetic acid.

### Combined Alcian Blue/Silver Stain

The protocol for alcian blue followed by silver stain using glutaraldehyde was adapted from that previously described (10). The length of time for each wash and

incubation was as that described for the combined Stains-all/silver protocol described above.

### Densitometric Analysis

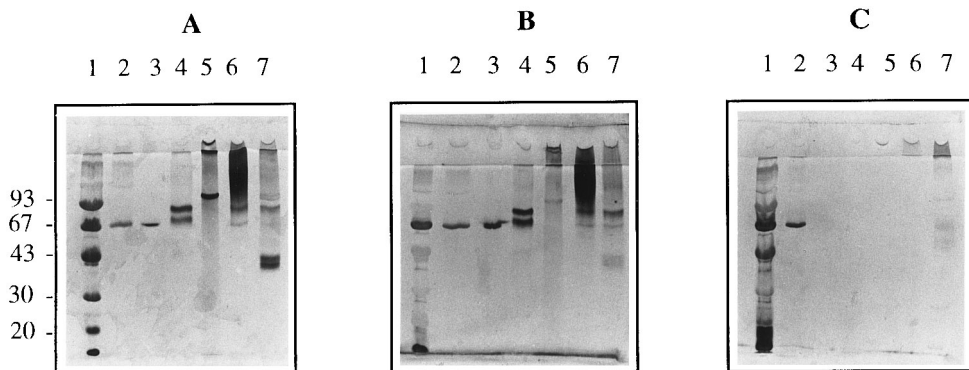
Densitometry was performed utilizing a Gel Print system (Biophotonics Corporation) with the Video Copy Processor P4OU (Mitsubishi). Analysis was performed utilizing the software package GPTools v3.0, 1-D Gel Analysis Software (BioPhotonic Corp.).

## RESULTS

### Staining with Stains-all and Silver

A number of purified proteins including osteopontin, BSP, dentin phosphophoryn, and osteoglycan were analyzed by the combined Stains-all/silver staining protocol. These proteins were electrophoresed and stained with silver nitrate (Fig. 1A), with Stains-all (Fig. 1B), or by the combined Stains-all/silver protocol (Fig. 1C). The only bands that were apparent from staining with silver nitrate alone (Fig. 1A) were those of the MW markers (lane 1), the BSA control (lane 2), and the albumin that was added to the enzyme chondroitinase ABC as a stabilizer (lane 7). Other silver-stained bands present in lane 7 are those from the chondroitinase ABC preparation (the PAGE of chondroitinase ABC alone, is not shown).

The cationic dye, Stains-all, showed a different staining profile which is not easily observed after photographic replication (Fig. 1B). BSA as well as the MW markers stained pink, but became translucent within 1 min on exposure to light. The highly acidic proteins stained blue with Stains-all. However, these bands for the most part were barely visible at the amounts loaded onto the gel. The dentin phosphophoryn, due in part to the high levels of aspartic acids and attached phosphates, was well stained.



**FIG. 2.** Comparison of protein staining with alcian blue and Stains-all in conjunction with silver nitrate enhancement. Triplicate samples ( $1 \mu\text{l}$ ) were loaded and electrophoresed on 12.5% acrylamide gels and stained with (A) the Stains-all/silver protocol, (B) the alcian blue/silver protocol, and (C) the silver nitrate protocol utilizing glutaraldehyde as the initial sensitizer. Lane 1, MW markers; lane 2, BSA (20 ng); lane 3, osteopontin (20 ng); lane 4, BSP (20 ng); lane 5, dentin phosphophoryn (50 ng); lane 6, osteoglycan (100 ng); lane 7, chondroitinase ABC-treated osteoglycan (100 ng).

Incubation of the polyacrylamide gel first with Stains-all followed by silver nitrate stained all of these acidic proteins more intensely (Fig. 1C). Strong, sharp bands for osteopontin and BSP are evident, whereas phosphophoryn, which appeared relatively homogeneous with Stains-all alone, actually consisted of a broad range of indistinguishable bands centered at 94 kDa. This profile is consistent with the relatively fragile nature of this phosphoprotein (A. Veis, personal communication). The bands corresponding to intact osteoglycan (lane 6) and osteoglycan core proteins ( $M_r$  35–40 kDa; lane 7), became more apparent with the combined Stains-all/silver protocol. However, additional bands with  $M_r$  of 90 and 70 kDa also became apparent in the lane corresponding to intact osteoglycan (lane 6). These protein bands, which would not normally have been observed with Stains-all alone, unless the gels were significantly overloaded with protein, are likely acidic in nature, since silver staining failed to reveal their presence (Fig. 1A, lane 6).

It is of interest that the MW protein markers stain well with the Stains-all/silver protocol. However, the relative staining is variable. Whereas BSA staining is lower in the combined protocol compared to silver staining alone, a few of the MW markers stained more intensely. In addition, whereas the gel stained with Stains-all alone was light sensitive (all staining disappeared within an hour after continuous exposure to light), the use of the combined staining protocol generated bands that were light-insensitive. However, as is true for silver-stained gels, a background yellow-brown shade slowly developed with time.

#### *Alcian Blue vs Stains-all Staining*

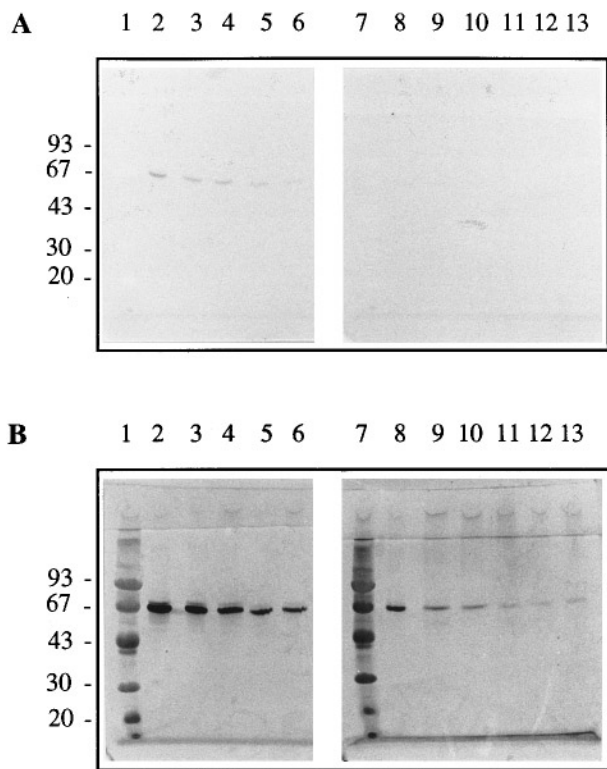
Previous studies have demonstrated that alcian blue in combination with silver staining resulted in an in-

creased sensitivity for proteoglycan detection on PAGE (10). To compare the two staining techniques, protein samples were electrophoresed and stained with Stains-all/silver nitrate (Fig. 2A), with alcian blue/silver (using glutaraldehyde as the sensitizer in the silver staining protocol, Fig. 2B), or with silver nitrate alone (using glutaraldehyde, Fig. 2C). The gel treated with the Stains-all/silver was similar to that shown in Fig. 1C, with minor differences due to the different levels of protein loaded and for the presence of a doublet band for BSP.<sup>5</sup> Prior staining with alcian blue followed by silver (Fig. 2B) resulted in strong staining of osteopontin (lane 3), BSP (lane 4), and intact osteoglycan (lane 6). These bands appear to be as intense as those dyed with Stains-all/silver (Fig. 2A). However, phosphophoryn (lane 5) and the osteoglycan protein cores (lane 7), which were poorly stained with alcian blue/silver, were more intensely stained by Stains-all/silver. Furthermore, the MW markers, which were intensely stained by Stains-all/silver (Fig. 2A), were variably stained by alcian blue/silver (Fig. 2B). While it is apparent that alcian blue/silver is equivalent to Stains-all/silver for staining some glycoproteins, the Stains-all/silver protocol appears more suitable for detecting a wider range of proteins.

#### *Limits of Sensitivity*

To quantify the sensitivity of staining, we used osteopontin over a range of 0.25–50 ng. The limits of visual sensitivity was 1–2.5 ng when directly observed on gels

<sup>5</sup> The BSP, which appears as a doublet on the PAGE gels depicted in Fig. 2, is derived from a different bone preparation than that of Fig. 1. Amino acid compositional analysis of this BSP doublet preparation and Western blotting with polyclonal antibodies to BSP confirm that both bands are BSP (results not shown).



**FIG. 3.** Staining of varying concentrations of osteopontin. Osteopontin was dissolved and diluted in sample buffer to yield solutions containing 0.25 to 50 ng osteopontin per microliter. The osteopontin samples (1  $\mu$ l) were applied and electrophoresed on 12.5% acrylamide gels. The gels were stained with (A) Stains-all alone or (B) the combined Stains-all/silver protocol. Lanes 1 and 7, MW markers; lane 2, 50 ng; lane 3, 40 ng; lane 4, 30 ng; lane 5, 20 ng; lane 6, 10 ng; lane 8, 10 ng; lane 9-5 ng; lane 10, 2.5 ng; lane 11, 1 ng; lane 12, 0.5 ng; lane 13, 0.25 ng.

stained with Stains-all alone. However for proper photographic reproduction more than 10 ng of protein was needed (Fig. 3A). Osteopontin stained more intensely using the combined Stains-all/silver technique (Fig. 3B). As little as 0.25 ng of osteopontin was readily observed directly on the gel (Fig. 3B, lane 13). Higher amounts yielded strong, darkly stained bands. Furthermore, these stained bands were light-stable, in that there was no significant loss of staining intensity with exposure to light with time over a one week period (not shown).

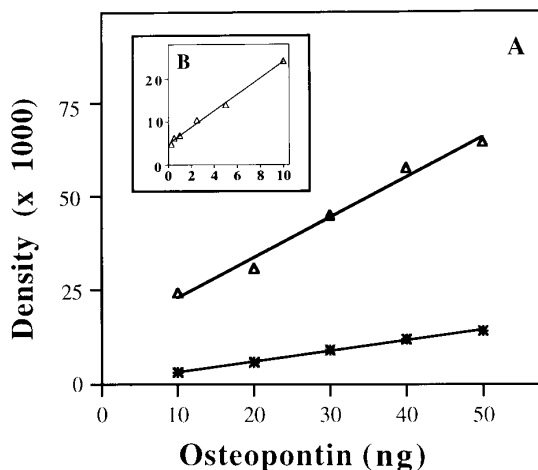
Densitometric analysis of the osteopontin bands of Fig. 3A, 3B was performed and is shown in Fig. 4. There is at least a fivefold increase in relative staining using the Stains-all/silver protocol compared to Stains-all alone. This increased intensity is much more evident on analysis of samples containing less than 10 ng of osteopontin. Of note, using Stains-all alone, densitometry was not possible for amounts lower than 5 or 10 ng, whereas using the combined staining protocol measurable, data were obtained from amounts as little as

0.25 ng (Fig. 4B, inset). Over the ranges chosen, from 0.25 to 10 ng and from 10 to 50 ng, the densitometric measurements were linear. However, it is important to note that the slopes of these two sets of data are different and that both do not intercept the  $y$  axis at the zero point. Nevertheless, by establishing a limited standard curves using three points, concentrations of specific protein can be determined as long as all the data were generated from a single gel.

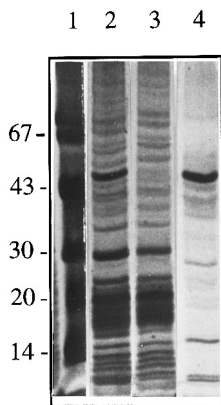
The combined staining protocol can also enhance staining of proteins in complex biological solutions. For example, staining of prokaryote-expressed recombinant porcine BSP using the combined Stains-all/silver staining protocol is shown in Fig. 5. The protein band at  $M_r$  47 kDa represents the C-terminal containing 75% of recombinant BSP. Staining with the combined staining protocol allows for the detection of low levels of acidic proteins of interest as well as the determination of the extent of contamination. Further purification by FPLC chromatography was necessary to purify this protein to homogeneity.

## DISCUSSION

Staining with Coomassie brilliant blue or silver nitrate stains have been well characterized and established (1) and are appropriate for the majority of known proteins. However, there are a number of proteins which stain poorly, if at all, with these stains. These include a group of proteins that are highly acidic in nature, such as those found in mineralized tissues. For these reasons, other stains which include Kenacid Blue



**FIG. 4.** Densitometric analysis of osteopontin staining on PAGE. Varying concentration of osteopontin which were electrophoresed and stained with either Stains-all alone or by the combined Stains-all/silver protocol (Fig. 3) were analyzed by densitometry. (A) Amounts of 10–50 ng of osteopontin stained with Stains-all (asterisks) or Stains-all/silver (triangles). B (inset) Amounts of 0.25–10 ng of osteopontin stained with Stains-all/silver.



**FIG. 5.** Combined Stains-all/silver staining of a complex biological solution. Recombinant porcine BSP peptide (P1L; corresponding to BSP residue 69-300) expressed as a his-tag fusion protein using the pET28b vector system was purified by nickel affinity chromatography, and column eluants were analyzed by SDS-PAGE as described under Materials and Methods. Lane 1, MW markers; lane 2, total insoluble bacterial proteins; lane 3, nonretarded proteins from nickel-affinity chromatography; lane 4, bound protein eluted with 100 mM imidazole. Note that the major 47-kDa band Stains-all/silver stained band is the recombinant BSP peptide.

R (23), Stains-all (8), and alcian blue and toluidine blue for proteoglycan staining (10) have been utilized. Alcian blue has also been used to stain the highly glycosylated BSP (24). However, in general, these stains are not appropriate for all classes of acidic proteins or are not sufficiently sensitive to detect nanogram levels on polyacrylamide gels.

Stains-all has increasingly been utilized for the detection of a variety of anionic compounds on gels. For example, Stains-all has been used for the detection of hyaluronan (25), mineral-associated proteins such as osteopontin and BSP (16), BAG-75 [bone acidic glycoprotein (26)] and dentin phosphophoryn (6), and the salivary mucins (27). In addition, Stains-all has been utilized for staining RNA loading on agarose gels (28). These studies would thus suggest that Stains-all is an appropriate stain for a wide spectrum of acidic glyco- and phosphoproteins.

The combination of Stains-all staining with silver nitrate as shown in this study has taken advantage of the inherent capability of Stains-all to stain a wide variety of proteins with the added feature of increased staining sensitivity and stability to light. With Stains-all alone, the acidic proteins can be detected in the sub-100-ng range; however, when used in combination with silver nitrate incubation and development, sharp, distinct, and light-stable bands were evident for all the acidic proteins studied and at far lower concentrations. In fact, this staining technique allows for the detection of the less-acidic proteins as shown by the staining of the MW markers. Furthermore, using osteopontin, we

have determined that even at the lowest amount studied, 0.25 ng, distinct and photographically reproducible bands can be attained. Due to the linear relationship between the densitometric analysis and protein amounts loaded onto the gels, the combined staining technique can also be used to determine specific protein concentration. However, due to the variability in staining with silver which exists between different gels and proteins even when processed at same time [discussed in (24)], controls must be included on each gel.

Important advantages of an increased staining sensitivity include the detection of proteins in biological fluids or chromatographic column effluents without the need for sample manipulation and the fact that protein purity can be more adequately assessed without sample overloading. Protein manipulations would include concentration by ultrafiltration, centrifugal filtration, or simply dialysis and freeze drying, all of which do invariably result in significant protein losses. With a sensitive detection technique, purity can be more easily determined. For example, the analysis by the combined staining protocol of just 50 ng of one protein in the absence of any other detectable bands on the gels can be assessed as having 99%+ purity (based on MW criteria). Furthermore, silver staining and the use of Stains-all/silver protocol on duplicate gels can provide some degree of characterization of the contaminating protein, if present, as well. As demonstrated under Results, the osteoglycan preparation was not pure, and the different staining profiles indicate that the "contaminants" are likely acidic in nature.

Combined staining protocols utilizing silver nitrate with other dyes have been used to improve on sensitivity of detection. Differential staining of proteins was noted with the use of a combined silver/Coomassie blue staining protocol (29). Erythrocyte membrane-derived sialoglycoproteins stained yellow while other proteins stained blue. However, this staining protocol did not result in reproducible staining of either osteopontin or BSP at 100 ng loading (results not shown). The Alcian blue/silver technique was originally described to improve on staining for mucins (9) and for proteoglycan (10). In this study, this combination was shown to be a useful staining protocol for BSP, osteopontin, and the intact osteoglycan, but was inferior to the Stains-all/silver technique in staining of proteins with a lower carbohydrate content, including dentin phosphophoryn, osteoglycan protein core, and the MW markers for SDS-PAGE. While it is apparent that Stains-all is useful in staining the protein core of osteoglycan, previous studies on bone-derived decorin, a ubiquitous proteoglycan containing a single attached GAG, revealed that the decorin core protein stained pink (18). This difference in staining of the two proteoglycans' protein cores may be attributed to the higher levels of acidic amino acid residues in osteoglycan.

The Stains-all staining protocol as described is relatively simple and does not require significantly more time or effort than staining by silver nitrate alone. In total, a rapid staining protocol can be accomplished in approximately 2.5 h when Stains-all staining is limited to 1 h. In summary, a new staining protocol for acidic proteins is described which increases both the sensitivity and stability of the stain. This use of Stains-all in combination with silver nitrate allows for the detection of subnanogram amounts of protein on SDS-PAGE.

#### ACKNOWLEDGMENTS

The authors thank Dr. Ying Lu, Faculty of Dentistry University of Toronto (present address), for assistance in the preparation of the dentin phosphophoryn; Nicole Harris for recombinant BSP; and Drs. S. Jeffrey Dixon and Graeme K. Hunter, University of Western Ontario, for the critical review of the manuscript. This study was supported by the Medical Research Council of Canada.

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