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Double Staining of Coomassie Blue-Stained Polyacrylamide Gels by Imidazole–Sodium Dodecyl Sulfate–Zinc Reverse Staining: Sensitive Detection of Coomassie Blue-Undetected Proteins

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The sensitivity, simplicity, and relative rapidity of Coomassie blue staining have made this technique the method of choice for routine detection and quantitative analysis of gel electrophoresis-separated protein bands in many applications. To extend the usefulness of this technique, we have developed a new double-staining method for visualizing SDS–PAGE-separated protein bands that were undetected by Coomassie blue staining of the gel. Coomassie blue-stained gels are washed in distilled water (15 min, two times) and then subjected to imidazole–zinc reverse staining. As a result of the method, a homogeneous white-stained background is generated and two types of protein bands can be observed: (a) typical Coomassie blue-stained bands, which appear superposed on larger transparent bands; and (b) reverse-stained (transparent) bands, which were previously undetected by the Coomassie blue staining. The method is rapid, simple, and reproducible and double-stained gels can be kept in distilled water for months without loss of the protein pattern. The overall sensitivity is high (e.g., 1.6 ng for recombinant streptokinase, 47 kDa) over a wide range of protein molecular weights (10 to 100 kDa) and independent of the degree of Coomassie blue destaining of the gel. Furthermore, a mechanism offering a consistent explanation for the role of imidazole, SDS, and zinc in the reverse staining of gels, particularly after Coomassie blue staining is proposed.

The sensitivity (500 to 50 ng protein per band), simplicity (two steps), and relative rapidity (2 to 8 h) of Coomassie blue staining have made this technique the method of choice for routine detection and quantitative analysis of gel electrophoresis-separated protein bands in many applications (1). Double staining of the Coomassie blue-stained gel with silver staining has been reported in order to increase the sensitivity of detection of trace proteins present in the same gel (2). In addition, protein bands within the general detection range of the Coomassie blue technique but with low affinity for the dye (e.g., acidic proteins) can also be visualized. Nevertheless, a number of drawbacks are encountered with the use of silver stains (3–5): (a) high backgrounds may result, especially if the water is not sufficiently pure; (b) the methods are laborious and time consuming (8 to 16 h); (c) the methods are significantly expensive; and (d) each version of silver stain differs in its ability to stain proteins; while some proteins can be perfectly silver stained, others may stain only weakly or not at all (6). Therefore, one cannot be sured that, after double staining, all proteins have been detected. Moreover, silver staining techniques act by modifying the protein (e.g., at cysteine residues) (3–5); thus, the detected protein may not be useful for later microanalysis.

From the above discussion, it follows that there is a need for a simple and rapid procedure that permits general detection of proteins undetected on gels already stained with Coomassie blue without recourse to silver stainings to circumvent their disadvantages. The use of a sensitive negative staining is a possible choice for the last step in the double-staining strategy. Negative (reverse) staining of the gel using heavy *metal* (copper, zinc) salts has been described (6,7). This method consists of the selective precipitation of these metal cations on the gel matrix, leaving protein bands unstained and transparent. In addition, this technique is very simple and rapid. However, its reproducibility is highly dependent on many physicochemical factors such as pH of the

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staining solutions, concentration of anions in the gel matrix (e.g., hydroxide, carbonate), and temperature. Therefore, it cannot be considered a routine technique as such. However, an alternative to the heavy metal salt staining is the reverse staining by imidazole–zinc salts. It is a very reproducible modification of the former, first developed for protein detection on SDS[2] gels (8) and later extended to other electrophoresis systems run without SDS (9). This method is highly sensitive (1 to 10 ng of protein per band), simple (two steps), and rapid (15 min at most) and allows high-efficiency microanalysis of the detected proteins, which are not chemically modified by the staining[3] (8). Moreover, it is compatible with subsequent restaining of the gel with Coomassie blue or silver if desired (8).

In this paper, we describe a reproducible nondifferential double staining of proteins by combining Coomassie blue and imidazole–zinc reverse staining. This high-sensitivity double-staining technique permits detection and potential microanalysis of Coomassie blue-undetected protein bands with the added advantage of being rapid and simple. We also propose a mechanism that offers a consistent explanation for the role of imidazole, SDS, and zinc in this double staining for (a) enhancing detection of Coomassie blue-stained bands and (b) permitting visualization of Coomassie blue-undetected bands. We believe, further, that the present Coomassie blue reverse-staining method will be useful in the rapid and high-sensitivity detection of proteins after electrophoresis, especially in laboratories where Coomassie blue staining is otherwise a routine technique.

MATERIALS AND METHODS

Electrophoresis and staining reagents were from Merck (analytical grade) or Bio-Rad (electrophoresis grade). Protein mixtures from *Bacillus stearothermophilus* Y 406 and recombinant streptokinase (47 kDa) were supplied by the Center for Genetic Engineering and Biotechnology (La Habana, Cuba).

Electrophoresis

SDS–PAGE was performed according to Laemmli (10).

Coomassie Blue (CB) Staining

The CB-staining solution was freshly prepared and consisted of 0.27% Coomassie blue R-250 in 54% methanol and 13.5% acetic acid. Staining was performed for 30–60 min; destaining was done in 30% methanol, 10% acetic acid for 15–30 min (brief destaining) or 3 h or longer (extensive destaining). Unless stated otherwise, CB gels were always subjected to extensive destaining.

Double Staining by Coomassie Blue Reverse Staining

CB-stained gels were washed in distilled water. Any remaining methanol and acetic acid were removed from the gels by two changes of water 15 min each or, alternatively, overnight. The gels were then incubated in 30–50 ml of 0.2 M imidazole, 0.1% SDS under gentle shaking for 15 min. This solution was discarded, replaced by 50 ml of 0.2 M zinc sulfate solution, and agitated for 30 to 60 s. Overstaining was avoided by discarding this solution and rinsing the gel with 50 ml of deionized water (3 × 1 min).

Spectrophotometric Analysis

The term imidazole–SDS–zinc is used to refer to the complexes formed when a zinc salt was added to imidazole solutions containing SDS. The light transmittance was measured in suspensions of imidazole–SDS–zinc at 550 nm using a Hitachi 330 spectrophotometer provided with a homemade magnetic stirrer. A small magnet placed in the bottom of the spectrophotometer glass cuvette (cell path length: 1 cm) allowed measurements to be performed under continuous stirring. In this way, any precipitate formed could be kept in suspension during the analysis. SDS altered the light transmittance of the imidazole solution by less than 2%. The light transmittance of 0.2 M imidazole was taken as reference (%T equals 100%).

Light transmittance measurement on addition of zinc to an imidazole–SDS solution. To 0.2 M imidazole solutions, zinc sulfate (from a 0.2 M stock solution) was added to yield increasing concentrations (from 0 to 50 mM). In some experiments the imidazole solution contained SDS (0.1, 0.5, or 1%; adjusted from a 20% stock solution). In all experiments, zinc was added dropwise and under constant stirring to the imidazole solution placed in the spectrophotometer cuvette. Light transmittance of the suspension was measured after each zinc addition.

Light transmittance measurement on varying the SDS concentration in the imidazole at a fixed zinc:imidazole ratio. To 0.2 M imidazole solutions, 20% SDS was added to yield increasing SDS concentrations (from 0 to 2%). To each imidazole–SDS solution, 0.2 M zinc sulfate was added to yield a zinc:imidazole ratio of 1.5:200. Zinc was added dropwise directly to the spectrophotometer cuvette under constant stirring. Light transmittance was measured before and after addition of zinc to the imidazole–SDS solutions. The experiment was repeated at zinc:imidazole ratios of 4:200, 15:200, and 22:200.

In a similar series of experiments, SDS was replaced with bovine serum albumin. The light transmission was

[2] Abbreviations used: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CB, Coomassie blue.

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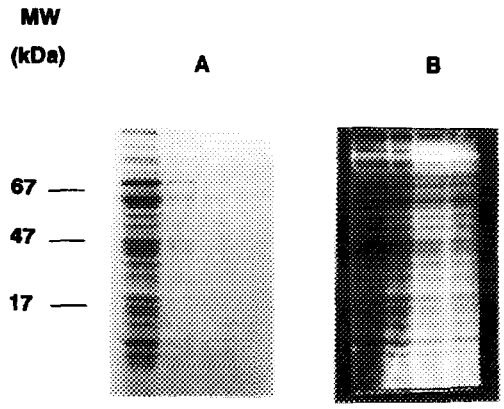

FIG. 1. Development of the new double staining. Dilutions (from left to right: 1:1, 1:10, 1:50, and 1:70) of total proteins (100 μg) from *Bacillus stearothermophilus* Y 406 were separated on two parallel 12.5% SDS–polyacrylamide gels. (A) Coomassie blue staining of the first gel. The gel was photographed on a white background to contrast the Coomassie blue-stained protein bands. (B) Double staining of the second gel. After Coomassie blue staining, the gel was washed with distilled water (15 min, two times) and reverse stained. High-sensitivity detection was obtained over the entire range of molecular weights at all dilutions. The gel was photographed on a glass plate held some centimeters above a black surface, while obliquely illuminating from both sides with fluorescent light. This photographic technique allowed us to record the total bands that emerged after double staining.

measured on addition of bovine serum albumin (from a 10 mg/ml stock solution) to yield increasing concentrations (from 0.025 to 2.5 mg/ml) at zinc:imidazole ratios of 1:200, 5:200, and 10:200.

RESULTS AND DISCUSSION

The New Double Staining

The procedure described here combined the Coomassie blue- and reverse-staining techniques. Two identical series of dilutions of *B. stearothermophilus* Y 406 proteins, separated on two parallel SDS–polyacrylamide gels, were stained with Coomassie blue (Fig. 1A). After CB staining, the replicate gel was washed in distilled water and later subjected to reverse staining (Fig. 1B).

Reverse staining of the CB-stained gel generated a homogeneous white-stained background and two kinds of protein bands: (a) typical Coomassie blue bands, which now appear superposed on larger transparent bands; and (b) reverse-stained (transparent) bands, undetected by CB staining.

The main result was the highly sensitive detection of protein bands in a wide range of molecular weights on the already stained gel, even at dilutions at which Coomassie blue failed to yield any detection at all (Fig. 1).

Figure 2 shows the electrophoretic separation of an ion-exchange chromatography (MonoQ HR 10/10) fraction of the *B. stearothermophilus* proteins. After Coomassie blue staining, only two protein bands were detected on the gel (Fig. 2A). The gel was kept in a sealed polyethylene bag. One month later, it was washed in distilled water and subjected to reverse staining. On the double-stained gel, eight new bands were easily visible (Fig. 2B) in addition to the two CB-stained bands. Even the two larger bands (shown by arrows in Fig. 2B) could not be observed with CB either before double staining (Fig. 2A) or after restaining of the double-stained gel with Coomassie blue (not shown).

The experiment shown in Fig. 2 is only one example of many instances in which detection on already stained gels was enhanced by the double-staining technique. This particular gel was run and stained with Coomassie blue in a laboratory that uses CB staining but does not use reverse staining as a routine technique. Two advantages of the double staining can be seen in this application: (a) it complemented the CB staining, giving a more realistic picture of the complexity of the sample analyzed; and (b) it was not affected by time after CB staining.

In the above examples, proteins from the *B. stearothermophilus* Y 406 were selected to evaluate the scope of the double-staining method because several components in these mixtures showed markedly low affinity for Coomassie blue dye. In the next section, we describe the use of recombinant streptokinase, a protein that stains well with Coomassie blue, to estimate the sensitivity of double staining.

Effect of the Extent of Coomassie Blue Destaining on the Sensitivity of Double Staining

A parameter that may vary from one CB-stained gel to another or from one laboratory to another is the ex-

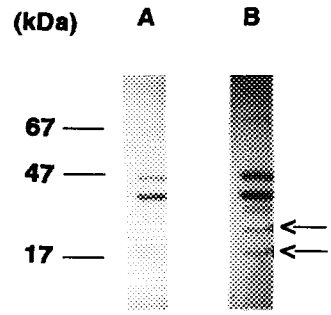

FIG. 2. Visualization of Coomassie blue-undetected bands on an already stained gel. (A) Ion-exchange chromatography fraction of the *Bacillus* proteins separated on a 12.5% SDS–polyacrylamide gel and stained with Coomassie blue. Only two bands were visible. (B) The same gel after double staining. Eight new transparent bands were detectable over the white-stained background, and the two CB-stained bands were enhanced. This enhancement effect is caused by the superposition of each blue band on a larger transparent (reverse-stained) band. According to size, the two larger transparent bands indicated by arrows in (B) should fall within the detection range of the Coomassie blue technique; however, they were practically undetected (see Fig. 3 for comparison with the detection limits of the Coomassie blue procedure). The photographic technique was as in Fig. 1.

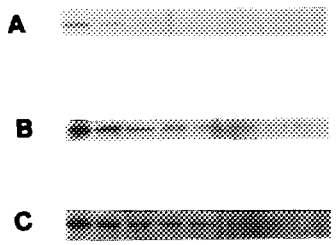

FIG. 3. Effect of extent of Coomassie blue destaining on sensitivity of the double staining. Serial dilutions of recombinant streptokinase (from left to right: 200, 100, 50, 25, 12.8, 6.4, 3.2, and 1.6 ng) were separated on 12.5% SDS gels. After electrophoresis, the gels were stained with Coomassie blue. The first and second gels were destained until the gel matrix was transparent (extensive destaining). (A) CB staining of the first gel. (B) Double staining of the second gel. For comparison, the third CB gel was only briefly destained. In this last gel, care was taken to leave a slightly transparent but still dark-blue-stained background, although CB-stained protein bands were already evident. (C) Double staining of the third gel. Note that due to the varying degree of CB destaining, the background in (B) is lighter than that in (C). However, the overall sensitivity in both gels was the same (1.6 ng). The photographic technique was as in Fig. 1.

tent of CB destaining. Thus, some CB-stained gels may show a dark blue background, whereas others may display an apparently colorless or weakly blue-stained background. The double-staining technique was applied to gels subjected to varying extents of CB destaining.

In our hands, the sensitivity of extensively destained Coomassie blue gels was 50 ng for streptokinase (Fig. 3A). A higher sensitivity (25 ng) was clearly observed when CB destaining was performed briefly; however, this high detection level was rather unstable upon storage in distilled water and decreased (to 50–100 ng) several days later when the gel was kept in distilled water. Extensive destaining led to low sensitivity, but gave a highly contrasted CB-stained pattern (Fig. 3A), while brief destaining produced highly sensitive but low-contrast patterns (not shown).

Coomassie blue gels subjected to brief or extensive destaining were subjected to the reverse-staining step (Figs. 3B and 3C). Independent of the degree of destaining attained in the CB gel, detection limits were extended to 1.6 ng after the reverse staining. Moreover, a superposition of backgrounds occurred, while the blue-stained bands kept their characteristic color and new transparent bands arose. In all cases, the enhanced contrast between CB protein bands and the resulting gel background, particularly on briefly destained CB gels, led to an improved sensitivity in visual detection of CB bands. In addition, the double-stained patterns were compatible with densitometric analysis of the CB-stained bands (not shown). Furthermore, the double-stained patterns were stable in distilled water for at least 3 months without appreciable loss of sensitivity (not shown).

The Influence of SDS and Zinc Concentrations on the Generation of an Insoluble Imidazole–SDS–Zinc Complex

The last step of the present method is the reverse staining of the gel. The formation in the test tube of the imidazole–SDS–zinc complex at different concentrations of SDS and zinc ions shed further light on the mechanism underlying the reverse-staining step.

A semiquantitative estimation of the imidazole–SDS–zinc precipitate was performed by measuring the light transmittance of its suspension. It was observed that, under continuous agitation, the transmittance values of the suspension were reproducible and independent of time. In subsequent results, then, a decrease in transmittance will be interpreted as an increase in the amount of insoluble imidazole–SDS–zinc complex.

The visible light transmittance of the imidazole–SDS–zinc suspension produced on the addition of zinc to 0.2 M imidazole solutions with varied SDS concentrations is illustrated in Fig. 4A. When zinc was added in the absence of SDS, the light transmittance decreased smoothly from 100 to 5–15%, remaining almost constant within this range for a zinc concentration of 5 mM or higher.

On the addition of zinc to imidazole solutions with SDS (0.1, 0.5, or 1%), the light transmittance remained almost unchanged (90 to 100%) at first and then showed a subsequent abrupt decrease to less than 0.5%. This decrease in transmittance was associated with the sudden formation of an abundant deep-white precipitate of particles apparently larger than those formed in the absence of SDS. The higher the SDS concentration in the imidazole solution, the larger the concentration of zinc ions required to form this precipitate (Fig. 4A). When zinc (up to a final concentration of 100 mM) was added to SDS alone (at concentrations of 0.1 or 1%), no precipitate formed. Similarly, in the absence of zinc no precipitate formed between imidazole (0.2 M) and SDS (varied from 0 to 2%).

The light transmittance after the SDS concentration in the imidazole was increased from 0 to 2% at zinc:imidazole ratios of 1.5:200, 4:200, 15:200, and 22:200 is shown in Fig. 4B. There was first a decrease in light transmittance. This decrease was followed by an abrupt increase in transmittance (90 to 100%) within a relatively narrow interval of SDS concentrations. At this point, a reduction in the precipitate was observed in the test tube, and a further increase in the SDS concentration seemed to prevent the formation of the precipitate observed at lower SDS concentrations. The critical SDS concentration required to inhibit complex precipitation increased as the zinc:imidazole ratio increased (Fig. 4B).

When SDS was replaced with bovine serum albumin in this experiment the imidazole–zinc precipitate could

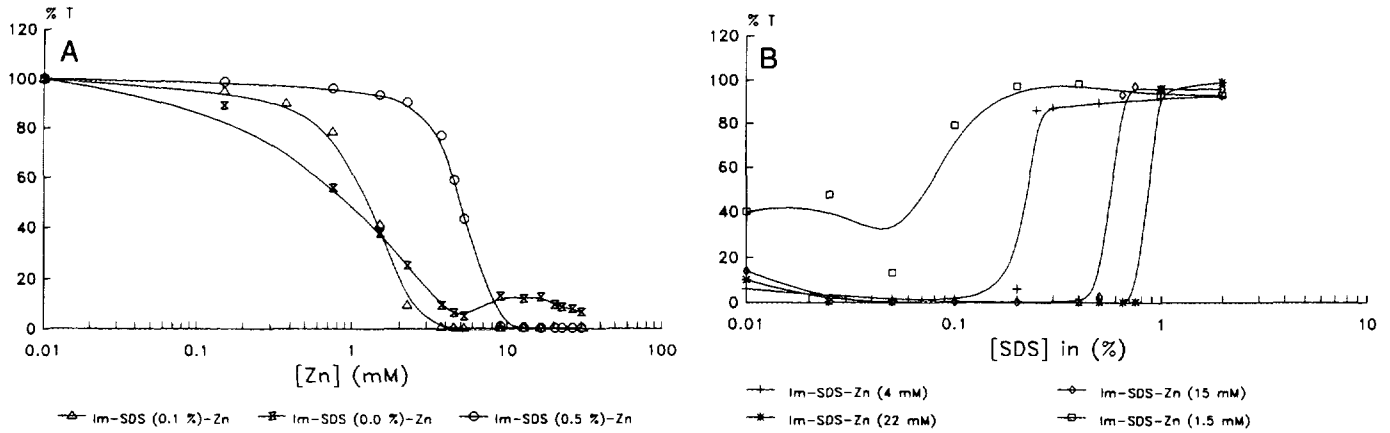

FIG. 4. Visible light transmittance (%T) of the imidazole–SDS–zinc (Im–SDS–Zn) suspension produced on addition of zinc to 0.2 M imidazole solutions with varied SDS concentrations. (A) Light transmittance of the suspension on addition of zinc at SDS concentrations of 0, 0.1, and 0.5%. (B) Light transmittance on an increase in the SDS content in the imidazole (from 0 to 2%) prior to addition of zinc at fixed zinc:imidazole ratios, 1.5:200, 4:200, 15:200, and 22:200. Zinc and SDS concentrations are noted on the figure. The experimental data are the average of two independent experiments.

not be redissolved nor could its formation be prevented (not shown).

In our previous work (8), fundamentals of reverse staining by imidazole–zinc salts of SDS–polyacrylamide gels were discussed. However, this method did not include SDS in the imidazole solution and a role of the dodecyl sulfate group (from electrophoresis reagents) in gel staining was not investigated. According to the proposed mechanism of staining, the dodecyl sulfate anion would account only for the transparency of protein bands. On this assumption, we included SDS in the imidazole solution to extend reverse staining to gels run without SDS (9). However, results suggested that SDS not only allowed negative staining but also significantly improved the background staining.

Given the present spectrophotometric results, we propose a general mechanism to explain the imidazole–SDS–zinc reverse staining of gels in protein detection that explains the reverse staining of gels already stained with Coomassie blue.

General Mechanism of Reverse Staining by Imidazole–SDS–Zinc Salts

Gel background staining. During the first step of reverse staining, imidazole and SDS diffuse into the gel matrix. At the same time, protein bands are loaded with SDS. When the gel is then soaked in the zinc staining solution, deposition of an insoluble complex occurs on the gel surface. This complex is insoluble in ammonia, so it is not mainly zinc hydroxide (8). This complex is not likely due to a precipitation of SDS micelles by zinc ions because SDS–Zn did not precipitate in a test tube over a wide range of zinc concentrations. Thus, although the precise nature of the insoluble complex that forms on the reverse-stained gel is still unknown, it apparently involves imidazole, SDS, and zinc. We speculate that SDS also forms part of the insoluble complex that stains the gel background white because (a) in the presence of SDS the formed particles were larger and apparently heavier and the light transmittance was lower than that observed at the same zinc:imidazole ratio in absence of SDS and (b) the addition of up to 10 times more zinc to the imidazole (e.g., from 3 to 30 mM), in the absence of SDS, did not reduce light transmittance as much as SDS did (e.g., at the concentration of 0.1%; Fig. 4A).

Transparency of protein bands. Bovine serum albumin, a protein that can be detected perfectly on imidazole–SDS–zinc reverse staining, did not prevent imidazole–zinc precipitation in the test tube in a range of protein concentrations that included those typical for a protein band (approx 10 μg of protein in a 10 × 2 × 1-mm gel band or 20-μl volume). This observation, together with the light transmittance characteristics of the imidazole–SDS–zinc suspensions, suggests that the local inhibition of staining observed on the protein bands cannot be attributed to the protein itself but mainly to the increase in the local SDS concentration that it induces. Although in gels run without SDS and stained according to the original imidazole–zinc method (8), visualization of certain proteins as transparent bands may not be excluded as a consequence of their particular interactions with the staining reagents.

Application of This Mechanism to Explain the Reverse Staining of Gels Already Stained with Coomassie Blue

On the Coomassie blue reverse-stained gels, the dodecyl sulfate bound to the blue-stained bands seemed to inhibit the precipitation of the complex in a way similar

to that in the absence of the dye, while imidazole–SDS in the gel matrix would account for background staining during the zinc step. This is supported by the observation that Coomassie blue-stained bands on double-stained gels appeared superposed on larger transparent bands, an effect that was more evident when the protein amount in the band decreased in the serially diluted samples: after a certain point (determined by the CB sensitivity), CB-stained bands became visually undetectable and only transparent bands were visible. As SDS strongly binds to most proteins (11), a wide variety of protein bands can be detected after the reverse-staining step. Moreover, the overall sensitivity of the new technique does not depend on the affinity of the particular protein for the Coomassie blue dye. Indeed, we have never found a protein detectable by Coomassie blue but undetectable by the reverse-staining technique. Therefore, the present method seems to provide a nondifferential double staining.

In practice, the staining intensity of the background which is essential for an optimal contrast after reverse staining of unstained gels is slightly sensitive not only to the actual concentration of the staining reagents in the gel matrix but also to the presence of glycine and, perhaps, of Tris from electrophoresis. This is especially true when the gel is treated with imidazole–SDS for too short a time (5 min or less), resulting in a somewhat uneven background that may tend to fade upon storage. However, this drawback of the reverse-staining technique, which can be circumvented by a longer incubation in imidazole–SDS, was overcome in the present double staining: double-stained backgrounds were reproducibly homogeneous after the Coomassie blue reverse-staining method. This improvement in background is likely due not only to the choice of an adequate incubation time in an optimized imidazole–SDS staining solution, but also to the substantial reduction in residual electrophoresis reagents from the gel matrix that takes place during the CB staining and destaining steps and the later wash in distilled water prior to the reverse-staining step.

Further conclusions of direct practical interest can be drawn from the solubility behavior of the imidazole–SDS–zinc complex:

(i) Due to the extensive removal of SDS during the CB staining and destaining steps, double staining of CB gels should be always performed using an imidazole–SDS solution (e.g., as described under Methods). In fact, pale backgrounds resulted when already stained gels were subjected to our original method (8), which employed only imidazole and zinc (not shown).

(ii) The abrupt decrease in the light transmittance of the imidazole–SDS solution from its maximal to its minimal level after addition of zinc (Fig. 4A) further reflects the high sensitivity of the double staining to the zinc incubation time. Indeed, too short an exposure (1 to 15 s) of the imidazole–SDS equilibrated gel to the zinc solution resulted in no staining at all but prolonged incubation (over 60 s), leading to overstaining of bands and decreased detection.

The proposed mechanism of staining also explains the reverse staining of gels run without SDS (e.g., isoelectric focusing gels) and the slightly improved backgrounds observed on reverse-stained SDS gels when imidazole–SDS was used instead of imidazole alone (9). This mechanism seems to be valid for at least another heavy metal, copper (II), which permits imidazole–copper reverse staining (8). In fact, the deep-blue imidazole–copper complex becomes insoluble in the presence of small amounts of SDS (not shown). However, on an increase in SDS concentration in the imidazole, the precipitation of this complex is prevented in a way similar to that shown for zinc in Fig. 4B.

Interestingly, the solubility behavior of this complex appears to be independent of the order of addition of the reagents (not shown). However, in this study we selected an order that would resemble the gel staining procedure, though in the test tube.

Finally, the new double-staining procedure has several advantages over the Coomassie blue–silver-staining methods: (a) It is a nondifferential staining. (b) It is simpler and faster. (c) The CB pattern is not affected by the reverse-staining step; therefore, it is compatible with densitometry. (d) After double staining, previously undetected bands will appear visible in a manner compatible with subsequent microanalysis; moreover, double-stained gels can be kept in distilled water for months and detected proteins subjected to analysis at any later time.

This simple procedure may prove useful for laboratories that routinely used the CB technique and kept their stained gels, but may be interested in knowing whether their preparations are actually as "they see" them. This may be the case for investigators involved in mapping biological systems, e.g., cells, by 2D PAGE, where a wide variety of proteins are separated for later microanalysis and the distribution of Coomassie blue-stained protein spots is recorded and integrated into databases. Detection of minor CB-undetectable proteins actually present in these preparations could, however, be accomplished in a way compatible with subsequent microanalysis by subjecting these already stained gels to a reverse-staining step, as described here.

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