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Imidazole-SDS-Zn reverse staining of proteins in gels containing or not SDS and microsequence of individual unmodified electroblotted proteins

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A reverse staining procedure is described for the detection of proteins in acrylamide and agarose gels with and without SDS. Protein detection occurs a few minutes after electrophoresis. The sensitivity on acrylamide gels is higher than that of Coomassie blue staining either on acrylamide gels or on electrotransferred membranes. Sequencing of protein bands only detected by reverse staining on the gel and not by Coomassie blue is demonstrated.

Imidazole; Reverse staining; Microsequencing; PAGE

1. INTRODUCTION

Electrophoresis in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) has been shown to be one of the most resolving techniques for separation and analysis of complex protein mixtures. A large number of sensitive staining procedures based on labeling the proteins with different dyes (Amido black, Silver, Coomassie blue, [1–3] etc.), used to detect proteins in acrylamide gels have been described, Coomassie blue (C.blue) staining being the routine procedure used. These methods stain the gels 'positively', fixing the protein and interfering with protein electroelution or blotting for further analysis. Fluorescent reagents (fluorescamine, *O*-phthalaldehyde [4, 5], etc) have also been used, although these reagents partially block the N-terminus.

Alternatively other less-sensitive staining procedures have also been described, based on the formation of insoluble metal (zinc, copper, potasium, etc.) hydroxides or salts, which produce a semiopaque background on the gel surface while the proteins are shown up as transparent bands, a technique known as reverse staining (RS) [6–10]. This method is limited to SDS-containing gels. More recently, the sensitivity of reverse staining in SDS-acrylamide gels has been increased by adding imidazole (Im), prior to metal staining. Reproducibility was substantially increased with respect to the previously reported zinc staining [11].

In this paper we report a modification of imidazole-zinc reverse staining (Im-Zn-RS) that extends its application to gel electrophoresis (acrylamide and agarose gels) without SDS, demonstrating that the sensitivity of RS detection on gels exceeds that of C.blue detection either on gels or on polyvinylidene difluoride (PVDF). This RS procedure has been used for the preparation of proteins for N-terminal amino acid sequence analysis.

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2. MATERIALS AND METHODS

2.1. *Gel electrophoresis*

SDS-PAGE (9 cm × 6 cm × 1 mm) containing 10–18% acrylamide was performed as reported by Laemmli [12]. Gels were stained in a solution of 0.1% (w/v) Coomassie blue R 250. Isoelectric focusing in homogeneous 5% polyacrylamide gels (5 × 5 cm) containing carrier ampholytes (pH range 5–8 or 3–9) were carried out by using a Phast-System (Pharmacia) apparatus. Gels were stained in 0.05% Coomassie blue R 350 by using an automatic program recommended by the PhastSystem manufacturer. Isoelectric focusing in Isogel (pH 3–10) agarose gel was performed using a Pharmacia apparatus.

2.2. *Reverse staining imidazole-SDS-Zn of acrylamide and agarose gels*

2.2.1. Imidazole step

Immediately after electrophoresis the gels were immersed and gently shaken in 0.2 M imidazole containing: (a) 0.1% SDS for SDS-acrylamide gels (18%), for 5 min; (b) 0.5% SDS for acrylamide gels (5%) without SDS, for 10 min; and (c) 0.1% SDS for isoelectric focusing agarose (1%) gels without SDS for 15 min. The incubation time can be modified depending on the % acrylamide or agarose.

2.2.2. Zn step

The imidazole containing gels were immersed and gently shaken in 20–30 ml of 0.2–0.3 M zinc sulfate [11]. After 5–15 s, the reaction was stopped and the zinc solution replaced by 20–30 ml of distilled water.

Visualization of the protein transparent bands was facilitated by placing the gels on a plate over a black surface with a fluorescent lamp from an oblique angle below.

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2.3. *Preparation of proteins from gels stained with Im-SDS-Zn for direct sequencing analysis*

2.3.1. Protein mobilization

The excised immobilized protein transparent bands (6–8 × 2–3 mm) were mobilized in 1.0 ml of 2% citric acid in an Eppendorf tube for 10 min [11].

2.3.2. Electroblotting

The mobilized protein containing gels were equilibrated in 1 ml of 50 mM Tris, 50 mM boric acid, pH 8.3, containing 0.1% SDS and 50 mM DTT for 40 min in an Eppendorf tube. The sandwich for electroblotting consisted of the following components, oriented from the negative to the positive pole: (i) 6 filters papers (15 × 10 mm) immersed in blotting buffer (the above buffer without SDS); (ii) the gel band in blotting buffer; (iii) the ellipsoidal PVDF (13 × 5 mm); and (iv) 6 filter papers (18 × 15 mm) in blotting buffer. The protein was electroblotted in a semidry transblot system (5 × 5 cm) (Pharmacia) for 2 h (for 18% acrylamide gels) or 30 min (for agarose gels) at a constant current of 12.5 mA. Proteins electroblotted onto PVDF were applied directly to a Knauer 810 modular liquid phase protein sequencer.

Proteins were also directly electroblotted from non-stained SDS-acrylamide gels as above. The protein bands were detected by staining with 0.2% w/v Coomassie blue R-250, for 1–2 min or with 0.1% Amido black for 1 min. Destaining was performed in distilled water [13,14]. The protein bands were cut out of the PVDF and applied to the sequencer.

3. RESULTS AND DISCUSSION

In order to extend the scope of the RS technique we have investigated increasing its sensitivity, as well as its applicability to protein detection on gels without SDS by incorporating SDS in the imidazole solution. After electrophoresis the SDS-acrylamide (18%) gels were incubated with 0.2 M Im containing variable amounts of SDS (0.02, 0.05, 0.1, 0.5 and 1% SDS). It was observed that, when incubation was made in a 0.1% SDS–0.2 M Im solution (Fig. 1, centre), the detection limits were extended in comparison with a replicate gel incubated in 0.2 M Im in the absence of SDS [11] (Fig. 1, left). The addition of SDS to the Im solution avoided loss of SDS from the SDS-gel during its incubation, and increased the sensitivity of the method. The best results were observed with 0.1% SDS in the Im solution.

Different incubation times were evaluated (from 1 to 20 min). The optimal RS pattern was obtained after 5 min, and no further improvement was seen with longer incubation times (data not shown).

Alternatively, the incorporation of 0.2 M imidazole in the gel and electrophoresis buffer allowed detection of proteins by placing the gel immediately after electrophoresis in a 0.2 M zinc solution (data not shown). However, this procedure, although very rapid, is much more expensive due to the high consumption of imidazole.

Replacement of zinc by alternative metals was evaluated (Mg, Fe, Cd, Ag, Ca) giving low detection or no detection at all (data not shown).

Fig. 2 shows a comparison of two-dimensional protein patterns on gels detected with Im-SDS-Zn RS and C.blue. Im-SDS-Zn detects minor components that are not detected by C.blue. Similar results are shown in Fig. 1B and C. These experiments demonstrate that protein detection in SDS-gels with Im-SDS-Zn RS is by far superior to C.blue staining.

Application of RS to the detection of proteins in non-SDS-containing gels was also investigated. After isoelectric focusing in 5% acrylamide or 1% agarose, gels were incubated for different periods of time (from 1 to 20 min) in 0.2 M Im containing 0.05, 0.1 or 0.5% SDS. The best RS patterns were observed by pre-incubation with an Im solution containing 0.1% SDS for 15 min for agarose and 0.5% SDS during 10 min for acrylamide gels.

Detection in acrylamide gels is again better with this RS technique than with C.blue (Fig. 3, top), while in agarose gels, RS detection is worse than with C.blue (Fig. 3, bottom). The poor results of RS on agarose gels could be related to factors associated with the gel matrix and electrophoresis buffers such as pH and the nature of ions [6,7]. After RS of the agarose gel, it is possible to recover the proteins by transfer to PVDF (Fig. 3, bottom right) following the same procedure reported for acrylamide gels as outlined in Fig. 4.

RS techniques are particularly useful in protein preparation for microsequencing. In the Im-SDS-Zn RS the immobilized proteins can be easily released from their Protein-SDS-metal complexes by chelating agents or citric acid [11]. A scheme of the sample preparation steps for further microsequencing from gels is illustrated in Fig. 4. Fig. 5 shows the sensitivity of protein detection with Im-SDS-Zn directly in gels and with C.blue after transfer from unstained gels to PVDF membranes. Detection by RS of protein bands on gels is much better, because it allows handling and analysis of those undetected on PVDF by C.blue. Serial dilutions from 450 to 10 pmol of a pure protein (17 kDa) were compared by both staining methods (Fig. 5, bottom). While all were detected by RS, only those corresponding to 450 and 150 pmol were detected by C.blue, confirming once more that the RS technique is much more sensitive. Sequence data have demonstrated that, although undetectable by C.blue, the 30 pmol band contained enough material for N-terminal automatic sequencing.

This result stresses once more the advantages of RS for protein isolation at the low pmol range, since it allows manipulation of protein bands for microsequencing that would otherwise be undetectable by C.blue on PVDF. Besides, the combined average of the initial yields from proteins transferred after RS of the gel, and proteins detected by C.blue or Amido black in the membrane are of the same order (data not shown).

In conclusion, the present method not only extends reverse staining to non-SDS-containing gels, including agarose gels, but also improves the sensitivity of the RS detection to SDS-containing gels. The sensitivity of the Im-SDS-Zn RS is much higher than that of C.blue acryl-

Fig. 1.

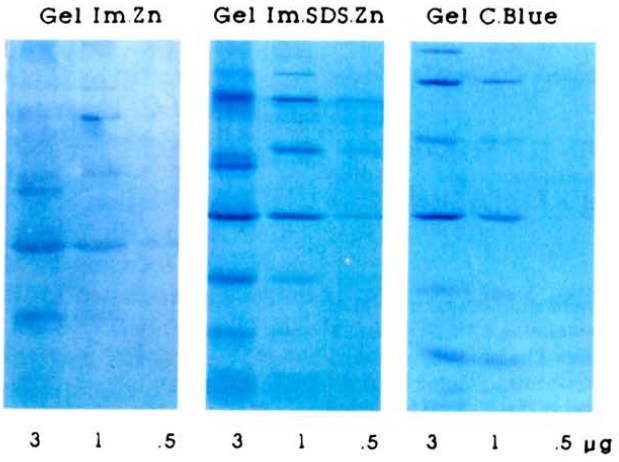


Fig. 2.

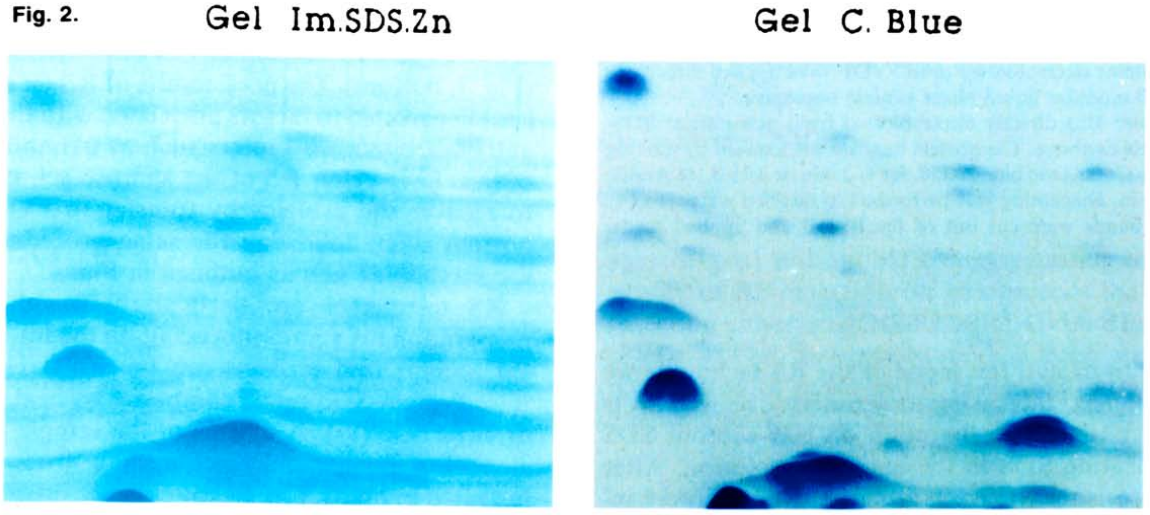
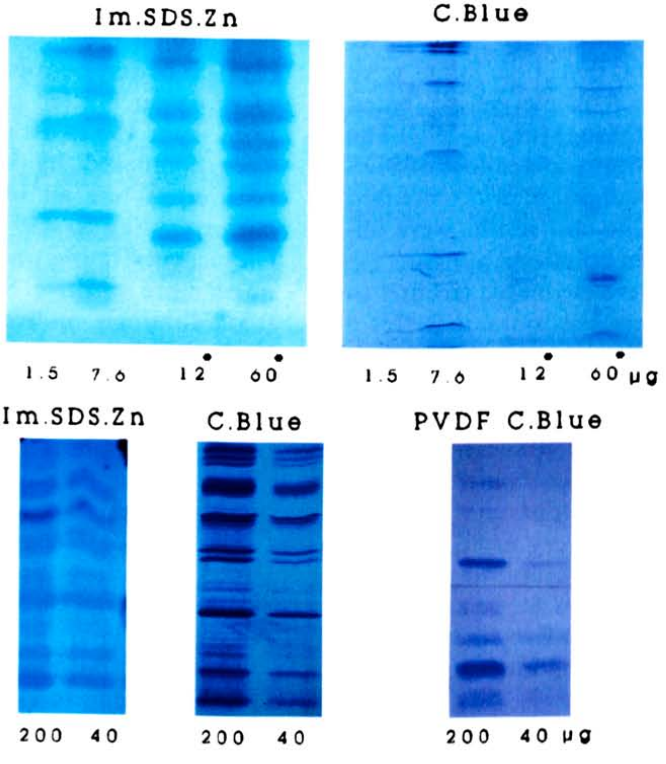


Fig. 3



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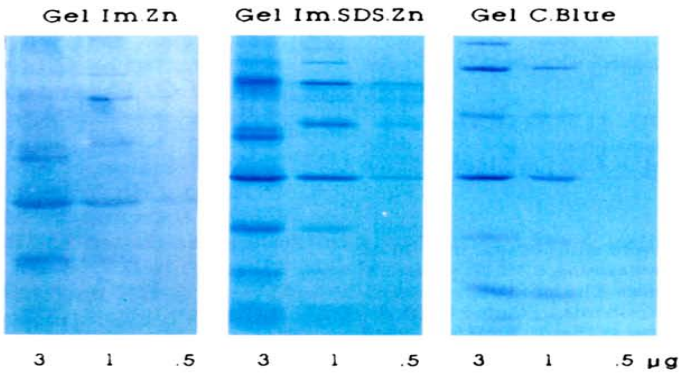

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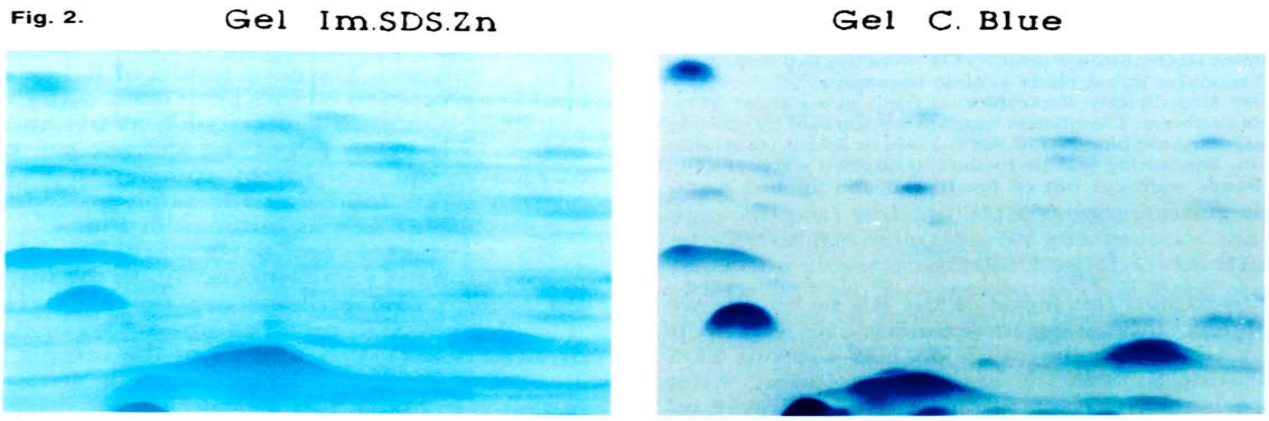

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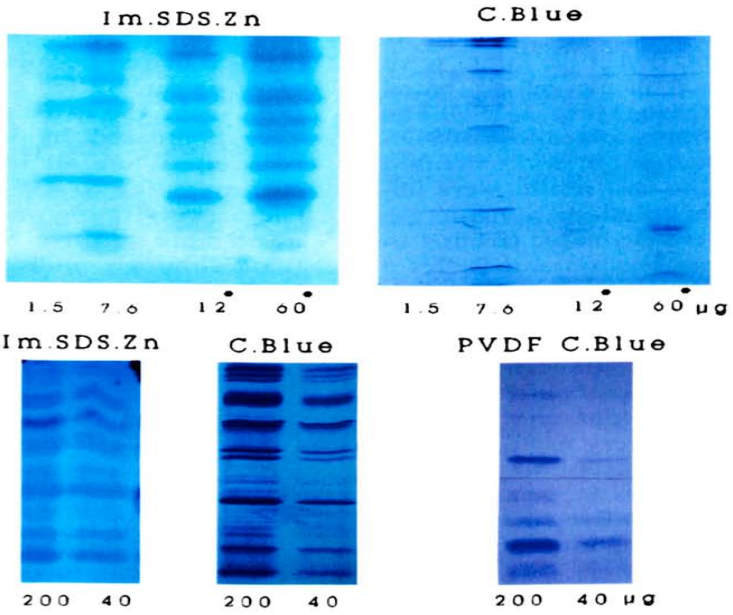

Fig. 1. Detection of proteins in SDS-acrylamide gels by the Im-Zn-RS after pre-incubation of the gels in an imidazole solution in the absence or presence of SDS. After electrophoresis the gels were incubated for; (left) 20 min in a solution of 0.2 M Im without SDS; (centre) 5 min in a solution of 0.2 M Im containing 0.1% SDS; (right) proteins detected on the gel by the C.blue staining. Total amount of a protein standard mixture loaded is indicated.

Fig. 2. Comparison of the sensitivity of the Im-SDS-Zn and the C.blue staining for detection of proteins on SDS-acrylamide gels. Two-dimensional isoelectric focusing/SDS-PAGE (10%) separation of a protein homogenate of *Drosophila thorax*. The gels were stained with Im-SDS-Zn (left) and C.blue (right).

Fig. 3. Detection of proteins on acrylamide (top) and agarose (bottom) gels by the Im-SDS-Zn compared with the C.blue staining. Total amount of loaded protein mixtures standard and from *rice endosperm is indicated.

Fig. 4. Schematic representation of the preparation for microsequence analysis of proteins separated by two-dimensional SDS-containing gels and detected by the Im-SDS-Zn RS. Protein recovery is indicated in brackets.

Fig. 5. Comparison of protein detection from SDS-acrylamide gels for direct sequence analysis. (A) Four dilutions of (A) a protein mixture standard and (B) a purified protein (restrictocine, 17 kDa) detected directly on the gel by the Im-SDS-Zn RS. (C and D) Transfer to PVDF from replicate non-stained gels and detected by C.blue on the membrane. The amount of protein loaded and the PTH-amino acid initial yield appears in brackets.

amide gel staining, allowing visualization of proteins within a few minutes, instead of hours, after electrophoresis. The method is adequate for sample preparation for microsequencing, and permits the handling of proteins, which are undetectable by C.blue staining on transferred membranes.

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