

lution (lanes 4–6). It is apparent, therefore, that maximal yields of nondegraded protein may be obtained simply and rapidly by boiling and resuspending total cellular material prior to electrophoresis.

Western blotting. Having established that this method produces significant yields of high-quality protein, we investigated whether the presence of additional cellular material would compromise subsequent immunodetection of recombinant protein. Indeed, we wished to test whether the improved yield of finely resolved protein would actually enhance such detection. Duplicate samples prepared as above were resolved by 12% SDS–PAGE and transferred to PVDF membranes for immunodetection with an anti-gal4 antibody (Fig. 1B). We calculated that Gal4-tagged E12 and E2-2 should generate fusion proteins of approximately 31 and 48 kDa, respectively. Proteins of the appropriate molecular weights are apparent in lanes 4 (E12) and 5 (E2-2), whereas no cross-reactive species of equivalent molecular weights are visible in the control lane (lane 6). In comparing lanes 1–3 and 4–6, it is apparent that preclearing leads to a reduction in protein concentration, thus compromising the detection of recombinant proteins. However, even when a correction for protein concentration with precleared lysates is made (lanes 7–9), poor resolution prevented unambiguous detection. Reducing the amounts of protein to levels between those shown in lanes 1–3 and 7–9 still failed to provide for detection of the fusion protein. Cross-reactivity with an unknown band of ca. 67 kDa is seen in each lane, but this does not hinder interpretation.

We have found that multiple cycles of freeze-thawing lead to even greater release of protein. However, this is accompanied by increased degradation which actually results in the diminution of signal intensity (data not shown). Thus, we recommend only a single initial freeze-thaw and the storage of lysates for subsequent analyses in small aliquots. Optimal results are also obtained from yeast colonies less than 2–3 days old.

The above report presents a simple and rapid technique for the preparation of protein from small cultures of yeast with consistent yield and protein integrity. The consistently large amounts of high-quality total yeast protein obtained with this method make it ideally suited to the simultaneous analysis of proteins from multiple samples. Indeed, the labor involved compares favorably with that expended in the preparation of bacterial lysates. Yeast lysates prepared in this way allow the unequivocal detection of relatively rare proteins by Western analysis. Indeed, we have used this method to detect the expression of a range of other yeast two-hybrid fusion proteins with both anti-yeast gal4 trans-activation domain and gal4 DNA binding domain antibodies (data not shown). The use of this technique is not necessarily limited to the study of yeast two-hybrid

fusion proteins. Rather, anyone wishing to expedite the preparation of multiple yeast lysates for Western blotting may find it of value.

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Detection of Calcium Binding Proteins by Two-Dimensional Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

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Calcium ion mediates various stimulus-induced cellular responses as a second messenger. Since calcium signaling pathways are thought to be triggered by binding of calcium ion to certain proteins such as calmodulin, development of a simple and sensitive method for detection of Ca^{2+} -binding proteins in crude cell extracts may be useful for studying calcium signaling. For this purpose, a ^{45}Ca overlay method on polyacrylamide gel (1) or on a blotted membrane (2) has been developed, and this method has been used for detection of high-affinity Ca^{2+} -binding proteins which exist abundantly in cells (1, 2).

In the present study, we describe a new simple method for detection of Ca^{2+} -binding proteins separated upon two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (2D·SDS–PAGE),¹ on a gel containing EGTA in one dimension and on a gel containing CaCl_2 in the other dimension. This

¹ Abbreviations used: 2D·SDS–PAGE, two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis; poly(Glu, Lys, Tyr)_{6:3:1}, poly(glutamic acid, lysine, tyrosine) containing 60% glutamic acid, 30% lysine, and 10% tyrosine; PSD, postsynaptic density.

method is based upon the fact that some Ca^{2+} -binding proteins exhibit different mobilities in the presence and absence of Ca^{2+} upon SDS-PAGE (3, 4), presumably owing to the conformational changes induced by the binding of Ca^{2+} . When PSD proteins from rat brain were analyzed by this method, several positive spots including calmodulin were detected. Since this method is very simple and does not require any radioactive material or special apparatus, it will be useful for detection of various proteins that interact with Ca^{2+} in crude tissue extracts.

Materials and Methods

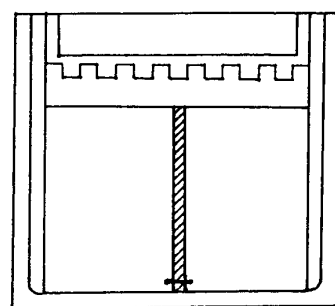
Materials. Poly(Glu, Lys, Tyr)_{6:3:1} and molecular weight protein standards for SDS-PAGE (SDS-7) were purchased from Sigma Chemicals. Calmodulin was prepared from rat testis by the method of Dedman *et al.* (5). PSD fraction was prepared from rat cerebral cortex as described by Sahyoun *et al.* (6).

2D-SDS-PAGE. Polyacrylamide gel electrophoresis in the presence of SDS was performed essentially according to the method of Laemmli (7) on slab gels consisting of a 10 or 12% acrylamide separation gel and a 3% stacking gel. CaCl_2 , MgCl_2 , or EGTA/EDTA was added to give a final concentration of 2 mM into a separation gel just prior to polymerization. Proteins, dissolved in Laemmli's sample buffer, were loaded onto a gel without heat treatment and electrophoresed on a gel containing EGTA/EDTA in a first dimension. Just before the bromophenol blue dye front reached the bottom of the gel, electrophoresis was stopped, and the lane of the gel which resolved the protein sample was cut out longitudinally approximately 1.5 mm in width (Fig. 1A). The gel strip was soaked in 2 ml of equilibration buffer comprising 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.002% bromophenol blue, and 2 mM CaCl_2 or MgCl_2 at room temperature for 10 min. Then, the gel was placed horizontally on top of a gel containing CaCl_2 or MgCl_2 (Fig. 1B), and electrophoresis in a second dimension was done. After electrophoresis, the gel was stained with Coomassie blue for detection of calmodulin or with a silver stain kit (Wako Chemicals) for detection of other proteins. In some experiments, gels for electrophoresis in a first and second dimension were reversed as indicated.

Results and Discussion

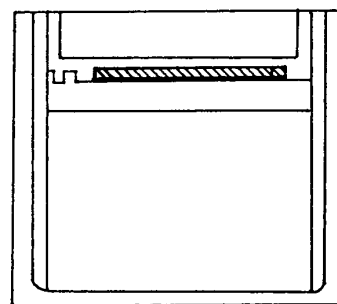
Analysis of rat brain PSD fraction by 2D-SDS-PAGE. If protein samples are subjected to 2D-SDS-PAGE under the same conditions in both the first and second dimensions, they are expected to give a diagonal straight line as shown in Fig. 1C, and PSD proteins actually gave such a straight line, when electropho-

A. 1st SDS-PAGE



Equilibration buffer

B. 2nd SDS-PAGE



C. Protein staining

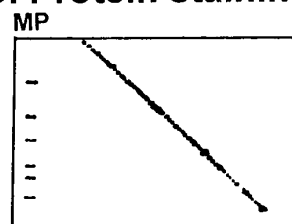


FIG. 1. Diagram of the major steps of 2D-SDS-PAGE. For details, see the text.

resed in the presence of EGTA/EDTA in both dimensions, as shown in Fig. 2A. On the other hand, when electrophoresis was carried out in the presence of EGTA/EDTA in the first dimension and in the presence of CaCl_2 or MgCl_2 in the second dimension, many spots deviating from the diagonal straight line were observed as shown in Figs. 2B and 2C, suggesting that many proteins in the PSD fraction interacted with Ca^{2+} and Mg^{2+} . Interestingly, most of the deviated spots were commonly observed in both Figs. 2B and 2C, indicating the possibility that most of the proteins which interact with Ca^{2+} can interact with Mg^{2+} . Such deviated spots were not clearly observed with ZnCl_2 or MnCl_2 added

in place of CaCl_2 or MgCl_2 (data not shown). Among many deviated spots, a few were observed only in the presence of Ca^{2+} , as indicated by the arrows (Fig. 2C), and not observed in the presence of Mg^{2+} . The spot of calmodulin was not clearly detected by silver staining, in agreement with the observation of Schleicher and Watterson (8).

Analysis of a purified calcium binding protein by 2D-SDS-PAGE. The answer to the question of whether a certain protein can interact with Ca^{2+} is

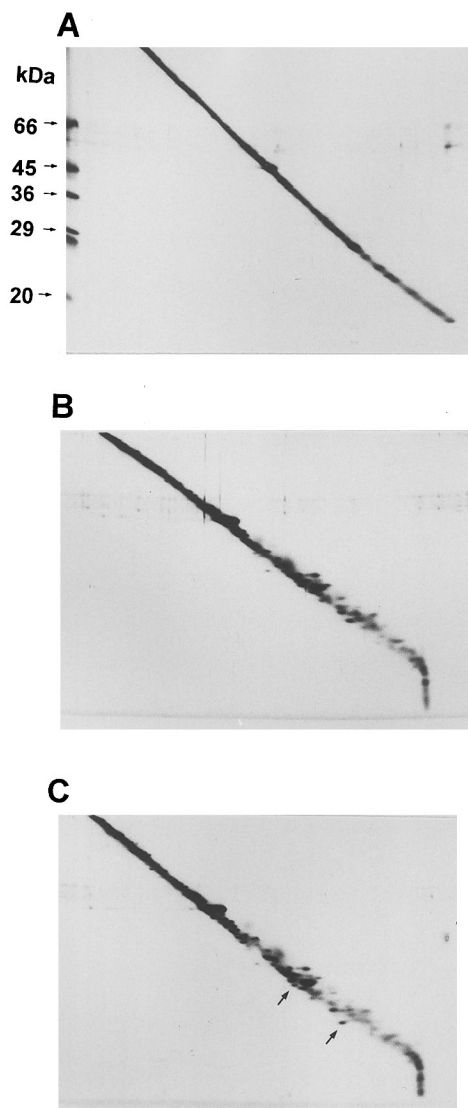


FIG. 2. Analysis of PSD proteins from rat cerebral cortex by 2D-SDS-PAGE. Approximately 2 μg protein of PSD fraction of rat cerebral cortex was electrophoresed on 10% acrylamide gels containing EGTA/EDTA in the first dimension, followed by SDS-PAGE on 10% gels containing EGTA/EDTA (A), MgCl_2 (B), and CaCl_2 (C) in the second dimension, as described under Materials and Methods. After electrophoresis, proteins were stained with silver. The arrows in (C) indicate the positive spots observed only in the presence of CaCl_2 .

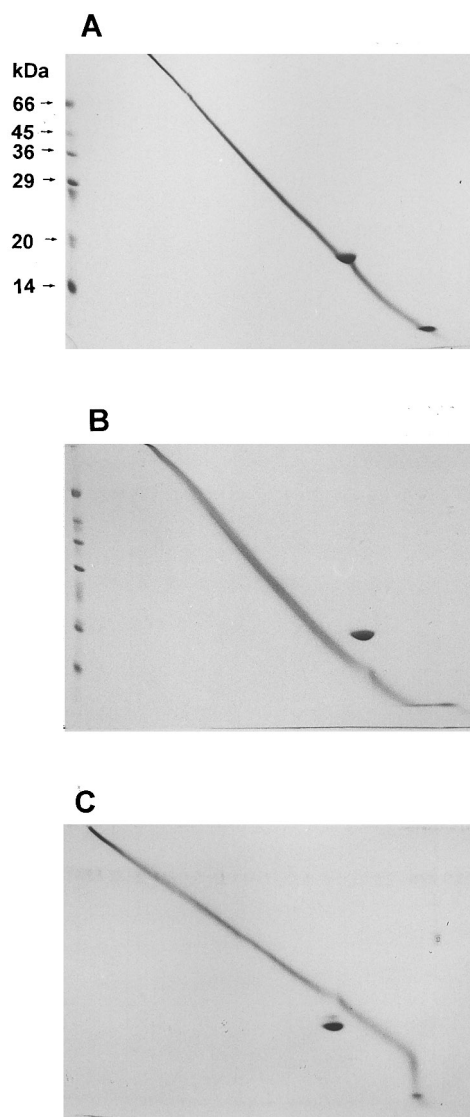


FIG. 3. Analysis of calmodulin by 2D-SDS-PAGE. Approximately 2.4 μg of purified calmodulin and 6 μg of poly(Glu, Lys, Tyr)_{6:3:1} were subjected to 2D-SDS-PAGE, on 12% gels containing EGTA/EDTA in both the first and second dimensions (A), on a gel containing CaCl_2 in the first dimension and a gel containing EGTA/EDTA in the second dimension (B), and on a gel containing EGTA/EDTA in the first dimension and a gel containing CaCl_2 in the second dimension (C), and stained with Coomassie blue.

sometimes important for studies of calcium signaling. Figure 3 shows that 2D-SDS-PAGE can also serve as a simple technique for examining this important question. Calmodulin, a typical Ca^{2+} -binding protein possessing EF-hands as Ca^{2+} -binding sites, was analyzed by 2D-SDS-PAGE. As a standard which does not interact with Ca^{2+} , random polymers of Glu, Lys, and Tyr in a ratio of 6:3:1 were employed. When a mixture of calmodulin and poly(Glu, Lys, Tyr)_{6:3:1} was subjected to 2D-SDS-PAGE in the pres-

ence of EGTA in both the first and second dimensions, a spot of calmodulin was observed on a diagonal straight line of poly(Glu, Lys, Tyr)_{6:3:1} (Fig. 3A). In contrast, when electrophoresis was carried out in the presence of CaCl₂ in the first dimension, a spot of calmodulin was observed above the diagonal standard line (Fig. 3B), and when electrophoresis was done in the presence of CaCl₂ in the second dimension, calmodulin was observed below the standard line (Fig. 3C), in agreement with earlier observations that calmodulin migrates more rapidly in the presence of Ca²⁺ than in the presence of EGTA (3, 4).

The question of whether electrophoretic mobilities of most Ca²⁺-binding proteins on SDS-PAGE differ in the presence and absence of Ca²⁺ remains unanswered, but many spots of proteins from PSD were actually shifted by the presence of Ca²⁺, as shown in Fig. 2C. Since each deviated spot upon 2D-SDS-PAGE can mostly be considered as composed of a single polypeptide, 2D-SDS-PAGE is a very convenient and powerful technique for not only detecting but also purifying Ca²⁺-binding proteins in crude tissue extracts.

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