TABLE 1

Primer Specificity Analyzed by Sex Determination on DNA Eluted from Filter-Paper Blood Spots

Sex	True number	Accurately sex-determined	Wrongly sex-determined (in duplicate)	Discrepancy between duplicate
M	48	48	0	0
F	48	45	1[a]	2
M		100%	0%	0%
F		94%	2%	4%
Total	96	93	1	2
		97%	1%	2%

Note. All samples were analyzed in duplicate.

[a] Contamination with male DNA in this sample was verified with Y-chromosomal specific primers published by Cui *et al.* (6) and Lo *et al.* (7).

ciated with nested-PCR applications, has been avoided, and the influence from polymerase inhibitors on the quantitation result has been controlled. We believe that it should be possible to increase the sensitivity of the assay further by using more sensitive detection methods, e.g., blotting techniques with enzymatic, chemiluminescent, or fluorescent detection.

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Recovery of Biologically Active Proteins Detected with Imidazole–Sodium Dodecyl Sulfate–Zinc (Reverse Stain) on Sodium Dodecyl Sulfate Gels

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Proteins separated by polyacrylamide gel electrophoresis can be detected in the gel with the imidazole–SDS–zinc staining procedure (reverse staining) (1–6). Detection occurs through precipitation of zinc salts on the gel surface everywhere except where proteins are located. This type of detection combines several desirable features: (i) simplicity (two steps), (ii) rapidity (15 min or less), (iii) sensitivity (1 to 10 ng protein/band), (iv) reproducibility, (v) low cost, (vi) temporary protein immobilization, (vii) reversibility, (viii) compatibility with subsequent protein analysis (e.g., with amino acid analysis, microsequencing, mass spectrometry, and immunoblotting), and (ix) nontoxicity. Here we show the compatibility of imidazole–SDS–zinc detection with analyses based on the biochemical activity of proteins.

METHOD

Recovery of Biochemically Active Proteins Detected by Imidazole–SDS–Zinc on SDS gels

(1) Gel staining. After SDS–PAGE, rinse the gel in distilled water for 30 to 60 s, incubate it in 0.2 M

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imidazole solution containing 0.1% SDS for 10 min, and then immerse it in 0.2 M zinc sulfate solution for 30–40 s until the gel background becomes deep white, leaving the protein bands transparent and colorless. Stop staining by rinsing the gel with abundant distilled water.

(2) Protein mobilization. Excise the protein band of interest and then rinse the gel slice in phosphate-buffered saline (PBS)[2] containing 100 mM EDTA (2 × 10 min) to complex zinc ions. Finally, wash the gel slice twice in PBS for 10 min to remove excess EDTA.

(3) On-gel renaturation. Soak the gel slice (3 × 10 min) in PBS containing 0.1% Triton X-100, and next wash it twice in PBS alone to remove excess detergent.

(4) Protein recovery. The protein may be passively eluted from crushed gel pieces (7) by incubation (two changes × 10 min) in PBS under vigorous shaking.

TABLE 1

Specific Biological Activities of Gel-Purified Proteins

	Specific activity (IU/mg)		
	rSK	rHuIFN-α2b	rHuIFN-γ
Untreated	5.35×10^4 (9.2)	2.3×10^8 (18.6)	9.0×10^6 (20.4)
Recovered from the reverse-stained gel	4.19×10^4 (14.1)	1.1×10^8 (20.0)	3.0×10^6 (23.8)
Recovered from the unstained gel	5.53×10^4 (13.7)	0.1×10^8 (21.2)	2.40×10^6 (24.6)

Note. As a control, samples of the three proteins were obtained from unstained lanes and assayed in parallel with the reverse-stained samples. For reference, the specific activity of equivalent amounts of pure proteins was determined in solution ("Untreated"). Values given in the table represent the averages for each set of three samples, followed by the relative error (%) in parentheses.

RESULTS AND DISCUSSION

Compatibility of Reverse Staining with Protein Micropurification for Biological Analysis

Many times, the gel-separated proteins are analyzed with techniques (e.g., determination of specific activity, receptor activity assays, enzyme-linked immunoabsorbent assays) requiring them in solution. However, because SDS causes protein unfolding, hindering further biological analysis, it is necessary to include a renaturation step in the purification strategy, as proposed by the present method. This strategy was verified at least for three distinct proteins [recombinant streptokinase (rSK), recombinant human interferon-α2b (rHuIFN-α2b), and recombinant human interferon-γ (rHuIFN-γ)]. These proteins were produced in transformed *Escherichia coli* as described elsewhere (8, 9). Total *E. coli* proteins from the transformed strains containing 30% rSK, 33% rHuIFN-α2b, or 35% HuIFN-γ were electrophoresed in a 12.5% polyacrylamide–SDS gel and detected by reverse staining. The proteins of interest were excised, renatured, and eluted as described above. Their specific activities were determined according to Refs. (10, 11) as shown in Table 1. Integrity, amount, and purity of the eluted proteins were determined by reelectrophoresis of an aliquot in a 12.5% polyacrylamide–SDS gel, followed by detection with Coomassie blue, and analysis by gel densitometry (not shown).

We formerly observed that these proteins dramatically lost their activity in the presence of SDS (from 0.1 to 1%) (not shown). Two principal reasons appear to account for the high specific activity values obtained: (i) zinc from the immobilized protein–SDS bands was effectively chelated with EDTA, as previously shown (5); the soluble zinc–EDTA complexes as well as free imidazole were rapidly removed from the zones of the proteins. (ii) A significant amount of the SDS bound to the proteins was extracted by simple diffusion after interchange with Triton X-100. Protein recovery by enhanced protein diffusion from crushed gels did not affect the integrity of the renatured samples nor did it interfere with their activity. This method allows protein recovery, over a wide range of physicochemical properties (mass, size, charge, hydrophobicity, etc.), to an extent larger than 90% (7).

Compatibility of Imidazole–SDS–Zinc with Enzyme Staining

One useful technique for the identification of proteins on gel is to stain for biological activity (zymogram) after electrophoresis [for review see (12)]. The combination of this technique with the reverse staining method allowed both a sensitive nonspecific and a specific protein detection to be performed on the same sample on this gel (Fig. 1). Note that the activity band of levansucrase on the reverse-stained lane is qualitatively indistinguishable from that of the unstained lane. Other examples were the successful application of the reverse stain before or after esterase and phosphatase staining (15) of total proteins from tilapia (*Oreochromis aureus*) serum (not shown). Typical activity and reverse-stained bands were visualized on the same lane, enabling the evaluation of the actual complexity of the protein sample at the same time.

In summary, we showed experiments verifying that reverse-stained proteins can be assayed for biological activity (in gel or in solution) with the same efficiency as unstained proteins. They strongly suggest that reverse staining can be generally applied in the nonspe-

[2] Abbreviations used: PBS, phosphate-buffered saline; rSK, recombinant streptokinase; rHuIFN-α2b, recombinant human interferon-α2b; rHuIFN-γ, recombinant human interferon-γ.

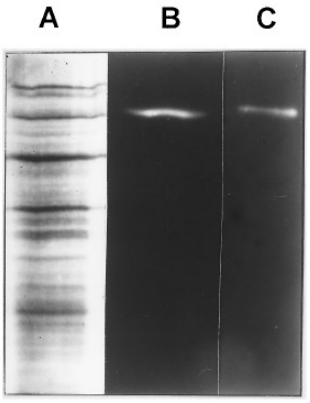

FIG. 1. Levansucrase (sucrose-2, 6-β-D-fructan 6-β-D-fructosyltransferase; EC 2.4.1.10) *in situ* activity staining on a SDS gel after reverse staining. Soluble intracellular proteins from *Acetobacter diazotrophicus* strain SRT4, a nitrogen-fixing bacterium that produces this levansucrase (13), were resolved in a 12% SDS–polyacrylamide gel (0.75 mm) as described by Laemmli (14). (A) Reverse staining of the lane. (B) Specific enzyme staining after reverse staining of lane A (see below). (C) Enzyme staining of an unstained (control) lane. Specific enzyme staining was according to Ref. (13): reverse stained (A) or unstained (C) lanes were incubated in sodium acetate buffer (pH 5.5) containing 5% sucrose, at 45°C. Destaining of the reverse-stained lane was unnecessary because the acid sodium acetate buffer effectively solubilized the imidazole-induced zinc precipitate in the gel matrix. The endpoint was judged from the appearance of a deep white-stained band, corresponding to the synthesis of levan from sucrose, at the position of the levansucrase band. It should be noted that levansucrase is normally detectable by zymogram staining after SDS gel electrophoresis; thus, in this case removal of SDS was not necessary.

cific detection of proteins prior to their recovery for biochemical analysis, as particularly evidenced here with rSK, rHuIFN-α2b, and rHuIFN-γ.

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A Method to Synthesize Strand-Specific Probes

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We describe here a method allowing synthesis of high specific activity probes for Southern and Northern assays as well as other techniques. Figure 1A summarizes our method for preparing strand-specific labeling DNA probes. The method employs immobilization (chemical crosslinking) of DNA fragments to chemically modified cellulose (1). DNA is crosslinked randomly via guanine residues. Immobilized DNA is used as a template for a primer extension reaction. The newly synthesized product can be eluted for further use and the template can be reused. Because the primer extension reaction is terminated at the sites of DNA crosslinking to cellulose, the newly synthesized DNA is represented by a set of fragments with a primer on

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