tions with hazardous organic solvents. A simple vortex adapter (like that available from Sigma) could aid in processing many samples simultaneously. The time required for the whole process, from picking up the colony for lysis to visualization of the amplified product on agarose gel, is only about 4 h, saving time in routine analysis of mycobacteria. We found that this method yields genomic DNA from common Gram-positive and -negative bacteria also without any noticeable disintegration and can be used to check for the presence of plasmids in certain cases. Moreover, the DNA obtained is amenable to restriction digestion. We hope this method will be useful in any laboratory screening a large number of bacteria by PCR, especially in developing countries.

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A Highly Active Immobilized Ribonuclease[1]

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By cleaving RNA, ribonucleases operate at the crossroads of transcription and translation. Ribonucleases are useful catalysts in procedures that call for the thorough degradation of single-stranded RNA. In such procedures, high ribonucleolytic activity is required in one step, but can be undesirable in subsequent steps. Available methods for eliminating ribonucleolytic activity suffer from a lack of specificity, efficacy, or permanence.

The most commonly used ribonuclease in biochemistry and biotechnology laboratories is ribonuclease A (RNase A[4]; EC 3.1.27.5) (1, 2). The T45G variant of RNase A, which cleaves efficiently on the 3′ side of adenosine (A) as well as cytidine (C) and uridine (U) nucleotides, degrades RNA more thoroughly than does the wild-type enzyme (3–5). Hence, we sought to develop a means to add T45G RNase A to a reaction mixture and then eliminate completely its ribonucleolytic activity.

A general and effective technique to add and then eliminate an enzyme is by attaching it covalently to a solid support (6). The enzymatic side chains that are most amenable to covalent modification are cysteine, lysine, and histidine. Unfortunately, native RNase A has no free cysteine residues (despite having four cystines), and 3 (Lys7, Lys41, and Lys66) of its 10 lysine residues and 2 (His12 and His119) of its 4 histidine residues are critical for ribonucleolytic activity (2). In addition, the side chain of Lys41 has an anomalously low pK_a of 9.0 (7) and is thus highly nucleophilic near neutral pH. Moreover, covalent modification of other

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[4] Abbreviations used: RNase A, ribonuclease A; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTB, 2-nitro-5-thiobenzoic acid; DTT, dithiothreitol.

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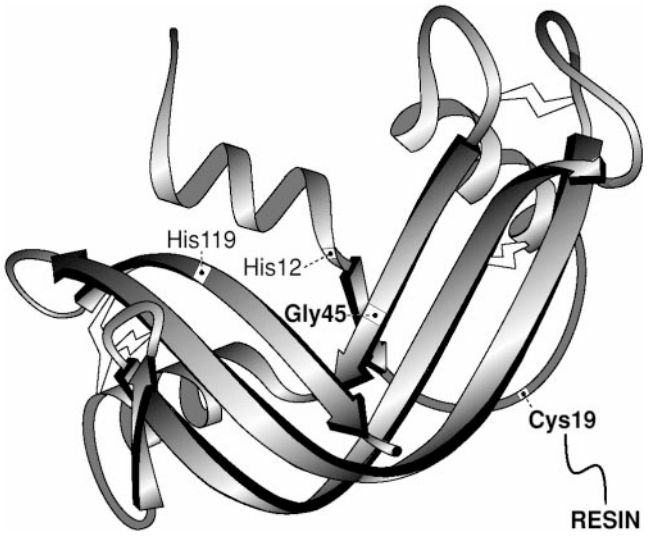

FIG. 1. Ribbon diagram of RNase A showing the location of the active-site histidine residues (His12 and His119), Gly45, and Cys19, which is used to attach the enzyme to a solid support. The diagram is based on the structure of crystalline T45G RNase A, which was determined by X-ray diffraction analysis [Protein Data Bank entry: 3RST (5)].

lysine or histidine residues could interfere with the binding of RNA to the active site. Thus, as expected, immobilization of wild-type RNase A severely compromises its activity against polymeric RNA (8–10). Here, we overcome this problem and describe the immobilization of fully active T45G RNase A.

Ala19 of RNase A has no known role in the structure or function of the enzyme. In most known homologs of RNase A, residue 19 is a serine, not an alanine (11). Ala19 of RNase A is located on a surface loop that is far removed from the enzymatic active site (Fig. 1). We reasoned that replacing Ala19 with a cysteine residue would insert a uniquely reactive function group into the enzyme without adverse consequences.

We created the A19C/T45G variant of RNase A from *Apa*I/*Cla*I fragments of DNA encoding A19C RNase A (12) and T45G RNase A (3). The enzyme was prepared and purified by methods described previously (3). To protect the sulfhydryl group of Cys19 from adventitious oxidation, purified A19C/T45G RNase A was reacted with a fourfold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in Tris–HCl buffer (pH 8.3) containing EDTA (10 mM). The solution rapidly became yellow as free 2-nitro-5-thiobenzoic acid (NTB) was produced in addition to a mixed disulfide involving the side chain of Cys19 and NTB. The resulting solution was dialyzed exhaustively against water and lyophilized.

We immobilized A19C/T45G RNase A via the side chain of Cys19. Lyophilized, NTB-protected enzyme was dissolved (to 5 mg/mL) in 20 mM sodium phosphate buffer (pH 8.0) containing NaCl (0.10 M). The side chain of Cys19 was deprotected at 25°C by adding a threefold molar excess of dithiothreitol (DTT). The solution became yellow, again indicating the production of free NTB. To remove small molecules, the deprotected A19C/T45G enzyme, which has the same ribonucleolytic activity as T45G RNase A, was applied to a Sephadex G25 gel-filtration column and eluted with degassed phosphate buffer. As a solid support, we chose SulfoLink resin from Pierce (Rockford, IL), which is crosslinked agarose [6% (w/v)] to which iodoacetyl groups are attached via a spacer. A suspension (1 mL) of SulfoLink resin was added to an empty Micro Bio-Spin chromatography column from Bio-Rad (Hercules, CA). After equilibration with 6 column volumes of phosphate buffer, the resin was incubated with enzyme (1 mL of a 5 mg/mL solution) for 30 min at room temperature. Adding a solution of higher enzyme concentration did not produce resin with greater ribonucleolytic activity. The column was drained by centrifugation, and the resin was washed with phosphate buffer (3 mL) before unreacted sites were blocked by incubation with 20 mM sodium phosphate buffer (pH 8.0) containing NaCl (0.10 M) and cysteine (50 mM). The resin was then washed extensively with 20 mM sodium phosphate buffer (pH 8.0) containing NaCl (1.0 M). For storage, the resin was suspended in water containing sodium azide [0.5% (w/v)] to inhibit microbial growth. No apparent loss of ribonucleolytic activity was observed after the resin was stored at 4°C for several weeks.

We assessed the ribonucleolytic activity of the resin with a fluorometric assay using the tetranucleotide substrate 6-FAM~dArCdAdA~6-TAMRA from Integrated DNA Technologies (Coralville, IA). This assay is based on the quenching of fluorescence of 6-FAM held in proximity to 6-TAMRA by a single ribonucleotide embedded within deoxyadenosine nucleotides. When RNase A cleaves the sole ribosyl unit, the fluorescein moiety is released from its proximity to the rhodamine moiety and manifests its intrinsic fluorescence (13). Fluorometric assays of coupled resin (5 μL of a suspension) indicated that a 1.0-mL volume of resin has ribonucleolytic activity equivalent to 5 μg of uncoupled T45G RNase A. The actual amount of enzyme coupled to the beads was determined by reducing its cystines with DTT in the presence of guanidinium chloride, washing extensively with buffer, reacting with DTNB, removing the resin by centrifugation, and measuring the absorbance of the supernatant at 412 nm [ϵ = 13,600 M^{-1} cm^{-1} (14)]. These data indicate that approximately 60 μg of enzyme is coupled to each milliliter of resin. Thus, the coupled enzyme retains close to 10% of its activity, in contrast to previous results (8–10).

A key advantage of an immobilized enzyme is the ability to remove its activity quickly, easily, and thor-

oughly. To assess this ability, we removed the highly active resin from an assay mix by centrifugation for a few seconds in a microcentrifuge. No ribonucleolytic activity was then detectable in the supernatant. Based on the sensitivity of our assay, the decrease in ribonucleolytic activity was $>10^5$-fold. Thus, the immobilization of T45G RNase A via a cysteine residue installed at position 19 allows for the enzyme to retain high ribonucleolytic activity but be removed rapidly and thoroughly from a solution. This immobilized ribonuclease could be of significant utility in the purification of DNA (which is not a substrate for RNase A) from cellular extracts as well as in a variety of analytical procedures, including the ribonuclease protection assay (15, 16).

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