Methods for Visualization of Enzymes in Polyacrylamide Gels

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White bands resulting from precipitation of dodecan-1-ol liberated by hydrolysis of sodium dodecyl sulfate and dodecan-5-ol released by hydrolysis of dodecan-5-yl sulfate produced zymograms of the primary and secondary alkylsulfatases from Pseudomonas C₁₂B. Gas-liquid chromatographic analyses of ether extracts of the precipitate-containing segments of the zymograms confirmed the identity of the alcohols which were not discerned in extracts of segments of the gels other than those containing precipitates. β-Galactosidase from Escherichia coli was marked on zymograms by the liberation of o-nitrophenol from o-nitrophenyl-β-D-galactoside, and arylsulfatase from Pseudomonas C₁₂B was marked in gels by liberation of p-nitrophenol from p-nitrophenyl sulfate. Membrane-associated dissimilatory nitrate reductases from a nitrate respirer (Enterobacter aerogenes) and a denitrifier (Pseudomonas perfectomarinus) did not penetrate either 6.8 or 3% polyacrylamide gel but were demonstrable at the top of the gels. In the membrane-bound state, formate served as electron donor for nitrate reductase from E. aerogenes, and reduced nicotinamide adenine dinucleotide (NADH) served as donor for nitrate reductase from P. perfectomarinus. Both enzymes reduced nitrate at the expense of reduced benzyl viologen as well. Assimilatory nitrate reductase from E. aerogenes moved easily into the 6.8% gels (Rₜ = 0.43 under the conditions of these experiments). The reduced dye served as electron donor for the assimilatory reductase, but formate and NADH did not. Incubation of the membrane-associated nitrate reductases with 2% Triton X-100 solubilized the enzymes and removed the capacity of formate and NADH to serve as electron donors. Both reduced the ability to retain nitrate at the expense of reduced benzyl viologen. The solubilized assimilatory reductase from E. aerogenes moved further in the gels (Rₜ = 0.49) than the soluble assimilatory reductase; the solubilized dissimilatory reductase from the denitrifier, P. perfectomarinus, moved further in the gels (Rₜ = 0.64) than either of the enzymes from E. aerogenes.

Soluble proteins can be located in polyacrylamide gels (PAG) after analytical electrophoresis (PAGE) by staining with amido black or Coomassie brilliant blue. If interest goes beyond separating proteins generally to include locating a specific enzyme, it is useful to be able to visualize that enzyme by specifically marking the location of products of enzymatic reactions. Gels so marked are called zymograms, and an increasing number of enzymes are separated from other proteins and located by this procedure (1).

Staining of dehydrogenases or dehydrogenase-coupled enzymes by the formation in situ of colored, water-insoluble, reduced nitro blue tetrazolium salts is the procedure most frequently used for obtaining zymograms (6).

Techniques for revealing other enzymes have also been described by Gabriel (5) and Shaw and Koen (15).

The purpose of this paper is to extend the range of phenomena useful for marking zymograms by showing that, whether colored or not, products such as long-chained alcohols that precipitate where they are enzymatically liberated in PAG may also mark the position of enzymes. In addition, evidence can now be presented for the usefulness of zymograms for demonstrating enzymes (such as nitrate reductase) that produce a chromogenic inorganic substance (such as nitrite).

MATERIALS AND METHODS

Bacteria, cultural conditions, and extract preparation. Pseudomonas C₁₂B was cultured on a minimal medium containing sodium hexan-1-yl sul-
fatetoinducesynthesisofprimaryalkylsulfatase(3),
onnutrientbrothtoprovidecellss-containingsecondary
alkylsulfatase(3),andontheminimalsupplemented
withNa₂SO₄,toyielcellsrepressedforsynthesisof
aryl sulfatase (4).

A stock strain of Escherichia coli K-12 (kindly
provided by G. J. Tritz) was cultured aerobically
on nutrient broth containing 0.5% lactose (lactose broth)
to induce synthesis of β-galactosidase. A nitraterespiring
strain of Enterobacter aerogenes (CDC 6566)
taken from this Department's stock was cultured to
elicit synthesis of assimilatory and dissimilatory nitrate
reductases (16) as follows: (i) anaerobically in
nutrient broth containing 0.5% KNO₃ (nitrate broth)
to induce synthesis of dissipimilatory (but repress
synthesis of assimilatory) nitrate reductase; (ii) aerobic-
ically in minimal salts-glucose medium (for each liter:
K₂HPO₄, 9.28 g; KH₂PO₄, 1.81 g; KNO₃, 1 g; FeCl₃,
12.5 mg; Na₂SO₄, 0.5 g; MgSO₄, 0.01 g; and

glucose, 2.5 g; pH 7.0) with nitrate as sole source of
nitrogen to induce polymerization of assimilatory nitrate
reductase; and (iii) anaerobically in the nitrate-glucose
minimal medium to induce concurrent syn-
thesis of assimilatory and dissimilatory nitrate reduc-
tases in the same population of cells. In addition,
batches of E. aerogenes were grown aerobically in
the minimal medium containing 0.5 g of NH₄Cl per liter
in place of KNO₃, to provide cells devoid of either
nitrate reductase.

Pseudomonas perfectomarinus (originally isolated
by Claude Zobell) was grown anaerobically on sea
salt-trypomone-yeast extract-nitrate medium (13) to
provide bacteria that contained a dissimilatory nitrate
reductase representative of a denitrifier. Addi-
tional batches of P. perfectomarinus were grown
aerobically in this medium lacking nitrate to provide
cells that did not contain nitrate reductase.

All cultures were incubated at 30°C for 18 h in 16-
to 24-liter lots in carboys. Aerobic cultures were ascepti-
cally sparged and agitated with filtered air, whereas
anaerobic cultures were continuously stirred by a
magnetically turned bar in completely filled, rubber-
stoppered carboys. Cells of the enteric bacteria were
harvested by centrifugation and washed with 0.1 M
phosphate buffer, pH 7.0. The marine bacteria were
harvested by centrifugation and washed with 0.052 M
MgCl₂ to prevent premature lysis. Washed cells of
each of these bacterial types were then suspended in
0.1 M phosphate buffer, pH 7.0, and ruptured by
several passes through a French pressure cell in the
cold. To avoid the inhibition of sulfatases by phos-
phate, Pseudomonas C₁₃B cells were washed and
suspended for rupture by the pressure cell in 0.01 M
tris(hydroxymethyl)aminomethane (Tris)-hydrochlor-
ide buffer, pH 7.5. The extracts were clarified by
centrifugation in the cold at 34,000 × g, dialyzed
against fresh Tris buffer, diluted, and analyzed.

For certain experiments, extracts of E. aerogenes
and P. perfectomarinus containing the membrane-
associated dissimilatory nitrate reductase were incu-
bated with 2% (vol/vol) Triton X-100 (7) for 30 min at
30°C. The detergent-treated extracts were centrifuged
for 1 h at 85,000 × g and then dialyzed overnight
in the cold against 0.1 M phosphate buffer, pH 7.0,
before analysis by PAGE.

Gel electrophoresis. The dialyzed extracts were
then subjected to electrophoresis in gel that contained
6.8% (wt/vol) Cyanogum 41 (Fisher Scientific Co.)
suspended in 1 M HCl (0.06 mL/ml); and N,N',N',N'-tet-
ramethylethylenediamine (Eastman) (0.75 µl/iter).
This mixture was filtered free of particles before use.
A stock buffer, pH 8.3, containing Tris (30 mg/ml)
and glycine (144 mg/ml) (1) was diluted 1:50 with
water and added to the buffer tanks with 0.001%
hromophenol blue included in the upper tank as
tracking dye. Glass tubes (6 cm by 5 mm inside
diameter and marked 1 cm from the top) were
employed as analytical columns. To polymerize the
gel, 1.0 mL of 3.5% (wt/vol) ammonium persulfate
was added to 24 ml of stock gel solution, and the mixture
was pipetted into the columns up to the 1-cm mark.

After polymerization, samples of the extract in 0.1 ml
of 20% (wt/vol) sucrose were placed on the surface
of the gels. The proteins were then subjected to
electrophoresis in a Canalco model 300 unit operating at 2.5
mA per centimeter for 1 h at 25°C. Protein content of
the extract fractions was routinely estimated by
the method of Lowry et al. (8). Bovine serum albumin
served as protein standard. The Rf values assigned
in the current studies indicate the mobility of the
enzymes relative to the tracking dye. Alkylsulfatases
were revealed by incubation of PAG in 3-ml lots of
unbuffered solutions of either 10 mM sodium dodecyl
sulfate (SDS) or 10 mM decan-5-yl sulfate (D-5-S).
β-Galactosidase was detected by incubation of PAG in
3 ml of 10 mM o-nitrophenyl-β-D-galactoside, and
aryl sulfatase was detected by incubation of PAG in
3 ml of 10 mM p-nitrophenyl sulfate. Eluates from
pooled segments of zymograms were assayed for
β-galactosidase by the method of Rickenberg et al.
(14).

Zymograms of nitrate reductases were prepared by
placing PAG in 3-ml reaction mixtures that were 0.01
M solutions of KNO₃ in 0.1 M phosphate buffer, pH 7.0.
These reaction mixtures were also either 0.1 M
with respect to sodium formate or 0.02 M with respect
to diithionite-reduced benzyl viologen (BV) and were
incubated in a desicator that was flushed with a
continuous stream of helium. For certain experi-
ments, PAG were placed in 3-ml reaction mixtures
that were 0.01 M with respect to KNO₃ and contained
1 µmol of reduced nicotinamide adenine dinucleotide
(NADH) and 0.5 µmol of flavine adenine dinucleotide
in place of formate or reduced BV.

After incubation, PAG charged with the sulfatases
and β-galactosidase required no further treatment to
mark the location of the enzymes. Those loaded
with nitrate reductases were removed from the reaction
mixtures, laid on a glass plate, and sprayed with the
dimethyl-α-naphthylamine-sulfanilic acid reagent for
detection of the nitrite that marked the location of the
nitrate reductases.

Gas-liquid chromatography (GLC). The white-
banded areas of 12 SDS-treated primary alkylsulfat-
asezymograms were cut out, pooled, and extracted with 5 ml of ether. The banded areas of 12 D-5-S-developed zymograms of secondary alkylsulfatases were treated identically. The remainder of these PAG and various control PAG were also extracted with ether. The extracts of each set were permitted to evaporate to 0.5 ml and were analyzed by GLC for linear alcohols and related compounds as previously described (10, 11).

RESULTS AND DISCUSSION

Primary (Fig. 1a) and secondary (Fig. 1b) alkylsulfatases were located in zymograms by white precipitates. The contact photographic technique used here is less than ideal but was used to show simultaneously both these white precipitates and the colored bands in Fig. 1c and d. More conventional pictorial representation of the precipitated bands for alkylsulfatases was presented by Payne and Painter (9).

The alkylsulfatase bands were discernible after 30 min at 30°C but became most intense after 1 to 2 h. In the other two zymograms, colored products marked the enzymes in much shorter time intervals. β-Galactosidase was revealed by a yellow band of liberated o-nitrophenol (Fig. 1c) that was most sharply defined after incubation for only 5 min at 30°C. Extracts of cells grown in the absence of an inducer exhibited minimal activity that developed only after incubation was prolonged for several hours. We found that β-galactosidase could be eluted with 0.1 M phosphate buffer, pH 7.0, and recovered from PAG after PAGE of the extracts of the lactose broth-grown cells. The enzyme was eluted only from segments of untreated PAG that matched up with the marked bands on the zymograms. Arylsulfatase activity was revealed most strongly by incubation for 2 to 4 min at 30°C (Fig. 1d), whereas activity in extracts of cells grown in the presence of excess repressor (sulfate) was barely perceptible in PAG even after protracted incubation. The difuseness of the band of p-nitrophenol liberated from p-nitrophenol sulfate probably indicates the presence of two isozymes like the arylsulfatases A and B produced by *Pseudomonas aeruginosa* (2).

Zymography is particularly useful in studies of the control of primary and secondary alkylsulfatase synthesis. Three to five protein species are involved (4, 9), although these are separable into only two groups by in vitro assays. Presumably, the white bands in zymograms of alkylsulfatases result from the localized and restricted release of the long-chained alcohols. Previously, alkylsulfatases were eluted only from those segments of untreated PAG that matched the positions of the white bands in zymograms developed with SDS and D-5-S (9). Detergency does not produce the white bands. Incubation of the charged PAG in solutions containing other

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**Fig. 1.** Zymograms of alkylsulfatases, β-galactosidase, and arylsulfatase. To obtain photographs free of the diffractive influence of the curved sides of the cylindrical gels, the PAG were submerged in water in a glass dish that was placed on the photographic film and strongly illuminated. The photographs reveal: (a) a white band produced by primary alkylsulfatase in PAG that were charged with extract (55 µg of protein) from *Pseudomonas C₁₁B* (grown on minimal medium containing hexan-1-yl sulfate) and then incubated in 10 mM SDS; (b) a white band produced by secondary alkylsulfatase in PAG that were charged with extract (90 µg of protein) from *Pseudomonas C₁₁B* (grown on nutrient broth) and then incubated in 10 mM D-5-S; (c) yellow band produced by β-galactosidase in PAG that were charged with extract (65 µg of protein) from *E. coli* (grown on lactose broth) and then incubated in 10 mM o-nitrophenol-β-D-galactoside; and (d) a yellow band produced by arylsulfatase in PAG that were charged with extract (95 µg of protein) from *Pseudomonas C₁₁B* (grown on methionine minimal medium) and then incubated in 10 mM p-nitrophenyl sulfate.
detergents and potential substrates or suspensions containing reaction products did not raise the bands (9). Despite these observations, the identity of the white material was not previously established. Ether extraction of the white material that marks primary alkylsulfatase on zymograms incubated with SDS has now yielded only dodecan-1-ol to analysis by GLC (Fig. 2A). Similarly, extraction of the white bands marking secondary alkylsulfatase incubated with D-5-S yielded only decan-5-ol (Fig. 2B). Under these experimental conditions, retention time for decan-5-ol and the hydrolytic product of D-5-S was 31 s, and that of dodecan-1-ol and the hydrolytic product of SDS was 106 s (Fig. 2C). Ether extraction yielded no alcohols (Fig. 2A and B) from the following positional, specificity, enzyme-lacking, and substrate-lacking control systems: (i) the remainder of the zymograms from which the white-banded segments were removed; (ii) segments that matched the position of primary alkylsulfatase in zymograms of secondary alkylsulfatase after incubation with D-5-S (and, conversely, segments that matched secondary alkylsulfatase in zymograms after incubation with SDS); (iii) blank PAG incubated with D-5-S or SDS after PAGE; and (iv) PAG bearing alkylsulfatases after PAGE but not incubated with D-5-S or SDS.

Further versatility is illustrated by our studies with \textit{E. aerogenes}, in which anaerobic

![Fig. 2. GLC of ether-extracted material from zymograms of alkylsulfatases. Chromatograms were prepared from: (A) extract of pooled banded segments from zymograms of primary alkylsulfatase developed with SDS; (B) extract of pooled banded segments from zymograms of secondary alkylsulfatase developed with D-5-S; and (C) authentic decan-5-ol and dodecan-1-ol in ether. Controls in A and B represent ether extracts of PAG from which banded areas were removed or other ether extracts as indicated in the text. Analytical procedures were previously described (10, 11). Other parameters were: carrier gas, helium; oven temperature, 132 C; flash heater, 160 C; and detector temperature, 170 C. Signal attenuated \times 2.](http://aem.asm.org/)

![Fig. 3. Diagrams of zymograms of assimilatory and dissimilatory nitrate reductases. Diagrams are presented because the red bands photographed poorly or not at all. PAG were charged with: (a) membrane-associated dissimilatory nitrate reductase \(R_{n} = 0\) in extract (315 \(\mu\)g of protein; electron donors, formate or reduced BV) from \textit{E. aerogenes} grown anaerobically on nitrate broth; (b) membrane-associated dissimilatory nitrate reductase \(R_{n} = 0\) in extract (315 \(\mu\)g of protein; electron donors, NADH or reduced BV) of \textit{P. perfectomarinus} grown anaerobically on sea salt-tryp- tone-yeast extract-nitrate (STYN) medium; (c) both membrane-associated dissimilatory \(R_{n} = 0\) and soluble assimilatory \(R_{a} = 0.43\) nitrate reductases in extract (150 \(\mu\)g of protein; electron donors, formate or reduced BV for top band, reduced BV for lower band) of \textit{E. aerogenes} grown anaerobically on glucose-minimal medium with nitrate as sole nitrogen source and electron acceptor; (d) soluble assimilatory nitrate reductase \(R_{a} = 0.43\) in extract (205 \(\mu\)g of protein; electron donor, reduced BV) of \textit{E. aerogenes} grown aerobically on glucose-minimal medium with nitrate as sole nitrogen source (minimal dissimilatory reductase appears with \(R_{n} = 0\)); e, detergent-solubilized dissimilatory nitrate reductase \(R_{n} = 0.49\) in extract (155 \(\mu\)g of protein; electron donor, reduced BV) from \textit{E. aerogenes} grown anaerobically on nitrate broth; (f) detergent-solubilized dissimilatory nitrate reductase \(R_{n} = 0.64\) in extract (285 \(\mu\)g of protein; electron donor, reduced BV) from \textit{P. perfectomarinus} grown anaerobically on STYN medium; and (g) extracts from cells that contained neither type of nitrate reductase, or incomplete control mixtures that lacked enzyme, electron donor, electron acceptor, or exposure to nitrate reagent, or PAG incubated with an ineffective electron donor.](http://aem.asm.org/)

growth in the presence of nitrate is known to derepress synthesis of dissimilatory nitrate reductase (16). Ammonia and amino acids do not
affect synthesis of this enzyme but repress synthesis of the assimilatory reductase when added to media containing nitrate as sole source of nitrogen irrespective of the presence or absence of oxygen (12, 16). In the current studies, extracts of E. aerogenes grown anaerobically on either nitrate broth or glucose-minimal medium containing both ammonia and nitrate thus yielded dissimilatory nitrate reductase that was immobile in 6.8% (or even 3.0%) PAG (Fig. 3a). Either formate or reduced BV served as electron donor for this membrane-associated reductase, but NADH did not (Fig. 3g). Incubation periods of 3 to 5 min were sufficient to produce detectable quantities of nitrite. Immobility of the dissimilatory nitrate reductase was expected because the enzyme is membrane bound along with formate dehydrogenase and a b-type cytochrome (7, 16).

Extracts of E. aerogenes cells grown anaerobically in glucose-minimal medium with nitrate as both sole nitrogen source and terminal electron acceptor yielded the soluble, assimilatory nitrate reductase (16), with an average Rf of 0.43, in addition to the complex dissimilatory reductase that remained at the origin (Fig. 3c). Reduced BV served as electron donor for the assimilatory reductase, but formate and NADH did not (Fig. 3g). As anticipated (16), extracts of cells grown aerobically in glucose-minimal medium containing nitrate as sole source of nitrogen contained the soluble assimilatory nitrate reductase, with but a trace of the complex dissimilatory enzyme discernible at the origin (Fig. 3d).

After treatment with Triton X-100, a portion of the dissimilatory reductase of E. aerogenes was solubilized (as expected from the work of Kiszkiw and Downey [7]) and moved into the PAG (Fig. 3e). The solubilized enzyme (Rf = 0.49) was separated from formate dehydrogenase and no longer utilized formate (Fig. 3g) but still transported electrons from reduced BV. The membrane-associated dissimilatory nitrate reductase from the denitrifier P. perfectomarinus did not move into the PAG (Fig. 3b). After treatment with detergent, this enzyme was also solubilized and moved through the PAG (Fig. 3f), further in fact (Rf = 0.64) than the solubilized reductase from E. aerogenes. Either NADH or reduced BV served as electron donor for the reductase from the denitrifier in the membrane-bound state (Fig. 3b). After solubilization, reduced BV supplied electrons for the reduction of nitrate (Fig. 3f), but NADH did not (Fig. 3g).

These results show that zymography may be used to demonstrate enzymes that yield inorganic as well as organic end products in PAG. Incubation periods must be brief to minimize band diffusion. Physical separation of the assimilatory and dissimilatory nitrate reductases may now be useful in surveys of the types of reductases produced by a variety of bacterial species. According to Pichinoty and Piechaud (12), detection of the assimilatory enzyme in the presence of highly active dissimilatory nitrate reductase is difficult and uncertain with a manometric assay of cell extracts.

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