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Highly Sensitive and Rapid Detection of Antibody Catalysis by Luminescent Bacteria

Hagit Shulman,[a] Anatol Eberhard,[a,†] Carolyn Eberhard,[b,‡] Shimon Ulitzur[b] and Ehud Keinan[a,c,*,§]

[a]*Department of Chemistry and Institute of Catalysis Science and Technology, Technion-Israel Institute of Technology, Technion City, Haifa 32000, Israel*

[b]*Department of Food Engineering and Biotechnology, Technion-Israel Institute of Technology, Technion City, Haifa 32000, Israel*

[c]*Department of Molecular Biology and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*

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Abstract—A highly sensitive, inexpensive, and facile bioluminescent assay for the detection of catalytic antibodies has been developed. This assay may be used for the early detection of antibody catalysis. The efficiency of this technique was exemplified by the use of the luminescent bacterium VhM42 for monitoring an antibody-catalyzed retroaldol fragmentation reaction with aldolase antibodies 38C2 and 24H6. © 2000 Elsevier Science Ltd. All rights reserved.

The most common practice in the design and production of catalytic antibodies for a given reaction involves immunization against a transition state analogue (TSA) and isolation of the best binding antibodies to this analogue.[1] While this approach was proven to be a powerful strategy to elicit efficient catalysts, not every antibody binds the TSA in such a way that will affect catalysis. Furthermore, many reactions involve more than one transition state that requires stabilization. Considering that only a small fraction of the immune response is typically sampled by the hybridoma technology, the commonly applied screening for best binders may not necessarily uncover the best catalyst in a given population of monoclonal antibodies. A better approach would involve the screening for actual catalytic activity rather than for TSA binding. Such a screening technique, however, should be sufficiently sensitive to allow detection of catalytic activity when the concentration of catalyst in the hybridoma supernatant solution does not exceed 10^{-7} M and the volume of the entire reaction mixture is 0.1 mL.[2] Previous efforts to detect catalytic activity involved detection of the reaction products using either UV spectroscopy[3] or anti-product antibodies (cat-ELISA).[4] Alternatively, the reaction products were selectively tagged with easily detectable groups. Thus, tagging with biotin allowed for detection with an avidin reagent,[5] tagging with DNA allowed for PCR detection,[6] and labeling with acridone allowed for fluorescence detection on TLC plates.[7]

Intact marine bioluminescent bacteria provide interesting opportunities for sensitive detection of specific compounds.[8] For example, *Vibrio harveyi* produces light as a result of the luciferase-catalyzed reaction of reduced flavin mononucleotide, with a long chain aldehyde and oxygen.[9] An aldehyde-negative mutant (VhM42) produces low or no light except in the presence of exogenous aldehyde. The addition of aldehyde to these bacteria induces a sharp increase in light emission within less than a second. The use of this mutant with a simple luminometer can therefore rapidly detect very small amounts of long chain aldehydes.

We reasoned that VhM42 could be used to detect the catalytic activity of an antibody in a reaction that produces a long chain aldehyde. The commercially available aldolase antibody 38C2, which was obtained via reactive immunization,[10] has already been shown to catalyze the aldol addition and retroaldol fragmentation

*Corresponding author. Tel.: +1-858-784-8511; fax: +1-858-784-8732; e-mail: keinan@scripps.edu

†Permanent address: Department of Chemistry, Ithaca College, Ithaca, NY 14850, USA.

‡Permanent address: Department of Plant Biology, Cornell University, Ithaca, NY 14853, USA.

§Incumbent of Benno Gitter & Ilana Ben-Ami chair of Biotechnology, Technion.

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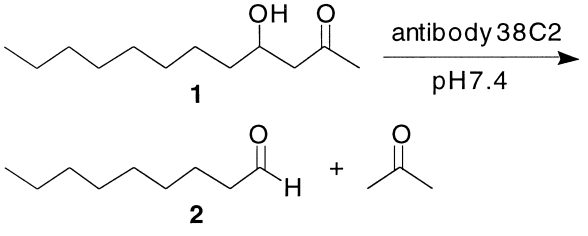
reactions with a broad variety of substrates.[11] Therefore, we anticipated that this antibody would also catalyze the retroaldol fragmentation of 4-hydroxy-2-dodecanone, **1**, to produce nonanal, **2**, and acetone (Scheme 1).[12]

Here we show that 38C2 indeed catalyzes the fragmentation of **1** to produce **2** and that this product is easily detected using VhM42. This assay, which is sensitive, inexpensive, and easy to perform, could be suitable for the early detection of catalytic antibodies in hybridoma cultures.

VhM42[13,14] was grown at 25 °C for approximately 24 h on Petri plates containing ASW (0.3 M NaCl, 0.01M KCl, 0.05 M $MgSO_4$, and 0.01 M $CaCl_2$) with 5 g/L of peptone (Difco), 3 g/L of yeast extract (Difco) and 15 g/L of agar (Difco). Bacterial colonies were dispersed in ASW to an OD_{600} of approximately 1.5 and kept on ice for the assays.

A calibration plot for nonanal was obtained using standard aqueous solutions (10^{-3}–10^{-6} M, containing 10% acetonitrile). These standard solutions were prepared from stock solutions (0.1 M in CH_3CN, made fresh daily) for which nonanal that had been distilled under reduced pressure was used. For each measurement, 1 mL of ASW was placed in a vial at 23 °C, 10 μL of the bacterial suspension was added and the light output was measured using a luminometer (Deltatox model PSI, Azur Environmental Co.). The standard solution of nonanal (10 μL) was added and the light output was measured immediately. The difference between these two readings was taken as the net light output caused by the aldehyde. The resultant calibration line (Fig. 1) indicates that nonanal can be easily detected at levels as low as 0.01 μM (10^{-11} mol).

In the absence of antibody no aldehyde was detectable in an aqueous solution of **1** (10^{-3} M containing 10% CH_3CN) by this bioassay even after several weeks at room temperature. All antibody-catalyzed reactions were carried out with **1** (10^{-3} M) in phosphate buffered saline (PBS, 0.05 M sodium phosphate, 0.1 M NaCl, pH 7.4) containing 5% acetonitrile at 25 °C, unless otherwise indicated. Aliquots were removed and assayed for nonanal as described above. On the basis of these measurements we can conclude that the detection of as little as 5 μg/mL ($\sim 3\times10^{-8}$ M) of this antibody is feasible under optimal conditions. The 38C2-catalyzed retroaldol fragmentation reaction of **1** was found to follow Michaelis–Menten kinetics. The kinetic parameters ($k_{cat} = 0.001\ min^{-1}$ and $K_M = 190\ \mu M$) were obtained from Michaelis–Menten analysis of the experimental data which were obtained via the bioassay (Fig. 2).



Scheme 1.

Samples of several nonrelevant antibodies[15] (2 mg/mL) as well as 38C2 at various concentrations (0.27 to 2.0 mg/mL) were tested for aldolase activity according to this procedure (Fig. 3). We have also tested the amount of aldehyde formed solely by the serum-based medium which is normally used to grow hybridomas. These results clearly show that the catalytic antibody, 38C2, can readily be distinguished from the other, non-catalytic antibodies as well as from serum proteins.

We examined our recently discovered aldolase antibody, 24H6,[15] under the assay conditions. Our previous attempts to detect catalysis of the retroaldol fragmentation with aliphatic substrates, such as **1**, using this antibody were unsuccessful, probably due to the high

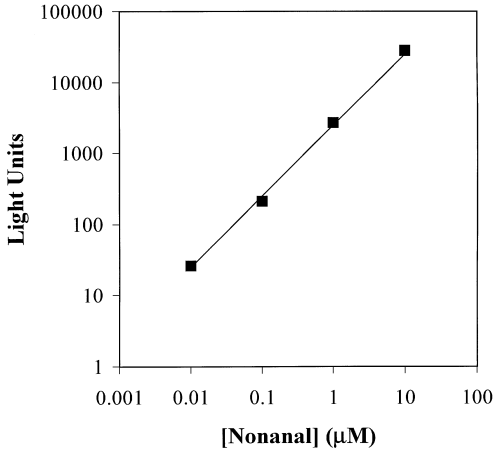


Figure 1. Calibration line for nonanal. Light emission is given in arbitrary units as a function of nonanal concentration in the assay vial. In this experiment, the practical detection limit was found to be 3 light units (10^{-12} mols aldehyde in the assay vial).

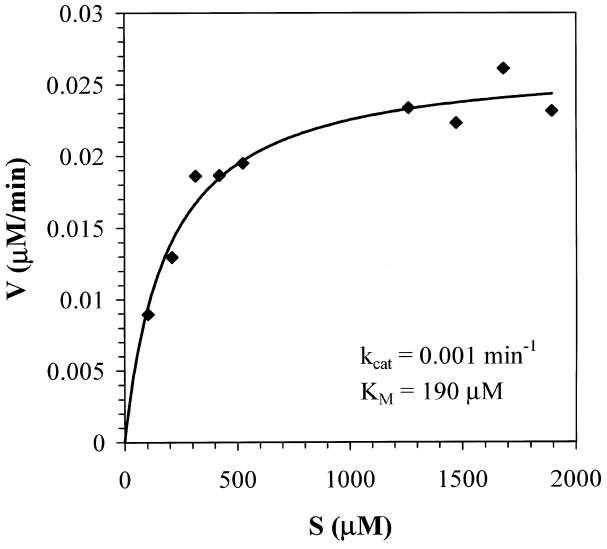


Figure 2. Michaelis–Menten kinetic analysis for the antibody 38C2-catalyzed retroaldol fragmentation of **1** at 25 °C. No background reaction could be detected.

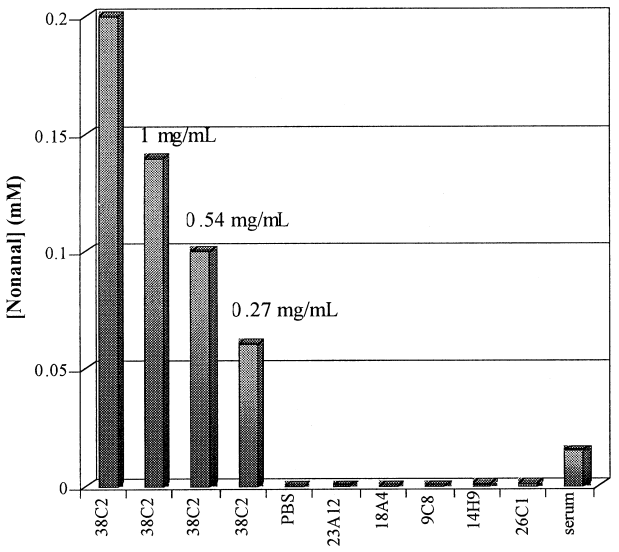


Figure 3. Nonanal formation from 4-hydroxy-2-dodecanone incubated with various proteins at 25 °C for 6 days. Concentration of all proteins was 2 mg/mL unless otherwise indicated. Serum: 10% FCS, 5% HCF, HAT.

preference of 24H6 for aromatic substrates. The increased sensitivity of our bioluminescence assay, however, allowed for detection of the 24H6-catalyzed fragmentation of **1** to produce **2** (2.3 μM of product with 24H6 versus 0.12 μM in the buffer-catalyzed reaction after an identical incubation time).

The usefulness of the bioluminescence detection technique is further exemplified by the fact that attempts to follow this antibody-catalyzed reaction by GC analysis (using a DB-5 column and an FID detector) gave irreproducible results, probably due to thermal decomposition of **1** within the GC injector port.

An important feature of the bioluminescence analysis is that it is a very facile and inexpensive bioassay. Detection is performed at room temperature, using simple instrumentation. Even when operated manually, approximately 60 samples can be measured per hour. This rate would allow for complete screening of an entire set of antibodies from a single fusion protocol (which typically yields 2000–3000 hybridoma clones).

In addition to the above-described procedure, the bioluminescent assay was examined with samples of **2** in a 96-well microtiter plate using concentrations that could occur in the early screening for catalytic activity in hybridoma clones. Solutions of **2** (10 μL each, at a concentration range between 0 and 0.001 M) were added to the wells of a microtiter plate. Each well contained 100 μL of buffer solution. The bacterial suspension (10 μL) was added rapidly to every well and light production was determined immediately with a luminometer (Lucy I, Anthos Co.). Samples containing 10^{-5} M nonanal were readily detected (data not shown). The sensitivity of this bioassay could be increased by using an aldehyde knock-out mutant, which is absolutely dark in the absence of **2**, in place of the somewhat leaky strain VhM42 used here.

In conclusion, we have provided evidence showing that the bioluminescent assay, which is sensitive, inexpensive, and easy to perform, could be used for the early detection of catalytic antibodies. The efficiency of this technique was exemplified by monitoring the antibodies 38C2- and 24H6-catalyzed retroaldol fragmentation reaction with the luminescent baterium VhM42. Luminescent bacteria with other specificities can be developed and used to assay other types of catalytic activities.

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12. The aldol adduct **1** was synthesized in one step from freshly distilled nonanal and the lithium enolate of acetone. Purification by flash chromatography (silica gel, hexane:ethyl acetate 85:15) afforded **1** in the form of a colorless oil. ^{1}H NMR: 3.9 (m, 1H), 3.1 (d, J = 3.6 Hz, 1H), 2.47 (m, 2H), 2.07 (s, 3H), 1.3 (m, 14H), 0.77 (t, J = 6.2 Hz, 3H). CI-MS: m/z 201 (MH^+). Its purity was not readily verifiable by GC due to its tendency to undergo a partial retroaldol reaction to give the starting materials. However, the bioassay as described above showed it to contain no detectable nonanal.
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