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CHARACTERIZATION OF AN IMMOBILIZED ANTIBODY–ENZYME COMPLEX

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Antiserum specific for propanediol dehydrogenase, an enzyme found in *Neisseria gonorrhoeae* cells, has been immobilized to glass. When mixed with *N. gonorrhoeae* cell lysates, the immobilized antibody (IMA) binds the enzyme. Over 70% of the calculated adsorbed activity can be recovered from the immobilized antibody-enzyme (IMA-E) complex. When mixed with bacterial lysates prepared from different organisms having propanediol dehydrogenase-like activity, the IMA specifically adsorbed the enzyme from the *N. gonorrhoeae* lysate. IMA-E complexes have been prepared and their kinetic, temperature and chemical stability, and antigenic properties investigated. These studies demonstrated the feasibility of using an immobilized antibody in the detection of the propanediol dehydrogenase enzyme.

INTRODUCTION

Neisseria gonorrhoeae cells contain an enzyme which utilizes propanediol as a substrate converting 1,2 propanediol to hydroxyacetone in the presence of NAD^+ (1). We have demonstrated a test for this propanediol dehydrogenase which uses this reaction as a marker for the detection of the organism. We have also prepared an antibody which, when in excess, completely inhibits the enzyme reaction (2). The incorporation of an antibody inhibition step in the detection system gives the test a two-fold check on specificity that occurs at the level of enzyme function (substrate) and enzyme structure (antigenic determinants).

This report investigates the possibility of using immobilized antibody (IMA) in the detection system. Unlike the inhibition test where antibody is used to neutralize the enzyme reaction, the IMA is used specifically to adsorb propanediol dehydrogenase in the bacterial lysate. Adsorbed enzyme exists in an enzymatically active immobilized antibody-enzyme (IMA-E) complex. In addition to conferring specificity, the IMA-E

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detection system purifies, concentrates, and converts soluble enzyme into a solid phase that can be separated from the reaction mixture, washed free of debris, and then reacted with substrate. This study characterizes the IMA-E complex formed with propanediol dehydrogenase, compares some of the properties of the bound enzyme with those of the free, and demonstrates the feasibility of using an IMA-assisted detection system.

MATERIALS AND METHODS

Culture Lysates

Neisseria gonorrhoeae culture F62 was obtained from the Center for Disease Control, Atlanta, Georgia, and cultivated in broth medium containing 15 g of protease peptone #3 per liter (Difco Laboratories, Detroit, Michigan), 1 g of cornstarch, 4 g of dipotassium phosphate, 1 g of potassium dihydrogen phosphate, 5 g of sodium chloride, and 10 ml of Isovitalex-enrichment (Baltimore Biological Laboratories, Baltimore, Maryland). Cells were cultured at room temperature with shaking for 24 to 48 h. Cell lysates were prepared by suspending the pellet obtained from 1 liter of cells in 100 ml of a 0.1 M Tris HCl, pH 9.0, buffer for 1 h at 4°C. Following centrifugation ($4000 \times g$, 30 min) to pellet cells and debris, the supernatant was frozen at −17°C. Cultures of other bacteria used in this study were prepared as described for *N. gonorrhoeae* but with lysozyme (1 mg/ml) added to aid disruption.

Antibody

Antibody to propanediol dehydrogenase was prepared (2) in goats immunized with enzyme purified by affinity chromatography on AMP columns (1). The antiserum contained 65 μg/ml of precipitable antibody protein that in excess could completely inhibit the enzyme reaction. Immunodiffusion analysis of antibody and enzyme showed one precipitin band that was enzymatically active. Immobilized antiserum was prepared according to Weetall and Filbert (3) using arylamine controlled pore glass 1 μ (ave.) pore diameter in a ratio of 1 g of glass per 4 ml of antiserum.

Enzyme Activity

Substrate was composed of the following reagents: 0.1 g of NAD^+; 1.0 ml of 1,2-propanediol; and 30 ml buffer (0.1 M Tris HCl, 0.25 M NH_4Cl, pH 9.0). One unit of enzyme activity was defined as the initial rate of reduction of 1.0 μmol of NAD^+ per minute at 23°C. K_m values were

determined graphically on the IMA-E complex. IMA (2 mg) saturated with enzyme was mixed with substrate containing varying concentrations of 1,2 propanediol or NAD^+ in a final volume of 3.0 ml. The sample tubes were capped and the reaction was incubated for 30 min at 25°C on a rocker platform. After centrifugation ($3000 \times g$, 5 min) at 23°C to pellet the complex, the absorbance of the supernatant fluid was read at 340 nm and corrected against a blank containing each substrate solution without enzyme. Under these conditions, the generation of NADH was linear with time for 45 min.

The IMA-assisted assay for propanediol dehydrogenase was performed as follows. Lysate (0.2 to 0.5 ml) to be tested for enzyme was added to a tube containing 200 μg of IMA and the reaction was incubated for 45 min at 37°C. The IMA-E complex was washed twice in 3 ml Tris buffer, substrate (0.2 to 2.0 ml) was added, and the reaction incubated at 37°C for 90 min. The NADH generated was read on a spectrophotometer at 340 nm or on a Turner Model III Fluorometer (Turner Associates, Palo Alto, California) with a neutral density filter, or under UV illumination using an SL-25 mineral light (Ultraviolet Products, Inc., San Gabriel, California). A blank was composed of IMA mixed with buffer in place of lysate and treated similarly.

Stability Studies

Heat stability was determined by treating samples of IMA-E complexes for 2 min at various temperatures. The effect of different chemical agents and antiserum was determined by mixing IMA-E complexes with each agent for 30 min at 23°C and then washing the complexes twice in Tris buffer. Age stability was determined by measuring the activity of a sample from a batch preparation of IMA-E complexes kept at various temperatures.

After each treatment, substrate was added and the reaction incubated for 30 min at 37°C. The absorbance was read at 340 nm and compared with an untreated sample.

RESULTS

Adsorption and Recovery of Enzyme Activity by an Immobilized Antibody

Figure 1 shows the results of a titration experiment performed by mixing dilutions of IMA with a constant amount of enzyme and then measuring the amount of enzyme activity adsorbed. The IMA performs the way an antiserum does; the less glass the less enzyme recovered. Table 1

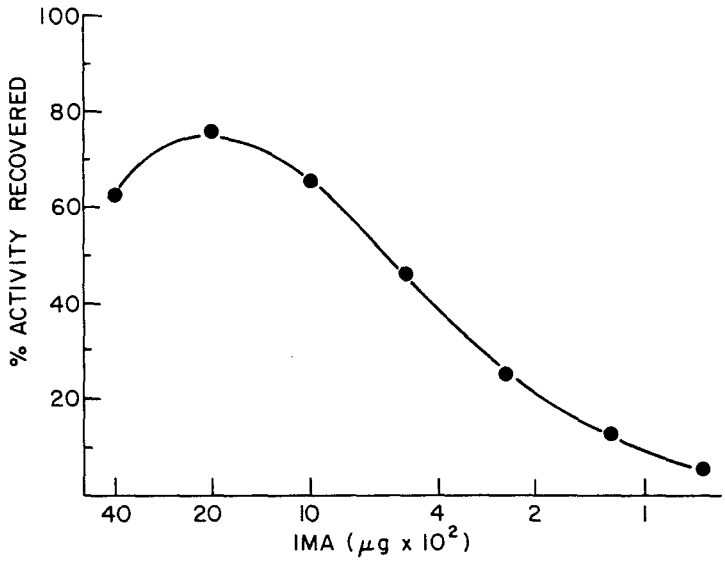

FIG. 1. Titration curve of immobilized antibody against a constant amount of lysate. Serial dilutions of IMA were incubated with 29 milliunits of enzyme activity in a total volume of 0.2 ml at 23°C for 30 min. After a wash step, 3.0 ml of substrate were added and the absorbance at 340 nm read after a 60-min incubation period at 23°C.

shows the results of three experiments in which the calculated amount of enzyme activity adsorbed is compared with the activity recovered. Of the calculated enzyme activity adsorbed 70 to 100% can be recovered when substrate is added to the IMA-E complexes. The inability to recover all the calculated adsorbed activity suggests some antibody coupling to catalytic site(s) or an effect on enzyme kinetics due to immobilization.

TABLE 1. **Adsorption and Recovery of Enzyme Activity by IMA**[a]

Experiment	Activity added	Activity in supernatant	Calculated adsorbed[b]	Activity recovered on IMA	% Calculated activity recovered
1	0.67	0.34	0.33	0.23	70
2	1.06	0.63	0.43	0.43	100
3	0.70	0.36	0.34	0.34	100

[a] Absorbance at 340 nm.
[b] The calculated adsorbed activity represents the difference between the activity added and the activity recovered in the supernatant fraction.

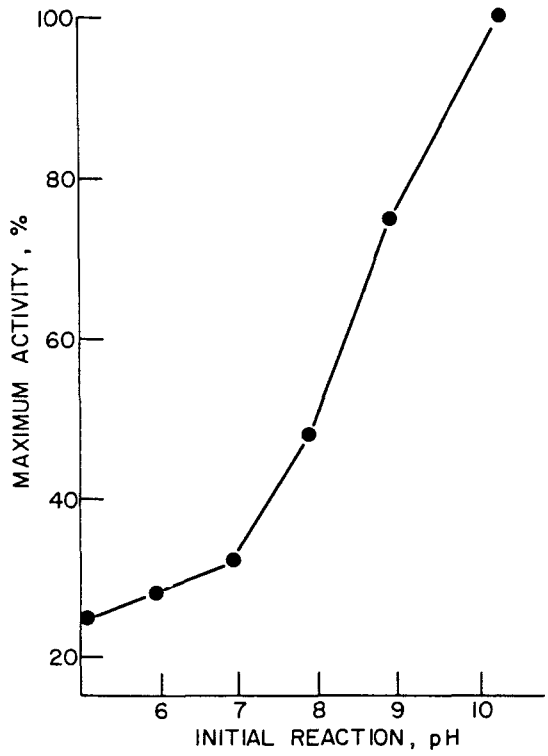

FIG. 2. Effect of pH on IMA-E activity.

Characterization of the IMA-E Complex

The characteristics of the immobilized enzyme were examined using IMA saturated with enzyme. A typical preparation of IMA-E complexes contained 10 to 20 milliunits of enzyme per milligram of glass. Figure 2 shows the effect of pH on IMA-E activity. Activity was best at pH 10. This was also the pH optima of the free enzyme (1). However, since antibody–antigen complexes dissociated at this pH, subsequent studies were performed at pH 9.0 where 75% of the maximum activity was found. Figure 3 shows a graphical determination of the K_m for the IMA-E complex. The apparent K_m for 1,2-propanediol was 40 mM; for NAD^+, 0.71 mM. The apparent K_m values for the free enzyme were 17 mM for propanediol and 0.37 mM for NAD^+ (1).

Table 2 shows the effect of heat treatment on the IMA-E complex. About 65% of the activity was lost after treatment at 70°C for 2 min, and all activity was lost after treatment at 95°C. Table 3 shows the results of

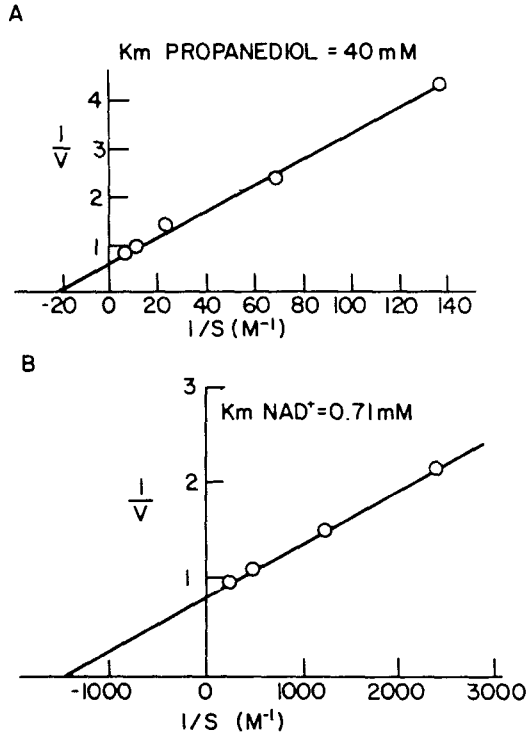

FIG. 3. K_m results for the IMA-E complex using propanediol (A) and NAD^+ (B).

chemical stability studies on the IMA-E complex. Greater than 70 % of the activity was lost after exposure to 0.1 N acid or 0.1 N base, 6 M guanidine hydrochloride, or a 2% solution of parachloromercuribenzoic acid, a dehydrogenase inhibitor. Enzyme activity was completely inhibited by goat antiserum but was not affected by equal amounts of normal goat serum. This result suggests that excess antibody can totally inhibit the enzymatic reaction

TABLE 2. **Effect of Heat Treatment on IMA-E Activity**

Temperature (°C)	Control Activity (%)
4 (control)	100
23	103
37	100
50	105
70	36
95	6

TABLE 3. **Effect of Chemical Agents and Antiserum on IMA-E Activity**

Treatment	% Control activity
PBS (control)	100
0.1 N HCl	5
0.1 N NaOH	5
95% ethanol	43
6 M guanidine hydrochloride	26
1% 2-mercaptoethanol	100
10 mg protease (Type III, sigma)	99
2% parachloromercuribenzoic acid	27
10% sodium dodecyl sulfate	58
Chlorox	54
Normal goat serum (0.1 ml)	105
Goat antiserum (0.1 ml)	0
Goat antiserum (0.01 ml)	6
Goat antiserum (0.001 ml)	47

and that immobilization does not substantially affect the antigenic determinants of the enzyme. Activity was partially destroyed by sodium dodecylsulfate solution, ethanol, and chlorox but not affected by protease or 2-mercaptoethanol, a strong reducing agent. The inability of protease to affect the reaction was probably due to the short incubation period used and the large amount of nonenzyme protein present on the glass.

Figure 4 shows the effect of time on IMA-E stability at four different temperatures. The half-life of the IMA-E complex was less than 1 day at

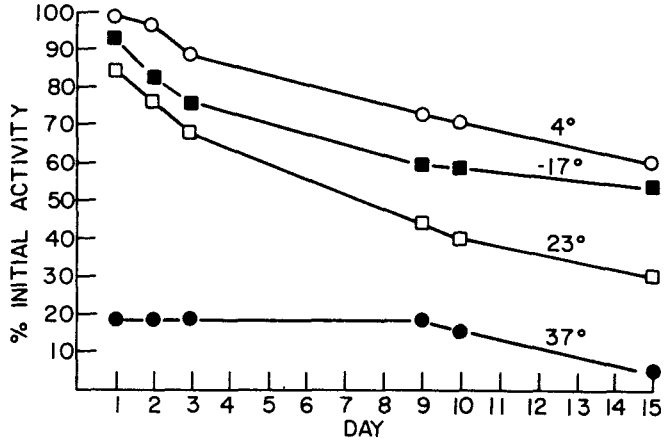

FIG. 4. Age stability of the IMA-E complex at different temperatures.

TABLE 4. **Detection of IMA-E Activity by Spectrophotometer, Fluorometer, and Visible Fluorescence Under UV Illumination**

Milliunits added	Activity recovered		
	340 nm	RFU[a]	Visual
50	1.7	>100	3+
5	<0.45	>100	3+
0.5	<0.02	>100	3+
0.1	<0.02	80	2+
0.05	<0.02	25	1+
0.02	<0.02	14	1
0.01	<0.02	8	0

[a]RFU-relative fluorometer units, neutral density filter, sensitivity ×3.

37°C, 8 days at 23°C, and greater than 15 days at 4°C or −17°C. In data not shown, the half-life of the free enzyme was less than 1 day at 37°C, 11 days at 23°C, and greater than 15 days at 4°C or −17°C. When the slopes of the stability curves at 4°C and −17°C for the free and adsorbed enzyme were compared, the free enzyme was more stable.

The IMA-E Detection System

When IMA is used as an isolation agent for an enzyme in bacterial lysates, the sensitivity of the assay depends on the detection method used. Table 4 compares the activity of the same samples by three detection methods. Detection of NADH generation by visual observation under UV illumination is almost as sensitive as the fluorometer which is 25 times more sensitive than the spectrophotometer. Table 5 shows the specificity of the IMA-E system. When lysates from organisms having similar propanediol activities were adsorbed with equal amounts of IMA, only the *N. gonorrhoeae* lysate was detected. This experiment demonstrates the ability of IMA to differentiate between enzymes that have the same substrate activity.

TABLE 5. **Specificity of IMA Adsorption of Propanediol Active Lysates**

Lysate	Relative fluorometer units	
	Added	Recovered
N. gonorrhoeae	46.5	30
S. epidermidis	38	2.8
E. cloacae	33	2.1
E. coli	48	2.8

DISCUSSION

This report investigates the possibility of using an immobilized antibody to differentiate between antigenically different enzymes with similar substrate activities. For an IMA-assisted detection system to work, the adsorbed enzyme must not be inhibited by the immobilized antibody. Since neutralization of propanediol dehydrogenase occurs in antibody excess (2), it is probable that an adsorbed enzyme would be active since immobilization probably occurs through only a few sites. In our system, the inability to recover 100% of the calculated adsorbed activity all the time indicates that some of the adsorbed enzyme might be attached via the catalytic sites(s). An alternate explanation is that the immobilization procedure has affected the enzyme's reaction kinetics. This is partially supported by higher K_m values for the adsorbed enzyme which is possibly related to diffusion effects on reaction velocity. Overall, however, when properties of the free and adsorbed enzymes are compared (Table 6), they appear to be similar. These results demonstrate the feasibility of using immobilized antibody in the detection of propanediol dehydrogenase.

The specificity of an IMA-assisted assay depends on the antiserum immobilized. In this study, the propanediol dehydrogenase like activity of extracts from *Staphylococcus epidermidis*, *Enterobacter cloacae*, and *Escherichia coli* were not adsorbed when mixed with IMA. The substrate activity in these extracts was probably due to glycerol dehydrogenase which also uses 1,2-propanediol as a substrate. An IMA-assisted detection system might also differentiate between isoenzymes if a suitably adsorbed antibody is used in the immobilization procedure.

An obvious advantage of IMA-assisted assays is the transfer of the target enzyme to the solid phase. This allows the enzyme to be separated from the sample melieu by a wash step and permits the reaction to be carried out in a partially purified state under optimal substrate conditions. We found

TABLE 6. **Comparison of the Properties of Free and Antibody Immobilized (IMA-E) Propanediol Dehydrogenase**

Characteristic	Free enzyme[a]	IMA-E
K_m apparent NAD^+	0.37 mM (1)	0.71 mM
K_m apparent propanediol	17 mM (1)	40 mM
Half-life at 23°C	11 days	8 days
pH optimum	10 (1)	10
Inhibition by excess antibody	complete (2)	complete

[a]The number in parenthesis indicates the reference from which the data was obtained.

this aspect quite important in the *N. gonorrhoeae* detection system where the test samples were cervical swabs which often contain tissue debris.

IMA-assisted assays might find particular use in systems like *N. gonorrhoeae* where a qualitative "yes" or "no" answer is sufficient and where enzyme activity can be detected by the formation of a colored reaction product. Dehydrogenase enzyme systems are good candidates since the generation of NADH can be detected visually by UV illumination or by coupling the system to a reducible tetrazolium dye.

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