CHARACTERIZATION OF GLUCOSE DEHYDROGENASE IMMOBILIZED ON ACRYLIC BEADS

James P. Eberhardt
Great Lakes College Association / Energy Research Undergraduate Laboratory Fellowship Program
Albion College
Oak Ridge National Laboratory
Oak Ridge, Tennessee 37831-2008

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Participant: _____
Signature

Research Adviser: _____
Signature

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Abstract

If a fuel cell can be constructed that uses hydrogen produced by an enzymatic pathway that oxidizes carbohydrates, its use as an alternative fuel is possible. Glucose dehydrogenase (GDH) oxidizes glucose to gluconic acid. The reducing equivalents generated are used by hydrogenase to generate molecular hydrogen. In order to construct an enzymatic fuel cell, these enzymes need to be immobilized. Different polyacrylamide materials with azlactone functionality were provided by the 3M Corporation and used in this investigation to immobilize GDH from *Thermosplasma acidophilum*. It was found that the properties of the polymer backbone effected the yield of active immobilized enzyme. The best yields of 50+% were achieved when the coupling reaction was carried out in the presence of a competitive binding reagent (BSA) on the 60:10:30 beads (10% vinyl-dimethyl azlactone). The use of different quenching solutions, which change the microenvironment of the immobilized GDH, altered the optimal pH of the immobilized enzyme. However, the immobilized enzyme did lose activity over time. The majority of activity was lost after 65h in all conditions tested to date with the 60:10:30 beads. This reduction in activity is most likely due to continued amide bond formation between the azlactone bead and the GDH rather than the GDH being released from the bead. Another sample of beads (55:5:40) containing 5% vinyl-dimethyl azlactone was tested. The preliminary data indicate that the yields were comparable with the 60:10:30 beads and the immobilized enzyme is stable.

Research Category
GLCA / ERULF: Chemistry Biology

School Author Attends: Albion College
DOE National Laboratory Attended: Oak Ridge National Laboratory
Mentor's Name: Jonathan Woodward
Phone: (865) 574-6826
E-mail: oop@ornl.gov

Author's Name: James P Eberhardt
Mailing Address: 1726 Oakwood
City/State/ZIP: Trenton, MI 48183
Phone: (734) 675-5035
E-mail Address: jeberhardt@albion.edu

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Introduction

Currently, fossil fuels are the world's primary source of energy. The US alone uses over 114 billion gallons of gasoline per year (1). With mounting environmental issues such as the global warming and other forms of pollution, and because only a limited amount of fossil fuels remain, there is a tremendous need for a renewable and environmentally friendly energy source. The answer to this energy dilemma could be the most abundant element on earth, hydrogen. Hydrogen can be used to fuel conventional combustion engines and can also be used in a fuel cell to generate electricity to power anything electric, from household appliances to vehicles. This could all be done with negligible pollution; the only emission from a hydrogen fueled system, either combustion or a fuel cell, is water. Though the benefits of hydrogen power are great, it is not being used today because of the lack of a hydrogen infrastructure. This creates a sort of "chicken and egg" dilemma. Companies are reluctant to build machines that use hydrogen power if no infrastructure to supply the hydrogen exists; likewise, companies are reluctant to build a hydrogen infrastructure if nothing exists to use the hydrogen. These two systems will have to expand and grow together for hydrogen to be a major fuel source.

Currently, hydrogen production is still dependent on fossil fuels. Most commercial hydrogen available today is produced at large scale steam methane reformer plants as this is the most cost efficient method of hydrogen production due to the low cost of methane (2). This method involves heating methane to 200°C, producing a mixture of H_2, CO, and CO_2. Through a series of catalyzed steps, this method produces a gaseous mixture of 70% H_2, 24% CO_2, 6% N_2 and trace amounts of CO (3). With hydrogen

production methods still dependent on fossil fuels, the hydrogen energy system would still have an environmental impact. Additionally, fossil fuels deposits are not equally distributed over the earth; the major reserves lie in a few discrete regions. Countries in which these reserves are not located are dependent on imports for their energy needs. The US alone imported an average of 8 million barrels of crude oil each day in 1997. This vast amount of imported oil was the main contributing factor of our 53.1% dependency on imports that year (1). This ever-increasing dependency on foreign nations can easily lead to economic instability. If a reliable method of hydrogen production with no environmental impact can be found, a truly zero emissions energy source would be possible and would lead to an indefinitely sustainable energy supply independent of foreign nations.

Hydrogen can easily be used as a safe fuel for combustion engines. It can be stored as either a gas or a liquid and provides much more power than conventional fossil fuels. A four-cylinder, 2.9L Ford Ranger converted to hydrogen fuel achieves 40% more power at a lower rpm than its conventionally fueled counterpart. It achieves this power increase even with an engine specifically designed to run on gasoline, not hydrogen. Additionally, the hydrogen vehicle has no carbon emissions and at its maximum power level, it shows less than a 100 ppm NO_x. Hydrogen fuel also provides an additional safety component when compared to gasoline. If the vehicle happens to be involved in a collision that liberated the stored hydrogen, the hydrogen would most likely burn harmlessly while rising from the vehicle. The only draw back of hydrogen fueled vehicles seems to be their lack of range. The hydrogen fueled Ford Rangers have a range of about 140 miles (4) and a converted Ford Taurus is estimated to have a range of about

290 miles (2). However, studies indicate that a combustion vehicle specifically designed for hydrogen fuel would have a range comparable to that of a standard gasoline vehicle. The range has to potential of being much greater than traditionally powered vehicles if the hydrogen was used to power a fuel cell instead of a combustion engine (2).

A fuel cell is an electrochemical device that converts the chemical energy into electrical energy by oxidizing a fuel. This electrical energy is then converted to mechanical energy to power the vehicle. In the situation described here, hydrogen is the fuel and oxygen the oxidant. A fuel cell has a cathode side and an anode side separated by an ion permeable membrane. The hydrogen flows into the anode side of the fuel cell where a catalyst facilitates the separation of hydrogen into a proton and two electrons. The proton is able to travel through the membrane where the catalyst again facilitates the formation of a water molecule using external oxygen. Unable to pass through the membrane, the electrons travel from the anode to the cathode via an external circuit bearing the electrical load. Since a fuel cell is not a mechanical process, its efficiency is not limited by the thermodynamics of the Carnot Cycle, as is the case with a combustion engine. A combustion engine uses a chemical reaction to produce mechanical energy; however, a large amount of heat is also produced in this reaction. The combustion engine cannot convert all of this heat into mechanical energy; it must transfer some of the energy to a lower temperature heat sink. Therefore, the efficiency of a combustion engine is limited by the temperature difference of the heat sinks. A fuel cell does not use heat to produce electrical energy; therefore, it can exceed the efficiency of a combustion engine (3). Fuel cells can be used in every place an electrical current is needed without the weight or recharge time a battery requires; whenever fuel is present, a fuel cell will

produce electricity. The key to successful fuel cell implementation is a sustainable source of hydrogen.

The use of carbohydrates as a source of hydrogen is a vast untapped fuel source. Hydrogen can be produced from carbohydrates via a novel enzymatic pathway by alternately reducing and oxidizing nicotinamide adenine dinucleotide phosphate ($NADP^+$). The enzyme glucose dehydrogenase reduces $NADP^+$ to NADPH and hydrogenase oxidizes NADPH to $NADP^+$ and produces molecular hydrogen (5). The complete pathway oxidizes sucrose to glucono-δ-lactone which spontaneously hydrolyzes to into gluconic acid. This system sets up a cycle continually oxidizing and reducing $NADP^+$, leaving molecular hydrogen and gluconic acid as the final products of the sucrose oxidation. The entire pathway is shown figure 1.

In order for enzymatic hydrogen production to be efficient, the enzymes must continually be in contact with their substrates. The efficiency of an enzymatic system can increase if the enzyme is continuously reused. The optimal method of enzyme reuse would be to find a reliable method of separating the enzyme from the reaction solution and products. One method of enzyme substrate separation is to immobilize the enzyme. This involves forming bonds between groups on the enzyme and the support, covalently attaching the enzyme to the support. The support is easily kept separate from the substrate solution. The substrate solution is then passed over the support and converted to product. Though a good technique, enzyme immobilization has its drawbacks.

The most destructive of these drawbacks is that if too many physical links are formed between the enzyme and the support, the conformation of the enzyme could change, causing it to lose activity. Immobilization also inherently changes the

microenvironment of the enzyme. This change can cause an increase or a decrease in enzymatic stability depending on the microenvironment provided. Other enzymatic properties, such as the optimal reaction conditions and kinetics, are also subject to change upon immobilization. To help prevent a loss of enzymatic function with immobilization, substrates can be used to protect the active sites of the enzyme during cross-linking. To prevent too many links between the enzyme and the support from forming, a competitive binding agent can be introduced. The competitive binding agent will also forms links with the support, providing less available binding sites for the enzyme, helping to prevent conformational changes due to multiple linkage sites with the enzyme.

Several polyacrylamide supports with azlactone functionality were used for the immobilization of glucose dehydrogenase (GDH) isolated from *Thermosplasma acidophilum*. The azlactone group on the support can form an amide linkage with the lysine groups of the GDH. This amide linkage attaches the enzyme to the polyacrylamide support, effectively immobilizing the enzyme (figure 2). Optimally, this immobilized enzyme will retain its activity and be easily be separated from the substrates and products.

This research focuses on the immobilization of GDH, a vital step in the development and implementation of the enzymatic hydrogen production. Other studies have successfully immobilized other enzymes in this pathway. Upon successful immobilization of GDH, the construction of an enzymatic reactor capable of producing hydrogen from biomass is one step closer to being realized.

Methods

Previous studies of the immobilization of glucose dehydrogenase (GDH) suggest that the 60:10:30 (10% vinyl-dimethyl azlactone) acrylic beads, provided by the 3M corporation, give the highest yield of active immobilized enzyme. These beads were used in all immobilizations unless otherwise indicated. *Thermosplasma acidophilum* GDH was purchased from Aldrich chemical company**.** The enzyme was washed several times with 50mM sodium phosphate buffer, pH 6.5, using a Centricon YM – 10 concentrator with a 10,000 MW cut off. The volume of supernatant recovered each time was used to calculate the salt concentration and washing was continued until the salt concentration was less than 40 nM. The desalted enzyme was stored at 4°C.

Enzyme activity is measured in units (U). In this case, one unit of enzyme is defined as the amount of enzyme required to produce 1 µmol NADPH per min at 45°C. Approximently one unit of GDH was used for each immobilization. Original units were based on a continuous assay of the desalted stock GDH solution. The immobilization procedure was carried out in a volume of 0.5 ml. Approximently one unit of GDH, 10 µl $NADP^+$, 25µl BSA, 25µl 0.5M glucose, 430µl 0.1M sodium phosphate / 0.6M sodium citrate /0.1% triton x100 at pH 7.5, and 10 mg of support were mixed by rotation for 2.5 hours at 25°C. The beads were then separated from the solution using a Millipore Ultrafree-MC Centrifugal Filter Unit and washed two times with 50mM sodium phosphate buffer at pH 6.5. The beads were then quenched for various times at using an amine-containing buffer at pH 8.0. The squench reaction was then stopped, washed twice more, and stored in 0.1M Tris pH 8.0. The same immobilization procedure was used for all trials and is described in figure 3.

The activity of the enzyme was measured by monitoring the formation of NADPH using a UV/vis spectrophotometer at 340 nm. An aliquot of enzyme containing solution, is combined with 0.5M glucose, 50mM $NADP^+$, and 50mM sodium phosphate buffer at the desired pH in a 1 ml assay at 45°C in a microassay cuvet. The reaction is started with the addition of $NADP^+$, inverted to mix, and the absorbance is measured continuously for one minute. The initial rate of the reaction is calculated, allowing for a determination of the number of units per volume of solution.

Conducting a continuous assay on the immobilized enzyme is impossible because the beads suspended in the liquid interfere with the light path. A fixed time assay was used for determining the activity of immobilized enzyme. An aliquot of immobilized enzyme containing solution, usually about 50µl, is combined with 0.5M glucose, 50mM $NADP^+$, and 50mM sodium phosphate buffer at the desired pH in a 1 ml assay. The reaction is placed in a 45°C water bath for a period of ten minutes. The reaction was stopped by separating the beads from the reaction solution using a Millipore Ultrafree-MC Centrifugal Filter Unit. The absorbance at 340 nm of a 5x dilution of the reaction solution was found, allowing for a calculation of the number of units.

To characterize and increase the yield of active immobilized enzyme, certain parameters of the immobilization procedure were varied. Five different quenching solutions 1.0M Glycine, 1.0M Tris, 1.0M Ethanol Amine, 3.0M Ethanol Amine, 1.0M Tris / 1.0M Glycine were tried. The storage temperature and the pH of various solutions, were also varied in an attempt to increase the yield of immobilized enzyme. For the 55:5:40 bead characterization, two-minute reaction times at 45°C were used. The only

quenching solution used with the 55:5:40 beads was 1.0M Tris / 1.0M Glycine / 0.1% Triton X100.

Results and Discussion

Enzyme Immobilization

Polyacrylamide materials with azlactone functionality provided by the 3M Corporation were used in this investigation to immobilize GDH from *Thermosplasma acidophilum*. These support materials varied in their azlactone loading as well as in the ratio of the two acrylamide based monomers. The properties of the polymer backbone affected the yield of active immobilized enzyme and the addition of a competitive binding reagent, such as bovine serum albumin (BSA) increased the yield of active immobilized enzyme. The best yields of active immobilized GDH were achieved when the coupling reaction was carried out in the presence of BSA on the 60:10:30 beads (10% vinyl-dimethyl azlactone). Yields of active immobilized GDH in excess of 50% were demonstrated as shown in figure 4. A variation in the yield of active immobilized GDH was noted.

Characterization of GDH immobilized on 60:10:30 Beads

Enzymatic stability over a range of pH values was assessed for immobilized GDH. Aliquots of GDH immobilized on 60:10:30 beads, using 1.0M Tris / 1.0M Glycine for the quenching solution, were incubated for a period of 18 hours in buffers covering a range of pH values. All were then assayed for activity at pH 6.5. Two of each of these trials was carried out, one at 25°C and the other at 55°C. The enzyme was stable over this time period at both 25°C and 55°C as seen in figure 5. However, when a stability

study was carried out storing the immobilized GDH over a range of temperatures for 65h, the enzyme was not stable as shown in figure 6. In this study all of the samples were assayed for activity at pH 6.5. 50mM sodium phosphate was used for the pH 6.5 buffer and 0.1M Tris was used for the pH 8.0 buffer. All of these activities are compared to the activity of the original enzyme assayed under the same conditions. The instability of the immobilized enzyme, even at low temperatures, can most likely be attributed to the continued binding of the GDH to the beads through still active azlactone sites.

Quenching

The purpose of the quench reaction is to react with the excess azlactone groups, blocking them from forming additional amide linkages with the protein after coupling. Different amine-containing quenching solutions were compared. The pH activity optimum of the immobilized enzyme was investigated using 1.0M Glycine, 1.0M Tris, 1.0M Ethanol Amine, 3.0M Ethanol Amine, 1.0M Tris / 1.0M Glycine as the quenching solutions. Quenching reactions were all conducted at pH 8.0. Aliquots of immobilized GDH were quenched with the various solutions. They were then assayed for activity at pH 6.5 and stored in 0.1M Tris pH 8.0. After a period of 24 hours they were again tested for activity. 1.0M Tris provided the optimal retention of activity after 24 hours as shown in figure 7. The optimal pH for the native enzyme is 6.5; however, the results indicate that the pH optimum of the immobilized enzyme varies depending on the quenching solution used (figure 8). This reflects a change in the microenvironment of the enzyme due to the introduction of ionizing groups from the reaction between the excess azlactone groups and the quenching solution. The ability to control the optimal pH of the enzyme

could prove to be a beneficial asset when using the immobilized GDH in a system with other enzymes, each with a different optimal pH.

Effectiveness of the Quenching Reaction

Only BSA was used in these trials since the binding capacity of the beads was the only parameter in question. All of the azlactone bead samples provided by the 3M Corporation at that time were evaluated in an attempt to find the best bead for enzyme immobilization. The optimal bead would not necessarily have the highest yield of active immobilized enzyme, but would have a high yield of immobilized enzyme along with a low recoupling capacity after the excess azlactone sites had been quenched, theoretically increasing the stability of the immobilized enzyme. It was thought that the structural polymer of the bead might be expanding and contracting with changing temperature, exposing more azlactone sites at higher temperatures. These sites would be active and able to bind to the GDH causing deactivation. It was thought that coupling initially at 25°C would allow the normal high yield of coupling to take place, and then by quenching at 50°C, the polymer would be allowed to expand, exposing the azlactone groups hidden at 25°C and allowing them to be quenched. This would block all of the azlactone sites preventing the continued binding that leads to deactivation of the immobilized enzyme.

To assess the effectiveness of the quenching solution, BSA was reintroduced to the beads after the quench in an attempt to couple additional protein to the bead. The temperature was kept at 50°C in order to evaluate the effectiveness of the quenching solution without the additional parameter of hiding azlactone sites within the shrinking polymer. The results of this study show that the quenching reaction does not effectively prevent coupling as shown in figure 9. The commercial beads and the 30:10:60 beads

have low recoupling capacity along with a high yield of immobilized protein; however, these beads do not bind GDH effectively. Quenching seems to have little effect on the binding capacity of other beads.

Characterization of GDH immobilized on 55:5:40 Beads

A 55:5:40 support was also test for its ability to bind GDH. These beads have given good active immobilized enzyme and stability results, significantly better than the 60:10:30 samples. The coupling reaction was carried out using the same procedure that was used with the 60:10:30 beads. Activity assays were carried out for 2 minutes in 50mM sodium phosphate pH 6.5 buffer at 45°C. The yields and retention of active immobilized enzyme are shown in figure 10. These results show that the immobilized enzyme is stable enough to be considered useful in an enzymatic pathway. A study fully characterizing the immobilized GDH has begun.

The native enzyme's K_m glucose value is 10.0mM (7). The K_m glucose value of the immobilized GDH was determined by varying the concentration of glucose while leaving the concentration of $NADP^+$ constant. A Lineweaver-Burk plot was constructed and the K_m glucose value was determined to be 7.58mM glucose as shown in figure 11. A shift in K_m is expected when immobilizing enzymes due to the conformational changes that arise from amide bond formation between the side groups of the protein and the support.

Other Immobilization Techniques

An attempt was made to immobilize the GDH in a sol-gel. The enzyme is introduced into the prepared sol-gel, which quickly polymerizes around the enzyme (6). This method of immobilization produced only 15% yield with the GDH alone and 5%

with the GDH already immobilized on 60:30:10 beads. This immobilization technique is not useful for GDH immobilization.

Stabilizing the immobilized enzyme with glutaraldehyde was also attempted. Glutaraldehyde forms a polymer at pH values above 3. It was thought that this polymer might stabilize the enzyme by forming a polymer around it. This increase in stability could help to retain activity. This was not the case. GDH was immobilized as usual, with a 21.68% yield. The introduction of glutaraldehyde to the system for only one hour caused the activity to fall to 0.8%. This technique is also not a viable method of preserving the activity of GDH.

Conclusion

Maintaining the activity of GDH immobilized on azlactone beads seems to be a function of the ratio of azlactone to the two polymers, not just the azlactone content alone. The 55:5:40 beads have a ratio of azlactone to the two polymers that allows for GDH binding to take place at a level which allows for the continued stability of the enzyme. Immobilization does alter the characteristics of the GDH, presumably by changing the microenvironment of the enzyme. Storage of GDH immobilized on 55:5:40 beads in alkaline pH at room temperature is sufficient to retain its activity.

Acknowledgements

I would like to thank Hugh O'Neill and Jonathan Woodward for inviting me to be a part of their lab and for all of their support, guidance and endless patience with me. I would also like the thank the Great Lakes College Association and the Department of Energy's, Energy Research Undergraduate Laboratory Fellowship for making my participation in this research possible and the Nation Science Foundation for organizing the program.

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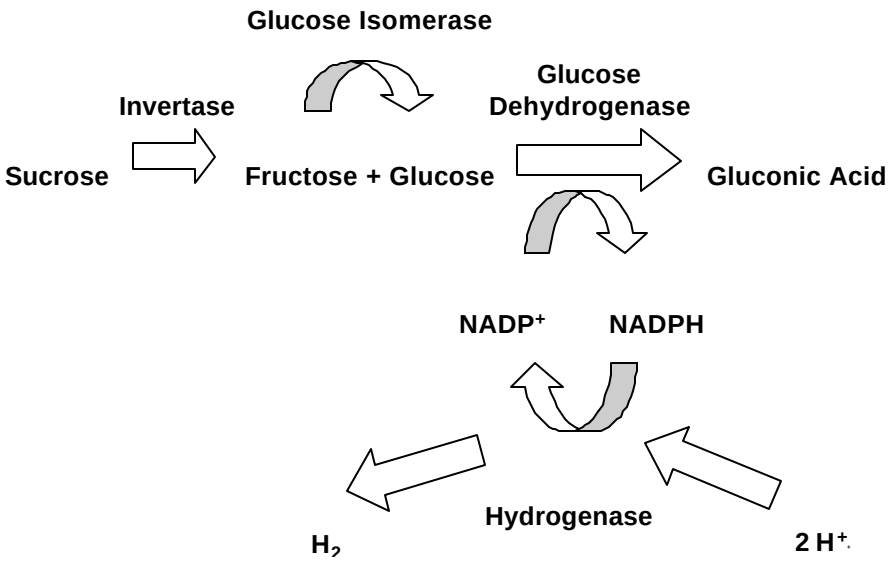
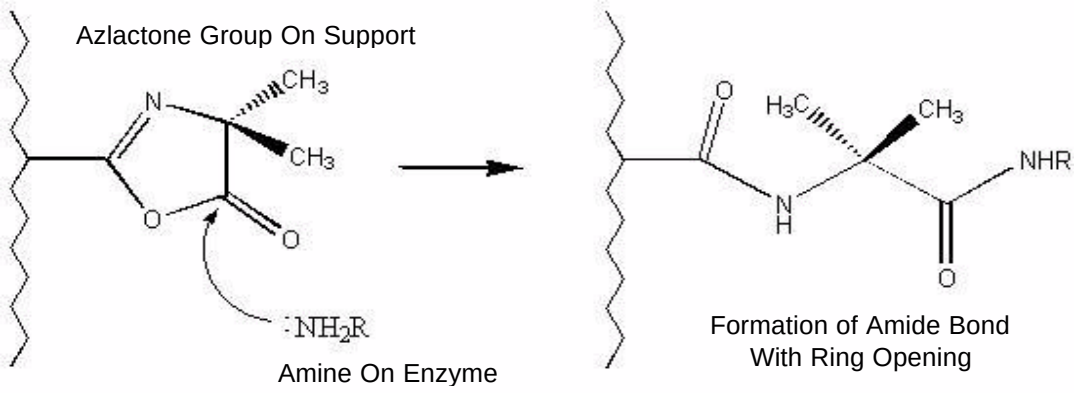
Figure 1



Pathway for enzymatic hydrogen production from sucrose.

Figure 2



Azlactone Coupling Reaction

Figure 3

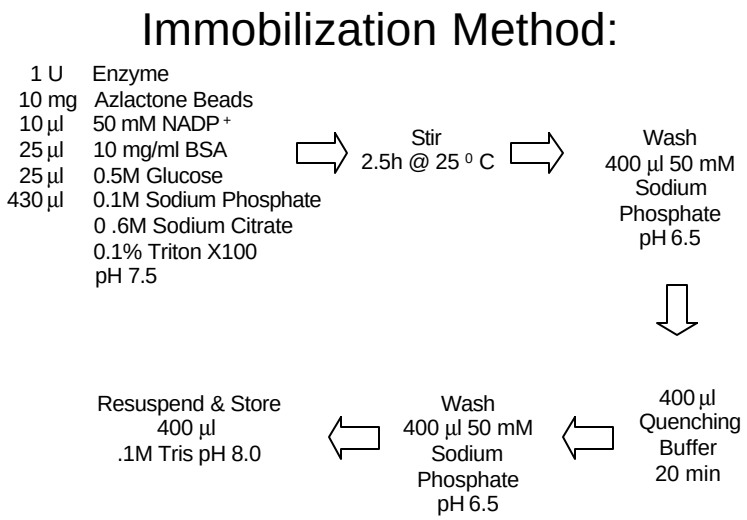


Figure 4

Date	Immobilized Activity	Highest % Yield in Experimental Conditions	
8/31/00	0.20	23.6	
9/1/00	0.48	56.6	
9/5/00	0.31	36.2	
9/7/00	0.32	49.4	
9/8/00	0.63	62.7	*
9/12/00	0.38	38	*
9/13/00	0.36	35.8	*
9/14/00	0.26	26	*
9/15/00	0.30	30	*
9/15/00	0.31	31.2	*
9/19/00	0.16	15.9	*
9/19/00	0.34	34.4	*
9/19/00	0.26	26	*
9/19/00	0.25	24.9	*
9/25/00	0.56	55.7	*
9/27/00	0.06	30.3	*
10/2/00	0.27	40.5	*

* stock assayed at r.t., immobilized assayed at 45
60:10:30 beads

Figure 5

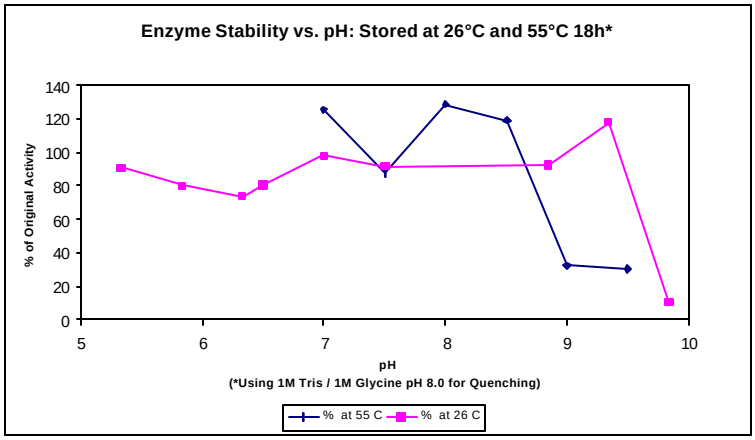


Figure 6

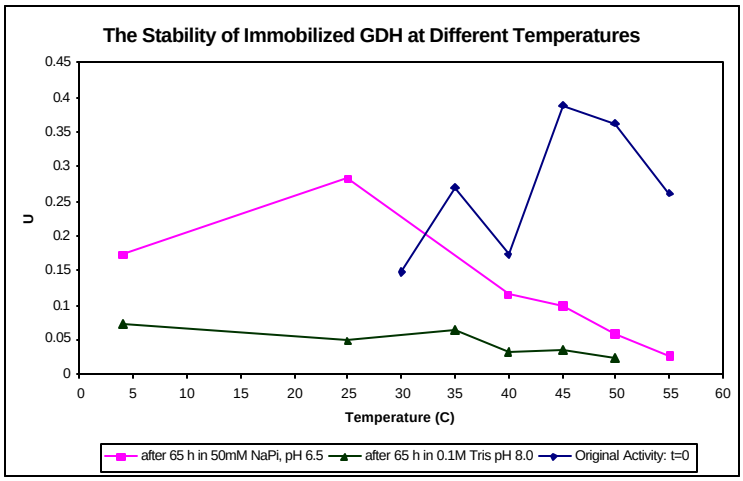


Figure 7

Quench	Ave % Yield	% Activity Retained (24h)
1.0M Glycine	20.23	72.3
1.0M Tris	28.94	99.7
1.0M Ethanol Amine	22.78	90.7
1.0M Tris / 1.0M Glycine	22.52	96.9

Figure 8

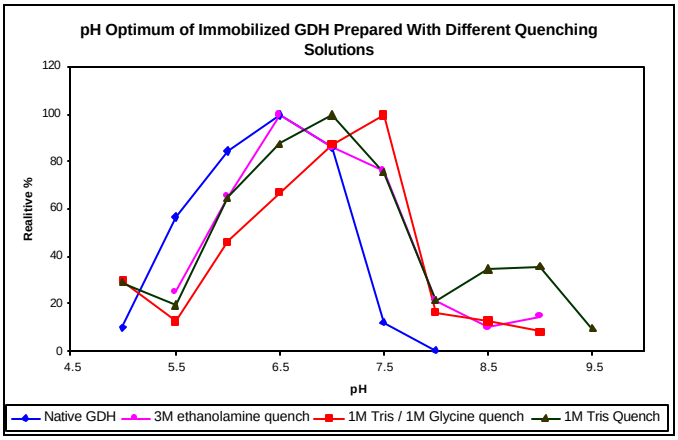


Figure 9

Trial 1 *0.1% Triton for 20 min*

	60:10:30	80:10:10	60:20:20	80:20:0	70:20:10	10:10:80	30:10:60	Commercial
mg Beads	10	10	10	10	10	10	10	10
mg/ml BSA	1	1	1	1	1	1	1	1
Coupling temp	25	25	25	25	25	25	25	25
Coupling time	2 h	2 h	2 h	2 h	2 h	2 h	2 h	2 h
ABS	0.05	0.02	0.19	0.09	0.31	0.37	0.34	0.37
mg/ml left	0.04	0.02	0.18	0.08	0.30	0.35	0.33	0.35
mg/ml coupled	0.96	0.98	0.82	0.92	0.70	0.65	0.67	0.65
% Coupled	95.73	98.14	81.84	92.05	70.33	64.69	67.30	64.74
Quench Solu.	*1.0M Tris pH 9.0 used in all reactions*							
Quench temp	50	50	50	50	50	50	50	50
Quench Time	16 h	16 h	16 h	16 h	16 h	16 h	16 h	16 h
Recouple mg/ml	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Recouple temp	50	50	50	50	50	50	50	50
ABS	0.07	0.08	0.11	0.01	0.02	0.22	0.28	0.31
mg/ml left	0.06	0.08	0.10	0.01	0.01	0.21	0.26	0.30
mg/ml coupled	0.24	0.22	0.20	0.29	0.29	0.09	0.04	0.00
% Recoupled	78.42	74.17	67.08	97.75	95.81	29.50	12.43	1.38

Trial 2 *1.0% Triton for 16 h*

	60:10:30	80:10:10	60:20:20	80:20:0	70:20:10	10:10:80	30:10:60	Commercial
mg Beads	10	10	10	10	10	10	10	10
mg/ml BSA	1	1	1	1	1	1	1	1
Coupling temp	25	25	25	25	25	25	25	25
Coupling time	1 h	1 h	1 h	1 h	1 h	1 h	1 h	1 h
ABS	0.36	0.41	0.39	0.31	0.34	0.40	0.38	0.38
mg/ml left	0.34	0.39	0.37	0.30	0.32	0.38	0.36	0.36
mg/ml coupled	0.66	0.61	0.63	0.70	0.68	0.62	0.64	0.64
% Coupled	65.68	61.07	62.99	70.02	67.66	61.87	64.01	63.65
Quench Solu.	*1.0M Tris pH 9.0 used in all reactions*							
Quench temp	50	50	50	50	50	50	50	50
Quench Time	1.5 h	1.5 h	1.5 h	1.5 h	1.5 h	1.5 h	1.5 h	1.5 h
Recouple mg/ml	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Recouple temp	25	25	25	25	25	25	25	25
ABS	0.12	0.06	0.15	0.08	0.06	0.23	0.17	0.26
mg/ml left	0.11	0.05	0.14	0.07	0.06	0.22	0.16	0.24
mg/ml coupled	0.19	0.25	0.16	0.23	0.24	0.08	0.14	0.06
% Recoupled	62.16	81.71	52.17	75.07	80.48	26.86	46.92	18.74

Figure 10

Coupled on 11/6/00

Date	Units	Yield	Retention
6-Nov	0.51	36.67	
7-Nov	0.33	23.86	65.6
8-Nov	0.59	42	177
13-Nov	0.50	35.6	84.1

Coupled on 11/7/00

Date	Units	Yield	Retention
7-Nov	0.51	37.7	
8-Nov	0.59	43.66	116
13-Nov	0.45	32.8	75.2

Figure 11