Free and Immobilized Glucose Isomerase from *Streptomyces phaeochromogenes*1

GERALD W. STRANDBERG AND KARL L. SMILEY

*Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604*

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Properties were determined of the glucose isomerase from *Streptomyces phaeochromogenes* NRRL B-3559. The enzyme exhibited a temperature optimum of 80°C and a pH optimum of about 8. The effect of various buffers on activity of the enzyme and the optimum pH were studied. Michaelis constants for glucose and Mg2+ were 0.25 and 0.025 mM, respectively. Co2+ enhanced enzyme activity. A functional polyacrylamide-entrapped glucose isomerase was prepared. The conditions for entrapment and use of the bound enzyme were examined.

The immobilization of enzymes on insoluble carriers is becoming increasingly important as evidenced by the number of reports of success in this area as well as the appearance of commercial insoluble enzymes and insolubilizing agents. One of the several immobilizing techniques employed is polyacrylamide gel entrapment (2, 3, 6, 8, 12). The enzyme is physically bound within the matrices of the gel network by forming the gel in the presence of the enzyme. Physical or chemical alteration of the enzyme (or both) appears to be much less with this treatment as compared to methods of insolubilization that employ chemical interaction between the enzyme and insoluble carrier (1, 2). One factor that must be considered with an enzyme immobilized by such a procedure is the size of the substrate molecule. The substrate should be small enough in size to penetrate the gel matrix and subsequently yield a product that can readily escape. Enzymes which have high-molecular-weight substrates (proteases, amylases) have been entrapped in polyacrylamide, but activity is limited to that portion of enzyme trapped at or near the surface of the gel (2).

Under the proper conditions the xylose isomerase (EC 5.3.1.5) from several organisms catalyzes the isomerization of D-glucose to D-fructose (11; all sugars referred to are of the D-configuration). This enzyme, herein referred to as glucose isomerase, requires a substrate of low molecular weight and is thus a suitable enzyme for immobilization in polyacrylamide gel.

This report describes the preparation and properties of the glucose isomerase from *Strepto-

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and 0.05 M MgCl₂·6H₂O in the buffer being used, normally 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride at pH 8.0. The enzyme reaction was carried out at 70 °C. At zero time and at desired intervals thereafter a sample of the reaction mixture was pipetted into 0.5 M HClO₄. Fructose was determined by an automated cysteine-sulfuric acid assay (4). One unit of enzyme produces 1 mg of fructose per hr under these conditions. Modifications of the enzyme assay are described wherever necessary.

Polyacrylamide gels were prepared by the procedure of Hicks and Updike (6). Acrylamide and N,N'-methylenebisacrylamide were dissolved in 8.0 ml of 0.05 M Tris, pH 8.0. To this solution were added 1.5 ml of enzyme in Tris buffer, 0.02 ml of 0.3% ammonium persulfate, and 0.02 ml of 0.3% aqueous riboflavin solution and the mixture was exposed to a Sylvania 230-w infrared lamp from a distance of 15 to 20 cm. The reaction vessel was kept in an ice bath during polymerization which took from 8 to 25 min depending upon the composition of the gel. The polymerized gel was passed through a stainless-steel sieve (24 1-mm holes/cm²) and washed by decantation with approximately 200 ml of buffer. The gel was stored at 4 °C until use. The gels were assayed for glucose isomerase activity by shaking them in a water bath (200 osc/min) at 70 °C in the glucose-MgCl₂·6H₂O-Tris mixture, at pH 8.0.

RESULTS AND DISCUSSION

Glucose isomerase is used industrially to produce high fructose corn syrups. This use plus the unusual properties of this enzyme prompted us to include glucose isomerase in the investigations of our laboratory on the insolubilization of enzymes of industrial significance. Japanese workers (11) have reported the properties of glucose isomerase from S. phaeochromogenes. As their strains were unavailable and the action of glucose isomerase somewhat novel, we felt it necessary to examine the enzyme from our culture to see if it was identical to that reported.

Seven strains of S. phaeochromogenes (NRRL B-1248, B-1266, B-1131, B-1517, B-2123, B-3010, B-3559) were inoculated into the xylose-glucose medium. Of the seven only strains B-1131, B-1517, and B-3559 grew sufficiently on this medium for further testing. These three strains possessed glucose isomerase activity. B-3559 had the highest activity in the initial assay and was chosen for detailed study.

The enzyme was not highly purified for our studies. Since glucose isomerase was essentially unretarded by Bio-Gel P150, it is probably a high-molecular-weight enzyme.

Glucose isomerase from B-3559 has optimal activity at 80 °C (Fig. 1). An Arrhenius plot of this data revealed an activation energy of about 25,000 cal/mole. This is not surprising as xylose, not glucose, is the natural substrate for this enzyme. For ease of handling, a temperature of 70 °C was used for routine assays since activity is near maximum at this temperature. The enzyme is very stable during extended heating. Fig. 2 shows that the enzyme loses about 40% of its activity after 24 hr at 70 °C in buffer (0.05 M Tris, pH 8.0) but in the absence of any substrate or reaction product. Experiments with other enzyme preparations demonstrated that the loss in activity was not always as great as 40% and that the stability of the enzyme apart from the reaction mixture was independent of protein concentration. Time course studies showed the rate of fructose forma-
tion to be nearly constant for 2 hr at 70 C (Fig. 3) but decreasing significantly after 3 hr in the presence of substrate and products. This was unexpected as equilibrium has been shown to be about 52% fructose with this enzymatic reaction (11). The conditions used in Fig. 3 (approximately 30 glucose isomerase units per g of glucose; standard reaction mixture) produced 6 to 7% fructose in 3 hr. However, 50 to 54% fructose was produced in about 3 hr (60 C) when enzyme levels were raised substantially (300 units per g of glucose). This effect was seen in both Tris and NH$_4$-NH$_4$Cl buffers. At lower enzyme levels substrate was not limiting, and it would be unlikely that fructose would be an inhibitor as the reaction goes well in either direction (11). High levels of enzyme must bring the reaction to equilibrium before inhibition of enzyme becomes significant.

Several experiments were done to determine the nature of the inhibition observed when low levels of enzyme are used. The addition of fresh glucose to a reaction mixture subjected to enzyme treatment for 24 hr did not promote renewed enzymatic activity. The addition of fresh enzyme to the same type of reaction mixture (enzyme treated for 24 hr) resulted in enzymatic activity but again the activity diminished rapidly. Enzyme added to a reaction mixture lacking enzyme (but heated for 24 hr at 70 C) showed the usual activity. These results suggested that the enzyme preparation may be producing something which inactivates it.

A polyacrylamide-entrapped enzyme was also tested under the same conditions described previously (the details of polyacrylamide entrapment of glucose isomerase are discussed later). Like the soluble enzyme, it was inactivated in a manner similar to that shown in Fig. 3. The polyacrylamide-enzyme complex was recovered, washed free of substrate and products, and retested for activity. It was found to be inactive. The mechanism of inactivation seems to be the same for both soluble and entrapped glucose isomerase and involves the enzyme and something resulting from its action on the reaction mixture.

The use of phosphate buffer at pH 8.0 was not feasible because a precipitate formed at the high reaction temperature. Activity in phosphate buffer was lower than in other buffers (Fig. 4) possibly owing to the depletion of Mg$^{2+}$ by conversion to insoluble magnesium salts. The optimum pH in phosphate appeared to be 7.0 but this is likely an artifact because at higher pH values available magnesium became limiting, thus

![Fig. 3. Effect of temperature on glucose isomerase activity.](image1)

![Fig. 4. Glucose isomerase activity in tris(hydroxymethyl)aminomethane (Tris) and potassium phosphate buffers.](image2)
causing a decrease in the rate of fructose formation. In Tris buffer from pH 7.5 to 9.0 the rate of fructose formation increases as the pH rises. Barbital buffer (not illustrated) showed an optimum pH range of 8.0 to 8.5. Tsumura and Sato (11) found a pH optimum of 9.3 to 9.5 using NH₂-NH₂Cl buffer, whereas Takasaki et al. (9) found 8.0 to 8.5 to be the pH optimum with the enzyme from S. albus (no buffer given). We found activity to be lower in NH₂-NH₂Cl buffer, pH 8.0, as compared to Tris. To further appraise the effect of buffer, five of Good's dipolar ionic buffers (Calbiochem, Los Angeles, Calif.) were tested covering a pH range of 6.0 to 9.5. A broad optimum between pH 7.5 and 8.5 was determined (Fig. 5). HEPES, TES, TRICINE, MOPS, and BICINE are Calbiochem's code names for these buffers.

An apparent Michaelis constant for glucose was determined in 0.05 M Tris, pH 8.0, at 70 °C (Fig. 6). A line of best fit (5) was calculated for a modified Lineweaver-Burk plot of the data. From the slope and intercept the velocity was calculated to be half maximal at 0.25 mM glucose.

Mg²⁺ was reportedly required for glucose isomerase activity (11). A Mg²⁺ requirement was found for the enzyme studied here. A Michaelis constant of 0.025 mM Mg²⁺ (Fig. 7) was determined in 0.8 mM glucose at 70 °C (0.05 M Tris, pH 8.0).

Although Co³⁺ is not required for either enzyme formation or activity, its presence stimulates both (10, 11). The data in Table 1 show that activity is stimulated about 30% at a concentration of $5 \times 10^{-4}$ mM Co³⁺. Even though cobalt alone did support some activity, it did not relieve the requirement for Mg²⁺.

The enzyme appears to be specific for xylose and glucose and was inactive with glucose-6-phosphate.

The glucose isomerase from S. phaeochromogenes NRRL B-3559 is thus quite similar to that from S. phaeochromogenes (11) and S. albus (9) with respect to temperature and pH optima, substrate specificity, metal requirements, and Michaelis constants for glucose and Mg²⁺.

Polycrylamide-entrapped enzymes have been generally used in columns (6, 8, 12). The compo-

![Fig. 5. Glucose isomerase activity in Good's dipolarionic buffers.](image)

![Fig. 6. Effect of glucose concentration on glucose isomerase activity.](image)

![Fig. 7. Effect of Mg²⁺ concentration on glucose isomerase activity.](image)

| Co³⁺ (mM) | Mg²⁺ (mM) | Specific activity |
|----------|-----------|------------------|
| 0        | 0         | 0.6              |
| 0        | 0.05      | 4.6              |
| 0.5 $\times 10^{-4}$ | 0.05 | 6.8 |
| 1.0 $\times 10^{-4}$ | 0.05 | 6.8 |
| 2.0 $\times 10^{-4}$ | 0.05 | 8.1 |
| 0.5 $\times 10^{-4}$ | 0 | 1.1 |
| 1.0 $\times 10^{-4}$ | 0 | 1.5 |
| 2.0 $\times 10^{-4}$ | 0 | 1.1 |
The entrapment of polyacrylamide gel is important because various ratios of acrylamide and \( N,N' \)-methylenebisacrylamide give gels of different properties. If a gel is too loose or too tight, its flow properties in a column will be undesirable. Hicks and Updike (6) reported on the entrapping capacity of different gel mixtures. They found a mixture of about 80\% acrylamide and 20\% \( N,N' \)-methylenebisacrylamide best for entrapping lactic dehydrogenase.

An assay based on the determined optimal conditions was used to analyze preparations of polyacrylamide-entrapped glucose isomerase (see Methods). The effect of the acrylamide-\( N,N' \)-methylenebisacrylamide ratio on enzyme entrapment and the effect of enzyme levels were examined.

It was not expected that all the enzyme trapped would be available physically to the substrate. The tightness of the gel network surrounding the enzyme could prevent the substrate from reaching the enzyme. We felt it necessary to infer the level of enzyme in the gel from residual enzyme in the wash. This was done by determining protein levels (7) in the solution from washing of the gel particles. The difference between the protein added to the polymerizing solution and that found in the gel rinses was considered entrapped in the gel lattice and therefore a measure of enzyme entrapment. The more direct method of measuring the isomerase present in the wash solution could not be used because for some undetermined reason the untrapped enzyme appeared to have greater specific activity than the original enzyme solution. Possibly there is more than one form or an isoenzyme which is not trapped but which has greater specific activity. Alternatively some inhibiting substance may have been separated from the residual activity in the wash solution allowing the enzyme to express a greater degree of activity. This phenomenon is being investigated further. Nevertheless, the washed gel does show good isomerase activity and the degree of activity has been calculated in terms of activity in the original enzyme solution.

About 30\% of the enzyme calculated to be in the gel, based on protein entrapped, assayed as isomerase (Table 2). The composition of the gel did not affect the amount of isomerase entrapped nor did it affect the activity of the enzyme. Although duplication of results from experiment to experiment was not as good as desired, it is evident that about 40 to 50\% of the enzyme added to the system was entrapped and about one third of this assayed as glucose isomerase.

A gel containing 90\% acrylamide was used to examine the effect of enzyme levels on glucose isomerase entrapment. The amount of enzyme entrapped increases with increasing levels added (Table 3), but the relationship is uncertain based on this data; there may be a linear relationship between the amount of enzyme added and the amount entrapped. The total activity in the gel

### Table 2. Effect of acrylamide concentration on the entrapment of glucose isomerase

| Acrylamide\( a \) | Enzyme units added | Enzyme units in gel | Per cent of enzyme entrapped\( b \) | Per cent of entrapped activity expressed\( d \) |
|-------------------|---------------------|---------------------|--------------------------|----------------------------------|
| 80                | 87                  | 10                  | 35                       | 40                               |
| 90                | 87                  | 9                   | 39                       | 45                               |
| 95                | 87                  | 14                  | 43                       | 49                               |
| 70                | 81                  | 8                   | 23                       | 38                               |
| 80                | 81                  | 11                  | 37                       | 46                               |
| 90                | 81                  | 12                  | 41                       | 51                               |
| 95                | 81                  | 13                  | 41                       | 51                               |
| 70                | 81                  | 8                   | 34                       | 42                               |
| 80                | 81                  | 10                  | 38                       | 47                               |
| 90                | 81                  | 11                  | 39                       | 48                               |
| 95                | 81                  | 8                   | 36                       | 44                               |

\( a \) N, \( N' \)-methylenebisacrylamide added to 100\%.

\( b \) Calculated units are based on enzyme units per milligram of protein and residual protein in wash.

\( c \) (Units calculated/units added) \( \times 100 \).

\( d \) (Units assayed/units calculated) \( \times 100 \).

### Table 3. Effect of enzyme levels on glucose isomerase entrapment\( a \)

| Protein added (mg) | Enzyme units added | Enzyme units in gel | Per cent of entrapped activity expressed\( d \) |
|-------------------|---------------------|---------------------|----------------------------------|
| 7.1               | 48                  | 4                   | 10                               |
| 10.6              | 42                  | 4                   | 10                               |
| 14.2              | 96                  | 8                   | 42                               |
| 7.6               | 61                  | 12                  | 21                               |
| 11.4              | 92                  | 10                  | 39                               |
| 15.2              | 122                 | 17                  | 67                               |
| 4.2               | 26                  | 0                   | 4                                |
| 8.5               | 53                  | 2                   | 27                               |
| 12.7              | 79                  | 8                   | 37                               |
| 17.0              | 106                 | 12                  | 65                               |
| 4.1               | 26                  | 2                   | 5                                |
| 8.2               | 53                  | 6                   | 24                               |
| 12.3              | 79                  | 13                  | 44                               |
| 16.4              | 106                 | 20                  | 61                               |

\( a \) Acrylamide, 90\%; 10\% \( N,N' \)-methylenebisacrylamide.

\( b \) Calculated units are based on enzyme units per milligram of protein and residual protein in wash.

\( c \) (Units assayed/units calculated) \( \times 100 \).
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also increases with increasing enzyme concentration, but a smaller percentage of the increase in entrapped enzyme assays as isomerase.

The polyacrylamide-entrapped enzyme was inactivated in the reaction mixture as discussed earlier. This sensitivity could limit the use of the entrapped enzyme as extremely large quantities of immobilized enzyme would have to be added to effect equilibrium. We have preliminary indications that if the products of the reaction mixture are continually withdrawn (as in a column), the immobilized enzyme retains activity for longer periods. This aspect is being investigated along with the binding of this enzyme to other support materials to determine whether the enzyme will function when chemically linked to solid supports.

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