0273-2289/91/3101—0011$02.00

Immunoaffinity Purification of Glucose/Xylose Isomerase from *Streptomyces*

MOHINI GHATGE, YOGESH MAWAL, SUSHAMA GAIKWAD, AND VASANTI DESHPANDE*

Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India

Received September 28, 1990; Accepted February 22, 1991

ABSTRACT

A procedure was developed to purify glucose/xylose isomerase from cell extract of *Streptomyces sp.* NCIM 2730 using immunoaffinity chromatography. High-titer polyclonal antibodies were raised in rabbit using electrophoretically homogeneous glucose/xylose isomerase as an antigen. The specificity of antibodies was confirmed by double immunodiffusion, rocket electrophoresis, and Western-blot ELISA, which revealed the presence of a single immunoreactive protein with an M_r of 40,000. The antibodies recognized 2-3 antigenic determinants/mol of enzyme and were found to partially neutralize the enzymatic activity in an immunotitration experiment. The affinity gel was prepared by coupling antibodies at pH 10.0 to divinyl sulfone-activated Sepharose CL-4B. The glucose/xylose isomerase purified by immunoaffinity chromatography yielded 75% recovery with a single enzymatically active protein band on gel electrophoresis and showed specific activity of 16 U/mg. The crossreaction of the antibodies with glucose isomerase from other actinomycetes indicated that they share common epitopes.

Index Entries: Glucose/xylose isomerase; antibody specificity, immunoaffinity purification; *Streptomyces*.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

D-Glucose/D-xylose isomerase (GI/XI) (E.C. 5.3.1.5) is generally an intracellular enzyme that catalyzes the reversible isomerization of D-glucose to D-fructose and D-xylose to D-xylulose. Both these reactions are important industrially, the former in the production of high fructose corn syrup and the latter in the production of ethanol from hemicelluloses. Despite its industrial importance, relatively little is known about the structural basis of its catalysis. Recently, based on the crystallographic data, the possibility of a 1,2-hydride shift mechanism in the isomerization of sugars by XI has been suggested (*1,2*). Involvement of a single histidine residue in the catalytic mechanism has been reported (*3,4*). Based on the kinetic analysis, we have reported that the enzyme has a single active site for the isomerization of both D-glucose and D-xylose (*5*). A limitation of more extensive investigations on the structure-function relationship of this system is imposed by the scarce quantities of the purified enzyme available with classical purification techniques.

In the present paper, we report a single step and easy-to-handle purification procedure for GI based on immunoaffinity chromatography. The enzyme purified by this method yielded 75% recovery with a specific activity (SA) of 16 U/mg, which is the same as that for the enzyme purified by conventional method of purification (*5*).

MATERIALS AND METHODS

Enzyme Production and Assay

The intracellular GI/XI from *Streptomyces* sp. NCIM 2730, *Chainia* and *Streptomyces lividans* TK24 were produced as described by Gaikwad et al. (*5*). GI/XI was estimated by colorimetric method of Takasaki and Tanabe (*6*). D-fructose/D-xylulose produced in the reaction was determined by the method of Dische and Borenfreund (*7*), as modified by Marshall and Kooi (*8*). The assay for GI was carried out at pH 7.5 at 70°C for 30 min, and that for XI at pH 7.5 at 35°C for 20 min. The unit of GI/XI activity is defined as the amount of enzyme that produces 1 μmol of D-fructose/D-xylulose under the assay conditions. The protein content of enzyme preparations was measured according to Bradford (*9*) and that of antibodies was measured by using the formula: IgG (mg/mL) = (λ 280– λ 310)/1.5 × dilution factor, where λ 280 and λ 310 represents the optical densities at respective wavelengths.

Antibody Preparation

Antibodies against electrophoretically pure GI/XI from *Streptomyces* sp. NCIM 2730 (*5*) were raised in a New Zealand white rabbit by injecting sc the enzyme (1 mg) emulsified with equal vol of complete Freund's adjuvant. Booster injections were given at fortnightly intervals for 10–12 wk. The rabbit was bled when the antibody titer reached 1:32.

Specificity of the Antibody

Oüchterlony double-diffusion was carried out in 1% agarose gel in PBS (phosphate buffered saline: 0.1*M* Na_2HPO_4, 0.4*M* NaH_2PO_4, 0.14*M* NaCl, pH 7.2) at 4°C for 24–48 h. Rocket electrophoresis was performed by incorporating the antibody into 1% agarose gel. Antigen was applied in a well, cut at one end and electrophoresis was carried out at 8 mA, 5–10 V/cm. Precipitin lines formed were visualized after drying the gels and staining with 0.25% Coomassie brilliant blue R-250. Western-blotting and the following immunodetection were performed in a modified version of the method of Towbin et al. (*10*). Proteins from SDS-PAGE (*11*) were electrophoretically transferred to nitrocellulose paper over a period of 2 h at 150 V in a buffer containing 20 m*M* Tris, 50 m*M* glycine, and 20% methanol at pH 7.8 at 4°C. The blot was treated overnight with 10% goat serum in PBS at 4°C and washed thoroughly with PBS containing 0.1% Tween-80. It was then treated with anti-GI antibodies diluted 250-fold with 1% BSA in PBS for 2 h at room temperature, followed by incubation with 1000-fold diluted goat antirabbit IgG horseradish peroxidase conjugate (Sigma Chemical Co., USA) in 10% goat serum, for 2 h. Bound peroxidase was detected with 0.5% 3,3-diaminobenzidine tetrahydrochloride in 0.5*M* sodium citrate buffer, pH 5, containing 0.03% H_2O_2.

Determination of Antigenic Valency

Varying amounts of GI ranging from 0 to 75 μg were added to a series of tubes containing 10 μL of antibody (22.4 mg/mL) to the final vol of 500 μL in PBS. The tubes were incubated at 37°C for 1 h, followed by an overnight incubation at 4°C. On centrifugation, the precipitate was washed twice with cold PBS and dissolved in 0.1N sodium hydroxide. The antigenic valency was determined according to Heidelberger and Kendall (*12*).

Calculation of Antigenic Valency

In an immunoprecipitation curve, at low antigen concentration, there is a relative antibody excess. Under such conditions, every antigenic determinant is likely to be covered by a separate antibody molecule and the

'antigenic valency' can be determined by calculating the molar ratio of antibody to antigen using following equation

$$\text{Antigenic Valency} = \frac{\text{Wt of antigen}}{\text{Mol wt of antigen}} \bullet \frac{\text{Wt of antibody}}{\text{Mol wt of antibody}}$$

The mol wt of antibody and antigen were assumed to be 1,50,000 and 1,60,000, respectively. The wt of antigen and antibody in the precipitate were calculated based on the extinction coefficients of 0.695 and 0.667 mg/mL, respectively.

Immunotitration

GI (20 μL, 10 U/mL) was added to antiserum (30–200 μL) in 0.05*M* sodium phosphate buffer, pH 7, in a total vol of 500 μL. After incubation at 37°C for 1 h, each reaction mixture was assayed for GI as well as XI activity.

Immunoaffinity Column Chromatography

Ten grams of Sepharose CL-4B (Pharmacia, Sweden) was activated with 2 mL of divinyl sulfone (DVS) (Aldrich Chemical Co., USA) for 80 min at room temperature. The activated resin was washed with 0.3*M* sodium carbonate, pH 10, to remove unreacted DVS. Two milliliters of antibody (15 mg/mL) dialyzed against 0.3*M* sodium carbonate, was added to the resin and was allowed to react for 6 h at room temperature. The excess of antibody was removed by washing successively with 0.3*M* sodium carbonate, pH 10, 0.3*M* sodium chloride, 0.3*M* glycine, and PBS.

The coupled resin equilibrated with 0.05*M* sodium phosphate buffer, pH 7, containing magnesium sulfate (1 m*M*) with cobalt chloride (0.5 m*M*) was packed in a column (10×2 cm). The crude enzyme preparation was allowed to adsorb on it for 1 h at 4°C. The column was washed with 0.05*M* sodium phosphate buffer, pH 7. The bound GI/XI was eluted with 0.3*M* glycine, pH 4, with a flow rate of 20 mL/h. Fractions (2 mL each) were collected and immediately neutralized with solid Tris to pH 7.2–7.4. The fractions were assayed for GI activity, dialyzed against 0.005*M* sodium phosphate buffer, pH 7.0, containing magnesium sulfate (1 m*M*) and cobalt chloride (0.5 m*M*), concentrated and checked for homogeneity on gel electrophoresis.

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis in nondenaturing gels (7.5%) was carried out using Tris-glycine buffer, pH 8.3 (*13*). The *in situ* staining for GI activity in the gel was performed according to Yamanaka (*14*).

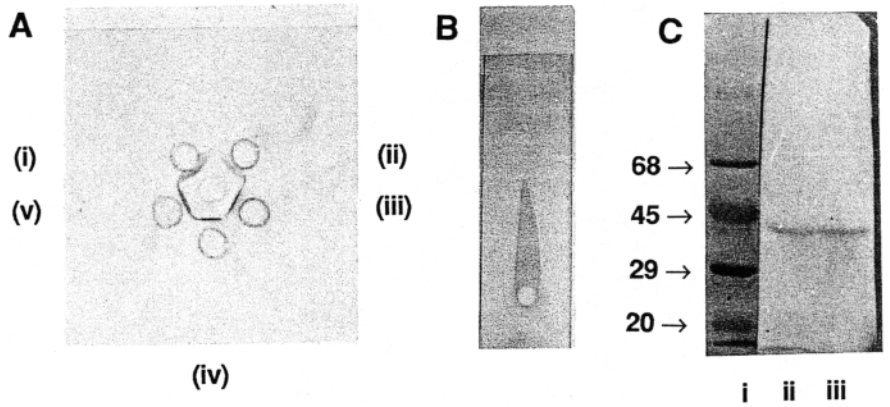


Fig. 1. Specificity of the antibody against GI/XI. A. Double immunodiffusion: The central well contained the antibodies. The peripheral wells contained (i) sonicated cell extract, (ii) heat treated, (iii) ammonium sulfate precipitated and dialysed, (iv) purified GI (10 μg), and (v) purified GI (20 μg). B. Rocket immunoelectrophoresis of crude GI/XI. C. Western-blot-ELISA of GI/XI. Lane i, mol wt markers, lanes ii and iii, immunoblots of crude and pure GI/XI.

RESULTS AND DISCUSSION

Specificity of the Antibody

The specificity of antibody toward GI/XI was determined using conventional immunological techniques. A single precipitin arc was obtained by Ouchterlony double-diffusion (Fig. 1A), as well as by Rocket electrophoresis (Fig. 1B), against both crude and purified GI/XI preparations. The sensitivity of Western-blot-ELISA technique is much higher than the gel-precipitin test, hence it was used to further confirm the specificity of the antibody. A single band at 40 K_d, corresponding to the M_r of the subunit of GI/XI, was immunoblotted from the crude and pure enzyme preparations (Fig. 1C).

Antigenic Valency of GI/XI

In a quantitative precipitin test, when the antigen concentration is low, there is a relative antibody excess wherein every antigen determinant is likely to be covered by a separate antibody molecule. Figure 2 shows the graph of antigen–antibody complex against the amount of GI/XI used. The calculations for the antigenic determinants were made in the antibody excess region (viz, at the antigen concentrations of 5, 15, and 20 μg) before the equivalence point was reached. The average value of molar ratios of antibody to antigen were found to be between 2 and 3, indicating that the antiserum recognizes 2–3 determinants/mol of enzyme.

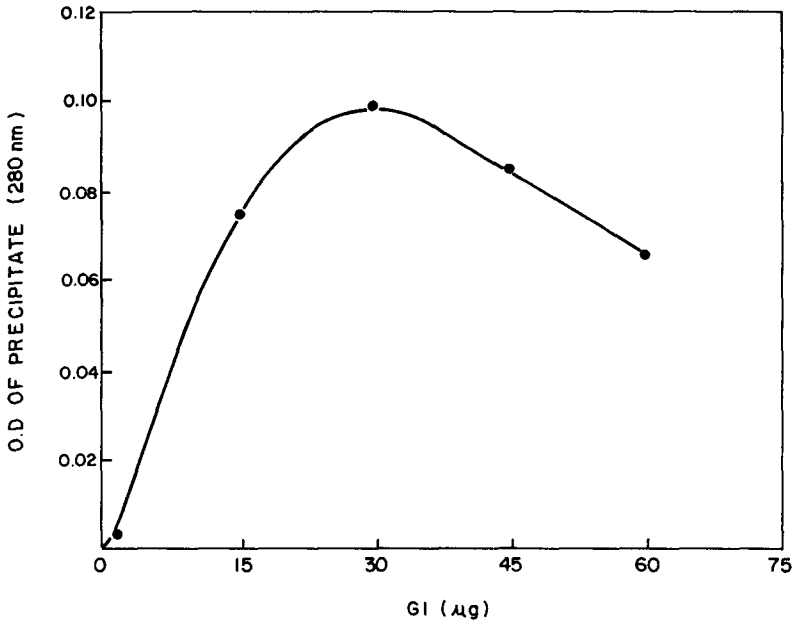


Fig. 2. Precipitation curve of GI/XI with anti GI/XI. Varying amounts of GI (0–75 μg) were added to 10 μL (22.4 mg/mL) of antibody. The precipitate was quantitated as described in Materials and Methods.

Immunotitration

The influence of the immunocomplex formation on the enzyme activity helps to monitor the resulting modification of the enzyme. Neutralization of GI activity with the antibodies resulted in nearly 30% loss in activity, whereas in case of XI the loss in activity continued to 50% (Fig. 3). Absence of complete inactivation of the enzyme after antibody reaction suggests that the antigen-binding site does not overlap the center of biological activity of GI. As the activity of the enzyme solely depends on the active site and the appropriate conformation of the protein, the partial loss in activity may be attributed to the change in conformation of the enzyme owing to the immunocomplex formation.

Purification of GI/XI by Immunoaffinity Chromatography

Since the antibodies recognized a single antigenic protein, viz, glucose/xylose isomerase, from the crude enzyme extract, they were used to purify the enzyme by coupling them to an insoluble matrix and passing the crude extract over it. Figure 4 shows the elution profile of bound enzyme. Seventy-five percent of the enzyme activity was recovered in fractions 4 and 5. When these two fractions were pooled and subjected to gel

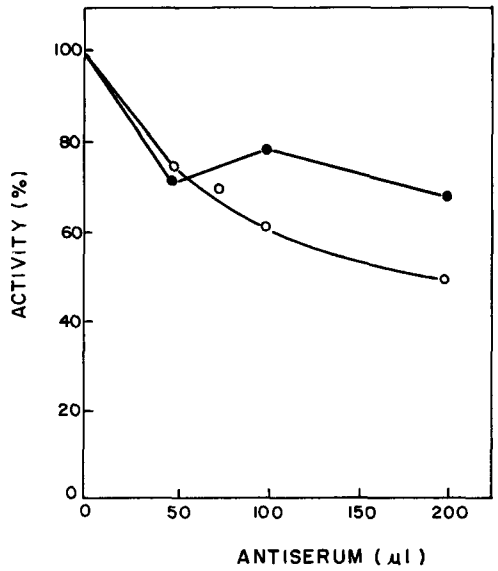


Fig. 3. Immunotitration of GI/XI. GI/XI (20 μL, 10 U/mL) was added to varying amounts (50–200 μL, 15 mg/mL) of antibody and the reaction mixture was estimated after 1 h at 37°C ● GI, ○ XI.

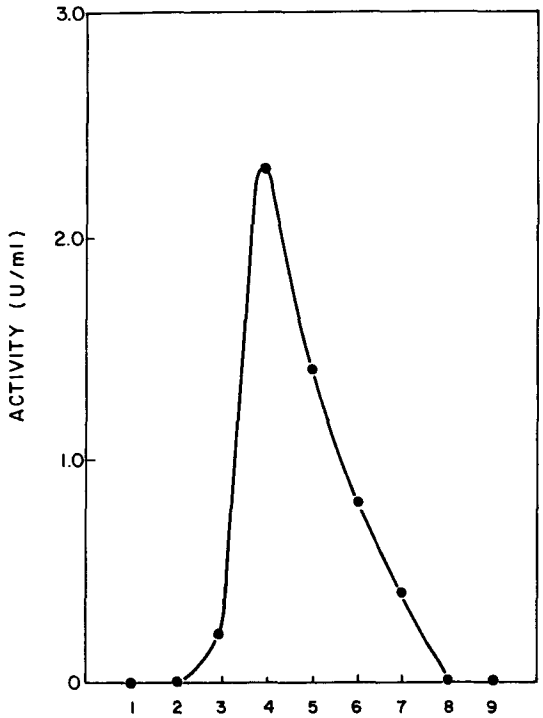
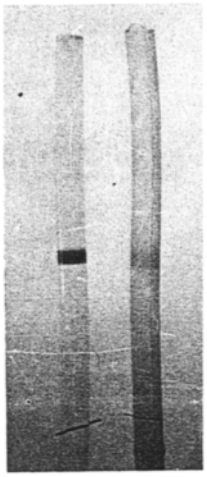


Fig. 4. Purification of GI/XI by immunoaffinity chromatography.



i ii

Fig. 5. Polyacrylamide gel electrophoresis of GI/XI purified by affinity chromatography stained for: i, protein and ii, activity.

Table 1
Purification of *Streptomyces* GI/XI

Method	Sample vol, mL	Total protein concn., mg	Total enzyme activity, U	SA	Recovery %
Crude extract	0.89	0.89	9.8	11.00	100
Affinity eluate	4.0	0.48	7.4	15.5	75.5
Crude extract	30	66	697	10.6	100
Gel[a] electroproduct	18	18	285	16.0	41

[a]Data from previous work (ref. 5).

electrophoresis, a single protein band that showed GI activity on *in situ* staining was observed (Fig. 5). The SA of the purified enzyme was 16 U/mg, which is similar to the enzyme purified by gel electrophoresis (*5*). As evident from Table 1, chromatography using immunoaffinity yields 75% of the purified enzyme as compared to 41% by gel electrophoresis, which requires an additional step of DEAE-cellulose chromatography to eliminate the acrylamide impurities. Although there was some loss in enzyme activity after immunotitration, the activity was regained after the dissociation of antigen–antibody complex by glycine, resulting in a reasonably good yield of purified enzyme.

Ours is the first report on the rapid, virtually instantaneous method for obtaining the large amount of purified GI/XI by immunoaffinity chromatography. The column after reequilibration with 0.05*M* sodium

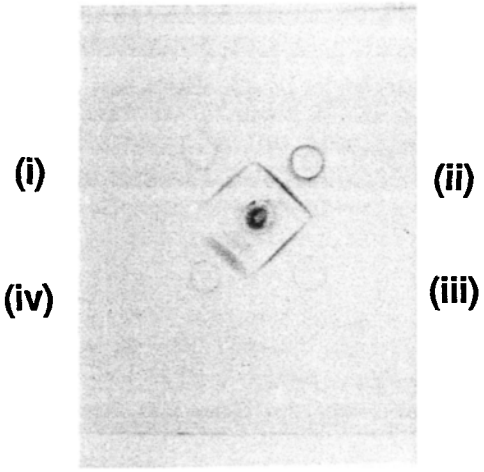


Fig. 6. Crossreaction of the antibody with other GI/XI preparations. The central well contained the antibodies. The peripheral wells contained crude extracts of (i) *Streptomyces* sp. NCIM 2730, (ii) *Chainia* (extracellular GI and XI), (iii) *Chainia* (intracellular GI/XI), and (iv) *S. lividans* TK24.

phosphate buffer, pH 7.0, was subsequently used three times without any observable loss in efficiency.

Crossreactivity of the Antibody with Other GI/XI Preparations

The antibodies raised against electrophoretically purified GI/XI of *Streptomyces* sp. NCIM 2730 reacted with (i) extracellular GI and XI of *Chainia* sp., (ii) intracellular GI/XI *Chainia* sp., and (iii) intracellular GI/XI of *S. lividans* TK24 (Fig. 6), indicating the presence of common antigenic determinants among the GI/XI preparations of different actinomycetes tested. As such, the antibodies raised against GI/XI from *Streptomyces* sp. NCIM 2730 can be used for screening high glucose isomerase producers among actinomycetes.

CONCLUSION

Polyclonal antibodies raised in rabbit against electrophoretically homogeneous glucose/xylose isomerase were used for preparing immunoaffinity matrix for purification of the enzyme. The specificity of the antibodies was confirmed by various immunotechniques, and antibodies were found to recognize 2–3 antigenic determinants/mol of enzyme. Purification using immunoaffinity chromatography yielded 75% recovery of electrophoretically homogeneous GI/XI of SA 16 U/mg.

ACKNOWLEDGMENTS

The authors are thankful to P. K. Ranjekar and D. A. Gadkari for their helpful suggestions during this work. Award of Senior Research Fellowship to Mohini Ghatge by the Council of Scientific and Industrial Research, New Delhi, is gratefully acknowledged.

REFERENCES

1. Farber, G. K., Glasfeld, A., Tirarby, G., Ringe, D., and Petsko, G. A. (1989), *Biochemistry* **28,** 7289–7297.
2. Collyer, C. A., Henrick, K., and Blow, D. M. (1990), *J. Mol. Biol.* **212,** 211–235.
3. Vangrysperre, W., Callens M., Kersters-Hilderson, H., and De-Bruyne, E. (1988), *Biochem. J.* **250,** 153–160.
4. Gaikwad, S. M., More, M. W., Vartak, H. G., and Deshpande, V. V. (1988), *Biochem. Biophys. Res. Commun.* **155,** 270–277.
5. Gaikwad, S. M., Pawar, H. S., Vartak, H. G., and Deshpande, V. V. (1989), *Biochem. Biophys. Res. Commun.* **159,** 457–463.
6. Takasaki, Y. and Tanabe, O. (1966), *Agric. Biol. Chem.* **30,** 220–225.
7. Dische, Z. and Borenfreund, E. (1951), *J. Biol. Chem.* **912,** 583–587.
8. Marshall, R. O. and Kooi, E. R. (1957), *Science* **125,** 648, 649.
9. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
10. Towbin, H., Staehelin, T., and Gordon, J. (1979), *Proc. Natl. Acad. Sci.* **76,** 4350–4354.
11. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
12. Heidelberger and Kendall (1980), in *Practical Immunology* (Hudson L. and Hay F. C., eds.), Blackwell Scientific Publications, Oxford, pp. 107–112.
13. Maurer, H. R. (1971), Disc gel electrophoresis and related techniques of PAGE, Berlin, Walter de Gruyter, New York
14. Yamanaka, K. (1975), *Methods in Enzymology* **41,** Wood, W. A., ed., Academic, New York, pp. 466–471.