Purification and Properties of Glucosaminephosphate Isomerase of *Proteus mirabilis*

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In Memoriam Prof. Florencio Bustinza *(Nov. 7, 1902–Jan. 10, 1982)*

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A glucosamine-P isomerase has been identified in *Proteus mirabilis*. The 113-fold purified enzyme exhibits a pH optimum of 7.5 with a secondary maximum at 8.5 and a temperature optimum at 37 °C. The apparent K_m was 13.3 mM for fructose-6-P and 18.8 mM for L-glutamine. Molecular weight of the enzyme has been estimated as 120000 and the protein can be dissociated in four subunits by SDS-polyacrylamide electrophoresis.

Introduction

Glucosamine-P isomerase (EC. 5.3.1.19) can synthesize glucosamine-6-P through a reaction of transamination using fructose-6-P and L-glutamine as substrats [1, 2]. The reaction is inhibited by 6-diazo-5-oxo-L-norleucine [3] and goes essentially to form L-glutamine.

The enzyme firstly lies fructose-6-P and secondly L-glutamine, showing two points of strong affinity for the hexose-P and only one point, of low affinity, for L-glutamine [4].

The enzyme is inhibited by an excess of L-glutamine [4, 5], being this inhibition increased by the presence of low concentrations of fructose-6-P [6]. Glucose alters several properties of the enzyme by inducing the appearance of two interconvertible forms and displacing the equilibrium to one or another of those as a function of the presence or absence of fructose-6-P [7]. Both forms of the enzyme have a different molecular weight and a distinct behaviour to trypsic diggestion.

The present paper reports a procedure of enzyme purification as well as a study of their main properties.

Experimental

Proteus mirabilis, NCIB 5887, was used throughout this work. Bacteria were grown routinely in batch culture, 250 ml of medium in 1-l flasks, and shaken vigorously at 37 °C. A minimal medium was used containing per liter of distilled water: glucose, 9.9 g; $(NH_4)_2SO_4$, 5.3 g; KH_2PO_4, 5.0 g; K_2HPO_4, 11.6 g; $MgSO_4 \cdot 7\,H_2O$, 0.04 g; NaCl, 4.0 g and nicotinic acid, 0.24 mg.

The cell (7.0 g in dry weight) were suspended in 50 ml of 0.15 M phosphate buffer, pH 7.5, containing 1 mM of both EDTA and DTT, and disrupted in a MSE 10 kc sonic oscillator at 8000 microns for 1 min with ice-cold protection. The suspension was centrifuged at 4 °C for 15 min at $29000 \times g$ and the pellet was discarded. Nucleic acids were removed from the supernatant by precipitation with polymixyn sulfate (10 ml of 2 per cent polymixyn sulfate per 50 ml of cell-free extract). The mixture was vigourously shaken for 30 min and then centrifuged at $40000 \times \dot{g}$ for 20 min at 4 °C. The pellet was also discarded and supernatant was dialyzed overnight against 4 l of 10 mM phosphate buffer, pH 7.5, containing 1 mM of both EDTA and DTT.

The protein was then adsorbed on calcium phosphate gel (75 mg in dry weight of gel per mg of protein) and eluted by increasing the molarity of the buffer. The most active fraction (50 ml in volume) was eluted with 50 mM phosphate buffer. This fraction was then concentrated at 5 ml by ultrafiltration through Amicon with a UM-2 membrane and loaded on a column of DEAE-cellulose (10 cm in height × 1 cm in diameter) prepared according to Seubert-Remberger [8], equilibrated with 10 mM phosphate buffer, pH 7.5, containing 20 mM sodium chloride. Protein was eluted by increasing the salt concentration. The most active fraction was eluted at 150 mM NaCl. Protein was measured

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Table I. Purification procedure of glucosamine-P isomerase of *Proteus mirabilis.*

Step	Volume [ml]	Protein [mg/ml]	Total protein [mg]	Total activity [units]	Specific activity [units]	Recovery [%]	Purification (-fold)
Cell-free extract	50	5.53	276.5	1274.66	4.61	100	–
Supernatant from polymyxin precipitation	50	5.01	250.5	1247.49	4.98	97.86	1.08
Calcium phosphate gel eluate	50	0.13	6.5	555.10	85.40	43.54	18.52
DEAE-cellulose eluate	5	0.21	1.05	546.36	520.35	42.86	112.87

according to Lowry *et al.* method [9] and the activity of the enzyme according to Ghosh *et al.* [10].

For molecular weight estimation, samples of the purified enzyme were treated for 5 min at 80 °C with SDS (6 μg of detergent per μg of protein) and 5 mM DTT in a final volume of 80 μl. Electrophoresis was performed on slabs from 5–15 per cent acrylamide gel [11]. Protein was observed after staining with Coomassie blue dye. Trypsin, pepsin, alcohol dehydrogenase, ovoalbumin, catalase, bovine serum albumin and urease were used as markers.

The absorption spectrum of the purified enzyme was determined in a quartz cuvette (1 cm pathlength) in a Varian 635 D spectrophotometer.

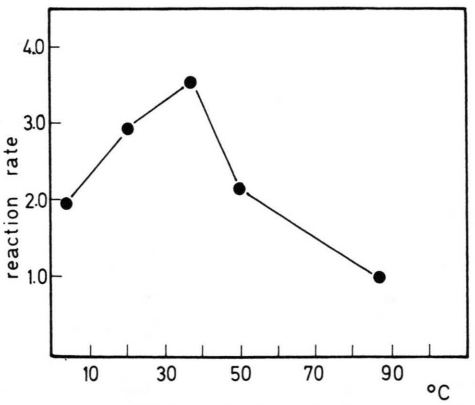


Fig. 2. Dependence of glucosamine-P isomerase activity on the temperature.

Results and Discussion

A summary of the enzyme purification procedure is given in Table I. Glucosamine-P isomerase was purified 113-fold with an overall yield of 43 per cent.

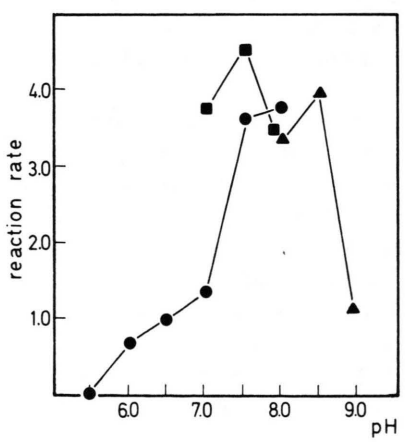
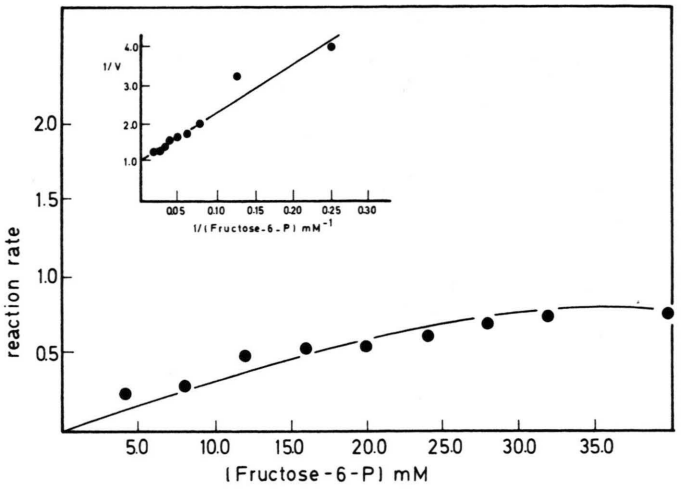


Fig. 1. Dependence of glucosamine-P isomerase activity on the pH values of incubation mixture. The assay is conducted in the presence of 0.15 M (●) Tris-maleate; (■) phosphate or (▲) borate-boric acid buffers.

Activity of the purified enzyme was determined with reaction mixtures maintained at pH values 5.5–8.0 with Tris-maleate, 7.8–8.0 with phosphate, and 8.0–9.0 with borate-boric acid buffers, all of them at 0.15 M concentration. The enzyme shows a pH optimum at 7.5 but a secondary maximum appears at pH 8.5 when borate-boric acid was used (Fig. 1). A double-optimal value of pH has been also described for the enzyme of *Escherichia coli*, being 7.6 when phosphate or 8.0 when Tris-maleate buffers were used. The enzyme exhibits a temperature optimum at 37 °C, being extremely heat-resistant (Fig. 2).

The enzyme kinetics as a function of the concentration of both substrats are shown in Figures 3 and 4. When the concentration of L-glutamine was fixed at 15 mM, isomerase showed the classical hyperbolic dependence on fructose-6-P concentration (Fig. 3). The K_m for the sugar-P was estimates as 13.3 mM. When the concentration of fructose-6-P was fixed at 20 mM, K_m for L-glutamine was estimated as 18.8 mM (Fig. 4). These values were higher than

Fig. 3. The effect of fructose-6-P concentration on the aminotransferase reaction. The insert shows the double-reciprocal plot.



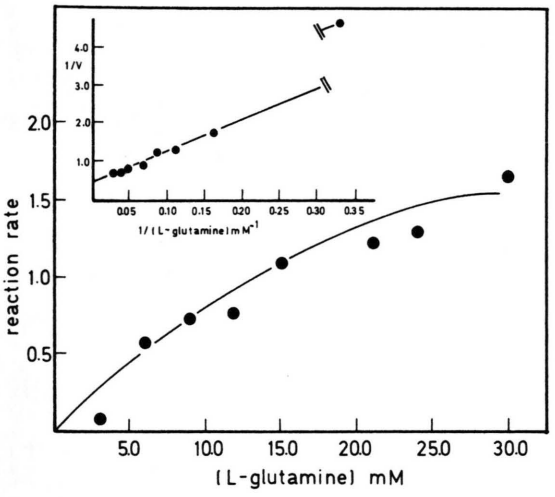


Fig. 4. The effect of L-glutamine concentration on the aminotransferase activity.

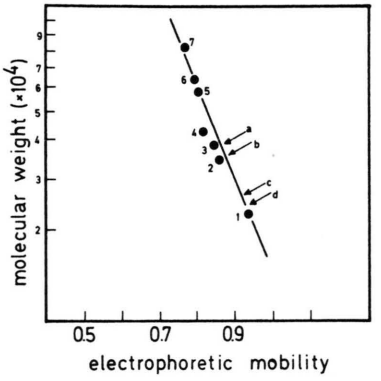


Fig. 5. Determination of molecular weight of the subunits of glucosamine-P isomerase on SDS-polyacrylamide gel electrophoresis. Molecular weights are estimated from standard plot of log molecular weight *versus* electrophoretic mobilities. Molecular standards are: 1, trypsin; 2, pepsin; 3, alcohol dehydrogenase; 4, ovoalbumin; 5, catalase; 6, bovine serum albumin; 7, urease. The arrows indicate the different subunits of isomerase.

those reported by Ghosh *et al.* [10] for the enzyme of *E. coli, Neurospora crassa* or rat liver.

The ultraviolet spectrum of *Proteus mirabilis* glucosamine-P isomerase shows a clear maximum at 272 nm.

Molecular weight of the enzyme has been previously reported as 120000 [12], this value being in accord to that reported for the enzyme of *E. coli* (97000) and *Bacillus subtilis* (107000) [13]. By SDS-polyacrylamide gel electrophoresis, isomerase is dissociated in four subunits, being determined their molecular weights as 38000, 35000, 26000 and 24000 respectively (Fig. 5). The dissociation pattern must be interpreted as opposite to the Kornfeld's theory about the nature of the bacterial enzyme, since he considers this protein as a subunit of the mammalian enzyme, which shows a molecular weight about 340000–400000 [13].

Acknowledgements

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