6-Phosphogluconolactonase from *Zymomonas mobilis*

An enzyme of high catalytic efficiency

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The enzyme 6-phosphogluconolactonase (EC 3.1.1.31) is present at high levels in *Zymomonas mobilis* cells. A simple procedure for its isolation involving dye-ligand chromatography and gel filtration has resulted in a 500-fold purification with high recovery. The purified enzyme is a monomer of 26 kDa, and has a high catalytic efficiency with k_{cat}/K_m of 9×10^7 $M^{-1} \cdot s^{-1}$ at 25° C. Two assay procedures for the enzyme are compared, and a simple method of obtaining a solution of 6-phosphoglucono-δ-lactone relatively free of other metabolites is presented.

6-Phosphogluconolactonase Zymomonas mobilis *Dye-ligand chromatography* *Enzyme efficiency* *6-Phosphogluconolactone*

1. INTRODUCTION

The product of the oxidation of glucose 6-phosphate, 6-phosphoglucono-δ-lactone is unstable and hydrolyse spontaneously to the open-chain 6-phosphogluconate. However, the rate of spontaneous hydrolysis is not sufficient in most tissues to prevent an undesirable accumulation of the lactone; an enzyme exists to catalyse the hydrolysis, 6-phosphogluconolactonase, EC 3.1.1.31 [1]. In *Zymomonas mobilis* the flux through the main fermentation pathway, which includes hydrolysis of 6-phosphogluconolactone, is 225 μmol·(g wet wt)$^{-1}$·min^{-1} at 30°C [2]. Even with the lactone present at 1 mM, its spontaneous hydrolysis could only account for about 0.1% of this flux [3]; the enzyme must be present at a level at least equivalent to the flux, which would be more than an order of magnitude greater amount than reported in other tissues [4,5].

This paper reports measurement and purification of *Z. mobilis* 6-phosphogluconolactonase, which proves to be an enzyme of particularly high catalytic efficiency. Two separate assay procedures are compared, and a simple way of preparing an authentic solution of 6-phosphoglucono-δ-lactone presented.

2. EXPERIMENTAL AND RESULTS

Z. mobilis cells strain ZM4 (ATCC 31821) were grown and extracted as described [6,7]. The buffer used for extraction was 10 mM Tris base. The pH of the extract, after standing overnight at 20–22°C, was lowered to 6.0 using 1 M Mes, and insoluble cell debris removed by centrifugation.

Glucose-6-phosphate dehydrogenase was isolated from *Z. mobilis* [6], and 6-phosphogluconate dehydrogenase from yeast (M. Zachariou, unpublished). Dye-ligand columns were prepared and screened as described in [8].

A Mes buffer was used for both the assays and the purification procedure. It consisted of 10 mM KOH adjusted to pH with 1 M Mes, and contained 30 mM NaCl and 2 mM $MgCl_2$. Assays were carried out at pH 6.5, and the isolation made use of buffers at pH 6.0 and 6.2.

The routine assay is based on the restoration of

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equilibrium of glucose-6-phosphate dehydrogenase when 6-phosphogluconolactone is removed [3]. To 1 ml of the pH 6.5 buffer containing 0.25 mM NAD^+ and 20 units $\cdot$ ml^{-1} glucose-6-phosphate dehydrogenase, 5 μl of 50 mM glucose 6-phosphate was added. Within 1–2 min the equilibrium position was attained, and the slow rate of increase of absorption at 340 nm was due to the spontaneous hydrolysis of the lactone. A sample containing about 0.05 units lactonase was added, the rate of increase in A_{340} noted and the spontaneous rate (0.008 A units min^{-1}) subtracted. Activity is expressed as μmol 6-phosphogluconolactone hydrolysed per min at 25°C, being μmol NADH formed per min multiplied by 3.6 (see below).

The amount of lactonase present in *Z. mobilis* was determined to be 500 ± 100 units $\cdot$ (g wet wt)$^{-1}$ at 25°C, which is about 3-times the minimum requirement for the in vivo metabolic flux. The spontaneous hydrolysis of 6-phosphogluconolactone at pH 6.5, and 25°C was equivalent to 5% per min, giving a first-order rate constant of 7.5×10^{-4} s^{-1}. In the assay using 6-phosphogluconate dehydrogenase the value was determined to be 6.0×10^{-4} s^{-1}.

To assay the enzyme using 6-phosphogluconate dehydrogenase, a solution of 6-phosphogluconolactone was prepared. Glucose-6-phosphate dehydrogenase was coupled to Eupergit C (Rohm Pharma, Darmstadt). Approx. 5 mg of immobilised enzyme beads was added to 200 μl of pH 6.5 buffer containing 10 mM glucose 6-phosphate, 1 mM NAD^+, 10 mM Na pyruvate and 1 unit rabbit muscle lactate dehydrogenase. The mixture was agitated for 5 min, centrifuged for 20 s, chilled on ice and used immediately. The residual amount of glucose 6-phosphate was about 10%, and was estimated using $NADP^+$ and yeast glucose-6-phosphate dehydrogenase. The amounts of lactone and open-chain 6-phosphogluconate were estimated in the subsequent assay procedure; 80–85% of the original glucose 6-phosphate was present as 6-phosphogluconolactone initially, which hydrolysed, at 0°C, at only 0.5% per min.

To pH 6.5 buffer containing 0.5 unit $\cdot$ ml^{-1} 6-phosphogluconate dehydrogenase and 0.25 mM $NADP^+$, a suitable amount of 6-phosphogluconolactone was added. A small, rapid burst of NADPH formation was due to open chain 6-phosphogluconate; a steady, slow, continuing production of NADPH was due to spontaneous hydrolysis of the lactone. A sample of lactonase was added and the increased rate of NADPH formation recorded. Occasionally, the reaction was allowed to go to completion to estimate the total substrate present. For kinetic measurements the concentration of lactone was estimated from the difference of the absorbance reading at the point where the rate was measured and the total absorbance change expected.

2.1. *Purification of 6-phosphogluconolactonase*

On screening of dye-ligand columns using 30 mM phosphate buffer, pH 6.0, I = 0.1 [6], it was found that only procion navy HE-R and yellow HE-4R bound the *Z. mobilis* enzyme well, though it was subsequently eluted by continued washing with the buffer. By using a buffer lacking phosphate, namely Mes (pH 6.0), I = 0.05, the enzyme adsorbed to several more dyes including Cibacron FG-A and procion green HE-4BD, and did not wash off with the buffer. The latter dye was selected as the adsorbent as affinity elution from this was very effective. Although a negative adsorption column [8] to remove much of the protein before running on the green dye column was occasionally used, it did little to improve the specific activity of the eluted fraction, so the simpler one-column procedure was adopted.

400–500 ml extract (~ 5000 mg protein) at pH 6.0 was run on a column of dimensions 16 cm^2 × 12 cm of green HE-4BD Sepharose CL-4B (reactive green 19, dye content 1.3 mg $\cdot$ g^{-1} wet wt), at 400 ml $\cdot$ h^{-1}. It was washed with 200 ml of pH 6.0 buffer and with 200 ml of pH 6.2 buffer. The flow rate was reduced to 200 ml $\cdot$ h^{-1} and 300 ml of pH 6.2 buffer containing 2 mM glucose 6-phosphate was applied, followed by more pH 6.2 buffer. 6-Phosphogluconolactonase was affinity eluted by the glucose 6-phosphate; active fractions were collected and concentrated by ultrafiltration to about 8 ml. This sample was applied to a 600 ml gel filtration column of Cellulofine GCL-2000 superfine (Amicon) or Sephacryl S-200 (Pharmacia). The enzyme clearly separated from the contaminants, which were all of higher molecular mass (fig.1). Gel electrophoresis indicated that this preparation was essentially homogeneous and had a specific activity of 3500 units $\cdot$ mg^{-1} in the routine assay. A summary of the procedure is given in table 1.

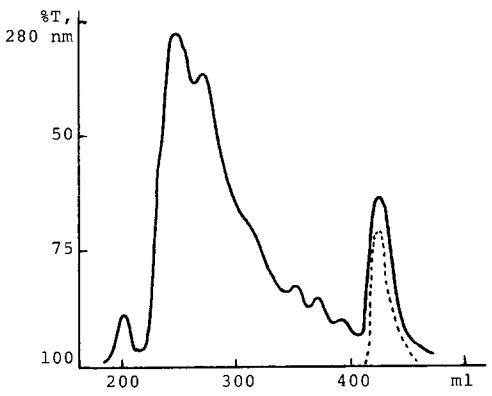

Fig.1. Gel filtration of glucose 6-phosphate-eluted fraction from the green HE-4BD column, on a column (8 $cm^2 \times 70$ cm) of Cellulofine GCL-2000 superfine grade, flow rate 30 $ml \cdot h^{-1}$ (– – –) 6-Phosphogluconolactonase activity.

2.2. *Enzyme assay and kinetic studies*

The routine assay is based on the fact that at pH 6.5 the equilibrium constant of the glucose-6-phosphate dehydrogenase reaction is close to unity, so a considerable proportion of glucose 6-phosphate remains unoxidized. On adding the lactonase, the removal of lactone perturbs the equilibrium so that more glucose 6-phosphate is oxidized and NADH produced. However, the rate of NADH production is considerably less than the rate of 6-phosphogluconolactone hydrolysis as the following calculation shows:

Starting with 0.25 mM each of NAD^+ and glucose 6-phosphate, the equilibrium level of NADH (extrapolated to zero time) was 0.12 ± 0.005 mM. So 6-phosphogluconolactone also equals 0.12 mM; NAD^+ and glucose 6-phosphate are 0.13 mM, $K_e = 0.90 \pm 0.1$.

If Δx mM 6-phosphogluconolactone is removed, Δy mM extra NADH is produced to restore equilibrium. Using $K_e = 0.90$ the ratio $\Delta x/\Delta y$ can be calculated. For a value of Δy giving ΔA_{340} between 0.05 and 0.1, the value of $\Delta x/\Delta y$ is 3.6. This means that the μmol NADH produced per min should be multiplied by 3.6 to give μmol 6-phosphogluconolactone hydrolysed per min. Different factors can be calculated for different starting concentrations of NAD^+ and glucose 6-phosphate.

The fact that the enzyme was successfully eluted from the dye column by glucose 6-phosphate indicates that this is an inhibitor of the enzyme; glucose 6-phosphate is present in the routine assay. Two sets of rates were measured with 11 different starting concentrations and ratios of NAD^+ and glucose 6-phosphate. After correcting for competitive inhibition by glucose 6-phosphate using a K_i of 0.3 mM (see below), the 2 sets gave K_m values for 6-phosphogluconolactone of 0.020 and 0.029 mM. V_{max} was 4400 $\mu mol \cdot min^{-1} \cdot mg^{-1}$.

Using enzymically produced 6-phosphogluconolactone in the virtual absence of glucose 6-phosphate, the V_{max} value came to within 10% of that estimated above (4000 $\mu mol \cdot min^{-1} \cdot mg^{-1}$), confirming that the computations are valid. The K_m

Table 1

Purification of 6-phosphogluconolactonase from 65 g (wet wt) *Z. mobilis* cells

	Volume	mg protein	Total activity*	Specific activity*
Extract	400	4600	31000	6.7
Not adsorbed on Green HE-4BD	450	1200	2000	–
Eluted by glucose 6-P from green HE-4BD	250	61	27000	440
Collected from gel filtration	15	5.5	25000	3500

* Activity measured by routine assay as described, including multiplication by the factor to relate 6-phosphogluconolactone removal to NADH production: not corrected for inhibition by the glucose 6-phosphate present

measured in this direct assay was 0.023 ± 0.002 mM, and assaying in the presence of added glucose 6-phosphate, a K_i value of 0.3 ± 0.1 mM was determined.

The pH optimum (6-phosphogluconate dehydrogenase assay) was 6.7, and activity dropped to 20% of the maximum at pH values 5.8 and 7.9. The activity at the optimum was 10% higher than in the routine assay at pH 6.5, so V_{max} at the pH optimum is 4600 ± 500 (mean for the 2 assay procedures) at 25°C.

The purified enzyme had no activity on gluconō-δ-lactone using the assay of Hucho and Wallenfells [9]. Crude *Z. mobilis* extract has about one-tenth activity of gluconolactonase compared with 6-phosphogluconolactonase.

2.3. *Molecular properties*

The enzyme is monomeric, with a molecular mass estimated to be 26 ± 1 kDa from SDS-polyacrylamide gel electrophoresis, and 25 ± 3 kDa from gel filtration on a calibrated Sephacryl S-200 column. This compares with the value of 30 kDa for the bovine erythrocyte enzyme [5]. The enzyme had a rapid reaction with 5,5′-dithiobis(2-nitrobenzoic acid), indicating 2 exposed sulphydryl groups, but it was not inactivated. The extinction coefficient for a 1 $mg \cdot ml^{-1}$ solution at 280 nm is 1.24, based on measurements at 280 and 205 nm [10].

3. DISCUSSION

Enzymes that catalyse reactions that occur spontaneously are sometimes neglected, as they are not absolutely essential for the reaction to occur. This neglect is certainly true for 6-phosphogluconolactonase of *Z. mobilis* [11], despite the fact that the spontaneous rate of hydrolysis of the lactone is far too slow to keep up with its production, In the cytoplasm of the cells the flux is approx. 5 $mM \cdot s^{-1}$, thus a typical metabolite at 1 mM has an average lifetime of 0.2 s. At this rate, 6-phosphogluconolactone is as stable as any other metabolite, and the enzyme to hydrolyse it is essential.

6-Phosphogluconolactonase has previously been isolated from bovine erythrocytes; using a synthetic γ-lactone substrate the specific activity of the purified enzyme was 55 units $\cdot mg^{-1}$ [5]. The specific activity of the *Z. mobilis* enzyme is much higher, though to what extent this is due to using the correct substrate is not clear. The affinity for its substrate is high, as has been indicated for the enzyme from other sources [3], and the value for k_{cat}/K_m is 9×10^7 $M^{-1} \cdot s^{-1}$, close to the theoretical maximum for the diffusion-controlled association rate [12]. It is notable that several of the enzymes with such high values of k_{cat}/K_m are of the type that catalyse reactions that occur spontaneously in mild conditions.

The purification procedure involving affinity elution from a selected dye-ligand column is widely applicable. We have now used it for isolating 12 out of 15 *Z. mobilis* enzymes; the other 3 procedures used dye-ligand columns without affinity elution. A 60-fold purification of 6-phosphogluconolactonase was achieved with high recovery, in a process that took 3 h to complete the low molecular size of the protein enabled gel filtration to effect total separation of the remaining impurities.

6-Phosphogluconolactonase should be a useful analytical enzyme in combination with glucose-6-phosphate dehydrogenase in enabling a rapid quantitative conversion of glucose 6-phosphate to 6-phosphogluconate even at pH values below 7, and without a large excess of $NAD(P)^+$ present.

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