Biochem. J. (1985) **228**, 627–634
Printed in Great Britain

Simultaneous purification and characterization of glucokinase, fructokinase and glucose-6-phosphate dehydrogenase from *Zymomonas mobilis*

Robert K. SCOPES,* Vittorio TESTOLIN, Ann STOTER, Kerry GRIFFITHS-SMITH and Elizabeth M. ALGAR
Department of Biochemistry, La Trobe University, Bundoora, Vic. 3083, Australia

(*Received 27 November 1984/21 January 1985; accepted 15 February 1985*)

The three enzymes glucokinase (EC 2.7.1.2), fructokinase (EC 2.7.1.4) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were isolated in high yield from extracts of *Zymomonas mobilis*. The principal steps in the isolation procedures involved the use of selected dye–ligand adsorbent columns, with affinity elution of two of the three enzymes. Glucokinase and fructokinase are dimeric proteins (2×33000 Da and 2×28000 Da respectively) and glucose-6-phosphate dehydrogenase is a tetramer (4×52000 Da). Some similarities in the structural and kinetic parameters of the two kinases were noted, but they have absolute specificity for their substrates. Fructokinase is strongly inhibited by glucose; otherwise non-substrate sugars had little effect on any of the three enzymes.

Zymomonas mobilis, a Gram-negative bacterium of uncertain generic relationship, but showing similarities to *Acetobacter* and pseudomonads, is probably the only strict anaerobe that ferments by the Entner–Doudoroff pathway exclusively (Gibbs & De Moss, 1954; Swings & De Ley, 1977). Sugar uptake (glucose and fructose) is by facilitated or possibly simple diffusion (Belaich *et al.*, 1968), and specific kinases are present for each monosaccharide (Doelle, 1982*a*). Previous work on glucokinase and fructokinase has reported their kinetic properties, and presented purification procedures, though without indicating the degree of homogeneity of the preparations (Doelle, 1982*a*, *b*). Bacterial glucokinases (EC 2.7.1.2) have been purified to homogeneity from *Aerobacter aerogenes* (Kamel *et al.*, 1966), *Streptococcus mutans* (Porter *et al.*, 1982) and *Bacillus stearothermophilus* (Hengartner & Zuber, 1973), but a totally specific fructose 6-kinase has not been reported substantially purified from any other bacterial source.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in *Z. mobilis* operates almost equally effectively with NAD^+ or $NADP^+$ (Dawes *et al.*, 1966), and in that respect resembles the much-studied *Leuconostoc mesenteroides* enzyme (Olive & Levy, 1966). Early studies on the *Zymomonas* enzyme (Sly & Doelle, 1968) established some of its kinetic properties with $NADP^+$; otherwise there has been little published information on it. Being part of the main catabolic fermentation pathway, glucose-6-phosphate dehydrogenase must be assessed to operate *in vivo* principally with NAD^+. The quantity of it in the *Zymomonas* cell is substantially higher than in most other organisms. The quantities of the kinases, and indeed of all of the Entner–Doudoroff-pathway enzymes, are also necessarily very high, as these bacteria have very fast fermentation rates (Rogers *et al.*, 1979).

The technique of enzyme purification by using differential adsorption with two dye–ligand adsorbents is becoming established as one of the more selective of adsorption-chromatographic methods. It is particularly suitable for large-scale preparations (Lowe *et al.*, 1981; Scawen *et al.*, 1984) and for use with the crude tissue extract without any prior fractionation step. We have presented simple procedures for isolating the Entner–Doudoroff-pathway enzymes 6-phosphogluconate dehydratase (EC 4.2.1.12) (Scopes & Griffiths-Smith, 1984) and 3-deoxy-2-oxo-6-phosphogluconate aldolase (EC 4.1.2.14) (Scopes, 1984) from *Z. mobilis* cells, and have also developed similar methods for many other enzymes (Scopes *et al.*, 1982). Qadri & Dean (1980) have presented a differential (tandem-column) technique for isolating 6-phosphogluconate dehydrogenase from *B. stearothermophilus*. Glucose-6-phosphate dehydrogenase from *L.*

* To whom correspondence and requests for reprints should be addressed.

mesenteroides has similarly been isolated by Hey & Dean (1983). In each case, after the screening of a large number of dye columns for their ability to bind the enzyme in question, two adsorbents are chosen. The first is selected so that, although it binds a large amount of protein from the sample applied, the enzyme in question is not bound. The second is chosen so that, in the same buffer conditions, it binds the enzyme totally, but adsorbs relatively small further amounts of protein. The selectivity of dye–ligands is such that pairs of dyes can often be chosen so that the first column binds more protein (from crude extract) than does the second, despite the fact that the enzyme in question is adsorbed in the opposite fashion.

The present paper presents a simple procedure for simultaneously isolating glucokinase, fructokinase and glucose-6-phosphate dehydrogenase by using dye–ligand adsorbents, and gives a brief summary of their structural and kinetic properties. All three enzymes (together with some others) can be purified from the one lysate by passage through a series of columns selected for optimum adsorption or rejection of each enzyme.

Materials and methods

ATP, NAD^+, $NADP^+$, NADH, phosphoenolpyruvate, glucose 6-phosphate, β-D-glucose, glucosamine hydrochloride, mannose, xylose, ribose, sorbitol, galactose, xylulose, sodium gluconate, calcium pantothenate, biotin, Nonidet P-40, lysozyme and deoxyribonuclease I were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Other chemicals were A.R. grade.

Sepharose CL-4B was from Pharmacia, Uppsala, Sweden, and DEAE-Trisacryl was from LKB Produkter, Bromma, Sweden. *Zymomonas mobilis* strains ZM1 (A.T.C.C. 10988), ZM4 (A.T.C.C. 31821), ZM6 (A.T.C.C. 29191) and ZM31 (Skotnicki *et al.*, 1983) were obtained from Dr. P. L. Rogers, University of New South Wales, Sydney, N.S.W., Australia. They were grown in media containing 15% (w/v) glucose, 0.5% yeast extract, 0.05% KH_2PO_4, 0.05% $MgSO_4,7H_2O$, supplemented with 1mg of biotin/l, 2mg of calcium pantothenate/l and 20mg of $Fe(NH_4)_2(SO_4)_2,6H_2O$/l. The pH was maintained at 5.0 with aq. NH_3, and the temperature was 30°C. On occasions up to half the glucose was replaced with fructose; this increased the specific activity of fructokinase in the extracts, but lowered the cell yield. Cells were harvested by centrifugation at 4000*g* for 25min, then resuspended in water, and the pH of the suspension was adjusted to 7.0 with 5M-imidazole. After re-centrifuging (4000*g* for 40min), the paste was collected and could be stored at −25°C.

Cells were lysed by mixing each g wet wt. of cell paste (approx. 0.26g dry wt.) with 6ml of 30mM-K_2HPO_4 containing 0.1% Nonidet P-40, 2mM-$MgCl_2$, 10mM-2-mercaptoethanol, 0.15mg of lysozyme/ml and 3μg of deoxyribonuclease I/ml. The pH of the suspension was adjusted to 8.2 with 1M-Tris, and the mixture was stirred for 2–3h at 30°C. The pH was then lowered to 6.0 with 1M-Mes in 5M-acetic acid, and the cell debris was removed by centrifugation at 10000–15000*g* for 15min. Satisfactory extraction was indicated by a protein content of 12±2mg/ml.

Enzyme activities were measured as follows: glucokinase, in a buffer consisting of 20mM-imidazole and 20mM-KH_2PO_4, adjusted to pH6.8 with HCl, containing 5mM-$MgCl_2$, 1mM-ATP, 10mM-glucose, 1mM-NAD^+ and 2 units of *Z. mobilis* glucose-6-phosphate dehydrogenase/ml; fructokinase, as for glucokinase, but replacing glucose with fructose and including 2 units of (rabbit muscle) glucosephosphate isomerase/ml. For testing the effects of other possible sugar substrates on the purified enzymes, glucokinase and fructokinase were assayed by using 0.15mM-NADH, 0.25mM-phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase (5 units/ml each) to detect ADP formation. Glucose-6-phosphate dehydrogenase activity was measured in a buffer consisting of 30mM-Tris, 30mM-KCl and 2mM-$MgSO_4$, adjusted to pH8.0 with HCl, containing 1mM-glucose 6-phosphate and 1mM-NAD^+. All enzyme assay solutions contained 0.2mg of bovine serum albumin/ml, and activity was monitored at 340nm. Reactions were initiated by addition of the enzyme and activities are expressed in units of μmol/min at 25°C. Protein was measured by the Coomassie Blue-binding method of Sedmak & Grossberg (1977).

Procion dyes were generously given by I.C.I. Australia, and Cibacron dyes by Ciba–Geigy Australia. Dyes were coupled to Sepharose CL-4B by the ambient-temperature procedure of Atkinson *et al.* (1981). Columns (2cm^3; Amicon Corp.) were used in the screening procedures, and the dye absorbents were cleaned with 6M-urea/0.5M-NaOH both before and after use. The buffer used in the screening consisted of 10mM-KOH, adjusted to pH6.0 with Mes, containing 30mM-NaCl and 2mM-$MgSO_4$ (Scopes, 1984); columns were equilibrated with this buffer before use.

Electrophoresis was carried out on 0.5mm-thick polyacrylamide gels in an LKB Multiphor apparatus, in a Tris chloride/Tris glycinate discontinuous-buffer system. Staining for activity of the enzymes in non-dissociating gels used Nitro Blue Tetrazolium linked through glucose-6-phosphate dehydrogenase (Fine & Costello, 1963).

Gel filtration for molecular size was carried out on a Sephacryl S-200 column (2cm² × 100cm) in phosphate buffer, pH 7 and *I* 0.15. The column was calibrated with rabbit muscle pyruvate kinase (M_r 228000), lactate dehydrogenase (M_r 140000), yeast enolase (M_r 88000), bovine serum albumin (M_r 66000) and yeast phosphoglycerate kinase (M_r 45000).

The sections of the present paper describing the extraction of *Z. mobilis* cells and the purification of the three enzymes are included in a patent application (Scopes, 1983).

Results

Screening of dye–ligand columns for adsorption of enzymes

Some 55 different dye–ligand adsorbents (Scopes, 1984) have been screened in the pH 6 buffer described in the Materials and methods section, by applying extract at a loading of 15mg/ml of column, at a flow rate of 30–40cm/h. After a washing with starting buffer, protein and enzymes were eluted from the columns with 1M-NaCl.

Glucose-6-phosphate dehydrogenase bound totally to 50 of these adsorbents and partially to the others. In most cases adsorbed enzyme was eluted with 1M-NaCl. Thus it was possible to choose a column for glucose-6-phosphate dehydrogenase that bound little protein, but retained the enzyme well. After several further trials it was concluded that, of a short list of Procion Blue HE-G, Red MX-5B, Turquoise MX-G and Scarlet MX-G adsorbents, the Scarlet MX-G–Sepharose was best, as it could retain all the enzyme even at loadings of up to 40mg of extract protein/ml of column. It was also (see below) an excellent adsorbent for glucokinase. No 'negative' column was used, as no dye–ligand adsorbent tested allowed all the glucose-6-phosphate dehydrogenase through.

Glucokinase was also found to bind to most of the dye–ligand adsorbents tested at pH 6.0, though to relatively few at pH 7 or higher. On the other hand, glucose-6-phosphate dehydrogenase bound to most columns even at pH 8. Up to loadings of 35mg of extract protein/ml of Scarlet MX-G–Sepharose adsorbent, all the glucokinase was retained on this column at pH 6.0. Under these conditions 70% of the applied protein passed through the column. Thus it was convenient to use Scarlet MX-G–Sepharose as the adsorbent for both glucose-6-phosphate dehydrogenase and glucokinase.

Fructokinase binds to far fewer columns. Under the stated screening conditions it bound to 21 columns totally, to 11 partially and to 22 not at all. Recovery of adsorbed fructokinase from the columns by elution with NaCl was satisfactory in most cases. At the higher protein loadings, such as described above for glucokinase and glucose-6-phosphate dehydrogenase, fructokinase 'broke through' and was not retained on many of the 21 columns mentioned above. But under these conditions it was still totally bound by Yellow MX-GR–Sepharose and some other dyes of higher protein-binding capacity. Of the several dye–ligand adsorbents that did not retain the enzyme at high loadings, but that held back a substantial proportion of the total protein, Brown H-5R–Sepharose was found to be particularly appropriate; it bound 64% of the protein from the original extract, whereas Yellow MX-GR–Sepharose bound only 50% in these conditions. Thus Brown H-5R–Sepharose was chosen as the 'negative' adsorbent, and Yellow MX-GR–Sepharose was used for the 'positive' column. Since fructokinase was not retained by Scarlet MX-G–Sepharose, the three columns could be coupled together and the extract passed through each successively, i.e. Scarlet MX-G–Sepharose, Brown H-5R–Sepharose and Yellow MX-GR–Sepharose.

Purification of glucokinase, fructokinase and glucose-6-phosphate dehydrogenase from the one extract

Extract from 60–70g of cells (400ml) at pH 6.0 was passed through the three columns, which were each 16cm² in cross-section. The Scarlet MX-G–Sepharose and Brown H-5R–Sepharose columns were 12cm tall and the Yellow MX-GR–Sepharose column was 8cm tall. Scarlet MX-G (Reactive Red 8, C.I. 17908) was coupled to Sepharose CL-4B at 5mg/g wet wt. of gel, Brown H-5R (Reactive Brown 7) at 1.5mg/g wet wt. of gel and Yellow MX-GR (Reactive Yellow 7) at 3.5mg/g wet wt. of gel. The flow rate was 400ml/h, and the extract was washed in with 500ml of the pH 6 buffer described in the Materials and methods section. The three columns were then separated; the Brown H-5R–Sepharose column was removed and the Yellow MX-GR–Sepharose column put aside while the Scarlet MX-G–Sepharose column was worked up for glucokinase and glucose-6-phosphate dehydrogenase; 500ml of a pH 7.0 buffer consisting of 30mM-potassium phosphate/2mM-$MgCl_2$ was run into the Scarlet MX-G–Sepharose column, and protein eluted by the change of pH was monitored. Glucokinase was released by the increase in pH, and was eluted in the latter part of the protein peak (Fig. 1). Then glucose-6-phosphate dehydrogenase was eluted by using 100ml of 0.1mM-$NADP^+$ plus 1mM-NAD^+ in the pH 7 buffer (alternatively, 0.2mM-$NADP^+$ could be used), followed by a further 200ml of buffer without nucleotides. This

affinity elution displaced the enzyme in a sharp peak, as illustrated in Fig. 1.

Both the glucokinase and the glucose-6-phosphate dehydrogenase fractions were concentrated by ultrafiltration (Amicon YM-10 membrane) to a protein concentration of about 5mg/ml, and further purified by $(NH_4)_2SO_4$ fractionation. Both enzymes, but particularly glucokinase, have a low solubility in $(NH_4)_2SO_4$. Glucokinase was precipitated by adding solid $(NH_4)_2SO_4$ to 1.5M, and glucose-6-phosphate dehydrogenase by adding solid $(NH_4)_2SO_4$ to 1.9M. Each was collected by centrifugation at 30000*g* for 20min, and the precipitates were redissolved in pH7 phosphate buffer. Trace impurities still remaining could be separated by gel filtration on Sephacryl S-200; otherwise both enzymes would crystallize when saturated $(NH_4)_2SO_4$ was added to turbidity and the mixture left in the cold. It was also equally effective to gel-filter on Sephacryl S-200 without prior $(NH_4)_2SO_4$ precipitation, although for glucokinase a larger gel-filtration column was needed, as much more protein was present if no $(NH_4)_2SO_4$ step had been carried out. A summary of the isolation procedures is given in Table 1.

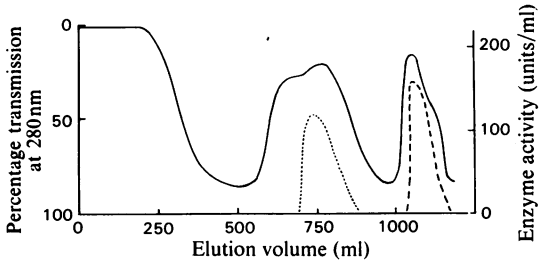

Fig. 1. *Pattern of elution of glucokinase and glucose-6-phosphate dehydrogenase from a Scarlet MX-G–Sepharose column*

The eluate was monitored at 280nm (——) in a 3mm-pathlength cell; much of the non-absorbed material was nucleic acid. Glucokinase (·····) was eluted by the stepwise increase in buffer pH from 6.0 to 7.0. Glucose-6-phosphate dehydrogenase (– – – –) was affinity-eluted by an $NAD^+/NADP^+$ mixture (1mM/0.1mM) in the pH7.0 buffer.

Fructokinase was purified from the Yellow MX-GR–Sepharose column by elution with ATP. After non-adsorbed proteins had been washed out with up to 500ml of pH6.0 buffer, the buffer was changed to 30mM-potassium phosphate/2mM-$MgCl_2$, pH6.5. After about 300ml of this buffer had passed into the column 2mM-ATP was added to a further 200ml of buffer, which was passed into the column, followed by more buffer without ATP. Fructokinase was eluted by the ATP, and the active fractions were collected. The enzyme was not pure at this stage, and a further step involving anion-exchange chromatography was necessary. The pH of the pooled fraction was adjusted to 8.0 with 1M-Tris, and after ultrafiltration to a protein concentration of about 5mg/ml it was run on a DEAE-Trisacryl column ($4cm^2 \times 10cm$), pre-equilibrated in 20mM-Tris/HCl, pH8.0. A linear gradient to 0.25M-NaCl was applied to the column, and the enzyme was eluted at about 0.15M-NaCl (Fig. 2). The isolation of fructokinase is summarized in Table 1.

Properties of glucokinase

Purified glucokinase had a specific activity of 240 ± 20 units/mg (mean ± S.E.M., five prepara-

Table 1. *Purification of glucokinase, fructokinase and glucose-6-phosphate dehydrogenase from one extract of Z. mobilis cells*

For this, 70 g wet wt. of strain-ZM6 cells was extracted and processed as described in the text.

Fraction	Protein (mg)	Glucokinase Activity (units)	Glucokinase Sp. activity (units/mg)	Fructokinase Activity (units)	Fructokinase Sp. activity (units/mg)	Glucose-6-phosphate dehydrogenase Activity (units)	Glucose-6-phosphate dehydrogenase Sp. activity (units/mg)
Extract	5100	13800	2.7	8100	1.6	28000	5.5
Not absorbed on Scarlet MX-G–Sepharose	3600	0		7900	2.2	0	
Eluted by pH7	122	10500	85	0		0	
Eluted by $NADP^+$	81	0		0		26000	320
Not adsorbed on Yellow MX-G–Sepharose	2300	0		0		0	
Eluted by ATP	103	0		5700	55	0	

Secondary processing

	Protein (mg)	Activity (units)	Sp. activity (units/mg)	Overall recovery (%)
Glucokinase: after gel filtration	38	9000	240	65
Fructokinase: eluted from DEAE-Trisacryl column	25	6000	240	74
Glucose-6-phosphate dehydrogenase: $(NH_4)_2SO_4$ precipitate	50	25000	500	89

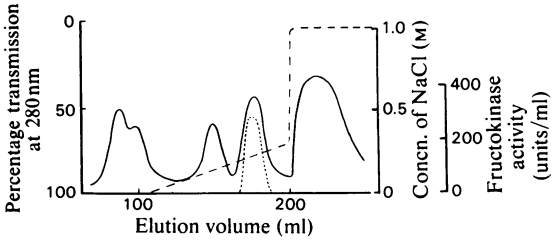

Fig. 2. *Elution of fructokinase (ZM6 strain) from a DEAE-Trisacryl column*

The applied sample was the ATP-eluted fraction from the Yellow MX-GR–Sepharose column after adjustment to pH 8.0 and concentration by ultrafiltration. The eluate was monitored at 280nm (——) in a 3mm-pathlength cell. The applied linear gradient in NaCl (– – – –) was followed by a stepwise change to 1 M-NaCl, which eluted a peak containing little protein, probably mainly nucleic acid. Fructokinase (·····) was eluted at about 0.15M-NaCl, preceded by a peak identified as phosphoglycerate mutase. When cells of strains ZM1 or ZM4 were used, the slightly less acidic character of fructokinase from these strains resulted in its elution closer to 0.1M-NaCl, causing overlap with the phosphoglycerate mutase peak.

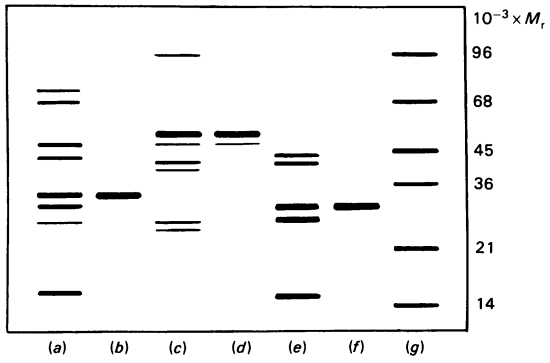

Fig. 3. *Diagram of sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms of glucokinase, glucose-6-phosphate and fructokinase preparations*

Fractions illustrated are: (*a*) glucokinase eluted from a Scarlet MX-G–Sepharose column; (*b*) glucokinase after gel filtration; (*c*) glucose-6-phosphate dehydrogenase eluted from a Scarlet MX-G–Sepharose column; (*d*) glucose-6-phosphate dehydrogenase after $(NH_4)_2SO_4$ purification; (*e*) fructokinase eluted from a Yellow MX-GR–Sepharose column; (*f*) fructokinase after DEAE-Trisacryl column chromatography; (*g*) standard proteins: rabbit muscle phosphorylase, bovine serum albumin, yeast phosphoglycerate kinase, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle adenylate kinase and hen egg-white lysozyme.

tions) at 25°C, measured at 10mM-glucose and 1mM-ATP. The $V_{max.}$ for saturating substrates is estimated to be 400 ± 40 units/mg. K_m values were determined to be 0.8mM for ATP at 10mM-glucose, and 0.22mM for glucose at 2mM-ATP. Compared with the values reported by Doelle (1982*b*), our K_m for ATP is significantly higher. Unlike him, we did not find any inhibition of the enzyme at the higher concentrations of ATP. The enzyme had no activity with, nor was it inhibited by, 10mM concentrations of fructose, 2-deoxyglucose, glucosamine, mannose, xylose, ribose, sorbitol, gluconate, glycerol, sucrose or galactose. It phosphorylated freshly dissolved α-D-glucose and β-D-glucose equally fast. In these examples activity was measured by the pyruvate kinase/lactate dehydrogenase method. Glucose 6-phosphate was weakly inhibitory, with an estimated K_i value of 15mM as a competitive inhibitor with ATP.

Whereas the glucokinase from *S. mutans* is activated by a number of bivalent cations other than Mg^{2+}, with Co^{2+} being the most effective (Porter *et al.*, 1980), the *Zymomonas* glucokinase had little or no activity with cations other than Mg^{2+} and Mn^{2+}, the latter giving about half the $V_{max.}$ with Mg^{2+}. However, we found that phosphate had a stimulatory effect of up to 50%, with optimal activity at 5mM-Mg^{2+} and 20mM-P_i.

Electrophoresis on dodecyl sulphate/polyacrylamide gels indicated a single subunit of size 33kDa (Fig. 3). Doelle (1982*b*) reported the molecular mass of the native enzyme from gel filtration to be 60kDa, and our gel-filtration results indicated 65kDa. Thus we conclude that the enzyme is dimeric, with almost the same size as the *B. stearothermophilus* enzyme (Hengartner & Zuber, 1973). Spectrophotometric studies of tryptophan and tyrosine (Goodwin & Morton, 1946) indicate three tryptophan and seven tyrosine residues per subunit, and a 1mg/ml absorption coefficient at 280nm of 0.80, based on protein determination at 205nm (Scopes, 1974). Treatment with 5,5′-dithiobis-(2-nitrobenzoic acid) did not inactivate the enzyme, but reacted slowly with two thiol groups per subunit. On addition of sodium dodecyl sulphate no more thiol groups were exposed to reaction with 5,5′-dithiobis-(2-nitrobenzoic acid).

Gel electrophoresis in non-dissociating conditions enabled staining of activity on the gel, giving a single band with the same mobility for the enzyme from all strains of *Zymomonas*.

Properties of fructokinase

The purified enzyme had a specific activity of 240 ± 30 units/mg (mean ± S.E.M., four preparations) at 25°C, measured at 10mM-fructose and 1mM-ATP. $V_{max.}$ is estimated to be 350 ± 40 units/mg with saturating substrates. K_m values were determined to be 0.45mM for ATP at 10mM-

fructose and 0.7mM for fructose at 1mM-ATP. These values are considerably higher than those reported by Doelle (1982*b*). The enzyme had no activity with, nor was inhibited by, 10mM concentrations of 2-deoxyglucose, xylose, ribose, sorbitol, gluconate, glycerol, sucrose, galactose, L-sorbose or xylulose. A trace of activity (approx. 0.5% that with fructose) was detected with mannose; however, contamination with fructose cannot be ruled out. Glucose is a powerful competitive inhibitor of fructokinase, with a K_i value of 0.14mM (Fig. 4).

Electrophoresis on dodecyl sulphate/polyacrylamide gels indicated a single subunit of size 28kDa (Fig. 3). In view of the fact that Doelle (1982*b*) reported a value by gel filtration of 85kDa for this enzyme, we undertook to repeat this work to see if the enzyme is a trimer. Gel filtration of our purified enzyme indicated that it emerged halfway between yeast phosphoglycerate kinase (45kDa) and bovine serum albumin (68kDa). Gradient-gel electrophoresis at pH9 in non-dissociating conditions indicated a size smaller than bovine serum albumin monomer. We thus conclude that *Zymomonas* fructokinase is a dimer, with a molecular mass of 56kDa. Spectrophotometric studies of tryptophan and tyrosine indicated three tryptophan and seven tyrosine residues per subunit (as with glucokinase), and the 1mg/ml absorption coefficient at 280nm was 0.90. 5,5'-Dithiobis-(2-nitrobenzoic acid) did not inactivate the enzyme, but it reacted with one thiol group per subunit. Treatment with sodium dodecyl sulphate exposed two further thiol groups per subunit.

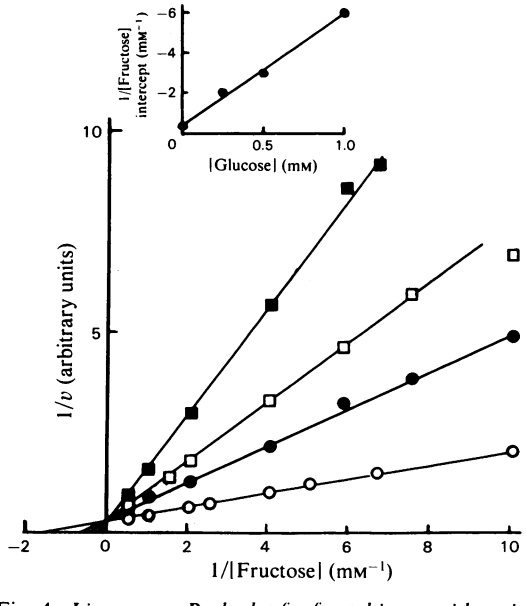

Fig. 4. *Lineweaver–Burk plot for fructokinase, with varied fructose concentration*

The diagram illustrates the competitive nature of glucose inhibition with respect to fructose. The ATP concentration was 1mM. ○, Zero glucose; ●, 0.25mM-glucose; □. 0.5mM-glucose; ■, 1.0mM-glucose. The re-plot of 1/[fructose] intercepts (inset) gives K_i 0.14mM for glucose.

Gel electrophoresis in non-dissociating conditions gave a single band on staining for fructokinase activity. However, strain-ZM6 fructokinase was more anodic in mobility than those from strains ZM1, ZM4 or ZM31 (which were identical, running at almost the same position as glucokinase). The same results were obtained whether the cells were grown on glucose or on glucose/fructose mixtures.

Properties of glucose-6-phosphate dehydrogenase

The purified enzyme had a specific activity of 500 ± 20 units/mg (mean ± S.E.M., eight preparations) at 25°C, measured with 1mM-glucose 6-phosphate and 1mM-NAD^+ at pH8.0. The $V_{max.}$ for saturating substrates is estimated to be 680 ± 50 units/mg. With $NADP^+$ at 1mM (and 1mM-glucose 6-phosphate), the specific activity was 390 ± 20 units/mg. K_m values were determined as listed in Table 1. These values are similar to those of the *L. mesenteroides* enzyme (Olive *et al.*, 1971), in that the affinity for $NADP^+$ is much greater than for NAD^+, and $V_{max.}$ is somewhat less with $NADP^+$. Efficient elution from the Scarlet MX-G–Sepharose column required an $NADP^+$ concentration of at least 0.2mM. Alternatively, NAD^+ at 1–2mM, but not less, could be used. We generally use a combination of each nucleotide. These concentrations reflect the relative affinity of the enzyme for the two nucleotides. It may be noted that Hey & Dean (1983) were unable to elute the *Leuconostoc* enzyme from their dye–ligand column even with 42mM-NAD^+, although $NADP^+$ at only 0.05mM was very effective.

The pH optimum is 8.0, as with most other glucose-6-phosphate dehydrogenases; it has 50% of its maximum activity with 1mM substrates at pH6.5, which is the likely pH inside *Zymomonas* cells (Barrow *et al.*, 1984). ATP is competitive with NAD^+, with K_i approx. 1.4mM (Table 2). Glucose is not inhibitory at 1M, and at this concentration is dehydrogenated at 1–2% of the rate of glucose 6-phosphate.

Electrophoresis on dodecyl sulphate/polyacrylamide gels indicated a single subunit of size 52kDa. The native size on Sephacryl S-200 gel filtration was 210 ± 10kDa, the enzyme emerging fractionally after rabbit muscle pyruvate kinase (228kDa). Thus, despite the kinetic similarities to the *Leuconostoc* enzyme, it differs in being tetra-

Table 2. *Kinetic constants for glucokinase, fructokinase and glucose-6-phosphate dehydrogenase from Z. mobilis strain ZM6*
Values (at 25°C) are apparent K_m values, except where indicated by *, in which case they are K_i values.

		K_m (or K_i*)					
	$V_{max.}$ (μmol/min per mg)	Glucose 6-phosphate	ATP	Glucose	Fructose	NAD^+	$NADP^+$
Glucokinase	400	15*	0.8	0.22	—	—	—
Fructokinase	350	—	0.45	0.14*	0.7	—	—
Glucose-6-phosphate dehydrogenase	680 (NAD^+) 390 ($NADP^+$)	0.17	1.4*	>500	—	0.21	0.04

meric rather than dimeric. Treatment with 5,5′-dithiobis-(2-nitrobenzoic acid) gave no reaction until the protein was denatured with dodecyl sulphate; two cysteine residues per subunit then reacted. The enzyme's tryptophan and tyrosine residues were estimated to be 10 and 19 per subunit respectively, giving an absorption coefficient at 280nm of 1.75 for a 1mg/ml solution.

Staining for activity after gel electrophoresis in non-dissociating conditions gave a single main band of activity in all strains. However, the enzyme in strain ZM31 was more anodic than those of strains ZM1, ZM4 and ZM6, which were not detectably different from each other.

Discussion

Measurements of enzyme activity in *Z. mobilis* have indicated that the fermentation rate is limited only by the amount of the first enzymes present, glucokinase (when growing on glucose) and glucose-6-phosphate dehydrogenase (Algar & Scopes, 1985). Similarly, when growing in fructose, the amount of fructokinase limits the overall rate of fructose fermentation to be approximately the same as that of glucose. In this case the intervening enzyme glucosephosphate isomerase is also present in amounts only just adequate for the maximum flux (V. Testolin & M. Zachariou, unpublished work). Nevertheless, the sugar uptake rate in *Z. mobilis* strains ZM4 and ZM6 is so great that the amounts of each of the enzymes exceed the amounts reported in any other bacterial species. This makes *Zymomonas* an excellent source of these enzymes and for others involved in the Entner–Doudoroff pathway.

The two kinases have certain similarities in their properties. Both are dimers with similar sizes, and have approximately the same kinetic properties, including $V_{max.}$ values. Each binds glucose with about the same avidity; for fructokinase this is inhibitory. But glucokinase is not inhibited by fructose. Both enzymes are constitutive, but are further induced by between 50% and 100% in the presence of their substrate, compared with cells grown in the absence of their substrate. Compared with the bacterial glucokinases previously described (Kamel *et al.*, 1966; Hengartner & Zuber, 1973; Porter *et al.*, 1982), the *Zymomonas* enzyme is about the same size as the *B. stearothermophilus* enzyme, but larger than that of *S. mutans* (M_r 41000). It does not have the wide metal-ion-specificity of the *S. mutans* enzyme, and its affinities for substrates are most similar to those of the *A. aerogenes* enzyme, although it does not phosphorylate glucosamine. Other fructokinases have not been described in such detail, although fructokinases of M_r 47000–49000 have been described that also phosphorylate mannose (Sapico & Anderson, 1967; Porter *et al.*, 1980). Glucose-6-phosphate dehydrogenase from *Zymomonas* shows considerable kinetic homology with the enzyme from *L. mesenteroides*. The functions of the enzymes are similar, in that they occupy key roles in anaerobic energy-producing metabolisms that may be regarded as inefficient; in each species only one ATP molecule is produced per molecule of glucose metabolized. The *Zymomonas* enzyme differs from most other glucose-6-phosphate dehydrogenases in being tetrameric; however, its subunit size is normal.

The use of dye–ligand chromatography for multiple enzyme purifications as illustrated here has many advantages, the most important being speed, since survival of enzymes in an impure state may be limited by the presence of proteinases. A further important benefit compared with most other types of isolation procedures is the ability to apply lysates and extracts directly to dye–ligand columns without prior processing. Choice of both 'positive' and 'negative' adsorption columns allows for high selectivity when they are operated in a differential mode, as illustrated here for the purification of fructokinase and elsewhere for other enzymes (Scopes, 1984; Scopes & Griffiths-Smith, 1984; Hey & Dean, 1983). Affinity elution further increases the chance of obtaining a high degree of purification, as is instanced by the present examples; usually a 20–50-fold purification can be achieved, with recoveries of at least 80%. In

the present example, three enzymes were purified simultaneously; with interposition of other dye–ligand columns, several other enzymes can be selectively removed from the extract without affecting the processing of the present three enzymes. For isolating only glucokinase or glucose-6-phosphate dehydrogenase, clearly only the Scarlet column needs to be used; for only fructokinase the Scarlet MX-G–Sepharose column can be omitted. Secondary processing by conventional techniques such as $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography and gel filtration can then be carried out (if necessary) on a sample that is relatively free of nucleic acids, carbohydrates and miscellaneous cell debris, which frequently create problems during the isolation of proteins from bacterial extracts.

This work was supported by the Australian Research Grants Scheme. E. M. A. was the recipient of a Commonwealth Postgraduate Award.

References

Algar, E. M. & Scopes, R. K. (1985) *J. Biotechnol.* in the press

Atkinson, T., Hammond, P. M., Hartwell, R. D., Hughes, P., Scawen, M. D., Sherwood, R. F., Small, D. A. P., Bruton, C. J., Harvey, M. J. & Lowe, C. A. (1981) *Biochem. Soc. Trans.* **9**, 290–293

Barrow, K. D., Collins, J. G., Norton, R. S., Rogers, P. L. & Smith, G. M. (1984) *J. Biol. Chem.* **259**, 5711–5716

Belaich, J. B., Senez, J. C. & Murgier, M. (1968) *J. Bacteriol.* **95**, 1750–1757

Dawes, E. A., Ribbons, D. W. & Large, P. J. (1966) *Biochem. J.* **98**, 795–803

Doelle, H. W. (1982*a*) *Eur. J. Appl. Microbiol. Biotechnol.* **14**, 241–246

Doelle, H. W. (1982*b*) *Eur. J. Appl. Microbiol. Biotechnol.* **15**, 20–24

Fine, I. H. & Costello, L. A. (1963) *Methods Enzymol.* **6**, 958–972

Gibbs, M. & De Moss, R. D. (1954) *J. Biol. Chem.* **207**, 689–694

Goodwin, T. W. & Morton, R. A. (1946) *Biochem. J.* **40**, 628–632

Hengartner, H. & Zuber, H. (1973) *FEBS Lett.* **37**, 212–216

Hey, Y. & Dean, P. D. G. (1983) *Biochem. J.* **209**, 363–371

Kamel, M. Y., Allison, D. P. & Anderson, R. L. (1966) *J. Biol. Chem.* **241**, 690–694

Lowe, C. R., Small, D. A. P. & Atkinson, A. (1981) *Int. J. Biochem.* **13**, 33–40

Olive, C. & Levy, H. R. (1966) *Biochemistry* **6**, 730–736

Olive, C., Gooch, M. E. & Levy, H. R. (1971) *J. Biol. Chem.* **246**, 2047–2057

Porter, E. V., Chassy, B. M. & Chester, E. H. (1980) *Infect. Immun.* **30**, 43–50

Porter, E. V., Chassy, B. M. & Holmlund, C. E. (1982) *Biochim. Biophys. Acta* **709**, 178–186

Qadri, F. & Dean, P. D. G. (1980) *Biochem. J.* **191**, 53–63

Rogers, P. L., Lee, K. R. & Tribe, D. E. (1979) *Biotechnol. Lett.* **1**, 165–170

Sapico, V. & Anderson, R. L. (1967) *J. Biol. Chem.* **242**, 5086–5092

Scawen, M. D., Hammond, P. M., Comer, M. J. & Atkinson, A. (1984) *Anal. Biochem.* **132**, 413–417

Scopes, R. K. (1974) *Anal. Biochem.* **59**, 277–282

Scopes, R. K. (1983) *Int. Patent Appl.* PCT/AU84/00069

Scopes, R. K. (1984) *Anal. Biochem.* **136**, 525–529

Scopes, R. K. & Griffiths-Smith, K. (1984) *Anal. Biochem.* **136**, 530–534

Scopes, R. K., Algar, E. M. & Griffiths-Smith, K. (1982) *Proc. Int. Congr. Biochem. 12th* 382

Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.* **79**, 544–552

Skotnicki, M. L., Warr, R. G., Goodman, A. E., Lee, K. J. & Rogers, P. L. (1983) *Biochem. Soc. Symp.* **48**, 53–86

Sly, L. I. & Doelle, H. W. (1968) *Arch. Microbiol.* **63**, 197–213

Swings, J. & De Ley, J. (1977) *Bacteriol. Rev.* **41**, 1–46