Purification and characterization of 5-ketofructose reductase from *Erwinia citreus*

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5-Ketofructose reductase [D(–)fructose:(NADP⁺) 5-oxidoreductase] was purified to homogeneity from *Erwinia citreus* and demonstrated to catalyse the reversible NADPH-dependent reduction of 5-ketofructose (D-threo-2,5-hexodiulose) to D-fructose. The enzyme appeared as a single species upon analyses by SDS/polyacrylamide-gel electrophoresis and isoelectric focusing with an apparent relative molecular mass of 40000 and an isoelectric point of 4.4. The amino acid composition of the enzyme and the N-terminal sequence of the first 39 residues are described. The steady-state kinetic mechanism was an ordered one with NADPH binding first to the enzyme and then to 5-ketofructose, and the order of product release was D-fructose followed by NADP⁺. The reversible nature of the reaction offers the possibility of using this enzyme for the determination of D-fructose.

INTRODUCTION

The utilization of D-fructose by acetic acid bacteria, notably of the genus *Gluconobacter*, involves conversion into 5-keto-D-fructose (D-threo-2,5-hexodiulose) using the enzyme D-fructose dehydrogenase (EC 1.1.99.11) [1]. This enzyme is membrane-bound in *Gluconobacter industrius*, catalyses the apparently irreversible oxidation of D-fructose to 5-ketofructose, and is suggested to have a cytochrome-*c*-like electron carrier [2]. The production of 5-ketofructose appears unique to acetic acid bacteria [1], which possess enzymes for its subsequent metabolism. In many cases, this involves the reconversion of the 5-ketofructose into D-fructose by an NADP(H)-linked 5-ketofructose reductase (5-KFR) (EC 1.1.1.124). Such an enzyme has been previously reported from *Gluconobacter* spp. [3] and, in contrast with the fructose dehydrogenase enzyme from the same organism, is located in the soluble protein fraction. The reversible cycling of D-fructose to 5-ketofructose may serve an important role in the reoxidation of NADPH during growth on D-fructose [1].

During a study of hexose interconversion in species of the genus *Erwinia*, we found that *Erwinia citreus* can use 5-keto-D-fructose as a carbon source, and possesses a soluble enzyme which functions as an NADPH-dependent 5-keto-D-fructose reductase, catalysing the reaction shown in Scheme 1. To compare this enzyme with those previously reported from *Gluconobacter* spp., we have purified the enzyme to homogeneity, as shown by a number of different criteria, and describe some physicochemical and catalytic properties. We also show that the enzyme, at suitable alkaline pH values, can oxidize D-fructose and can thus be used to assay D-

Scheme 1.

fructose. The apparent specificity of the enzyme for D-fructose as an oxidizable substrate makes it particularly suitable for this purpose.

MATERIALS AND METHODS

Chemicals, standards and enzyme substrates

NADP⁺, NADPH, NAD⁺, NADH, buffer salts, phenazine methosulphate and Nitro Blue Tetrazolium chloride were all obtained from Sigma Chemical Co. 2,5-Diketogluconate, 2-keto-D-gluconate, 5-keto-D-gluconate and 2-keto-L-gulonate were gifts from Shionogi and Co., Osaka, Japan. 5-Ketofructose was obtained from Chemalog (South Plainfield, NJ, U.S.A.) and other sugars and sugar analogues were from Sigma Chemical Co.

Abbreviations used: 5-KFR, 5-ketofructose reductase; PAGE, polyacrylamide-gel electrophoresis.

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Growth of *Erwinia citreus*

E. citreus was grown in a 10-litre fermenter (Chemap A.G., Volketswil, Switzerland) in a medium containing casein hydrolysate (20 g/litre), yeast extract (3 g/litre) and glycerol (40 g/litre) in a basal salts medium. Growth was at 30 °C and pH 7.0 (agitation, 1200 rev./min; aeration, 15 litres/min) for 18 h. Cells were harvested in an industrial centrifuge (Sharples T16; flow rate, 20 litres/h; 14000 g) and stored at -20 °C.

Enzyme purification

All stages in the purification of 5-KFR were performed at 4 °C. *E. citreus* cells (50 g wet wt.) were resuspended in 250 ml of 100 mM-sodium phosphate (pH 7.0) containing 0.5 mM-phenylmethanesulphonyl fluoride. The cells were passed twice through a French pressure cell (Aminco, Kontron, Basel, Switzerland) at 124 MPa. The suspension was centrifuged at 15000 g for 30 min and the supernatant was re-centrifuged for 1 h at 60000 g. The supernatant (260 ml) was dialysed for 4 h against 10 litres of 10 mM-sodium phosphate (pH 7.5) containing 5 mM-benzamidine hydrochloride and then applied to a 10 cm × 2.5 cm column of Q-Fast Flow (Pharmacia), equilibrated with 40 mM-sodium phosphate (pH 7.5). The column was washed with 100 mM-sodium phosphate (pH 7.5) until the absorbance at 280 nm of the effluent reached a base-line value. The column was eluted with a 1-litre linear gradient of sodium phosphate (100–300 mM, pH 7.5). Fractions containing 5-KFR, eluted at a phosphate concentration of 200 mM, were pooled (107 ml), and solid ammonium sulphate was added (0.52 g/ml) over a period of 30 min. The solution was stirred for 1 h and then centrifuged at 10000 g for 30 min. The pellet was dissolved in 10 ml of 50 mM-Tris/HCl, pH 7.8 (buffer A), containing 100 mM-NaCl and applied to a 90 cm × 2.5 cm column of Ultrogel AcA54 (LKB), equilibrated with the same buffer. Fractions (5 ml) containing 5-KFR activity were pooled (35 ml) and dialysed for 4 h against buffer A. The dialysate was applied (20 ml/h) to a 10 cm × 1.6 cm column of Matrex Green A (Amicon) equilibrated with buffer A, and the column was washed with the same buffer (200 ml). 5-KFR was eluted from the column with 50 ml of 0.2 mM-NADP⁺ in column buffer. Enzyme-containing fractions were pooled (12 ml), concentrated to about 2 ml, and applied to a 20 cm × 1.6 cm column of Sephadex G-50 (Pharmacia), equilibrated with 30 mM-Tris/HCl (pH 7.8).

Protein determinations

Protein was measured by the method of Lowry *et al.* [4], with bovine serum albumin as a standard.

Analytical separation methods

SDS/PAGE and isoelectric focusing were carried out as previously described [5].

The purity of carbohydrate substrates and reaction products was analysed by h.p.l.c. with an Aminex HPX-87H column (25 cm × 1.5 cm) and a Waters 510 chromatograph and Wisp 710 injection system. The column effluent was monitored using a refractometer. The samples were chromatographed at 30 °C using 5 mM-phosphoric acid (pH 3.0) as the solvent, and a flow rate of 400 µl/min.

Activity staining for 5-KFR

Following isoelectric focusing, the polyacrylamide gel containing 5-KFR was placed in a reaction mixture containing 450 mg of D-fructose, 25 mg of NADP, 15 mg of Nitro Blue Tetrazolium chloride, 1 mg of phenazine methosulphate and 5 ml of 1 M-glycine (pH 9.5), in a final volume of 50 ml. After incubation for 30 min at 22 °C, the gel was placed in a solution containing 7.5 % (v/v) acetic acid and 5 % (v/v) methanol. As a control a similarly prepared gel was incubated in a reaction mixture without D-fructose.

Amino acid analysis of reduced and alkylated protein

5-KFR (0.5 mg/ml) in 100 mM-Tris/HCl, pH 8.0, containing 4 M-guanidinium chloride and 5 mM-EDTA was incubated under N₂ with 10 mM-dithiothreitol for 1 h at 25 °C. Solid iodoacetamide was added (30 mM) and the solution was incubated for 30 min at 25 °C in the dark. The solution was then dialysed against 50 mM-NH₄HCO₃ and freeze-dried.

Protein samples were hydrolysed in constant-boiling HCl at 110 °C for 24, 48 and 72 h in N₂-flushed evacuated tubes in the presence of 1 mg of phenol/ml and 100 mM-ethanethiol. Hydrolysates were analysed on a Beckman 6300 amino acid analyser.

Tryptophan was determined as previously described by Edelhoch [6].

N-Terminal amino acid sequence determination by Edman degradation

Edman degradation was performed with a gas-phase sequencer (Applied Biosystems). Identification and quantification of phenylthiohydantoin derivatives was by reverse-phase h.p.l.c. with an external standard for quantification.

Analytical ultracentrifugation

Enzyme (0.45 mg/ml) in 50 mM-Tris, pH 7.8 (20 °C), containing 1 % (w/v) NaCl was brought to sedimentation equilibrium by centrifugation at 13 500 rev./min (15 °C) for 20 h. Sedimentation-velocity measurements were made at 50 000 rev./min and at 20 °C. All other details are as previously described [5].

Enzyme assays

Protein solution (1–10 µl) was added to spectrophotometer cells (1.5 ml, 10 mm light-path length) containing 0.1 mM-NADPH, 50 mM-5-ketofructose and 50 mM-Hepes (pH 7.0) in a total volume of 1.0 ml. The reaction mixture at 30 °C was monitored at 340 nm. The pH optimum of the reaction was determined as previously described [7].

RESULTS

Purification of 5-KFR

The purification of the enzyme is summarized in Table 1. The enzyme is most likely to be cytoplasmic in origin as all of the initial activity detected in the crude extract was recovered in the soluble fraction, i.e. the supernatant fraction following high-speed centrifugation of crude lysates (see the Materials and methods section). About 0.7 mg of pure protein was recovered from 50 g (wet wt.) of cells, which represents a recovery of 28 % of the original enzymic activity detected in cell extracts (Table

Table 1. Purification of 5-KFR

The cell extract was prepared from 50 g wet wt. of bacteria cells. Recovery values are relative to cell extract. One unit of enzyme activity produces 1 μmol of NADP⁺/min and D-fructose from 5-ketofructose and NADPH under conditions of maximum velocity.

Step	Volume (ml)	Concn. (units/ml)	Total activity (units)	Protein (mg/ml)	Specific activity (units/mg)	Recovery (%)	Purification
Cell extract	260	24.4	6330	25.6	0.952	100	(1.0)
Anion-exchange chromatography	107	35.4	3790	1.1	32.7	60	34
Gel filtration	35	71.4	2500	0.19	385	40	404
Dye-ligand chromatography	12	148.3	1780	0.06	2450	28	2573

Fig. 1. SDS/PAGE (12.5% acrylamide) of 5-KFR

Lane A contains 20 μg of the purified enzyme. Lane B contains standard proteins, the M_r values of which are indicated.

1). Based on the specific activity of the purified enzyme, we estimate that 5-KFR represents about 0.05 % of total *E. citreus* cell protein when the organism is grown under the conditions described.

There was no loss of activity when the enzyme (0.1–1 mg/ml) was stored for 4 weeks in 30 mM-Tris buffer at pH 7.8 and 4 °C, or when it was stored for at least 3 months in the same buffer at –20 °C. However, dilute protein solutions (1–10 $\mu\text{g}/\text{ml}$) lost about 20 % of their activity per week when stored at 4 °C.

The purified protein gave a single band on SDS/PAGE (Fig. 1) with an estimated molecular mass of 38 000–40 000. Isoelectric focusing (Fig. 2) of the enzyme also gave a single band with an estimated isoelectric point of 4.4. Activity staining of the isoelectric focusing gel (see the Materials and methods section) resulted in

Fig. 2. Electrofocusing of 5-KFR

Lane A contains 10 μg of enzyme subjected to activity staining for the oxidation of D-fructose. Lane B contains 10 μg of the enzyme subjected to Coomassie Blue staining. Lane C contains standard proteins with known isoelectric points.

the detection of a single band which was coincident with the Coomassie Blue-stained protein band (Fig. 2). These results, and that of N-terminal sequencing (see below), indicate that the enzyme was homogeneous and that the protein was responsible for catalysis of the reactions studied.

Chemical analysis of 5-KFR

The amino acid composition of the enzyme is given in Table 2. The *N*-terminal sequence of the first 39 residues is shown below:

where X indicates unidentified residues and residues in parentheses are tentative assignments.

The first cycle of Edman degradation indicated only alanine; no methionine derived from initiating *N*-formylmethionine was detected.

M_r

As described above, the M_r of the enzyme estimated by SDS/PAGE was 38 000–40 000. On the other hand, an M_r of 50 000 was determined by sedimentation-equilibrium measurements using the partial specific volume ($\bar{v}$) of 0.73 (Table 3). Sedimentation-velocity and size-exclusion chromatography measurements indicated a Stokes radius close to that predicted for a hydrated sphere of M_r 50 000 (Table 3). These data are consistent with the M_r of 50 000 determined by sedimentation equilibrium and indicates association of monomers.

Identification of D-fructose as the reaction product of 5-KFR

A reaction mixture containing 5-ketofructose, NADPH and purified 5-KFR was incubated at 30 °C for

15 min and then analysed by h.p.l.c. (Fig. 3). The h.p.l.c. profile of the reaction products, by comparison with appropriate standards, identified the reaction product as

D-fructose (Fig. 3). No other reaction products were detected.

Steady-state kinetic analysis

The steady-state kinetic parameters of the reaction are summarized in Table 4. Reactions involving the reduction of 5-ketofructose were performed at pH 7.0, and those for the oxidation of D-fructose or D-arabinose at pH 9.5, values found to be optimal for the respective reactions (results not shown). The K_m values obtained for NADPH, NADP⁺, 5-ketofructose and D-fructose are markedly different from those obtained by Avigad *et al.* [3] for a soluble 5-KFR from *Gluconobacter cerinus*. Furthermore, NADH did not substitute for NADPH in the reaction catalysed by the *Gluconobacter* enzyme, but does for the *Erwinia* enzyme reported here. We cannot relate the differences in enzymic properties between the two enzymes, as the purity and physicochemical properties of the *Gluconobacter* enzyme are unknown.

Product inhibition

A summary of the product inhibition studies with 5-KFR is presented in Table 5. The competitive type of inhibition observed for NADP⁺ as an inhibitor of D-fructose synthesis with respect to NADPH, and for NADPH as an inhibitor of D-fructose oxidation with respect to NADP⁺, indicates that the oxidized and reduced cofactor binds to the same site on the enzyme. These results, and the fact that all other product inhibition experiments resulted in non-competitive-type inhibition, suggests that in the synthesis of D-fructose, NADPH binds first to the enzyme followed by 5-ketofructose, with the order of product release being D-fructose followed by NADP⁺. However, these results do

Table 2. Amino acid composition of 5-KFR

The composition was estimated from 24 h acid hydrolysates and is the average of three estimations. The values of serine, threonine, leucine and isoleucine were extrapolated from the analysis of 24 h, 48 h and 72 h acid hydrolysates. The cysteine content was determined as carboxymethylcysteine (CmCys), derived from protein reduced with dithiothreitol and alkylated with iodoacetamide (see the Materials and methods section). The tryptophan content was estimated by u.v. absorption spectroscopy. The residue numbers were based on a protein M_r of 40 000, estimated by SDS/PAGE.

Residue	Composition	
	(mol %)	(mol/mol of 5-KFR)
CmCys	1.78	7
Asx	15.26	54
Thr	5.87	22
Ser	3.44	16
Glx	12.04	38
Pro	3.35	14
Gly	5.63	40
Ala	8.06	46
Val	4.86	20
Met	2.91	9
Ile	5.86	21
Leu	9.49	34
Tyr	2.82	7
Phe	3.35	12
His	0.68	2
Lys	6.64	21
Arg	4.62	12
Trp	2.30	5

Table 3. Sedimentation-velocity measurements on 5-KFR

The partial specific volume ($\bar{v}$) was estimated from the amino acid composition [8]. The Stokes radius (r_s^h) was calculated as previously described [9] using the experimentally determined M_r of 50 000 and a $\bar{v}$ of 0.73. r_s^2 was estimated by size-exclusion chromatography using Ultrogel AcA54 and r_s^3 was calculated for a sphere using the values indicated above. The value in parenthesis is for a hydrated sphere assuming 0.34 g of water/g of protein. The frictional ratio (f/f_0) compares r_s^h of the protein as given above with that of r_s^3 for non-hydrated and hydrated (value in parenthesis) spheres with M_r 50 000 and $\bar{v}$ 0.73.

Parameter	Value
$\bar{v}$	0.73 ml · g ⁻¹
$S_{20,w}^h$	4.20 S
r_s^h	2.84 nm
r_s^2	3.00 nm
r_s^3	2.44 nm (2.77 nm)
f/f_0	1.17 (1.03)

Fig. 3. H.p.l.c. identification of the product of the 5-KFR reaction

The three elution profiles show the reaction mixture at time zero (a) and after 15 min (b). (c) shows the sample in (b) with pure D-fructose added to verify the identity of the product. The peaks correspond to NADP(H) (a), 5-ketofructose (b) and D-fructose (c). The reaction mixture for the synthesis of D-fructose contained 23.6 μmol of NADPH, 9.6 μmol of 5-ketofructose, 20 μmol of sodium phosphate and 30 units of 5-KFR in a final volume of 960 μl (pH 7.2). At various times, 300 μl portions were heated for 1 min in a boiling-water bath, and then frozen at -80°C until analysis by h.p.l.c. (see the Materials and methods section). The reaction was monitored by dilution of 10 μl of the reaction mixture to 1 ml with water and reading the absorbance at 340 nm, where an absorption coefficient of $6.22\text{ mm}^{-1}\cdot\text{cm}^{-1}$ was used to determine NADPH.

not rule out other possibilities. The observation that cofactor binds to the enzyme before other substrates, and is released last after other products, is common for NADPH/NADP oxidoreductases.

Substrate specificity

It has been suggested that 5-KFR may be responsible for the degradation of 2,5-diketo-D-gluconate by acetic acid bacteria [2]. We therefore tested this compound as a possible substrate for the enzyme. The result was that no NADPH oxidation was observed. Identical results were also obtained with 2-keto-D-gluconate, 5-keto-D-gluconate and 2-keto-L-gulonate.

Table 4. Steady-state kinetic parameters for substrates of 5-KFR

Values are given $\pm$ S.E.M. Where appropriate, the nicotinamide nucleotide given in parenthesis represents the second substrate used for that determination. All steady-state kinetic experiments in the direction of the synthesis of D-fructose were conducted in the presence of 50 mM-Hepes (pH 7.0) at 30°C with 23 ng of enzyme. Reactions in the direction of the oxidation of D-fructose were conducted in the presence of 100 mM-glycine (pH 9.5) at 30°C with 0.92 μg of enzyme. The concentrations of the variable substrates used were from one-fifth to 5 times their respective K_m values, except in the case of NADPH (and NADH), where the maximum concentration used was 0.2 mM. Kinetic parameters were determined by using non-linear-regression-analysis computer programs [7] from which the maximum velocity constant (V) and the K_m values were obtained.

Substrate	K_m (mM)	$V_{\max}$ ($\mu\text{mol}/\text{min}$ per mg)
5-Ketofructose	12.7 ± 0.9	2900 ± 300 (NADPH) 2700 ± 500 (NADH)
D-Fructose	9.1 ± 0.7	26.5 ± 0.5 (NADP $^+$) 23.8 ± 0.1 (NAD $^+$)
D-Arabinose	140 ± 10	0.36 ± 0.01 (NAD $^+$)
NADP $^+$	0.053 ± 0.005	
NADPH	0.071 ± 0.004	
NAD $^+$	1.97 ± 0.08	
NADH	0.32 ± 0.07	

With the aim of using the reductase to enzymically measure D-fructose, we checked the specificity of the enzyme with a number of five- and six-carbon carbohydrates, including the epimers of D-fructose (D-psicose, D-tagatose and L-sorbose), for activity in the direction of NADP $^+$ reduction. In addition, the five-carbon ketoses, D-ribulose and D-xylulose, and the six-carbon aldo sugars, D-glucose, D-mannose, D-galactose and D-altrose, were also tested. No activity was detected with any of these compounds, confirming a high specificity for D-fructose. In contrast, the five-carbon aldo sugar D-arabinose was found to be a substrate for the enzyme. However, the dissociation constant was 15 times higher and the maximum velocity was over 5-fold lower than for D-fructose.

The configuration of the ring hydroxy groups of the two 5-KFR substrates D-fructose and D-arabinose are similar in both the pyranose and furanose ring forms, which may account for the above-mentioned ability of D-arabinose to serve as an alternative substrate. Although the major forms in solution of both D-fructose [10] and D-arabinose [11] are the pyranose forms, the furanose ring is also present. Further studies would be necessary to prove which form binds to the reductase.

DISCUSSION

We have described the purification of an enzyme from *E. citreus* with the properties of an NADP-dependent 5-KFR, which produces D-fructose as a product. The reverse reaction also occurs to a significant extent, but only at non-physiological pH values (pH optimum 10.3). Thus it seems likely that the function of the enzyme

Table 5. Inhibition patterns and constants obtained for product inhibition of the reaction catalysed by 5-KFR

The inhibition constants K_{ii} and K_{is} were determined as described in [7]. Abbreviations: C, competitive; NC, non-competitive.

Variable substrate	Constant substrate	Inhibitor	Inhibition type	K_{ii} (mM)	K_{is} (mM)
NADPH	5-Ketofructose (13 mM)	NADP ⁺	C	—	0.0698 ± 0.0099
NADPH	5-Ketofructose (13 mM)	Fructose	NC	210 ± 34	832 ± 83
5-Ketofructose	NADPH (0.1 mM)	NADP ⁺	NC	0.233 ± 0.13	0.981 ± 0.031
5-Ketofructose	NADPH (0.1 mM)	Fructose	NC	750 ± 110	102 ± 16
NADP ⁺	Fructose (20 mM)	NADPH	C	—	0.011 ± 0.002

in vivo is the conversion of 5-ketofructose into D-fructose. The oxidation of D-fructose *in vivo* presumably requires an additional D-fructose dehydrogenase, such as the membrane-bound enzyme reported for strains of *Glucobacter* [1].

Kinetic analyses using the purified enzyme indicated a high degree of substrate specificity. This suggests that the enzyme may be used to assay fructose in biological fluids. We have shown that this is possible (results not shown), and recombinant DNA technology could be used to produce large amounts of the enzyme for this purpose.

We thank Anke Filter, Ariane Bourquin and Gerardo Turcatti for expert technical assistance, and Nadine Huber for careful typing of the manuscript.

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Received 30 October 1987/8 January 1988; accepted 6 April 1988