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Purification of a stereospecific 2-ketoreductase from *Gluconobacter oxydans*

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The 2-ketoreductase from *Gluconobacter oxydans* (SC 13851) catalyzes the reduction of 2-pentanone to (*S*)-(+)-2-pentanol. The 2-ketoreductase was purified 295-fold to homogeneity from *G. oxydans* cell extracts. The purified 2-ketoreductase had a molecular mass of 29 kDa with a specific activity of 17.7 U/mg. (*S*)-(+)-2-pentanol was prepared on a pilot scale (3.2 kg of 2-pentanone input) using Triton X-100-treated *G. oxydans* cells. After 46 h, 1.06 kg (32.3 M%) of (*S*)-(+)-2-pentanol of >99% enantiomeric excess (ee) was produced. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 171–175.

Keywords: *Gluconobacter oxydans*; (*S*)-(+)-2-pentanol; 2-ketoreductase; enzyme purification

Introduction

The stereospecific reduction of carbonyl groups is of interest for the production of chiral alcohols. Enantiomerically homogeneous chiral secondary alcohols are useful intermediates for pharmaceuticals, agrochemicals, liquid crystals and perfumes and may be prepared from ketones using NADH/NADPH-dependent secondary alcohol dehydrogenases. Several other biochemical and chemical approaches have been used in the synthesis of enantiomerically pure alcohols, e.g., stereospecific chemical reduction of ketones, enzymatic hydrolysis of racemic esters or enzymatic esterification of racemic alcohols. Microbes and enzymes have been extensively used for the synthesis of chiral alcohols at laboratory, pilot, and production scales [10,13]. Such reactions can be carried out using resting cells, isolated enzymes and/or cloned and overexpressed enzymes. The carbonyl reductase isolated from *Rhodococcus erythropolis*, an NADH-dependent enzyme has a broad specificity in accepting a wide range of substrates such as 2-ketones, 3-ketones, keto-esters and aromatic ketones [14,15]. Baker's yeast has been widely used for the asymmetric reductive biotransformation of a variety of 2-ketones and 3-ketones [4–8,11,12]. *Gluconobacter oxydans* cells have been used in the reduction of various ketones to (*S*)-alcohols with high enantiomeric excess (ee) [1,2]. The present study describes the large-scale synthesis of (*S*)-(+)-2-pentanol catalyzed by a NADH-dependent reductase from *G. oxydans* in the presence of formate dehydrogenase for regeneration of cofactor (Figure 1). However, the process requires a large cell mass (the ratio of 2-pentanone to cell mass is 1 kg:50 kg) for the synthesis of the chiral alcohol. In order to facilitate the use of enzyme in an industrial process for the production of (*S*)-(+)-2-pentanol, the enzyme was purified for purposes of cloning and overexpression.

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Materials and methods

Chemicals

2-Pentanone, (*S*)-2-pentanol, and (*R*)-2-pentanol were purchased from Aldrich. DEAE cellulose was purchased from Whatman Inc. Phenylsepharose was obtained from Pharmacia Biotech.

Microorganisms

G. oxydans (SC 13851) was obtained from the Bristol-Myers Squibb (BMS) culture collection. The SC number denotes the number in the BMS culture collection.

Fermentation

G. oxydans was grown on a glycerol-containing medium as follows. The culture was grown in 500-ml shake flasks for 24 h in 100 ml of medium A (5% glycerol, 0.5% yeast extract, 0.05% ammonium sulfate, 0.3% peptone, 0.05% K_2HPO_4, 0.05% KH_2PO_4, and 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 7H_2O$). After 24 h the flasks were used to inoculate (1% inoculum) a 15-l fermentor containing medium A. The fermentor was operated at 28°C for 24 h. A 4000-l fermentor was inoculated with 10 l inoculum from the 15-l fermentor. The 4000-l fermentor contained medium A with 0.05% antifoam SAG 5693. The fermentor was operated at 28°C, 100 LPM airflow, 690 mbar pressure and 620 rpm agitation for 48 h.

Cell recovery

The fermentor broth was cooled to 8°C at the harvest. The tank was pressurized to 15 psig and broth was diverted to a Sharples centrifuge running at 18,000×g. The broth was processed at 3.2 l/min. The recovered cells were stored at −70°C until used.

Analysis of enantiomeric alcohols by gas chromatography

Samples were extracted in ethyl acetate and dried over anhydrous magnesium sulfate. Samples were applied onto an Astec Chiraldex

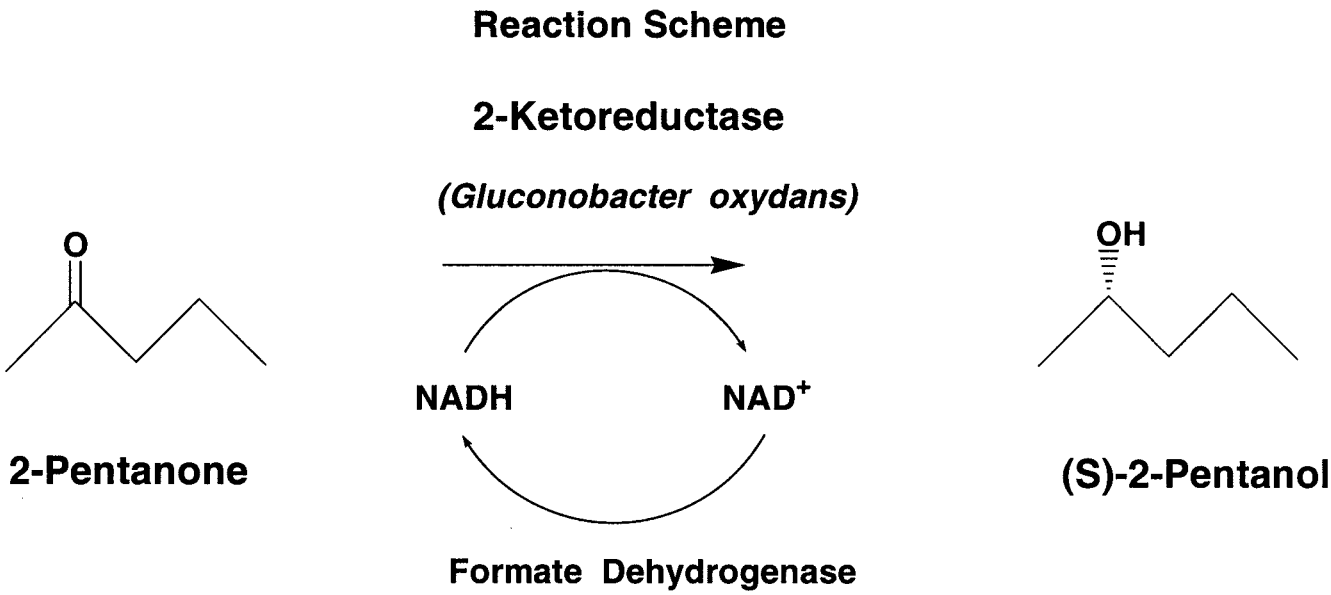

Figure 1 Reduction of 2-pentanone to (*S*)-(+)-2-pentanol by 2-ketoreductase.

G-TA, gamma cyclodextrin column (20 m×0.25 mm×0.125 μm thickness) equipped with a guard column (Hewlett-Packard Ultra II, 5% phenyl methyl silicone, 5 m×0.32 mm×0.17 μm thickness). The temperature of the injector was set at 150°C and the detector of the chromatograph (Hewlett-Packard 5890) was set at 200°C. Detection was with a flame ionization detector. The separation was carried out by a gradient with the following conditions (28°C for 15 min, 5°C/min to 50°C and hold 5 min). The helium flow rate was maintained at 22 cm/min. Under these conditions, the retention times for (*S*)-2-pentanol, (*R*)-2-pentanol, and 2-pentanone were 10.85, 11.67 and 17.84 min, respectively.

2-Pentanone reductase assay

2-Pentanone assays were carried out using resting cells and the cell extract (soluble enzyme) of *G. oxydans*.

Whole cell assays

Toluene treatment of whole cells: Three grams of wet cell paste were suspended in 15 ml buffer containing 0.1 M Tris–HCl, pH 8 and 5 mM EDTA. The cell suspension was treated with 0.36 ml toluene. The cell suspension was shaken gently for 30 min in a 50-ml Erlenmeyer flask. The cells were then collected by centrifuging them at 18,000×g for 20 min.

Triton X-100 treatment of whole cells: Three grams of wet cell paste were suspended in 15 ml buffer containing 0.1 M Tris–HCl, pH 8 and 5 mM EDTA. The cell suspension was treated with 0.45 g of Triton X-100. The cell suspension was shaken gently for 60 min in a 50-ml Erlenmeyer flask. The cells were then collected by centrifuging them at 18,000×g for 20 min.

On a large scale, the wet cell paste (10–20%) was suspended in 0.2 M Tris–HCl buffer pH 7.5 containing 1% w/w of the nonionic detergent Triton X-100, 10 mM $CaCl_2$ and 10 mM $MgCl_2$. After detergent treatment for 1 h, the cells were frozen, held at least overnight at −70°C, and then thawed before use in the bioconversion of 2-pentanone to (*S*)-(+)-2-pentanol.

Bioconversion procedure: Toluene-treated or Triton X-100-treated cells (0.25 g) were suspended in 10 ml of 0.2 M Tris–HCl buffer pH 7.5, containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$ in a 25

Table 1 Purification of 2-ketoreductase

Steps	Volume (ml)	Enzyme activity units	Protein (mg)	Specific activity units (mg)	(*S*)-2-pentanol (ee)	Purification (fold)
Cell extract	300	39.00	729.00	0.05		1.0
DEAE cellulose	180	235.80	185.40	1.27		25.4
Phenylsepharose	150	186.00	72.00	2.58		51.6
Amicon concentration (10 K membrane)						
Sephacryl S200 gel filtration	22	27.28	4.40	6.20	>99	124.0
Mono Q column	5.6	35.39	3.64	9.72		194.4
Centricon concentration (10 K membrane)						
Sephadex-75 gel filtration	0.75	13.28	0.90	14.76	>99	295.2

A unit (U) of enzyme activity is defined as 1 μmol of (*S*)-2-pentanol formed in 1 h under the conditions described in Materials and Methods. All purification steps were carried out at 4°C.

ml Teflon flask. To the reaction mixture were added 7 mg of NAD, 0.136 g of sodium formate, 1.5 units of formate dehydrogenase and 0.025 ml of 2-pentanone. The reaction mixture in the flask was incubated in a shaker at 28°C with agitation at 200 rpm. At various time intervals (2–18 h), 2 ml of ethyl acetate was added to 0.5 ml of reaction mixture to stop the reaction. The organic layer was separated by centrifugation and was used to analyze both the substrate and product.

Soluble enzyme assays: The assay was carried out by incubating the enzyme in a reaction mixture (5 ml) containing 0.35 mg of NAD^+, 68 mg of sodium formate, 0.75 units of formate dehydrogenase, and 5 mg of 2-pentanone. Reactions were carried out in a Teflon flask at 28°C on a shaker at 200 rpm. At the end of 18 h, the reactions were quenched with 10 ml of ethyl acetate and analyzed by gas chromatography.

Protein assay: The Bio-Rad protein assay was used to determine protein concentration. The assay was performed according to the manufacturer's (Bio-Rad) protocol. Samples containing 1–10 μl of enzyme fraction were brought up to 0.8 ml with water. Bio-Rad reagent (0.2 ml) was added and the solution was mixed thoroughly. The absorbance of the solution was measured at 595 nm. The protein concentration (mg/ml) was calculated from the standard curve with BSA as standard.

Enzyme activity units: A unit (U) of enzyme activity was defined as 1 μmol of (*S*)-2-pentanol formed in 1 h under the conditions described above. The specific activity was expressed as units per milligram (U/mg) of protein.

Purification of 2-ketoreductase

All purification steps were carried out at 4°C. Forty-four grams of cells were suspended in 0.3 l of buffer A (50 mM Tris–HCl, pH 7.5, 1 mM $CaCl_2$, and 1 mM Mg Cl_2). After 30 min of homogenization, the cell suspension was passed through a microfluidizer twice at 12,000 psi. The supernatant obtained by centrifugation at 30,000×g for 30 min was loaded onto a DEAE cellulose column (400 ml), which had been pre-equilibrated with buffer A. The enzyme activity was eluted with a 0 to 0.8 M NaCl gradient in buffer A. The active fractions were pooled, and ammonium sulfate at 132 g/l was added to the extract before loading it onto a phenylsepharose column (350 ml), which had been pre-equilibrated with buffer A containing 1 M ammonium sulfate. The column was then washed with buffer A containing 1 M ammonium sulfate and the enzyme was eluted with 1.2 l using a 1 to 0 M ammonium sulfate gradient. The fractions containing the active enzyme were pooled (150 ml) and concentrated with an Amicon YM-30 membrane to 8 ml. The enzyme was then loaded onto a Sephacryl S-200 gel-filtration column (400-ml column). The enzyme was eluted with buffer A containing 0.1 M NaCl with a flow rate of 0.8 ml/min. The active fractions from the gel-filtration column were pooled, and loaded onto a mono Q ion-exchange (BioRad Q2) column. The enzyme activity was eluted with a 0 to 0.8 M NaCl gradient in buffer A. The fractions containing the active enzyme were pooled (5.6 ml) and concentrated with a Centricon-30 membrane to 0.6 ml. The enzyme was then loaded onto a Superdex-75 gel-filtration column (FPLC) and eluted with buffer A containing 0.1 M NaCl. The enzyme present in fractions 13–15 was analyzed by SDS-PAGE.

Results and discussion

Fermentation

To prepare (*S*)-(+)-2-pentanol using resting cells, a 4000-l fermentor was inoculated with *G. oxydans* from a 15-l fermentor. The cell density increased for 36 h, and then remained constant. During fermentation, the pH dropped from 6.5 to 4.0 in 24 h and remained constant. After 24 h, the activity of the enzyme remained constant. At the time of harvest, the broth was cooled to 8°C, and

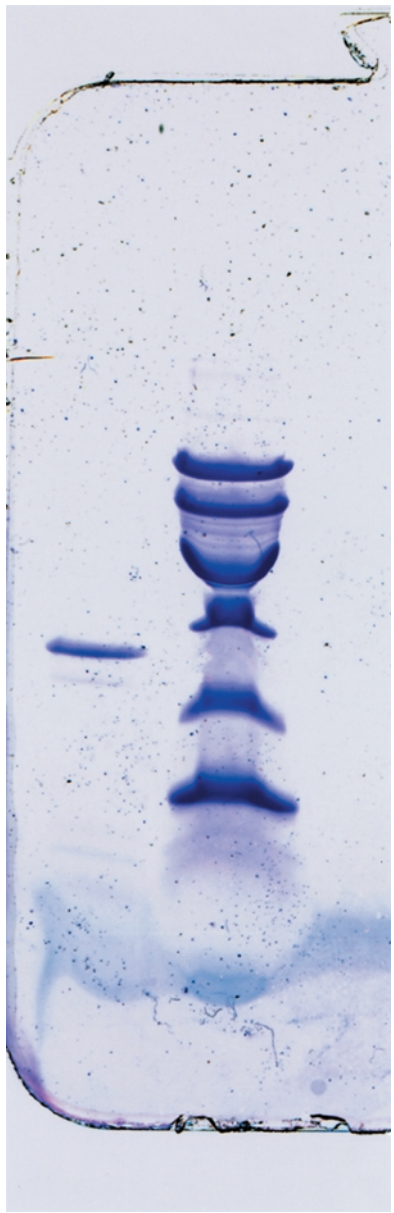

Figure 2 SDS-PAGE analysis of 2-ketoreductase from *G. oxydans* SDS-PAGE analysis on a Pharmacia phastgel homogeneous 20% gel and Coomasie Blue-R stained. Right lane: molecular mass standards (top to bottom: 94,000; 67,000; 43,000; 21,000; and 14,400 Da). Left lane: purified 2-ketoreductase.

the cells were recovered by centrifugation. Approximately 38.4 kg of wet cell paste was recovered from a 4000-l fermentor and stored at -70°C until used.

Bioconversion

Toluene treatment of *G. oxydans* cells increased permeability of the cells to the substrate and product [1]. The following simple and reproducible procedure was developed to increase the permeability of *G. oxydans* cells for the reduction of 2-pentanone to (*S*)-(+)-2-pentanol. Freezing and thawing the cell suspensions (10–20%) or wet cell paste, in the presence of 1% w/w of the nonionic detergent Triton X-100, increased their permeability. After detergent treatment, the cells were frozen, held at least overnight at -70°C, and then thawed before use in the bioconversion of 2-pentanone to (*S*)-(+)-2-pentanol. The freeze/thaw cycle was independent of cell concentration and is equally effective with a 10% or a concentrated wet cell paste. This procedure is highly reproducible and produces cells that catalyze the above reduction with reaction yields of 80–90 M% and 99% ee in the flask studies. Results compare favorably with those achieved by an earlier procedure that used toluene treatment and lyophilization [1].

The reduction of 2-pentanone required NADH cofactor which was regenerated during the biotransformation. The regeneration system involved the use of NADH-dependent formate dehydrogenase, which oxidizes formate to CO_2 [7]. The purity of formate dehydrogenase was a key factor in producing (*S*)-(+)-2-pentanol in high ee. Use of formate dehydrogenase from crude cell extracts of *Candida boidinii* SC 13822 produced racemic 2-pentanol. The formation of racemic 2-pentanol in the reaction might be due to the presence of other dehydrogenases in the cell extracts of *G. boidinii*. However, the use of partially purified formate dehydrogenase by phenylsepharose column chromatography from *C. boidinii* cell extract in the bioconversion process, produced (*S*)-(+)-2-pentanol in high ee. (*S*)-(+)-2-pentanol was prepared in a 1500-l reaction volume by microbial reduction of 2-pentanone using Triton X-100-treated *G. oxydans* cells. With an input of 3.2 kg of 2-pentanone and 10% w/v of Triton X-100-treated cells, the total (*S*)-(+)-2-pentanol produced after 46 h reaction time was 1.06 kg, for a reaction yield of 32.3 M%. The ee of the alcohol was >99%.

Purification of 2-ketoreductase

The purification of 2-ketoreductase from *G. oxydans* was initiated to clone and overexpress the enzyme. Expressing 2-ketoreductase and formate dehydrogenase in the same organism should facilitate a better system for producing (*S*)-(+)-2-pentanol.

The purification involved the following chromatography steps, DEAE cellulose ion-exchange, phenylsepharose, Sephacryl S200 gel filtration, mono-Q ion exchange and Sephadex-75 gel filtration (Table 1). With these steps, the resulting enzyme was purified 295-fold with a specific activity of 14.8 U/mg. The purified enzyme appeared as a single band on SDS-PAGE, with a molecular mass of 29 kDa (Figure 2).

The N-terminal and internal peptide sequences of the purified enzyme were determined in the Protein Chemistry Laboratory at the University of Pennsylvania. The N-terminal sequence of 2-ketoreductase (Figure 3) has homology with that of ribitol 2-dehydrogenase of *Klebsiella aerogenes*, which catalyzes the reduction of a five-carbon sugar, ribulose (2-ketone), to ribitol (2-OH), using NADH as a cofactor [8] and with the N-terminal sequence of trihydroxynaphthalene reductase [3]. The ribitol 2-dehydrogenase from *Enterobacter agglomerans* is a tetramer with four identical subunits and a molecular mass of 25 kDa for each subunit [9], while the enzyme purified from *G. oxydans* is a monomer with a molecular mass of 29 kDa. The secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* catalyzes the reduction of 2-pentanone to (*S*)-2-pentanol with a low ee and uses NADPH as a cofactor. In contrast, *G. oxydans* uses NADH as a cofactor and produces >95% ee for (*S*)-(+)-2-pentanol [1,16]. The use of the N-terminal and the internal peptide sequences should facilitate cloning and overexpression of the 2-ketoreductase in a suitable microbe that can be used on an industrial scale.

The N-terminal and internal peptide sequences of the purified 2-ketoreductase from *G. oxydans* SC 13851: N-terminal sequence: NH_2-Ser-Leu-Ser-Gly-Lys-Ile-Ala-Ala-Val-Thr-Gly-Ala-Ala-Gln-Gly-COOH; Internal peptides: Peptide 1: NH_2-Lys-Arg-Met-Ala-Glu-Ile-Thr-Gly-Thr-Glu-Ile-COOH; Peptide 2: NH_2-Lys-Val-Glu-Ala-Leu-Gly-Arg-Arg-Ala-Val-COOH.

Figure 3

Acknowledgements

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