J. Biochem. **136**, 447–455 (2004)
DOI: 10.1093/jb/mvh141

Purification, Characterization, Cloning and Expression of Pyruvate Decarboxylase from *Torulopsis glabrata* IFO005

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Received May 11, 2004; accepted June 26, 2004

In the production of pyruvate and optically active α-hydroxy ketones by *Torulopsis glabrata*, pyruvate decarboxylase (PDC, EC 4.1.1.1) plays an important role in pyruvate metabolism and in catalyzing the biotransformation of aromatic amino acid precursors to α-hydroxy ketones. In this paper, we have purified and characterized PDC from *T. glabrata* IFO005 and cloned the corresponding gene. A simple, rapid and efficient purification protocol was developed that provided PDC with high specific activity. Unlike other yeast or higher plant enzymes, known as homotetramers (α_4 or β_4) or heterotetramers ($\alpha_2\beta_2$), two active isoforms of PDC purified from *T. glabrata* IFO005 were homodimeric proteins with subunits of 58.7 kDa. We isolated the *T. glabrata* PDC gene encoding 563 amino acid residues and succeeded in overproducing the recombinant PDC protein in *Escherichia coli*, in which the product amounted to about 10–20% of the total protein of the cell extract. Recombinant PDC from *E. coli* was purified as a homotetramer. Targeted gene disruption of PDC confirmed that *T. glabrata* has only one gene of PDC. This PDC gene showed about 80% homology with the genes of other yeasts, and amino acid residues involved in the allosteric site for pyruvate in other yeast PDCs were conserved in *T. glabrata* PDC. Both native PDC and recombinant PDC were activated by pyruvate and exhibited sigmoidal kinetics similar to those of *Saccharomyces cerevisiae* and higher plants. They also exhibited the similar catalytic properties: low thermostability, similar pH stability and optimal pH, and complete inhibition by glyoxylate.

Key words: active dimmer, active staining, allosteric regulation, pyruvate decarboxylase, *Torulopsis glabrata*.

Abbreviations: PDC, pyruvate decarboxylase; ScePDC1, *Saccharomyces cerevisiae* pyruvate decarboxylase isoenzme 1; TglPDC, *Torulopsis glabrata* pyruvate decarboxylase; ZmoPDC, *Zymomonas mobilis* pyruvate decarboxylase; ThDP, thiamine diphosphate; SD, defined synthetic medium; ADH, alcohol dehydrogenase; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; DAB, diaminobenzidine; Bis-tris, bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane; IPTG, isopropylthio-β-D-galactoside; OPD, *o*-phenylenediamine; ELISA, enzyme-linked immunosorbent assay.

Wild-type and non-conventional yeasts are considered to have a more versatile secondary metabolism than *Sacchramyces cerevisiae* strains (baker's or brewer's yeast), which have been cultivated over decades to optimize product yield (*i.e.*, ethanol and carbon dioxide) at the expense of product diversity. *Torulopsis glabrata* was a superior species for pyruvate production and optically active α-hydroxy ketones production (*1*, *2*). For pyruvate production, it is important to keep balance of pyruvate metabolism in balance by regulating the activity of pyruvate decarboxylase (PDC, EC 4.1.1.1) (*1*, *3*). PDC plays a great role in *T. glabrata* during α-hydroxy ketones production. Cells of *T. glabrata* produced α-hydroxy ketones from α-keto acid and aromatic amino acid precursors by the action of PDC during fermentation (Fig. 1) (*2*). Thus, a detailed characterization of *T. glabrata* PDC is helpful for understanding its metabolic function and enzyme reaction mechanism in this organism.

PDC has been found in yeasts (*4*, *5*), fungi (*6*), bacteria (*7*, *8*) and plants (*9–11*). Dependent on the species from which the enzyme was isolated, either one (bacteria, haploid yeast and non-conventional yeast) or two (diploid brewer's yeast, pea, maize and wheat) types of subunits were detected by SDS-PAGE with molecular masses of about 60 kDa. Generally, the enzyme is a tetramer, with each monomer containing one molecule of the cofactor, thiamine diphosphate (ThDP), anchored to the subunit by coordination to a magnesium ion (Mg^{2+}). All these PDCs consist of subunits containing about 552–610 amino acid residues. Enzymes from plants (pea, maize, rice) have somewhat longer chains than those from yeasts, molds and bacteria. During the past decade, more studies of PDCs including their structure and function relationships have focused on *S. cerevisiae* and *Zymomonas mobilis* (*12*). PDC has two different catalytic properties: non-oxidative decarboxylation of α-keto acids and carboligase side-reaction leading to the formation of α-hydroxy ketones (*12*). PDC mainly catalyses the conversion of pyruvate to acetaldehyde, a key step in ethanol fermentation. In the glycolytic pathway, glucose is first broken down to form two molecules of pyruvate, supplying energy to cells in the form of ATP and NADH. The pyruvate formed has several metabolic fates (*13*).

Genes for PDC were also isolated from yeasts and fungi such as *S. cerevisiae* (*14–16*), *Hanseniaspora uvarum* (*17*), *Klyveromyces marxianus* (*18*), *K. lactis* (*19*),

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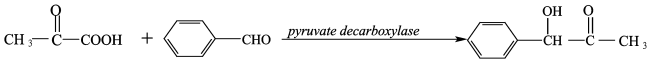

Fig. 1. **Formation of acromatic α-hydroxy ketones by pyruvate decarboxylase (PDC).**

Zygosaccharomyces bisporus (*20*), *Neurospora crassa* (*21*), *Aspergillus parasiticus* (*22*), *A. nidulans* (*23*), *A. oryzae* (*24*), from plants, like maize (*Zea maize*) (*25*), rice (*Oryza sativa*) (*26*), pea (*Pisum sativum*) (*27*) and from bacteria, such as *Z. mobilis* (*28*), *Zymobacter palmae* (*29*), *Acetobacter pasteurianus* (*30*) and *Sarcina ventriculi* (*31*). One way to confirm the homomeric form of active PDC is to clone and express the enzyme gene in a heterologous host. The same system could then be used to study structure-function relationships of this protein. So far, only a few PDC genes have been actively expressed (*30–32*).

In this study, we have established the presence of PDC in *T. glabrata* by cloning the gene and purifying the enzyme. Biochemical and kinetic properties of the enzyme are reported here and compared with those of homologues from yeasts, molds, bacteria and plants. Differences in properties among them might provide a useful guide for development of biocatalysis for optically active α-hydroxy ketone production and improvement of pyruvate production by regulating the activity of the enzyme.

EXPERIMENTAL PROCEDURES

Materials—Phenyl-Sepharose 6 Fast Flow, Q-Sepharose 6 Fast Flow, Superdex 200 and HMW native marker kit were from Amersham Pharmacia Biotech. NADH, CAPS, DAB and Bis-Tris were from Amresco Inc. (Solon, OH). Alcohol dehydrogenase was from Worthington Biochemical Corporation (Lakewood, NJ); 1,2-dianilionehtane was from ICN Pharmaceuticals Inc. (Irvine, CA). IPTG was from BBI International (Cotopaxi, CO). Sodium pyruvate, thiamine diphosphate, OPD and ampicillin were from Sigma. Bovine serum albumin was from Roche Molecular Biochemicals. Anti-Rabbit IgG conjugated horseradish peroxidase was from Vector Laboratories (Burlingame, CA). YNB (yeast nitrogen base) was from Difco Laboratories (Detroit, MI). All other chemicals were of the highest grade commercially available.

Strains, Plasmids, and Growth Media—T. glabrata IFO005 was a gift from Dr. Liu Jiquan (Southern Yangtze University, P.R. China). *S. cerevisiae* YSH 901(alpha leu2-3/112 ura3-52 trp1-92 SUC GAL pdcΔ::LEU2) was a gift from Prof. Stefan Hohmann (Göteborg University, Sweden). pRUL129 was obtained from Dr. H. Yde Steensma (Leiden University, The Netherlands); pMD18-T was from Takara; *E. coli* strain DH5α was from Stratagene; pET21a(+) and *E. coli* strain BL21(DE3) were from Navogen. Yeasts were grown in YEPD (1% yeast extract, 2% tryptone, 2% glucose, pH5.5) or SD (0.67% YNB, 2% glucose) and *E. coli* in LB (*33*) with 100 μg/ml ampicillin, if necessary.

Assay of Enzyme Activity—In the purification steps, PDC activity was measured by a coupled enzyme assay (*34*) with minor modification. Pyruvate is decarboxylated by PDC, producing acetaldehyde, which is reduced by an excess of ADH. The oxidation of NADH was followed by the decrease in absorption at 340 nm using a spectrophotometer. Assays were performed at 30°C and pH 6.0 in 100 mM citrate buffer, 33 mM sodium pyruvate with 1.5 units of yeast ADH in 3 ml volumes, unless otherwise noted. Enzyme activity was measured for the first 1–3 min. One unit of enzyme activity is defined as the activity to decarboxylate 1 μmol of pyruvate per min.

Purification of PDC—All purification procedures were performed at 4°C. *T. glabrata* IFO005 was grown overnight (18 h) in YEPD at 30°C. Cells were harvested by centrifugation at 5,000 × *g* for 10 min, washed twice in 20 mM phosphate buffer (pH 6.0), containing 5 mM $MgSO_4$ and 2 mM EDTA (buffer A) and resuspended (15 g cell paste in 50 ml) in buffer A. The cells were disrupted by sonication in ice-bath at 600 W for 20 min. The intact cells and debris were removed by centrifugation (15,000 × *g*, 30 min). The enzyme solution obtained was adjusted to 40% saturation with solid ammonium sulphate, stirred for 30 min and centrifuged (15,000 × *g*, 30 min). The supernatant solution was made up to 70% ammonium sulphate saturation and stirred for 8–10 h. After centrifugation (15,000 × *g*, 30 min), the pellet was resuspened in 10 ml of 20 mM phosphate buffer (pH 6.0) containing 5 mM $MgSO_4$ and 2 mM EDTA, 1 M ammonium sulphate (buffer B) and applied to a 10 mm × 20 cm column of phenyl-Sepharose 6 Fast Flow equilibrated with buffer B. The column was washed with 250 ml of buffer B, and then a decreased gradient 250 ml of buffer B and 250 ml of buffer A was applied at 1 ml/min. Fractions containing high activity were pooled and concentrated with PEG20000. The concentrated enzyme solution was dialyzed against 20 mM Bis-Tris containing 5 mM $MgSO_4$ and 2 mM EDTA (buffer C) and applied to a 10 mm × 20 cm column of Q-Sepharose 6 Fast Flow equilibrated with buffer C. The column was washed with 150 ml of buffer C, then an increased gradient of 150 ml buffer C and 150 ml of 20 mM Bis-Tris containing 5 mM $MgSO_4$, 2 mM EDTA and 0.4 M NaCl was applied at 1 ml/min. Fractions containing high activity were pooled and dialyzed against buffer A. The dialyzed enzyme solution was lyophilized and stored at –20°C. The recombinant PDC from *E. coli* was purified with the same procedure.

Gel Filtration—The purified enzyme was applied to a Superdex 200 column (10 mm × 30 cm) and fractionated at a flow rate of 0.5 ml/min with a fast protein liquid chromatography system (Amersham Pharmacia Biotech). Bovine pancreas chymotrypsinogen A (M_r 25 kDa), hen egg ovalbumin (M_r 43 kDa), bovine serum albumin (M_r 66 kDa), rabbit muscle aldolase (M_r 150 kDa), horse spleen ferritin (M_r 440 kDa) were used as molecular weight standard proteins.

Amino Acid Sequencing—The purified enzyme was subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) with SDS-Tris system using 10% polyacrylamide gel according to the procedure described by Laemmli (*35*) using a Mini-Protean III apparatus (Bio-Rad), then electrotransferred onto PVDF membrane (Millipore Corp.). The transferred protein was visualized with Commassie Brilliant Blue (0.25% Coomassie Blue R-250 in 45% methanol, 10% acetic acid). The band at about 60 kDa was excised and used for N-terminal amino acid sequencing with a Procise® Protein Sequencing System

(Applied Biosystems, Inc.) following the manufacture's procedures.

PAGE and Enzyme Activity Staining—The purified enzyme was subjected to native gradient PAGE using 5–12.5% linear gradient slab gel with a running buffer consisting of 25 mM Tris and 192 mM glycine (pH 8.8) at 4°C. Gels were fixed and stained in 0.25% Coomassie Blue R-250 in 45% methanol, 10% acetic acid. Activity staining with 1,2-dianilinoethane (*36*) was utilized for native gel.

Antibody Production and Western Blotting—Antibodies against PDC II from *T. glabrata* were raised in rabbits by four injections of emulsion containing 2 mg of enzyme (purified enzyme powder) in 40 d. The antibody titer was determined by diluting the antibody on an ELISA microtiter plate and by using the anti-rabbit IgG horseradish peroxidase conjugate system with OPD as staining reagent for detection. Western blotting was performed according to Candy *et al*. (*32*) with some modifications, and blots were stained with dissolved DAB (50 ml of phosphate buffer containing 30 mg of DAB and 75 μl of 30% H2O2).

Isolation and Sequencing Genomic DNA Clone Encoding PDC from T. glabrata—Based on the sequences of various yeast PDC genes in Genbank, we designed and synthesized oligonucleotides as primers to obtain partial DNA fragments encoding the structural gene of PDC by PCR against genomic DNA from *T. glabrata.* The oligonucleotides were 5′-GCGGATCCATGTCTGAAATTACTTTGG-3′ and 5′-GTTCTCGAGCTTATTGCTTAGCGTTGG-3′. The genomic DNA of *T. glabrata* was prepared by the glass-beads method (*37*). PCR was performed using *EX Taq* DNA polymerase (Takara) under following conditions: 94°C for 2 min, 30 cycles of 94°C for 40 s, 57°C for 40 s, and 72°C for 2 min, then 72°C for 5 min. The PCR product was directly cloned into a pMD18-T vector and sequenced by ABI 373A DNA sequencer (Perkin Elmer Life Sciences). Based on the obtained sequence of cloned PDC, the upstream sequence (199 bp) of the starting codon of *T. glabrata* PDC gene was cloned specifically following the instruction manual with TaKaRa LA PCR™ *in vitro* cloning kit. The specific oligonucleotides (S1, S2) were 5′-GTTCTCGAGCTTATTGCTTAGCGTTGG-3′ and 5′-CGTTGTTCAAGACGAACAAG-3′, respectively. From the sequence of the 5′ non-coding region, we designed and synthesized oligonucleotides to obtain the DNA fragments containing the 5′ non-coding region and coding region of the PDC gene by PCR against genomic DNA from *T. glabrata*. The oligonucleotides were 5′-CAGAAAGAAAAATTTTCTTC-3′ and 5′-GTTCTCGAGCTTATTGCTTAGCGTTGG-3′. PCR was performed using *EX Taq* DNA polymerase under the following conditions: 94°C for 2 min, 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 2 min, then 72°C for 10 min. The PCR product was directly cloned into a pMD18-T vector and sequenced by the same procedure as above. The whole sequence was submitted to GenBank.

Gene Disruption—To generate *T. glabrata* strains suitable for genetic transformation, strains bearing mutations in the gene (*URA3*) encoding orotidine 5′-phosphate decarboxylase were isolated by ethyl methansulfonate (EMS, Sigma Chemical Co., St. Louis, MO.) mutagenesis (*37*). The PDC-derived homologous DNA fragment I (Fig. 8A) was constructed. Then this DNA fragment was transfected into *T. glabrata ura3* strain by modified lithium acetate method (*37*), and *PDC* gene disruption was carried out by homologous recombination *in vivo* with *S. cerevisiae URA3* gene as selective marker (Fig. 8A). Targeted *PDC* gene disruption in *T. glabrata* was also confirmed by PCR amplification and sequencing of the *PDC* gene and its mutants (Fig. 8B). The primers for PCR amplification were 5′-CAGAAAGAAAAATTTTCTTC-3′ and 5′-GTTCTCGAGCTTATTGCTTAGCGTTGG-3′.

Expression of Recombinant PDC in E. coli and S. cerevisiae—To analyze expression in *E. coli*, the PCR product of the PDC gene was digested with *Bam*HI and *Xho*I to obtain a 1.7-kb fragment containing *T. glabrata* PDC coding region. This fragment was then cloned directly into the expression vector pET21a(+) downstream of the T7 promotor to produce pETP1, which was transfected into BL21(DE3) host cells for protein production. Transformed host cells were induced with IPTG (100 μM) and expressed the recombinant protein. Recombinant PDC was extracted from bacteria by votex-shaking (10 min) after adding glass beads and buffer A to the bacterial pellet. The lysate was cleared by centrifugation (15,000 × *g*, 30 min).

To analyze expression in *S. cerevisiae*, pRTP1 was constructed. Briefly, the PCR product of the PDC gene was digested with *Bam*HI and *Xho*I and cloned directly into pRUL129 to produce recombinant plasmid pRTP1. *S. cerevisiae* strain YSH 901 was transformed with pRTP1 by electroporation, followed by uracil selection on SD agar plates containing tryptophan and leucine. Transformed host cells were grown overnight at 30°C in SD medium containing tryptophan and leucine, and expressed the recombinant protein. PDC (containing ScePDC and recombinant TglPDC) was extracted from yeast by votex-shaking (10 min) after adding glass beads and buffer A to the yeast pellet. The lysate was cleared by centrifugation (15,000 × *g*, 30 min).

Other Methods—Protein concentration during the purification procedure was determined by the Bradford method (*38*) using bovine serum albumin as a standard. The basic molecular biology techniques were performed according to manufacture's instructions or standard procedures (*33*). Preparation of plasmid DNA from *S. cerevisiae* was performed by Zhao's method (*39*).

RESULTS

Purification of PDC—Cells of *T. glabrata* were harvested after 18 h of incubation in YEPD medium, when the maximum catalytic activity of PDC in *T. glabrata* was detected. Two pools of fractions with PDC activity were isolated by ammonium sulphate precipitation of *T. glabrata* IFO005 crude extract after hydrophobic interaction chromatography (Fig. 2). The two pools were purified to homogeneity by ion-exchange chromatography on a column of Q-Sepharose. As shown in Fig. 3, two purified isoforms of PDC (PDC I and II) both showed a single band with an apparent M_r of about 58.7 kDa on SDS-PAGE. Table 1 summarizes the purification of PDC from *T. glabrata* extract. Yields of 1.8 mg of PDC I protein and 3.5 mg of PDC II protein were obtained from 1 liter culture of *T. glabrata* with 18.1-fold and 33.3-fold purification and

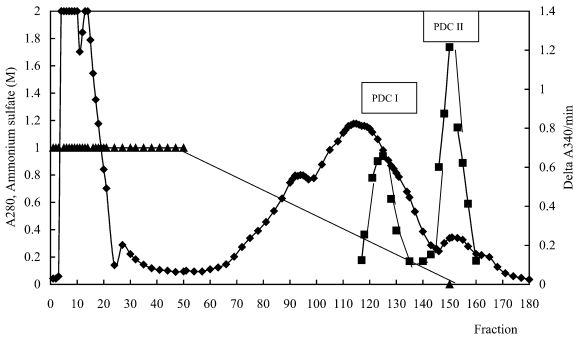

Fig. 2. **Phenyl-Sepharose 6 Fast Flow hydrophobic interaction.** A_{280} was the absorption of enzyme fraction at 280 nm; A_{340} is the decrease in absorption at 340 nm per min during enzyme reaction. PDC I: Fraction 118–135; PDC II: Fraction 143–160. open squares, protein concentration; solid squares, PDC enzyme activity; solid triangles, ammonium sulfate.

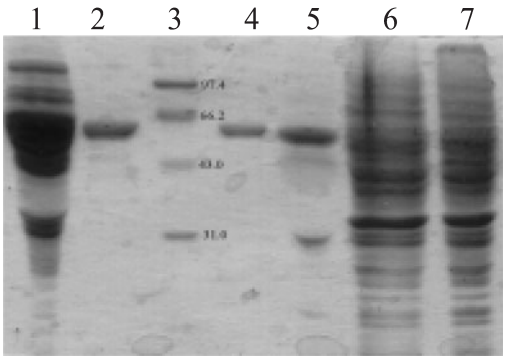

Fig. 3. **SDS-PAGE at various steps of PDC purification from *T. glabrata*.** Lane 7: Crude extract of *T. glabrata*; lane 6: $(NH_4)_2SO_4$ precipitation; lanes 1, 5: Phenyl-Sepharose 6 FF column (PDC I and PDC II, respectively); lanes 2, 4: after Q-Sepharose 6 FF column (PDC I and PDC II, respectively); lane 3: protein marker (97.4 kDa, 66.2 kDa, 43.0 kDa, 30.0 kDa, from top). The samples were analyzed by SDS-PAGE on 10% gel and stained with the Coomassie Brilliant Blue R-250.

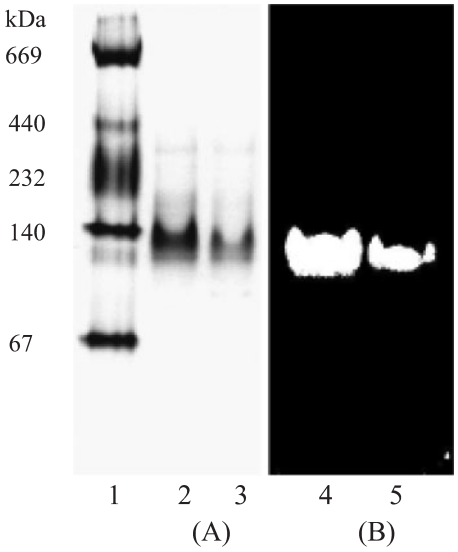

Fig. 4. **Native PAGE of PDC from *T. glabrata* after purification and activity stained native PAGE of PDC.** The samples were analyzed by gradient (5–12.5%) native PAGE and stained with the Coomassie Brilliant Blue R-250 (A) or activity staining (B) (41). Lane 1: protein marker; lanes 2, 4: PDC II; lanes 3, 5: PDC I

5% and 17% enzyme activity recovery, respectively (averaged values of 5 preparations). With lyophilization, the enzyme powder could be stored at –20°C for 3 months without loss of activity.

Physical Characterization, Activity Staining and Cross Reactivity—The M_r of both purified enzymes was estimated to be about 150 kDa by gel filtration and 120 kDa by native gradient PAGE (Fig. 4A) respectively. These results show that the two isoforms of native PDC from *T. glabrata* have a dimeric structure composed of two identical subunits. After activity staining, two white activity bands appeared in gradient native gel (Fig. 4B), indicating that both PDC I and II from *T. glabrata* have PDC activity. This is thought to be the first report of active dimeric proteins of PDC purified from yeast. Polyclonal antibodies against *T. glabrata* PDC II could be obtained from rabbits. The non-purified antibodies reacted with 58.7-kDa proteins (PDC I and II) (data not shown).

Kinetic Parameters of Native PDC—Steady-state analysis of the purified PDC was carried out. *T. glabrata* PDC I and II both showed the same sigmoid dependence of reaction rate on pyruvate concentration as the enzyme from *S. cerevisiae* (*40*), but differed from the Michaelis-Menten kinetics found with PDC from *Z. mobilis* (*7*). The kinetic parameters for PDC I and II were determined with respect to pyruvate. As shown in Fig. 5, the experimental data were analyzed with a modified Lineweaver-Burk plot (1/v versus 1/S2) (*20*, *41*). The values of K_m and k_{cat} from pyruvate were 0.8 mM and 43.6 s^{-1} for PDC I, and 0.9 mM and 77.0 s^{-1} for PDC II, respectively (Table 2). These K_m values were similar to those of many yeast and plant PDCs, including those purified from *S. cerevisiae* (1–3 mM) (*4*, *40*), *Z. bisporus* (1.73 mM) (*20*), *H.*

Table 1. **Purification of PDC from *Torulopsis glabrata* IFO005.**

Steps	Total protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	687.1	825	1.2	100	—
Ammonium sulphate precipitation	361.2	593	1.6	72	1.3
Phenyl-Sepharose 6 FF					
Pool I	28.6	127	4.4	15	3.7
Pool II	22.1	369	16.7	45	13.9
Q-Sepharose 6 FF					
Pool I	1.8	39	21.7	5	18.1
Pool II	3.5	140	40.0	17	33.3

The data shown here are the mean results of five purifications.

Table 2. **Kinetic parameters of purified PDC.**

Enzymes	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
Native PDC I	43.6	0.8	5.4×10^4
Native PDC II	77.0	0.9	8.5×10^4
Recombinant PDC	60.8	2.0	3.0×10^4

Kinetic constants were determined under standard assay conditions described in "EXPERIMENTAL PROCEDURES," k_{cat} was calculated from V_{max}, assuming that one subunit contains one active site. The molecular mass of PDC I and II was 120 kDa, and that of the recombinant PDC was 240 kDa.

uvarum (0.75 mM) (*17*), wheat germ (3.0 mM) (*9*), and pea (1.0 mM) (*11*). However, they are several-fold higher than those reported for the PDCs of *Z. mobilis* (0.3–0.4 mM) (*7*) and rice (0.25 mM) (*10*).

Stability of Temperature and Effect of pH—The stability of the native enzymes was examined at various temperatures. After samples of the enzymes had been preincubted for 60min in buffer A, their PDC activities were assayed as described in EXPERIMENTAL PROCEDURES. The activities relative to that at 30°C were as follows: (*1*) PDC I: 60°C, 3%; 50°C, 23.9%; 45°C, 43.7%; 40°C, 73.2 %; 30°C, 100%. (*2*) PDC II: 60°C, 2.7%; 55°C, 8.4%; 50°C, 43.1%; 45°C, 75.8%; 40°C, 93.2%; 30°C, 100%. The T_{50} value of these two isoforms of PDC was 44.3°C and 49.1°C, respectively. If the cofactors (ThDP and Mg^{2+}) were added into the enzyme solution during preincubation, the temperature stability of these enzymes increased. The T_{50} values increased by 4.6°C and 5.3°C, respectively. Storage at –20°C in a buffered solution containing their cofactors (ThDP and Mg^{2+}) also increased stability. The activity of PDC stored at –20°C for 24 h in the buffered solution not containing the cofactors decreased about by 34% and 28%, respectively. The stability of the two isoforms of PDC was also examined at various pH values. The enzyme was incubated at 30°C for 30 min in the following buffers (300 mM): sodium citrate (pH 4.0–6.0) and sodium phosphate (pH 6.0–8.0). Then a sample of the enzyme solution was taken, and the PDC activity was assayed under the standard conditions. The activities of two isoforms of PDC were stable in the range of pH 6.5–7.5 and decreased gradually at lower pH. At pH 4.0, almost no activity could be detected. The optimal pH for the activity of the two isoforms was determined in the buffers described above. The two enzymes showed their maximum activity at pH 6.0–6.5 (Fig. 6). According the product manual (Worthington Biochemical), yeast ADH has an optimum pH nearer to 7.0 for the reduction of acetaldehyde. However, in our PDC assay, the optimal pH of the two isoforms was still pH 6.0–6.5 when the amount of ADH was varied at the different pHs. Therefore, the pH-dependence of ADH activity did not affect the optimal pH of PDC.

Inhibition Studies—Various compounds were investigated for their inhibitory effect on enzyme activity. We measured the enzyme activity under standard conditions after incubation at 30°C for 30 min with various compounds added at different concentrations. The fact that the enzymes were completely inhibited by glyoxylate indicated that they were ThDP-containing enzymes (*42*). High concentration of ThDP also completely inhibited the enzyme activity (75 mM for PDC I, 100 mM for PDC II).

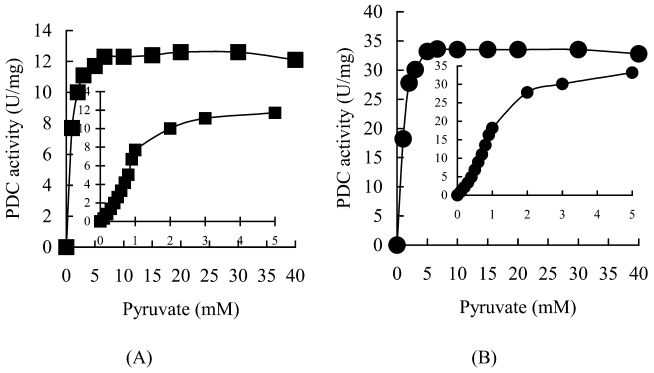

Fig. 5. **Dependence of the PDC I (A) and PDC II (B) reaction rate on the substrate concentration.** Measurements were done at a protein concentration of 0.8 mg/ml at 30°C in 0.1 M sodium citrate, pH 6.0.

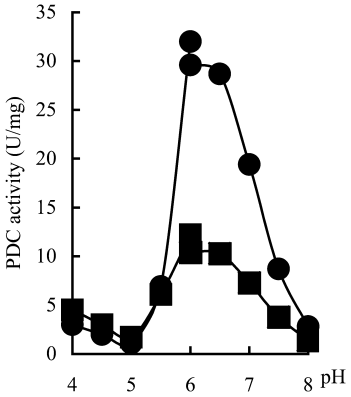

Fig. 6. **pH dependence of PDC activity.** Measurements were made at a protein concentration of about 0.8 mg/ml at 30°C in the following buffers: sodium citrate (0.1 M, pH 4.0–6.0) and sodium phosphate (0.1 M, pH 6.0–8.0). PDC I (filled square), PDC II (filled circle).

The enzymes were also completely inhibited by Ag^+ and Cu^{2+} (20 mM $AgNO_3$ and 20 mM $CuSO_4$). Other inorganic compounds (20 mM) such as $NiSO_4$, $FeSO_4$, $ZnCl_2$, $ZnSO_4$, Li_2SO_4, $CoCl_2$, $BaCl_2$, KCl, $CaCl_2$, $FeCl_3$, $Al_2(SO_4)_3$, $CrCl_2$, and $MnSO_4$ did not influence the activity. High concentration (1 M) of dithiothreitol could completely inhibited the enzyme activity. 2-Mercaptoethanol, urea and ethanol had no significant effect on the enzymes.

Cloning and Disruption of the PDC Gene—Three clones were isolated and sequenced. The nucleotide sequence of *T. glabrata* PDC (GenBank accession number AF545432) is shown in Fig. 7. The gene contains a 1692-base pair open reading frame (47.9% GC content) encoding a protein of 563 amino acid residues. The deduced amino acid sequence of PDC is also shown in Fig. 7. The molecular mass of 58.7 kDa obtained by SDS-PAGE was lower than the value of 62 kDa (average molecular weight) calculated from the deduced amino acid sequences of PDC. The sequence of the 5′ non-encoding region of the *T. glabrata* PDC gene contained a number of features: a TATA box at –115 and two CAAG boxes at –93 and –147. Targeted gene disruption of PDC confirmed that *T. glabrata* only has one PDC gene (Fig. 8)

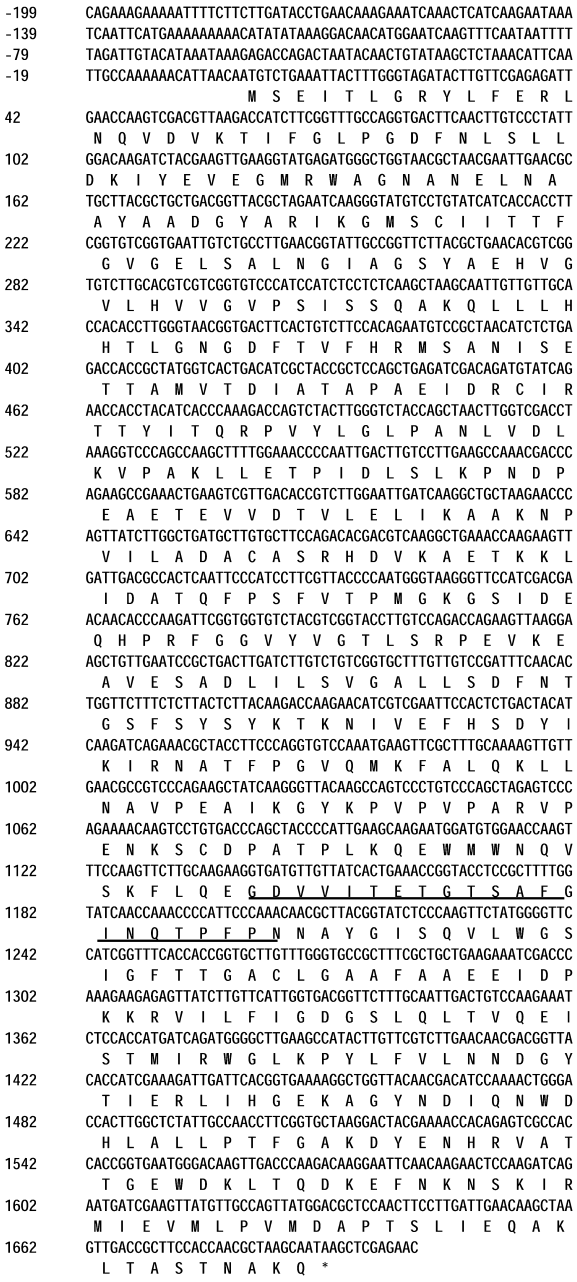

Fig. 7. **Nucleotide and deduced amino acid sequences of *T. glabrata* PDC gene.** The deduced amino acid is given below the nucleotide sequence. *Marks the termination codon. The underlined sequence is a conserved motif identified in TPP-dependent enzymes (*45*)

Sequence Comparisons—The non-redundant databases at the National Center for Biotechnology Information were searched for amino acid sequences similar to the *T. glabrata* PDC using BLAST algorithm. PDC genes from many other yeasts such as *S. cerevisiae*, *H. uvarum*, *K. marxianus*, *K. lactis*, *Z. bisporus* and *S. kluyveri* have high homology with that from *T. glabrata*. About 80% or more identity at the amino acid level was found for PDC from *S. cerevisiae* isoenzyme 1 (ScePDC1; 88%), *K. lactis* (KlaPDC; 85%), *K. marxianus* (KmaPDC, 87%), *H. uvarum* (HuvPDC, 77%), *S. kluyveri* (SklPDC, 86%) and *Z. bisporus* (ZbiPDC, 83%). In comparison with the *S. cerevisiae* PDC1 gene, the *T. glabrata* PDC gene possessed a greatly different 5′-noncoding region. The homology between *S. cerevisiae* and *T. glabrata* was very low (below 30%). However, sequences similar to the *S. cerevisiae* TATA concensus and initiator CAAG sequences were seen between –93 and –147 with an AT-rich area (*18*).

Expression of Recombinant PDC in E. coli and S. cerevisiae—The recombinant PDC produced was functional in *E. coli* and *S. cerevisiae* (Table 3), and the products amounted to about 10–20% of the total protein in the crude extract of *E. coli* (Fig. 9A). The recombinant enzyme in *E. coli* was purified to homogeneity using the same procedures as in *T. glabrata*. The recombinant PDC showed a 59 kDa band on SDS-PAGE (Fig. 9C). Polyclonal antibodies raised against purified *T. glabrata* PDC II recognized the bacterially produced recombinant protein (Fig. 9B). The N-terminal sequence of the purified recombinant enzyme was ASMTGGQQMGRGSMSEITLGRYLFE. The lack of N-terminal methionine suggested the cleavage by a native methionine aminopeptidase. The sequence of T7 fusion tag was ASMTGGQQMGRGS (pET System Manual, 9th ed., Novagen). Thus the recombinant PDC was a fusion protein containing a T7 tag, which should be useful upon purification with an affinity column. The sequence after T7 tag agreed with the deduced protein sequence. Hence, the molecular weight of recombinant PDC in SDS-PAGE was a little larger than the native enzyme. Attempts to determine the N-terminal amino acid sequence of the two isoforms of the native PDC failed because they were blocked. The catalytic properties of the recombinant PDC were similar to that of the native PDC, and no significant difference between the two enzymes was found in their steady-state kinetic parameters (Table 2).

DISCUSSION

Pyruvate decarboxylase in *T. glabrata* plays a key role in the production of pyruvate and optically active α-hydroxyketones (*1–3*). To study the properties of PDC, we isolated the enzyme and sequenced its gene from *T. glabrata*. This study established the presence of a functional PDC gene in *T. glabrata*. Together with alcohol dehydrogenase (ADH), PDC directs pyruvate to CO_2 and ethanol as the end-products in *T. glabrata* fermentation. After disruption of the PDC gene in *T. glabrata*, no PDC activity could be detected by activity staining (Fig. 8C). The accumulation of pyruvate increased markedly, and the level of ethanol decreased during fermentation (Wang, Q., and Jiang, N., unpublished data). The results of gene disruption showed that only one PDC gene existed in *T. glabrata*, unlike *S. cerevisiae*, which has several PDC genes (*13*)

PDC purified from *T. glabrata* had high specific activity (about 40 U/mg). PAGE and re-chromatography demonstrated homogeneity. The most interesting finding was that we purified active dimers of PDC from *T. glabrata*. Tetramers of ScePDC are known to reversibly dissociate into dimers under alkaline conditions, and those dimers could indeed be active (*43*). Moreover, the X-ray structure of ScePDC (*44*) indicated that the tetramer could potentially dissociate into two different dimers, depending on

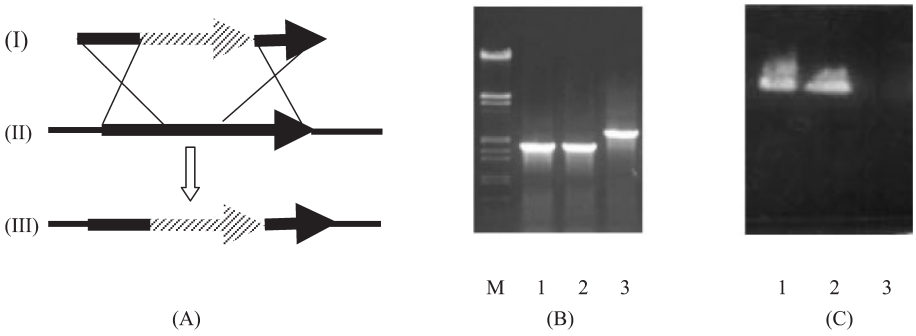

Fig. 8. **Schematic representation of the disruption of *PDC* by homologous recombination (A), PCR analysis (B) and activity staining (C) of *T. glabrata* mutants *T. galbrata PDC* gene or fragments (black), *S. cerevisiae URA3* gene (*ScURA3*, hatched).** (I) PDC-derived homologous DNA fragment; (II) *T. galbrata PDC* locus before disruption; (III) *T. galbrata PDC* locus after disruption. 1, *T. glabrata* IFO005; 2, *T. glabrata ura3*; 3, *T. glabrata pdc*; M, DNA maker (λ DNA/*Eco*RI + *Hind*III: 21,227, 5,148, 4,973, 4, 268, 3,530, 2,027, 1,904, 1,584, 1,375, 947, 831, 564, 125 bp).

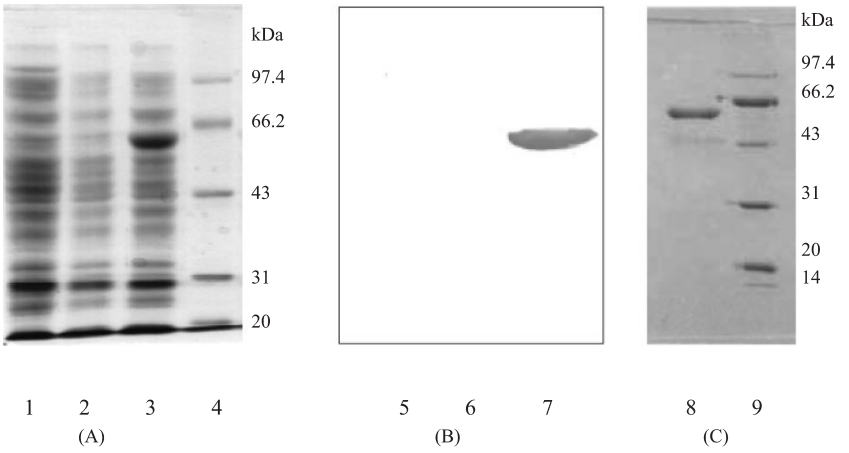

Fig. 9. **Expression of the cloned yeast PDC gene in *E. coli* (A), Western blot (B), SDS-PAGE of purified recombinant PDC from *E. coli* (C).** Lanes 1, 5: crude extract of *E. coli* BL21(DE3); lanes 2, 6: crude extract of *E. coli* BL21(DE3) /pET21a; lanes 3, 7: crude extract of *E coli* BL21(DE3)/pETP1; lane 8: purified recombinant PDC; lanes 4, 9: protein marker (97.4, 66.2, 43, 31, 20, 14 kDa).

the interface between subunits along which it was cleaved. However, to our knowledge, no work has been reported on directly purifying active dimers of PDC from microorganisms or plants. The two active dimers from *T. glabrata* were different in specific activity and kinetic parameters. Therefore, they could have formed through dissociation of a tetramer with two different cleavage patterns: to separate subunits interacting via α and γ domains, or to separate subunits interacting via β domains (*44*). Thus, we postulated that these two dimers were composed of the same subunit but different in three-dimensional structure, which could result in the minor difference in enzymatic kinetics. As shown in Fig. 10, most PDC in the crude extract from *T. glabrata* was dimeric, but a small percentage was present as the tetramer. Although some tetramer PDC existed in *T. glabrata*, purified homotetramer was not obtained, probably because the tetramer readily dissociates or is unstable. However, recombinant PDC was homotetramer in engineered *E. coli*. The molecular mass of the purified recombinant PDC by gel filtration was 240 kDa. It is possible that the T7 tag in recombinant PDC stabilized the tetramer in *E coli*. Consequently, it is necessary to analyze the structures of these three PDCs (two dimers and one recombinant tetramer) to further explore their structure and function.

In our purification processes, the most important and efficient step was phenyl-Sepharose hydrophobic interaction chromatography. This step was chosen after many failed attempts with various other column chromatography strategies. Only with this column chromatography could two different active dimers be obtained. The kinetic behavior of the two isoforms of *T. glabrata* PDC was very similar to that of PDC from other yeast and plants. These *in vitro* data supported the suggestion that PDC activity is controlled by pH and the pyruvate concentration *in vivo*. The optimal pH for PDC (including native dimers and recombinant tetramer) enzymatic activity was about 6.0–6.5. Thus, almost all PDCs from different sources had similar optimal pH. Although we did not detect that

Table 3. **PDC activity in engineered *E. coli* and *S. cerevisiae*.**

Strains	Protein concentration (mg/ml)	Enzyme activity (U)	Specific activity (U/mg)
E. coli BL21(DE3)	2.31	0.96	0.42
E. coli BL21(DE3) /pET21a(+)	1.93	1.04	0.54
E. coli BL21(DE3) /pETP1	1.36	10.14	7.46
S. cerevisiae YSH901	5.78	7.97	1.38
S. cerevisiae YSH 901-1/pRUL129	4.44	6.67	1.50
S. cerevisiae YSH 901-3/pRTP1	3.89	11.87	3.05

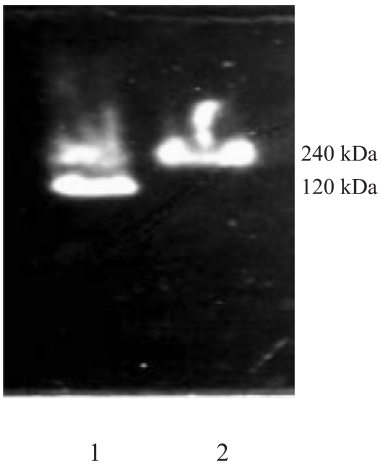

Fig. 10. **Activity Staining with crude extract of *T. glabrata* and engineered *E. coli* (*E coli* BL21(DE3)/pETP1).** Protein concentration: lane 1: yeast crude extract (10 mg/ml); lane 2: engineered *E. coli* crude extract (10 mg/ml).

the coenzyme (ThDP) could promote PDC activity, and a high concentration of ThDP actually inhibited the PDC activity, no white precipitation band was produced if ThDP was omitted from buffer during enzyme activity staining. This experiment showed that ThDP was the coenzyme of PDC. Glyoxylate, which was an inhibitor of ThDP-containing enzymes, formed a non-cleavable bond with the catalytic center of PDC and could completely inhibit the enzyme activity of PDC (*42*).

Sequence comparison between the *T. glabrata* PDC and other yeast PDCs showed that conserved amino acids were distributed throughout the entire sequence. The conserved motif of ThDP-dependent enzymes identified by Hawkins *et al*. (1989) (*45*) and known to be involved in Mg^{2+}-ThDP cofactor binding was highly conserved in these yeast PDC proteins. Amino acid residues located within 0.4 nm of the binding site for Mg^{2+} and ThDP in the PDC proteins crystallized from *S. cerevisiae* and *Z. mobilis* (*6*, *46*) were conserved in primary sequences with the *T. glabrata* PDC protein. These included residues similar to the aspartate (SceD444, ZmoD440) and asparagines (SceN471, ZmoN467) residues involved in binding Mg^{2+}. The *T. glabrata* PDC appeared to be similar to *S. cerevisiae* PDC in binding the diphosphates of ThDP, where serine and threonine side-chains (SceS446 and T390) as well as the main chain nitrogen of iosleucine (SceI476) are conserved. *T. glabrata* PDC also possessed aspartate, glutamate, threonine and histidine residues (SceD28, E477, T388, H114, H115; ZmoD27, E473, T388, H113, H114), which might potentially interact with intermediates during the decarboxylation reaction mediated by *Z. mobilis* and *S. cerevisiae* PDC. Furthermore, the iosleucine (Sce and Zmo I415) side-chain that appeared to stabilize the V conformation of ThDP through Van der Waals interactions, as well as the glutamate (SceE51, ZmoE50) that might donate a proton to the N1′ atom of TPP, were conserved in the *T. glabrata* PDC protein sequence. These results suggested that residues of the *T. glabrata* PDC protein might allosterically bind the substrate activator with a mechanism common to the majority of yeast PDC proteins. Cluster analysis of PDC proteins from microorganisms (data not shown) also suggested that the *T. glabrata* PDC was most closely relately to the *S. cerevisiae* PDC1.This was consistent with the evolutionary relationships among *Candida* (*Torulopsis*) species and relatives (*47*).

We have succeeded in constructing a PDC overproduction system in *E. coli*. The growth rate of *E. coli* was not inhibited even after the expression was induced, and the overproduced PDC was catalytically active even though the recombinant enzyme was in the form of fusion protein containing a T7 tag. Thus, this system could then be used to study structure-function relationships of the protein. In addition, due to the low expression level of recombinant PDC in yeast, no further studies could be carried out.

This work also expands the database of PDC sequences. In the future, we hope to use this information to guide experiments to determine the mec hanism of enzyme reaction and to determine what controls the substrate specificity of the enzyme. Further studies will also be needed to clarify the reasons for the production of active dimers in *T. glabrata*.

This work was supported by the National Knowledge Innovation Project of the Chinese Academy of Sciences. We thank Dr. Martina Pohl and Dr. Keqian Yang for their advice and constructive criticisms throughout our investigation. We thank Drs. Liu Jiquan, Stefan Hohmann and H. Yde Steensma for their gifts of strains, *Torulopsis glabrata* IFO005, *S. cerevisiae* YSH 901, and plasmid pRUL129.

REFERENCES

1. Yonehara, T. and Miyata, R. (1994) Fermentative production of pyruvate from glucose by *Torulopsis glabrata*. *J. Ferment. Bioeng.* **78**, 155–159
2. Miyata, R. (2000) Manufacture of optical active alpha-hydroxy ketone for use in pharmaceutical and agrochemicals, involves adding aldehyde into culture of microorganism with pyruvic acid producing ability-also useful for the preparation of I-ephedrine and as a flavor enhancer. JP2000093189
3. Miyata, R. and Yonehara, T. (1999) Breeding of high-pyruvate-producing *Torulopsis glabrata* with acquired reduced pyruvate decarboxylase. *J. Biosci. Bioeng.* **88**, 173–177
4. Killengerg-Jabs, M., König, S., Hohmann, S., and Hübner, G. (1996) Purification and characterisation of the pyruvate decarboxylase from a haploid strain of *Saccharomyces cerevisiae*. *Biol. Chem. Hoppe Seyler* **377**, 313–317
5. Krieger, F., Spinka, M., Golbik, R., Hübner, G., and König, S. (2002) Pyruvate decarboxylase from *Kluyveromyces lactis*. An enzyme with extraordinary substrate activation behaviour. *Eur. J. Biochem.* **269**, 3256–3263
6. König, S. (1998) Subunit structure, function and organisation of pyruvate decarboxylases from various organisms. *Biochim. Biophys. Acta* **1385**, 271–282
7. Miczka, G., Vernau, J., Kula, M.R., Hofmann, B., and Schomburg, D. (1992) Purification and primary structure of pyruvate decarboxylase from *Zymomonas mobilis*. *Biotechnol. Appl. Biochem.* **15**, 192–206
8. Lowe, S.E. and Zeikus, J.G. (1992) Purification and characterization of pyruvate decarboxylase from *Sarcina ventriculi*. *J. Gen. Microbiol.* **138**, 803–807
9. Zehender, H., Trescher, D., and Ullrich, J. (1987) Improved purification of pyruvate decarboxylase from wheat germ. Its partial characterisation and comparison with the yeast enzyme. *Eur. J. Biochem.* **167**, 149–154

10. Rivoal, J., Ricard, B., and Pradet, A. (1990) Purification and partial characterization of pyruvate decarboxylase from *Oryza sativa* L. *Eur. J. Biochem.* **194**, 791–797
11. Mücke, U., König, S., and Hübner, G. (1995) Purification and characterisation of pyruvate decarboxylase from pea seeds (*Pisum sativum* cv. Miko). *Biol. Chem. Hoppe Seyler* **376**, 111–117
12. Pohl, M. (1997) Protein design on pyruvate decarboxylase (PDC) by site-directed mutagenesis. Application to mechanistical investigations, and tailoring PDC for the use in organic synthesis. *Adv. Biochem. Eng.* **58**, 15–43
13. Zimmerman, F.K. and Entian, K. (1997) *Yeast Sugar Metabolism* pp.187–233, Technomic Publishing Co., Inc., Lancaster
14. Kellermann, E., Seeboth, P.G., and Hollenberg, C.P. (1986) Analysis of the primary structure and promoter function of a pyruvate decarboxylase gene (PDC1) from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **14**, 8963–8977
15. Seeboth, P.G., Bohnsack, K., Hollenberg, C.P. (1990) pdc1^0 mutants of *Saccharomyces cerevisiae* give evidence for an additional structural PDC gene: cloning of PDC5, a gene homologous to PDC1. *J. Bacteriol.* **172**, 678–685
16. Hohmann, S. (1991) PDC6, a weakly expressed pyruvate decarboxylase gene from yeast, is activated when fused spontaneously under the control of the PDC1 promoter. *Curr. Genet.* **20**, 373–378
17. Holloway, P. and Subden, R.E. (1994) The nucleotide sequence and initial characterization of pyruvate decarboxylase from the yeast *Hanseniaspora uvarum*. *Yeast* **10**, 1581–1589
18. Holloway, P. and Subden, R.E. (1993) The isolation and nucleotide sequence of the pyruvate decarboxylase gene from *Kluyveromyces marxianus*. *Curr. Genet.* **24**, 274–277
19. Bianchi, M.M., Tizzani, L., Destruelle, M., Frontali, L., and Wesolowski-Louvel, M. (1996) The 'petite-negative' yeast *Kluyveromyces lactis* has a single gene expressing pyruvate decarboxylase activity. *Mol. Microbiol.* **19**, 27–36
20. Neuser, F., Zorn, H., Richter, U., and Berger, R.G. (2000) Purification, characterisation and cDNA sequencing of pyruvate decarboxylase from *Zygosaccharomyces bisporus*. *Biol. Chem.* **381**, 349–353
21. Alvarez, M.E., Rosa, A.L., Temporini, E.D., Wolstenholme, A., Panzetta, G., Patrito, L., and Maccioni, H.J. (1993) The 59-kDa polypeptide constituent of 8–10-nm cytoplasmic filaments in *Neurospora crassa* is a pyruvate decarboxylase. *Gene* **130**, 253–258
22. Sanchis, V., Vinas, I., Roberts, I.N., Jeenes, D.J., Watson, A.J., and Archer, D.B. (1994) A pyruvate decarboxylase gene from *Aspergillus parasiticus*. *FEMS Microbiol. Lett.* **117**, 207–210
23. Lockington, R.A., Borlace, C.N., and Kelly, J.M. (1997) Pyruvate decarboxylase and anaerobic survival in *Aspergillus nidulans*. *Gene* **191**, 61–67
24. Lee, D.W., Koh, J.S., Kim, J.H. and Chae, K.S. (1999) Cloning and nucleotide sequence of one of the most highly expressed genes, a pdcA homologue of *Aspergillus nidulans*, in *Aspergillus oryzae*. *Biotechnol. Lett.* **21**, 139–142
25. Kelley, P.M., Godfrey, K., Lal, S.K. and Alleman, M. (1991) Characterization of the maize pyruvate decarboxylase gene. *Plant Mol. Biol.* **7**, 1259–1261
26. Hossain, M.A., Huq, E., Hodges, T.K., and Huq, E. (1994) Sequence of a cDNA from *Oryza sativa* (L.) encoding the pyruvate decarboxylase 1 gene. *Plant Physiol.* **106**, 799–800
27. Mücke, U., Wohlfarth, T. Fiedler, U., Baumlein, H., Rucknagel, K.P., König, S. (1996) Pyruvate decarboxylase from *Pisum sativum*. Properties, nucleotide and amino acid sequences. *Eur. J. Biochem.* **237**, 373–382
28. Reynen, M., and Sahm, H. (1988) Comparison of the structural genes for pyruvate decarboxylase in different *Zymomonas mobilis* strains. *J. Bacteriol.* **170**, 3310–3313
29. Raj, K.C., Talarico, L.A., Ingram, I.O., and Maupin-Furlow, J.A. (2002) Cloning and characterization of the *Zymobacter palmae* pyruvate decarboxylase gene (pdc) and comparison to bacterial homologues. *Appl. Environ. Microbiol.* **68**, 2869–2876
30. Raj, K.C., Ingram, I.O., and Maupin-Furlow, J.A. (2001) Pyruvate decarboxylase: a key enzyme for the oxidative metabolism of lactic acid by *Acetobacter pasteurianus*. *Arch. Microbiol.* **176**, 443–451
31. Talarico, L.A., Ingram, I.O., and Maupin-Furlow, J.A. (2001) Production of the Gram-positive *Sarcina ventriculi* pyruvate decarboxylase in *Escherichia coli*. *Microbiology* **147**, 2425–2435
32. Candy, J.M., Duggleby, R.G., and Mattick, J.S. (1991) Expression of active yeast pyruvate decarboxylase in *Escherichia coli*. *J. Gen. Microbiol.* **137**, 2811–2815
33. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* 2nd ed., Cold Spring Harbor Laboratory Press, New York
34. Ulrich, J. (1970) Yeast pyruvate decarboxylase (2-oxoacid carboxy-lase, EC4.1.1.1) assay of thiamine pyrophosphate. *Methods Enzymol.* **18**, 109–115
35. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
36. Zehender, H., trescher, D., and Ullrich, J. (1983) Activity stain for pyruvate decarboxylase in polyacrylamide gels. *Anal. Biochem.* **135**, 16–21
37. Burke, D., Dawson, D., and Stearns, T. (2000) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual* pp. 113–114, Cold Spring Harbor Laboratory Press, New York
38. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
39. Zhao, Y. and Lu, Y. (1998) *Practical Protocols in Molecular Biology* (Li, Y. and Zhao, Y., eds) pp. 161–163, Science Press, Beijing
40. Boiteux, A. and Hess, B. (1970) Allosteric properties of yeast pyruvate decarboxylase. *FEBS Lett.* **9**, 293–296
41. Segel, I.H. (1975) *Enzyme Kinetics*, John Wiley & Sons, Inc., New York
42. Flatau, S., Fischer, G., Kleinpeter, E., and Schellenberger, A. (1998) ^{31}P NMR investigations on free and enzyme bound thiamine pyrophosphate. *FEBS Lett.* **233**, 379–382
43. Killenberg-Jabs, M., Jabs, A., Lilie, H., Golbik, R., and Hübner, G. (2001) Active oligomeric states of pyruvate decarboxylase and their functional characterization. *Eur. J. Biochem.* **268**, 1689–1704
44. Sergienko, E.A. and Jordan, F. (2002) Yeast pyruvate decarboxylase tetramers can dissociate into dimers along two interfaces. Hybrids of low-activity D28A (or D28N) and E477Q variants, with substitution of adjacent active center acidic groups from different subunits, display restored activity. *Biochemistry* **41**, 6164–6169
45. Hawkins, C.F., Borges, A., and Perham, R.N. (1989) A common structural motif in thiamin pyrophosphate-binding enzymes. *FEBS Lett.* **255**, 77–82
46. Dobritzsch, D., König, S., Schneider, G., and Lu, G. (1998) High resolution crystal structure of pyruvate decarboxylase from *Zymomonas mobilis*. Implications for substrate activation in pyruvate decarboxylases. *J. Biol. Chem.* **273**, 20196–20204
47. Barns, S.M., Lane, D.J., Sogin, M.L., Bibeau, C., and Weisburg, W.G. (1991) Evolutionary relationships among pathogenic Candida species and relatives. *J. Bacteriol.* **173**, 2250–2255