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Purification and Characterization of Two Dihydroxyacetone Kinases from *Schizosaccharomyces pombe* IFO 0354

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Two dihydroxyacetone kinases (DHAKs), DHAK I and DHAK II, were purified to homogeneity from *Schizosaccharomyces pombe* IFO 0354. They were immunologically different from each other. Although both of the enzymes had some affinity for glycerol and DL-glyceraldehyde in addition to dihydroxyacetone and glyceraldehyde, V_{max} values for dihydroxyacetone were much higher than those for glycerol and DL-glyceraldehyde. On the basis of the K_m values of both enzymes for dihydroxyacetone, DHAK II plays a more important role than DHAK I in dissimilation of glycerol via dihydroxyacetone.

Many microorganisms grow on glycerol or dihydroxyacetone (DHA). Two different pathways have been proposed for dissimilation of glycerol in yeasts. *Candida utilis* and *Saccharomyces cerevisiae* are known to contain the pathway by which glycerol is converted to DHA phosphate via glycerol-3-phosphate (G-3-P) by the successive action of glycerol kinase and G-3-P dehydrogenase (3, 13). On the other hand, *Schizosaccharomyces pombe* and a number of glycerol-utilizing yeasts were assumed to produce DHA phosphate via DHA on the basis of the observation of the absence of glycerol kinase and the presence of glycerol dehydrogenase and DHA kinase (DHAK) (11). Marshall et al. (10) purified a DHAK from *S. pombe* 972h$^-$ which is a key enzyme in the main pathway of glycerol dissimilation and showed that its native form was a molecule composed of four identical subunits with a molecular mass of 45,000 Da. We have found that *S. pombe* IFO 0354 produces two immunologically distinct DHAKs, and we have characterized these enzymes.

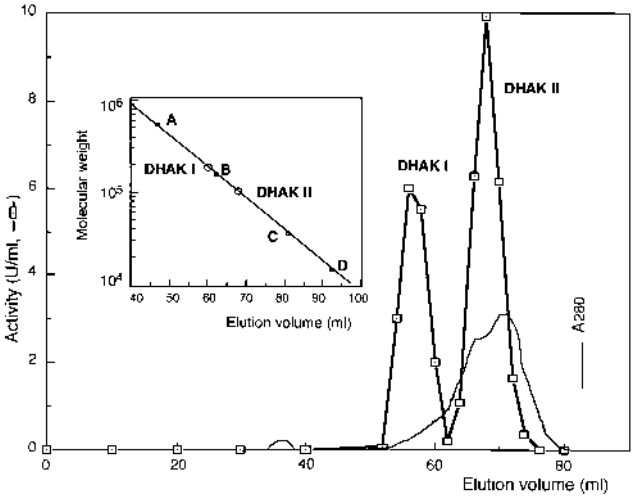

FIG. 1. Chromatography of DHAKs on Superdex 200pg. The inset shows a semilogarithmic plot of molecular weight versus elution volume for estimation of the molecular weights of native DHAK I and DHAK II. A, thyroglobulin (bovine); B, gamma globulin (bovine); C, ovalbumin (chicken); D, myoglobin (horse).

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Purification of DHAKs. *S. pombe* IFO 0354 cultures were incubated in Erlenmeyer flasks (500 ml) containing 200 ml of DHA medium (pH 6.2; composed of 1.5% peptone, 1.2% yeast extract, 0.5% glycerol, 0.8% maltose, 0.1% potassium dihydrogen phosphate, 0.05% magnesium sulfate, 0.05% potassium chloride, and 0.001% ferrous sulfate) for 48 h at 28°C on a rotary shaker. Cells were harvested from 2 liters of the cultures by centrifugation, washed once in PMG buffer (10 mM potassium phosphate, 10 mM 2-mercaptoethanol, 10% glycerol [pH 8.0]) and ground with aluminum oxide powder. The sample was suspended in 20 ml of the same buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and centrifuged at 15,000 × *g* for 10 min to remove cell debris and aluminum oxide powder. The supernatant thus obtained was used for the purification of DHAK as a crude enzyme solution. All the purification procedures were performed at 4°C. DHAK activity was determined with DHA as a substrate by the method of May and Sloan (12). The assay mixture (total volume, 1.0 ml) consisted of 900 μl of 0.1 M triethanolamine buffer (pH 7.3) containing 8 mM magnesium sulfate, 5.5 mM DHA, and 10 U of G-3-P dehydrogenase; 33.3 μl of 5.24 mM NADH solution; 33.3 μl of 82 mM ATP solution; and 33.3 μl of an enzyme solution. The decrease in the A_{340} of the reaction mixture was

TABLE 1. Summary of purification of DHAK I and DHAK II from *S. pombe* IFO 0354

Step	Vol (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	13.3	55.1	86.5	0.64	1	100
DEAE-Toyopearl 1	20.0	53.9	78.0	0.69	1.1	98
DEAE-Toyopearl 2	18.8	46.6	64.8	0.72	1.1	85
Superdex 200pg 1						
DHAK I	8.0	11.8	2.32	5.09	8.0	21
DHAK II	8.0	16.0	2.42	6.61	10	29
Superdex 200pg 2						
DHAK I	6.0	3.96	0.90	4.40	6.9	7.2
DHAK II	8.0	3.68	0.80	4.60	7.2	6.7
Phenyl-Toyopearl						
DHAK I	5.0	3.92	0.28	14.3	22	7.1
DHAK II	6.0	3.30	0.24	13.8	22	6.0

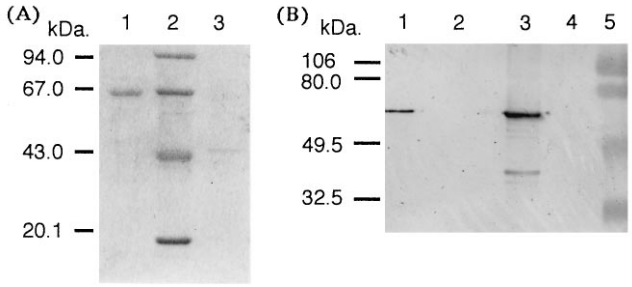

FIG. 2. SDS-polyacrylamide gel electrophoresis (A) and Western blot (with anti-DHAK II antibody) (B) analyses of DHAK I and DHAK II. (A and B) Lanes 1, DHAK II (65 kDa); lanes 2, protein size standards; lanes 3, partially purified DHAK II (first peak of the first Superdex 200pg column). (B) Lane 4, partially purified DHAK I (second peak of the first Superdex 200pg column); lane 5, prestained protein size standards.

due to the oxidation of NADH coupled with the reduction of DHAK by G-3-P dehydrogenase. DHAK was measured at 25°C for 5 min. One unit of DHAK was defined as the amount of enzyme that produced 1 μmol of DHA phosphate from DHA and ATP per min. Protein concentrations were determined by the method of Bradford (1) with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as a standard. The crude enzyme solution was applied to a DEAE-Toyopearl column (1.5 by 20 cm; Tohso Co., Tokyo, Japan) and eluted with a linear gradient of sodium chloride (0 to 1 M) in PMG buffer. Fractions containing DHAK activity were collected, desalted by ultrafiltration, and rechromatographed on the DEAE-Toyopearl column under the conditions described above. Active fractions were collected, concentrated by ultrafiltration, and loaded onto a Superdex 200pg column (1.5 by 60 cm; Pharmacia Biotech, Uppsala, Sweden). When the column was eluted with PMG buffer containing 300 mM sodium chloride, DHAK activity gave two sharp peaks (Fig. 1). The enzyme in the first peak was referred to as DHAK I, and that in the second peak was referred to as DHAK II. DHAK I and DHAK II were separately rechromatographed on the Superdex 200pg column. The pooled eluates from the second Superdex 200pg column were dialyzed against 200 mM PMG buffer containing 1.0 M ammonium sulfate, loaded onto a phenyl-Toyopearl column (1.0 by 7.0 cm; Tohso) previously equilibrated with the same buffer, and eluted from the column with a decreasing linear gradient of ammonium sulfate (1.0 to 0 M). The purification of DHAK I and DHAK II is summarized in Table 1. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the purified enzyme preparations indicated that the apparent molecular mass of DHAK I was 45,000 Da and that of DHAK II was 65,000 Da (Fig. 2A). On the other hand, the molecular weights of DHAK I and DHAK II determined by gel filtration were 180,000 and 130,000, respectively (Fig. 1). These results suggest that the native DHAK I enzyme has a tetrameric structure composed of identical 45,000-Da subunits and that the native DHAK II enzyme has a dimeric structure composed of identical 65,000-Da subunits. Differences between DHAK I and DHAK II in the molecular mass and arrangement of the subunits suggested that DHAK I and DHAK II did not originate from a common parental molecular species through proteolysis or modification.

TABLE 3. Kinetic properties of DHAK I and DHAK II from *S. pombe* IFO 0354

Substrate	DHAK I		DHAK II	
	K_m (mM)	V_{max} (μmol · min^{-1} · mg of $protein^{-1}$)	K_m (mM)	V_{max} (μmol · min^{-1} · mg of $protein^{-1}$)
DHA	3.3	5.6	0.052	6.7
DL-Glyceraldehyde	8.4	3.3	9.1	5.0
Glycerol	8.9	0.19	8.9	0.37

Characterization of DHAKs. DHAK I and DHAK II were optimally active at pH 6.0 and 7.0, respectively, when the enzyme activity was measured in a mixed buffer (50 mM Tris, 50 mM 2,2-dimethylglutaric acid, 50 mM 2-amino-2-methyl-2,3-propanediol). Both of them were stable over the pH range of 6 to 8 in 10 mM potassium phosphate buffer at 4°C for 24 h. The optimum temperatures were found to be 50 and 60°C for the activities of DHAK I and DHAK II, respectively, although both enzymes were stable at up to 30°C when incubated for 15 min. The discrepancy in optimum temperatures and the temperature stabilities may be due to the different incubation periods in those experiments. Since all the DHAKs reported were known to require metal ions such as Mg^{2+} and Ca^{2+} for their activity (see Table 4), 8 mM magnesium sulfate was routinely added to the enzyme assay mixture used in this study as a source of Mg^{2+} ions. Therefore, the effect of metal ions other than Mg^{2+} ions on DHAK I and DHAK II was examined. DHAK I was fully active with Ca^{2+} ions (Table 2), and Mg^{2+} ions were somewhat replaceable with Co^{2+} and Ni^{2+} ions and slightly replaceable with Mn^{2+} ions. By contrast, DHAK II was slightly activated with only Ca^{2+} or Co^{2+} ions instead of Mg^{2+} ions. Neither DHAK I nor DHAK II was active in the absence of effective metal ions. The substrate specificities of DHAK I and DHAK II were examined as follows with DHA, DL-glyceraldehyde, glycerol, DL-glyceric acid, and G-3-P as substrates. The assay mixture (total volume, 1.0 ml) consisted of 900 μl of 0.1 M triethanolamine buffer (pH 7.3) containing 8 mM magnesium sulfate, one of the substrates tested (1 mM), 1.0 mM phosphoenolpyruvate, and 6 U each of pyruvate kinase and lactate dehydrogenase; 33.3 μl of 5.24 mM NADH; 33.3 ml of the purified DHAK solution; and 33.3 μl of 82 mM ATP. In this enzyme reaction, the rate of phosphorylation of each substrate by DHAKs was estimated as the rate of ADP formation, which was determined by measuring the decrease in the A_{340} of the reaction mixture over 5 min at 25°C

TABLE 2. Properties of DHAK I and DHAK II from *S. pombe* IFO 0354

Enzyme	M_r (subunit M_r)	Optimum temp (°C)[a]	Optimum pH	Thermal stability[b]	Activity (%) with divalent cation (7.2 mM)					
					Mg^{2+}	Ca^{2+}	Co^{2+}	Mn^{2+}	Ni^{2+}	None
DHAK I	180,000 (45,000)	50	6.0	Up to 30°C	100	98	54	7	49	0
DHAK II	130,000 (65,000)	60	7.0	Up to 30°C	100	20	10	0	0	0

[a] Activity was measured between 0 and 70°C at pH 8.0.
[b] Activity was measured after 15 min of heat treatment (0 to 70°C gradient).

TABLE 4. Comparison of the properties of DHAKs from various microorganisms

Organism	Purity of enzyme prepn[a]	M_r (1,000)		Optimum pH	K_m (mM) for DHA	Divalent cation(s) and effect(s) (%)[b]	Reference
		Native	Subunit				
Dunaliella salina	C	78–100		7.5			8
Dunaliella parva	C			6.5	0.01	Mg^{2+} (100)	7
Klebsiella pneumoniae	EH	100	50	7.5–8.0	0.004–0.005	Mg^{2+} (100), Ca^{2+} (150)	5
Schizosaccharomyces pombe							
972h$^-$	EH	160	45	6.5	0.016	Mg^{2+} (100), Ca^{2+} (80), Mn^{2+} (15)	10
IFO 0354 (DHAK I)	EH	180	45	6.0	3.3	Mg^{2+} (100), Ca^{2+} (98), Mn^{2+} (7)	This work
IFO 0354 (DHAK II)	EH	130	65	7.0	0.052	Mg^{2+} (100), Ca^{2+} (20), Mn^{2+} (0)	This work
Candida methylica	C			7.5			4
Candida boidinii	EH	139	71	7.2–8.2			2
Gluconobacter suboxydans	EH	260		6.5	0.03	Mg^{2+} (100), Mn^{2+} (0)	14
Hansenula polymorpha	EH	150	72	6.5	0.011	Mg^{2+} (100)	6

[a] C, crude; EH, electrophoretically homogeneous.
[b] Percentages shown are calculated against maximum activity in the presence of 8 mM magnesium sulfate.

due to the oxidation of NADH coupled with the reduction of pyruvate formed from phosphoenolpyruvate. Preliminary experiments showed that DHAK I and DHAK II had similar substrate specificities, i.e., both were active on DL-glyceraldehyde and glycerol as well as DHA but not on DL-glyceric acid and G-3-P. Therefore, the kinetic studies were carried out with DHA, glyceraldehyde, and glycerol as substrates (Table 3). From double-reciprocal plots, the K_m values of DHAK I for DHA, glyceraldehyde, and glycerol were estimated to be 3.3, 8.4, and 8.9 mM, respectively. On the other hand, the K_m values of DHAK II for the same substrates were 0.052, 9.1, and 8.9 mM, respectively. Both of the enzymes had their highest V_{max} values for DHA.

Until now, three different types of kinases which are active on DHA have been found. The first is DHAK, which is highly specific for DHA (8, 9). However, several enzymes capable of phosphorylating both DHA and glyceraldehyde but with much higher affinity for DHA than for glyceraldehyde are also classified as DHAKs (2, 4, 6, 14). The second is triokinase (EC 2.7.1.28), which is capable of catalyzing the phosphorylation of DHA and glyceraldehyde with similar rates for both substrates but cannot catalyze the phosphorylation of glycerol at all. The third is glycerol kinase (EC 2.7.1.30). This enzyme preferentially phosphorylates glycerol but is also active on DHA and glyceraldehyde. The K_m values of glycerol kinase purified from *Escherichia coli* were reported to be 0.0013, 0.5, and 0.5 mM for glycerol, DHA, and DL-glyceraldehyde, respectively (9). Although two kinases from *S. pombe* IFO 0354 have activity toward glycerol, phosphorylation of glycerol is a very minor activity of DHAK I and DHAK II, with V_{max} values for glycerol that were only 3.3 and 5.5%, respectively, of the velocities for DHA as the substrate (Table 3). We concluded that these kinases should be classified as DHAKs on the basis of higher affinities and higher V_{max} values for DHA than for the other substrates. The K_m value of DHAK II for DHA (0.052 mM) was comparable with those of the reported DHAKs (Table 4), but that of DHAK I was unexpectedly high, suggesting that DHAK II is more important than DHAK I in the utilization of DHA under circumstances in which DHA is limiting. The immunological properties of DHAK I and DHAK II were examined by Western blot (immunoblot) analysis with antiserum raised against purified DHAK II. As shown in Fig. 2B, DHAK I did not cross-react with the anti-DHAK II antibody, showing that DHAK I and DHAK II definitely did not originate from the same parental molecular species through proteolysis.

Some properties of microbial DHAKs are listed in Table 4. These enzymes can be divided into two types on the basis of the oligomeric structure of the native form; i.e., the enzymes from *Klebsiella pneumoniae*, *Candida boidinii*, and *Hansenula polymorpha* had dimeric structures, and the enzyme from *S. pombe* 972h$^-$ was a tetramer. DHAK I of *S. pombe* IFO 0354 was quite similar to the enzyme from *S. pombe* 972h$^-$ with respect to the subunit size of 45,000 Da and its tetrameric structure, while the K_m value of the former for DHA was much higher than that of the latter. We could not determine whether DHAK I has a physiological function in the glycerol dissimilation pathway on the basis of its K_m value. On the other hand, the structural and kinetic properties of DHAK II were similar to those of DHAKs purified from other yeasts (Table 4). In light of its K_m value for DHA, which was comparable to those of other microbial DHAKs, DHAK II may play a role in the glycerol dissimilation pathway via DHA in *S. pombe* IFO 0354.

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