PURIFICATION AND PROPERTIES OF DIHYDROXYACETONE SYNTHASE FROM THE METHYLOTROPHIC YEAST *CANDIDA BOIDINII*

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1. Introduction

Yeasts assimilate methanol after its oxidation to formaldehyde via a novel xylulosemonophosphate (dihydroxyacetone) cycle [1–4]. The first enzyme of this cycle which has been given the trivial name dihydroxyacetone synthase (DHAS) catalyses the transfer of a glycolaldehyde fragment from xylulose-5-phosphate to formaldehyde resulting in the formation of dihydroxyacetone and glyceraldehyde-3-phosphate.

According to preliminary data [5,6] DHAS and transketolase (TK) of methylotrophic yeasts differ in their ability to bind with anion exchangers. So far the properties of DHAS were studied using partially purified preparations only [3,5]. This work is concerned with the purification and some properties of homogeneous DHAS from the methylotrophic yeast *Candida boidinii*.

2. Materials and methods

2.1. *Yeast growth and cell-free extract preparation*

Candida boidinii KD1 was grown on mineral salt medium with 1% methanol and cell-free extract was prepared as in [6].

2.2. *Chemicals*

Sugar phosphates, $MgCl_2$, EDTA and $(NH_4)_2SO_4$ were obtained from Sigma; SP-Sephadex C-50, Sephadex G-15, DEAE-Sepharose CL-6B, Pharmalyte 3-10 from Pharmacia, Bio Gel HTP, reagents and protein standards for SDS–disc-gel electrophoresis from Bio-Rad, Tris, *N*-ethylmaleimide and protein standards for gel-filtration from Serva, dihydroxyacetone and *p*-chloromercuribenzoate from BDH, thiamine pyrophosphate from Merck. Formaldehyde was prepared from aqueous suspension of paraform (Apolda, GDR) by heating in a sealed ampoule overnight at 115°C.

2.3. *Enzyme assays*

The methods for the assay of TK in [7] and dihydroxyacetone kinase in [1] were used.

Reaction mixture for the assay of DHAS contained: K-phosphate buffer (pH 6.8) 0.1 M; $MgCl_2$, 5 mM; thiamine pyrophosphate (TPP), 0.5 mM; xylulose 5-phosphate, 2 mM; formaldehyde, 2 mM and enzyme preparation. Reaction was started with formaldehyde and carried out at 30°C. The quantity of trioses formed was determined as in [6]. Protein was estimated according to [8].

2.4. *Acrylamide gel electrophoresis*

SDS–disc-gel electrophoresis in 10% polyacrylamide gel and isoelectrofocusing in 5% polyacrylamide gel were done by standard procedure [9].

2.5. *Enzyme purification*

Potassium phosphate buffer (pH 6.8) (KP-buffer) was used during the purification procedure. All the steps were carried out at 0 – +2°C.

The crude extract was fractioned with solid $(NH_4)_2SO_4$. Material precipitating at 30–60% $(NH_4)_2SO_4$ saturation was redissolved in 0.01 M KP-buffer and desalted on a Sephadex G-25 column (2.6 × 70 cm). Protein was applied to a column (5 × 10 cm) of SP-Sephadex C-50 pre-equilibrated with the same buffer. In contrast to DHAS, dihydroxyacetone kinase and TK did not bind with this ion-exchanger. DHAS was eluted with 0.1 M KP-buffer containing 5 mM $MgCl_2$ and applied to a column

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Table 1
Purification of dihydroxyacetone synthase from *Candida boidinii*

Steps	Volume (ml)	Protein (mg)	Spec. act. (units/mg protein)	Total units	Yield (%)
Crude extract	100	1600	0.08	128	100
$(NH_4)_2SO_4$ (30–60% satd.)	30	600	0.18	108	84
SP–Sephadex C50	25	20	31	62	48
DEAE–Sepharose CL-6B	15	4.5	9.2	41	32
Bio-Gel HTP	4	4.3	4.0	17	13

(2.6 × 30 cm) of DEAE–Sepharose CL-6B equilibrated with the same buffer. KP-buffer (0.2 M) with 5 mM $MgCl_2$ served as eluent of the enzyme and active fractions were combined and applied to a column (1.6 × 15 cm) of hydroxylapatite Bio-Gel HTP pre-equilibrated with the same buffer. The column was washed with 0.4 M KP-buffer + 5 mM $MgCl_2$ and DHAS was eluted with 0.5 M KP-buffer containing 5 mM $MgCl_2$.

The purification procedure lasts ⩽12 h and allows one to obtain a homogeneous preparation of DHAS (fig.1) with spec. act. 4 U/mg. Typical results are presented in table 1.

The activity of the final DHAS preparation is lower (0.5–1 U/mg) and the enzyme is less stable when KP-buffer without $MgCl_2$ is used during purification.

3. Results

3.1. *Homogeneity and properties of DHAS*

The DHAS preparation gave a single symmetrical protein peak coinciding with that of enzymic activity during gel-filtration on a Sepharose CL-6B column (1.6 × 90 cm). The M_r of the enzyme as estimated with this column is 145 000. When the enzyme was subjected to SDS–disc-gel electrophoresis a single protein band of M_r 76 000 was found (fig.1). A single protein band with pI 7.1 was also obtained after isoelectrofocusing in polyacrylamide gel. The pH optimum for DHAS activity and stability was at 6.8–7.1.

3.2. *Effect of cations*

The influence of salts on DHAS depends on cations rather than on anions since $(NH_4)_2SO_4$ and NH_4Cl have a similar effect, DHAS is not stabilized by Ca^{2+} as demonstrated for yeast TK [10], whereas NH_4^+ and Mg^{2+} stabilize DHAS for ⩾80 h.

3.3. *Effect of thiamine pyrophosphate*

Thiamine pyrophosphate (TPP) is known to be the cofactor of TK [7]. Since it is suggested that the DHAS and TK reactions have similar mechanisms, we have studied the effect of TPP on DHAS. Using the fluorescent method [11] we have found TPP in our DHAS preparation. Mg^{2+} is essential for the effective binding of the cofactor to the enzyme molecule. The highest DHAS activity is observed in the presence of 5 mM $MgCl_2$ and 0.5 mM TPP. TPP also protects DHAS from inhibition by EDTA (table 2). A similar effect was demonstrated for TK from brewer's yeast [12].

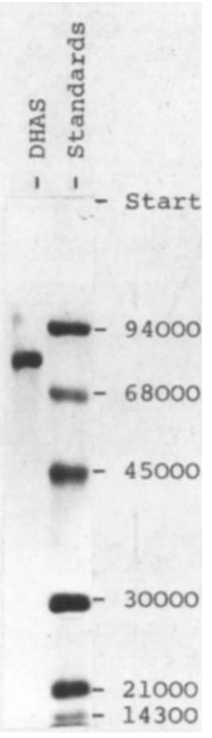

Fig.1. SDS–disc-gel electrophoresis of DHAS.

Table 2
Protection of DHAS by $MgCl_2$ and TPP from EDTA inactivation

EDTA (mM)	DHAS activity (%) after preincubation with	
	$MgCl_2$	TPP
0	51 (100)	65 (100)
2	22	64
4	4	71
6	0	76
8	0	98
10	0	100

DHAS was preincubated in the presence of 5 mM $MgCl_2$ or 0.5 mM TPP for 30 min at 30°C. The activity without preincubation is given in parentheses

3.4. *Substrate specificity*

It is known that TK exhibits a low specificity both for donors and acceptors of the glycolaldehyde radical [7,14,15]. The specificity of DHAS is higher. The best donor for the reaction is xylulose 5-phosphate. The reaction rate with fructose 6-phosphate and sedoheptulose 7-phosphate is 10-times lower than that with xylulose 5-phosphate. In contrast to work with partially purified DHAS [5], we did not detect dihydroxyacetone formation with ribulose 5-phosphate as a donor. Formaldehyde was the only acceptor of all substances tested. The reaction catalyzed by DHAS seems to be irreversible since we have not detected formaldehyde formation in a reaction mixture containing dihydroxyacetone, glyceraldehyde 3-phosphate and enzyme.

3.5. *Effect of formaldehyde concentration*

Substrate inhibition is observed at >1 mM formaldehyde (fig.2). Preincubation with xylulose 5-phosphate protects DHAS against the inhibitory effect of formaldehyde.

3.6. *Inhibition studies*

The DHAS activity was inhibited by *p*-chloromercuribenzoate and *N*-ethylmaleimide (both at 1 mM) by 100% and 88%, respectively. The inhibitory effect of *N*-ethylmaleimide is partially removed by reduced glutathione (GSH). However, a 2–3-fold excess of GSH in the reaction mixture with regard to formaldehyde completely blocks dihydroxyacetone formation (fig.3).

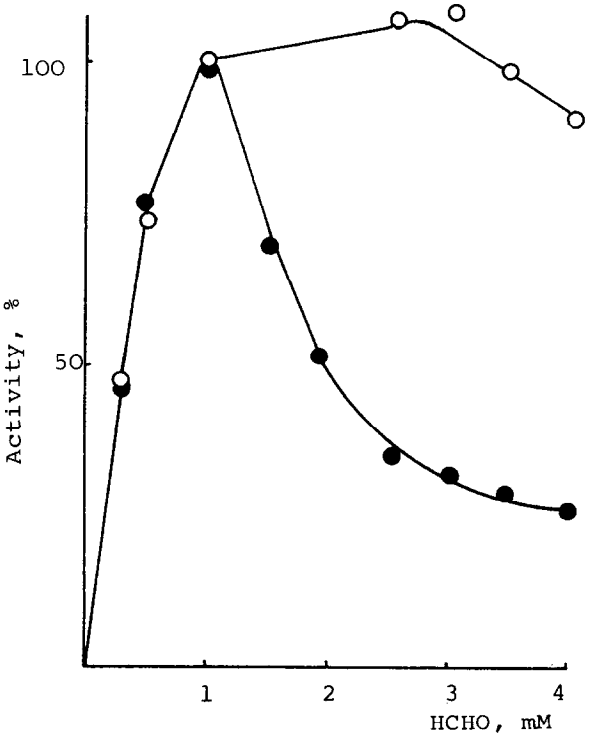

Fig.2. Effect of formaldehyde on DHAS. Reaction was started with formaldehyde before (-●-) or after (-○-) preincubation with xylulose 5-phosphate.

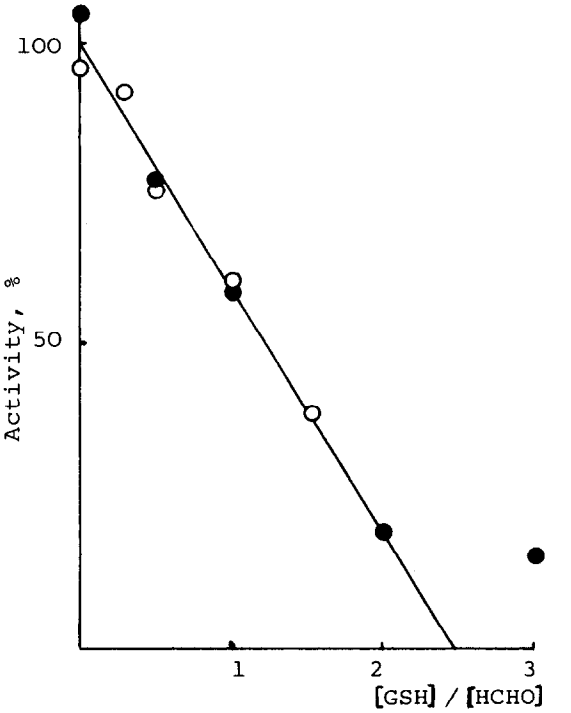

Fig.3. Effect of GSH on DHAS: formaldehyde at 1 mM (-●-) or 2 mM (-○-).

4. Discussion

The exact mechanism of the DHAS reaction is not known. It is assumed that this enzyme is a type of transketolase [1,3]. In fact, both enzymes contain TPP, require divalent cations, possess similar molecular structure and close specificity towards donors of the glycolaldehyde radical. However, these enzymes have significant differences (table 3). It is known, that substrate-acceptors of transketolase are aldoses with the D-configuration at the second carbon atom [15,16]. Formaldehyde cannot be referred to this class of compounds. In aqueous solutions formaldehyde is almost fully hydrated and exists in the form of methylene glycol and not the free aldehyde [18]. Although baker's yeast TK catalyzes the transfer of the glycolaldehyde radical to formaldehyde [14] the rate of this reaction is very slow. DHAS uses formaldehyde as substrate-acceptor much more effectively. Moreover, TK and DHAS are markedly distinguished by pH optimum and sensitivity to sulfhydryl reagents. *p*-Chloromercuribenzoate and *N*-ethylmaleimide do not affect TK [7] but inhibit DHAS activity. It seems likely from these facts that the structure of the active centers in both enzymes is rather different. These data show that DHAS is a new enzyme belonging to the class of transferases. The proposed systematic name for this enzyme is 'xylulose 5-phosphate:formaldehyde-glycolaldehyde transferase' (EC 2.2.1-).

The results obtained with the highly purified enzyme confirm our suggestion [6] that DHAS and TK fulfil different functions in methylotrophic metabolism of the yeasts. DHAS catalyzes the first step of formaldehyde assimilation whereas TK is involved in the following regeneration of the substrate-donor, i.e., xylulose 5-phosphate.

It is known that GSH plays an important role in the oxidation of the formaldehyde to CO_2 by yeasts,

Table 3
Properties of DHAS and yeast transketolases

Properties	DHAS [this research]	TK [7,17–19]
M_r	145 000	158 000 (baker's yeast) 100 000 (brewer's yeast) 163 000 (*C. utilis*)
Number of subunits	2	2
Presence of TPP in enzyme molecule	yes	yes
Mg^{2+} requirement	yes	yes
pH optimum	6.8	7.6
Donors:		
xylulose 5-phosphate	good	good
fructose 6-phosphate	poor	good
sedoheptulose 7-phosphate	poor	good
Acceptors:		
formaldehyde	good	poor
glyceraldehyde	nil	poor
glyceraldehyde 3-phosphate	nil	good
erythrose 4-phosphate	nil	good
ribose 5-phosphate	nil	good
glucose 6-phosphate	nil	poor
Inhibition by thiol reagents (*p*-CMB, NEM)	yes	no

since the true substrate of formaldehyde dehydrogenase is *S*-hydroxymethylglutathione, i.e., the product of non-enzymic condensation of formaldehyde and GSH [19]. Therefore, the hindering of the DHAS reaction by GSH is of particular interest. We suppose that the distribution of the formaldehyde flux between assimilation and dissimilation sequences depends on the ratio [GSH]/[HCHO] in vivo. Further studies of the kinetic and regulatory properties of the purified DHAS are needed to clarify the control mechanisms of C_1-metabolism in yeasts.

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