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PURIFICATION AND CHARACTERIZATION OF *S*-FORMYLGLUTATHIONE HYDROLASE FROM A METHANOL-UTILIZING YEAST, *KLOECKERA* SP. No. 2201

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Summary

S-Formylglutathione hydrolase (EC 3.1.2.12), a glutathione thiol esterase, was purified from a methanol-utilizing yeast, *Kloeckera* sp. No. 2201, to homogeneity as judged by polyacrylamide gel electrophoresis. The molecular weight of the native enzyme was determined to be 58 000 by gel filtration. The enzyme appeared to be composed of two identical subunits (M_r = 31 000). The apparent K_m for *S*-formylglutathione was 0.077 mM. The optimum temperature was 50°C and the optimum pH was 6.4—6.6. The enzyme was inhibited by several types of sulfhydryl reagents. The purified enzyme preparation contained no activity of formaldehyde dehydrogenase or of formate dehydrogenase. It is thought that three enzymes, formaldehyde dehydrogenase, *S*-formylglutathione hydrolase and formate dehydrogenase, participate in the oxidation of formaldehyde to CO_2 in *Kloeckera* sp. No. 2201.

Introduction

In methanol-utilizing yeasts, methanol is successively oxidized to CO_2 by alcohol oxidase, catalase, formaldehyde dehydrogenase and formate dehydrogenase [1]. The true substrate and product of formaldehyde dehydrogenase (the forward reaction) are *S*-hydroxymethylglutathione, the adduct of formaldehyde and GSH, and *S*-formylglutathione, respectively [2—5]. Uotila and Koivusalo [5,6] demonstrated that in human liver, *S*-formylglutathione was hydrolyzed to formate and GSH by *S*-formylglutathione hydrolase (EC 3.1.2.12). van Dijken et al. [3] reported that the formate dehydrogenase from

methanol-grown *Hansenula polymorpha* could catalyze hydrolysis of *S*-formylglutathione as well as oxidation of formate to CO_2. On the other hand, Schütte et al. [2] suggested the possible presence of *S*-formylglutathione hydrolase in *Candida boidinii*, as in human liver.

In the present work, *S*-formylglutathione hydrolase has been purified to an electrophoretically homogeneous form from methanol-grown *Kloeckera* sp. No. 2201. It is shown that the purified enzyme has no formate dehydrogenase activity.

Materials and Methods

Materials

DEAE-Sephacel, Sephadex G-75 and G-100, and 5'-AMP-Sepharose 4B were products of Pharmacia Fine Chemicals (Uppsala, Sweden). Hypatite-C was purchased from Clarkson Chemical Co. Inc. (PA, U.S.A.). NAD^+ and NADH were products of Oriental Yeast Co. Ltd. (Osaka, Japan). Formaldehyde was prepared by heating 0.5 g of *p*-formaldehyde in 5 ml of water at 100°C in a sealed tube for 15 h. All other chemicals used were of the highest purity available from commercial sources.

Organisms and cultivation

Kloeckera sp. No. 2201 was mainly used throughout this work. Other methanol-utilizing yeasts were as follows: *C. boidinii* [7] (kindly supplied by Professor H. Sahm), *H. polymorpha* DL1 [8] (kindly supplied by Professor C.L. Cooney), *Pichia trehalophila* (IFO 1282) and *Torulopsis pinus* (IFO 0741). The cultivation on methanol (1%, v/v) or other carbon sources (1%, w/v) was carried out in 2-l shaking flasks containing 500 ml of medium as described previously [9].

Preparation and standardization of S-formylglutathione

S-Formylglutathione was synthesized from acetic anhydride, formic acid, thioglycolic acid and GSH by the method of Uotila [10] which was modified by van Dijken et al. [3]. *S*-Formylglutathione was also prepared enzymically [5]. The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.5), a variable amount of formaldehyde, the molar equivalent of GSH to formaldehyde, 0.08 mM NAD^+, 2 mM sodium pyruvate, 50 units/ml of lactate dehydrogenase (from rabbit muscle, product of Sigmal Chemical Co., St. Louis, U.S.A.) and 0.1 unit/ml of the purified formaldehyde dehydrogenase from *Kloeckera* sp. (described below). Chemically synthesized *S*-formylglutathione was standardized enzymically according to van Dijken et al. [3], with the formaldehyde dehydrogenase from *Kloeckera* sp. The concentration of enzymically prepared thiol ester was determined photometrically by using ϵ_M at 240 nm = 3300 [10].

Enzyme assay

Formaldehyde dehydrogenase (EC 1.2.1.1) and formate dehydrogenase (EC 1.2.1.2) were assayed spectrophotometrically by using a Shimadzu double-beam spectrophotometer UV 300 equipped with a constant-temperature bath

and a recorder as described previously [11,12]. The assay mixture for the *S*-formylglutathione hydrolase contained 60 mM potassium phosphate buffer (pH 6.5), 0.2 mM *S*-formylglutathione and the enzyme in a total volume of 3.0 ml. Hydrolysis of the thiol ester bond was followed at 240 nm at 30°C. Activities were calculated as μmol/min (U) by using $\Delta\epsilon_M = 3300$ for *S*-formylglutathione as described by Uotila and Koivusalo [6].

Analysis

The formaldehyde solution was standardized with alcohol dehydrogenase according to Bernt and Gutman [13]. The protein was estimated by the method of Lowry et al. [14], with bovine serum albumin as a standard. Proteins were precipitated with 10% trichloroacetic acid before measurement to remove dithiothreitol. Acrylamide gel electrophoresis was carried out according to Jovin et al. [15]. To locate the enzyme activity, a gel was cut into 40 pieces with a stainless-steel knife. The pieces were put into 0.2 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol and left to stand overnight at 5°C, then enzyme activity in the supernatant was assayed under the standard conditions. The molecular weight of the subunit(s) was determined by polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate (SDS) according to Shapiro et al. [16], with molecular weight markers in the range of 14 300—71 500 (BDH Chemicals Ltd, Poole, U.K.). The molecular weight of the native enzyme was determined by gel filtration on Sephadex G-100 (column size, 0.9 × 60 cm) according to the method of Andrews [17], with cytochrome *c*, α-chymotrypsinogen A, hemoglobin, β-lactoglobulin, ovalbumin, bovine serum albumin and alcohol dehydrogenase (products of Sigma Chemical Co., St. Louis, U.S.A.) as marker proteins.

Purification of formaldehyde dehydrogenase and formate dehydrogenase

Both enzymes were purified from *Kloeckera* sp. as described previously [11,12]. Each enzyme preparation obtained contained a small amount of protein which did not exhibit the activity. Each enzyme solution from the Sephadex G-200 gel filtration was placed on a 5′-AMP-Sepharose 4B column (2.6 × 14 cm) which had been equilibrated with 100 mM Tris-HCl buffer (pH 7.5). After the column had been washed with 150 ml of equilibration buffer, protein was eluted with a linear gradient formed from 100 ml of the equilibration buffer and 100 ml of the same buffer containing 0.5 mM NAD^+. The eluate was collected in 2-ml fractions and those exhibiting the highest enzyme activity were pooled. The purified preparations of formaldehyde dehydrogenase and formate dehydrogenase each gave a single protein band on polyacrylamide gel electrophoresis that coincided with the enzyme activity. Each enzyme solution was mixed with an equal volume of glycerol and stored at −20°C.

Purification of S-formylglutathione hydrolase from Kloeckera sp.

All manipulations were carried out at 0—5°C. Potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol was used throughout the purification procedures.

Step 1: cell-free extract. The methanol-grown cells were suspended in the 10

mM buffer to give about 20% cell concentration and were disrupted for 3 h with a Kajio Denki ultrasonic oscillator (19 kHz). Cell debris was removed by centrifugation at 16 000 × *g* for 30 min.

Step 2: $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to 50% saturation to the cell-free extract, the mixture was centrifuged at 16 000 × *g* for 30 min, and solid $(NH_4)_2SO_4$ was added to the supernatant to 90% saturation. The resulting precipitate was dissolved in a small volume of the 10 mM buffer and dialyzed against three 2-l volumes of the same buffer.

Step 3: DEAE-Sephacel column chromatography. The dialyzed solution was applied to a DEAE-Sephacel column (5 × 45 cm) previously equilibrated with the 10 mM buffer and washed with 3 l of the same buffer. The enzyme was eluted with the 50 mM buffer. The active fractions were combined and concentrated by ultrafiltration with a Mini-module® (product of Asahikasei Co. Ltd., Tokyo, Japan).

Step 4: hypatite-C column chromatography. The concentrated enzyme solution was dialyzed against 2 l of the 10 mM buffer, then placed on a hypatite-C column (3.4 × 27 cm) which had been equilibrated with the same buffer. The enzyme was eluted with the 50 mM buffer. The active fractions were pooled and concentrated as described above.

Step 5: 1st gel filtration. A column (2.5 × 100 cm) of Sephadex G-75 was equilibrated with 50 mM buffer. The enzyme solution was layered ont the column and eluted with the same buffer. The active fractions were pooled and concentrated by ultrafiltration.

Step 6: 2nd gel filtration. The concentrated enzyme solution was layered on a column of Sephadex G-100 (2.5 × 100 cm) equilibrated with 50 mM buffer. The enzyme was eluted with the same buffer and the active fractions were pooled. The purified enzyme solution was stored at −20°C in small portions.

Results

S-Formylglutathione hydrolase activity in several yeasts

Table I lists the specific activities of *S*-formylglutathione hydrolase in the identically prepared cell-free extract of methylotropic yeasts, *Kloeckera* sp., *C. boidinii*, *H. polymorpha*, *P. trehalophila* and *T. pinus*, and non-methylotrophic yeasts, *Saccharomyces cerevisiae* and *Candida utilis*. The enzyme activity was detected only in the extract of cells grown on methanol but not on

TABLE I

SPECIFIC ACTIVITY OF *S*-FORMYLGLUTATHIONE HYDROLASE IN CELL-FREE EXTRACT OF METHANOL-GROWN YEASTS

Yeast	Specific activity (U/mg)
Kloeckera sp. No. 2201	16.5
C. boidinii	15.6
H. polymorpha	5.8
P. trehalophila	1.9
T. pinus	3.7

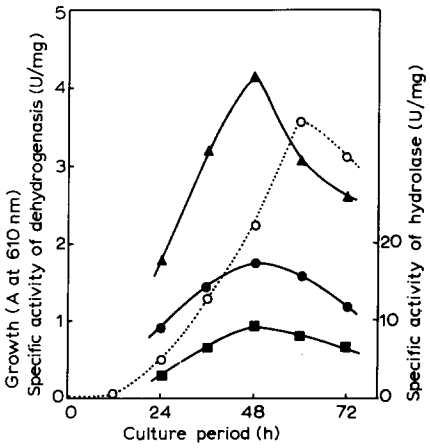

Fig. 1. Growth (○) and change in specific activity of formaldehyde dehydrogenase (▲), *S*-formylglutathione hydrolase (●) and formate dehydrogenase (■) in *Kloeckera* sp. during the growth on methanol.

glucose, glycerol and ethanol. Fig. 2 shows the change of the specific activities of formaldehyde dehydrogenase, *S*-formylglutathione hydrolase and formate dehydrogenase in *Kloeckera* sp. during the growth on methanol. The specific activity of *S*-formylglutathione hydrolase, as well as those of formaldehyde dehydrogenase and formate dehydrogenase, was maximal at the logarithmic phase. These results indicate that *S*-formylglutathione hydrolase also is involved in the oxidation of methanol by the methylotrophic yeasts.

Purification of S-formylglutathione hydrolase from methanol-grown Kloeckera sp.

As shown in Fig. 2, the formate dehydrogenase and *S*-formylglutathione hydrolase could be completely separated by the DEAE-Sephacel column

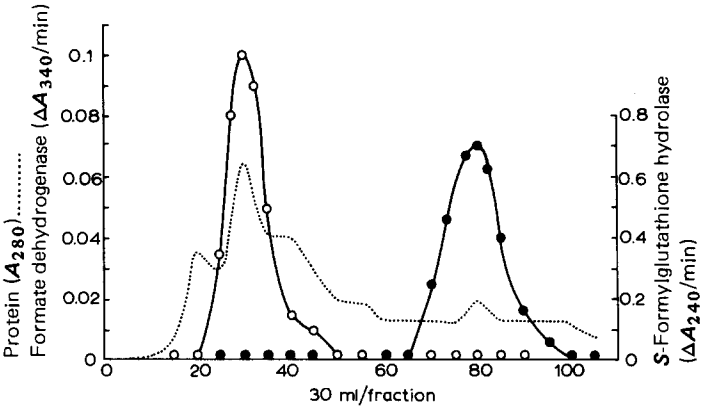

Fig. 2. Separation of formate dehydrogenase (○) and *S*-formylglutathione hydrolase (●) by DEAE-Sephacel column chromatography. Elution was carried out with 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol. The details are described in Materials and Methods.

TABLE II

SUMMARY OF PURIFICATION OF *S*-FORMYLGLUTATHIONE HYDROLASE FROM METHANOL-GROWN *KLOECKERA* SP.

Procedure	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification	Yield (%)
Cell-free extract	44 300	2 560	17.3	1	100
50—90% $(NH_4)_2SO_4$ saturation	39 900	1 840	21.7	1.3	90.1
DEAE-Sephacel	27 400	17.2	1 590	91.7	61.9
Hypatite-C	20 000	4.6	4 390	254	45.2
Sephadex G-75	18 000	3.4	5 260	304	40.6
Sephadex G-100	10 400	1.8	5 800	335	23.6

chromatography (step 3). Under the column-chromatographic conditions, formaldehyde dehydrogenase was eluted with the 100 mM buffer.

The summary of purification of *S*-formylglutathione hydrolase (Table II) shows that 335-fold purification and 23.5% yield from the cell-free extract were obtained. The purified enzyme gave a single protein band on polyacrylamide gel electrophoresis that coincided with the hydrolase activity.

Michaelis constant

The Michaelis constant was determined by double-reciprocal plots [18] of initial velocity vs. substrate concentration. The apparent K_m for *S*-formylglutathione was 0.077 and 0.070 mM, using the thiol esters prepared by the chemical and enzymical methods, respectively.

Optimum temperature and heat stability

The optimum temperature of the enzyme activity was found to be 50°C. The heat stability of the enzyme was studied using 50 mM potassium phosphate buffer (pH 7.5) with or without dithiothreitol. Almost full activity was retained after incubation at 45°C for 10 min regardless of the presence of dithiothreitol in the buffer. The remaining activity at 50°C indicated that the enzyme was stabilized by dithiothreitol.

Optimum pH and pH stability

The enzyme exhibited maximal activity at pH 6.4—6.6 and was stable at pH 7.2—7.6 in the buffer solution containing 1 mM dithiothreitol at 5°C for 4 days. The recovery of activity was much lower in the absence of dithiothreitol.

Inhibition of enzyme

The enzyme was strongly inhibited by several sulfhydryl reagents (Table III). The reversibility of the inhibitions was examined by adding 1 mM dithiothreitol to the enzyme after treatment with the sulfhydryl reagents. After incubation for 10 min at 30°C, the almost full enzyme activity was restored. This indicates that the enzyme contains a reactive sulfhydryl group.

None of the chelating agents tested, EDTA, *o*-phenanthroline and α,α'-dipyridyl, was inhibitory at concentrations up to 0.5 mM. The enzyme was

TABLE III

INHIBITION OF *S*-FORMYLGLUTATHIONE HYDROLASE BY SULFHYDRYL REAGENTS AND METAL SALTS

Inhibitor (mM)	Inhibition (%)
N-Ethylmaleimide	
0.5	100
0.1	50.0
p-Chloromercuribenzoate	
0.001	100
0.0005	66.3
$HgCl_2$	
0.005	100
0.001	75.6
$CuSO_4$	
0.01	100
0.003	64.6
$FeSO_4$	
0.05	100
0.02	83.0
$ZnSO_4$	
0.5	23.9

inhibited strongly by $CuSO_4$ and $FeSO_4$ (Table III). NaCl, KCl, $(NH_4)_2SO_4$, $MgCl_2$ and $CaCl_2$ at 0.2 mM all had no effect on the enzyme activity.

Oxidation of formaldehyde to CO_2 catalyzed by the purified enzyme system

Fig. 3 shows that absorption increase at 240 nm which indicates the formation of the thiol ester, *S*-formylglutathione, by the formaldehyde dehydrogenase reaction. When a small amount of the purified *S*-formylglutathione hydrolase was subsequently added to the cuvettes, *S*-formylglutathione was rapidly decomposed, as is seen from the decrease of the absorbance to the

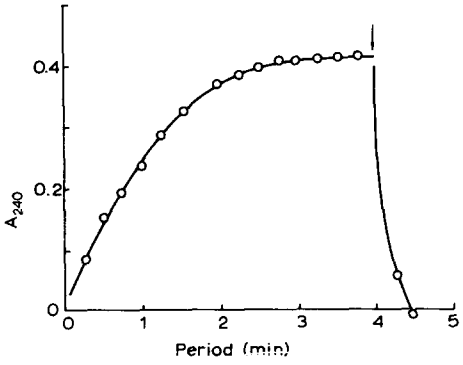

Fig. 3. Reaction catalyzed by purified formaldehyde dehydrogenase and *S*-formylglutathione hydrolase followed at 240 nm. The reaction system contained 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM formaldehyde, 0.1 mM GSH, 0.08 mM NAD^+, 2 mM sodium pyruvate, 50 units/ml of lactate dehydrogenase and 0.1 U/ml of formaldehyde dehydrogenase in a total volume of 3 ml. The arrow indicates the addition of 0.1 U of *S*-formylglutathione hydrolase.

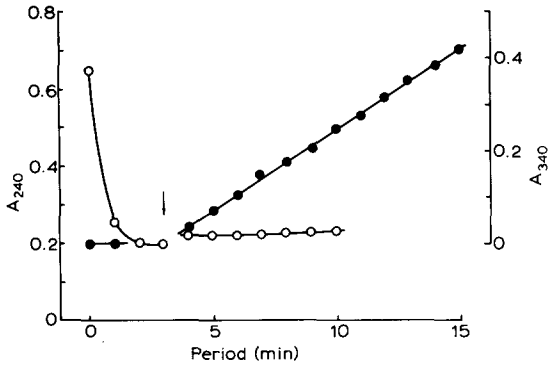

Fig. 4. Reaction catalyzed by purified *S*-formylglutathione and formate dehydrogenase followed at 240 (○) and 340 nm (●). The reaction system contained 60 mM potassium phosphate buffer (pH 7.5), 0.15 mM *S*-formylglutathione (prepared chemically) and 0.05 U/ml of *S*-formylglutathione hydrolase in a total volume of 3 ml. The arrow indicates the addition of 0.3 μmol of NAD^+ and 0.2 U of formate dehydrogenase.

original level. No absorption change appeared on additions of the purified formate dehydrogenase (0.5 U/ml in final volume) and NAD^+ instead of *S*-formylglutathione hydrolase.

Fig. 4 shows the oxidation of *S*-formylglutathione to CO_2 catalyzed by the purified hydrolase and formate dehydrogenase. The chemically synthesized thiol ester was hydrolyzed by the purified hydrolase. When the absorption decrease at 240 nm had almost stopped, the purified formate dehydrogenase and NAD^+ were added to the cuvettes. Formate formed by the hydrolysis of *S*-formylglutathione was oxidized to CO_2 by the formate dehydrogenase, as is seen from the reduction of NAD^+ reflected in the increase in absorbance at 340 nm. The results presented in Figs. 3 and 4 indicate that the three enzymes, formaldehyde dehydrogenase, *S*-formylglutathione hydrolase and formate dehydrogenase, are required for the oxidation of formaldehyde to CO_2.

Discussion

Some physicochemical and enzymic properties of the *S*-formylglutathione hydrolase from *Kloeckera* sp. are summarized in Table IV with those of the enzyme from human liver for comparison. The molecular weights of the native enzyme of *Kloeckera* and its subunit(s) are similar to those of the enzyme from human liver. One marked difference between the two enzyme is in their pH optima for activity: the maximum activity of the *Kloeckera* enzyme was expressed at lower pH than that of human liver enzyme. Since the cell fluid of yeast is slightly acidic, the enzyme probably exhibits maximum activity under the physiological conditions. Uotila and Koivusalo [6] reported that the human liver enzyme was sensitive to several types of sulfhydryl reagents. The *Kloeckera* enzyme was also inhibited by *N*-methylmaleimide, *p*-chloromercuribenzoate and $HgCl_2$, and the inhibited activity was restored by the addition of dithiothreitol.

TABLE IV

COMPARISON OF PROPERTIES OF *S*-FORMYLGLUTATHIONE HYDROLASE FROM HUMAN LIVER [6] AND *KLOECKERA* SP.

Property	Enzyme from:	
	Human liver	*Kloeckera* sp.
Molecular weight		
Native enzyme	52 500	58 000
Subunit	30 800	31 000
Specific activity	4 100	5 800
(μmol/mg per min)	(at 25°C)	(at 30°C)
K_m for *S*-formylglutathione	—	0.077 mM
Optimum temperature	—	50°C
Heat stability *	—	50°C
Optimum pH	6.9—7.1	6.4—6.6
pH stability	<8.0 **	7.2—7.6
Inhibition		
Sulfhydryl reagents	yes	yes
Chelating agents	no	no
Amino group reagents	yes	—
Metal ions	Ca^{2+}, Zn^{2+} Cu^{2+}, Co^{2+}	Cu^{2+}, Fe^{2+} Zn^{2+}

* Initial activity of the enzyme was lost at this temperature for 10 min.
** The enzyme remained stable at the pH cited at 4°C for 1 day.

van Dijken et al. [3] reported that the formate dehydrogenase from *H. polymorpha* catalyzed the hydrolysis of *S*-formylglutathione in the presence of NAD^+, and concluded that the thiol ester, a product of formaldehyde dehydrogenase reaction, was the substrate of formate dehydrogenase in methanol-utilizing yeasts. In general, the formate dehydrogenase from methanol-utilizing yeasts shows low affinity for formate (apparent K_m values are 6—40 mM) [2,3,19]. However, the possibility that such low affinity has physiological significance seems to be ruled out by the results of van Dijken et al. [3] that the enzyme from *H. polymorpha* showed approximately 40-fold higher affinity for *S*-formylglutathione than for formate.

In the present work, the activity of *S*-formylglutathione hydrolase could be detected in the absence of NAD^+ in the cell-free extract of some methanol-utilizing yeasts. The formate dehydrogenase from *Kloeckera* sp. is appreciably heat stable [12]; over the 90% of activity was recovered after heat treatment at 55°C for 10 min, whereas the *S*-formylglutathione hydrolase was almost completely inactivated by this treatment. Moreover, the activities of the two enzymes could readily be separated by normal enzyme purification procedures, such as a DEAE-Sephacel column chromatography (Fig. 2) and an affinity chromatography for NAD-linked enzymes using 5'-AMP-Sepharose 4B (unpublished data). The *S*-formylglutathione hydrolase purified from *Kloeckera* sp. in an electrophoretically homogeneous form exhibited no activity of formate dehydrogenase even in the presence of NAD^+. In contrast to the case of *H. polymorpha* [3], therefore, the oxidation of *S*-formylglutathione to CO_2 in *Kloeckera* sp. is catalyzed by two enzymes: *S*-formylglutathione hydrolase,

which hydrolyzes the thiol ester to formate and GSH, and formate dehydrogenase, which catalyzes the oxidation of formate.

The apparent K_m for *S*-formylglutathione of the hydrolase from *Kloeckera* sp. (0.077 mM) is though to be adequate for an enzyme reaction which occurs under physiological conditions. However, the question of the physiological significance of the low affinity of the formate dehydrogenase for formate remains open.

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