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CRYSTALLINE SERINE HYDROXYMETHYLTRANSFERASE FROM AN OBLIGATE METHYLOTROPH, *HYPHOMICROBIUM METHYLOVORUM*

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SUMMARY: Optimal culture conditions of a methylotrophic Hyphomicrobium methylovorum and improved purification of serine hydroxymethyltransferase from the bacterium were established for the large-scale preparation of the enzyme. The first crystalline serine hydroxymethyltransferase from the microbial source was obtained in the apo form and found to be homogeneous. Amino acid analysis revealed that the enzyme had higher value per subunit for acidic and neutral amino acids than that from rabbit liver. The carboxy-terminal amino acid analysis suggested the sequence -Ile-Ala-Tyr.

Serine hydroxymethyltransferase (EC 2.1.2.1) catalyzes the reversible interconversion of serine and glycine, with tetrahydrofolate as the one-carbon (C_1) acceptor. This enzyme from mammals has been extensively studied (1). Its major functions are to provide glycine for protein and purine synthesis and N^5,N^{10}-methylenetetrahydrofolate for the one carbon pool (2). On the other hand, a number of methylotrophic bacteria possess the serine pathway for the assimilation of C_1 compounds (3). In such bacteria, serine hydroxymethyltransferase plays an important role as the first enzyme in the assimilation of C_1-compounds through the addition of formaldehyde to glycine, which yields the main intermediate of the pathway, serine.

In a previous paper (4), we described the purification and the molecular properties of serine hydroxymethyltransferase from a serine-producing methanol-utilizing bacterium, Hyphomicrobium methylovorum GM2 (5). This communication describes the large-scale preparation and the improved purification of the enzyme from the bacterium, which allow the first crystallization of the enzyme from the microbial source.

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MATERIALS AND METHODS

Materials All chemicals were purchased from commercial sources and used without further purification except formaldehyde and tetrahydrofolate, which were prepared as described previously (4). Carboxypepetidase Y was from Oriental Yeast (Japan).

Microorganism The organism used was H. methylovorum GM2 (5).

Media and cultivation The media and cultivation conditions of the organism in a 100-liter jar fermentor were described previously (6).

Enzyme assay Serine hydroxymethyltransferase activity was assayed by two methods: 1. The synthesis of L-serine was assayed by measuring the disappearance of HCHO. 2. The degradation of β-threo-phenylserine was assayed by measuring the increase of benzaldehyde. The assay conditions for both methods were previously described (4). One unit of the enzyme activity was defined as the amount of enzyme that catalyzes the disappearance of 1.0 μmol HCHO h^{-1} at 37°C or that catalyzes the appearance of 1.0 μmol benzaldehyde per min at 25°C. Specific activity was expressed as units per mg protein.

Protein determination The protein concentration was determined by measuring the absorbance at 280 nm or by the method of Bradford et al. with Bio-Rad standard solution (7). The absorption coefficient of 4.8 $mg^{-1}cm^{-1}ml$ was calculated using absorbance and dry weight determination.

Amino acid analysis The solution of serine hydroxymethyltransferase (0.25 mg/ml) was hydrolyzed in 6 N HCl. The solution was sealed under vaccum in glass tubes, containing 10 μl thioglycolic acid in 1 ml enzyme solution. The sample was hydrolyzed at 110°C for 24-, 48- and 72-h, and then dried under vaccum. The hydrolyzed dried sample was dissolved in 20 mM potassium phosphate buffer, pH 7.5, and then analyzed in a Kyowa Seimitsu K-101 (Japan) amino acid analyzer. Cysteine and cystine were determined as cysteic acid after performic oxidation of the sample according to the method of Moore (8). Tryptophan was determined spectrophotometrically (9).

The carboxy-terminal amino acid was determined with carboxypeptidase Y (9). Carboxypeptidase Y (1.7 nmol) was added to 60 nmol SDS-denatured enzyme in 1.0 ml 0.1 M Tris-maleate buffer (pH 6.0), and the mixture was incubated at 25°C for 22 h. At various intervals, 30-μl aliquots were withdrawn and put into small tubes containing 60 μl 30% trichloroacetic acid. The released amino acids were quantitavely analyzed with an amino acid analyzer.

Absorption spectra The absorption spectrum of the crystallized apoenzyme was determined in cuvettes of 1-cm path length with a Shimadzu model UV-240 spectrophotometer at 25°C connected to a Shimadzu PR-1 computer-controlled graphic recorder.

Purification of serine hydroxymethyltransferase The cells in culture broth were pelleted by centrifugation with a CEPA centrifuge, Switzerland, and stored at -20°C. The thawed cells were resuspended in 50 mM potassium phosphate buffer, pH 7.5. All the purification steps were carried out at 5°C, using potassium phosphate buffer with 50 mM pyridoxal-P, 10 μM 2-mercaptoethanol and 10 μM Na_2EDTA, unless otherwise indicated.

Step 1. Preparation of crude cell-free extract. The thawed cells (around 1 kg as wet weight) were resuspended in 2 liters of 50 mM potassium phosphate buffer (pH 7.5), and cell-free extracts were prepared by passing the cells twice through a Dyno®-mill type KDL desintegrator (bellow 10°C for 30 min) and then the cell debris removed by centrifugation at 10,000 x g for 1 h and the supernatant was used as the cell-free extract.

Step 2. Protamine sulfate treatment. A 1% solution of protamine sulfate from salmon was added drop-wise to the cell-free extract to give a final ratio of 0.1 mg protamine to 10 mg protein. After 1 h, the precipitate was removed by centrifugation at 10,000 x g for 1 h. The supernatant was dialyzed against 10 times the volume of 50 mM potassium phosphate buffer (pH 7.3).

Step 3. $(NH_4)_2SO_4$ fractionation. To the dialyzed enzyme solution, ammonium sulfate was added to 25% saturation adjusting the pH to 7.3 with 10% ammonia water. After stirring for 1 h the precipitated protein was removed by centrifugation at 10,000 x g for 30 min and the precipitate was discarded. The ammonium sulfate was increased to 70% saturation by further addition of solid ammonium sulfate. After stirring for 1 h, the precipitate was collected by centrifugation at 10,000 x g for 30 min. The precipitate was dissolved in 50 mM potassium phosphate buffer, pH 7.3. The solution was then dialyzed overnight against 4 changes of the same buffer.
Step 4. First DEAE-cellulose ion exchange chromatography. The dialyzed enzyme from the previous step was layered on a column (6x66 cm) of DEAE-cellulose that had been equilibrated with 50 mM potassium phosphate buffer (pH 7.5). The column was eluted with the same buffer and 10-ml fractions were collected; the active fractions were pooled and concentrated by salting out with $(NH_4)_2SO_4$ at 80% saturation. The precipitate was resuspended in a minimum amount of 2 mM potassium phosphate buffer, pH 7.3, and dialyzed against the same buffer.
Step 5. Second DEAE-cellulose ion exchange chromatography. The protein from step 4 was charged on a column (5x50 cm) of DEAE-cellulose previously equilibrated with 2 mM potassium phosphate buffer (pH 7.3). The column was washed with the same buffer and was eluted with 10 mM potassium phosphate buffer (pH 7.3). The 10-ml fractions with high enzyme activity were pooled.
Step 6. Phenyl-Sepharose column chromatography. The enzyme solution from step 5 was loaded on a phenyl-Sepharose column (1.5x20 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.3) containing 10% $(NH_4)_2SO_4$. The enzyme was eluted with 20 mM potassium phosphate buffer (pH 7.3). The active 10-ml fractions were collected and concentrated by salting out with $(NH_4)_2SO_4$ at 80% saturation. The precipitate was resuspended in a minimum amount of 20 mM potassium phosphate buffer, pH 7.3, and dialyzed against the same buffer.
Step 7. First Sephadex G-100 superfine column chromatography. The protein from step 6 was layered on a Sephadex G-100 superfine column (1.7x100 cm) that had been equilibrated with 20 mM potassium phosphate buffer (pH 7.3); 2-ml fractions were collected and the fractions with high activity were combined and concetrated by salting out with $(NH_4)_2SO_4$ at 80% saturation. The precipitate was resuspended in a minimum amount of 20 mM potassium phosphate buffer, pH 7.3, and dialyzed against the same buffer.
Step 8. Second Sephadex G-100 superfine column chromatography. The concentrated protein from step 7 was placed on a gel filtration column of Sephadex G-100 superfine (1.7x100 cm) that had been equilibrated with 20 mM potassium phosphate buffer (pH 7.3). The enzyme was eluted with the above buffer, and the 2-ml fractions with high enzyme activity were combined. Ammonium sulfate was added to the solution up to 80% saturation, and the precipitate was collected by centrifugation (10,000 x g, 30 min) and dissolved in a minimum amount of the same buffer.
Step 9. Crystallization Finely powdered $(NH_4)_2SO_4$ was carefully added to the enzyme solution, until it became slightly turbid. Crystallization was virtually completed within 15 days at 5°C.

RESULTS AND DISCUSSION

Formation of serine hydroxymethyltransferase Optimal culture conditions were examined for the large-scale production of serine hydroxymethyltransferase by H. methylovorum GM2 grown in a 100-liter jar fermentor. The cultivation was carried out with pH control and methanol

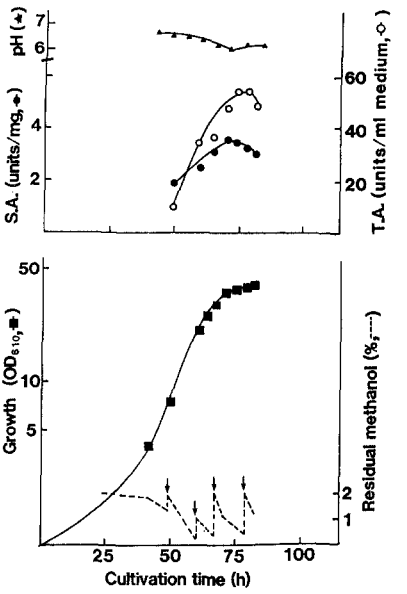

Fig. 1. Time course of serine hydroxymethyltransferase production by H. methylovorum during cultivation in a jar fermentor. Cultivation was carried out at 28°C in 60 liters medium in a 100-liter jar fermentor with aeration (1vvm) and with the lower limit of pH continuously controlled at pH 6.2 with 4 N ammonia water. Methanol was fed as indicated with arrows. S.A., Specific activity; T.A., total activity.

feeding. Figure 1 shows the time course of a typical cultivation. The specific activity and total activity of serine hydroxymethyltransferase reached a maximum at the end of the logarithmic phase, 70 to 80-h cultivation, and thereafter rapidly decreased. The maximum specific activity was 40 times higher than that in our previous paper (4), where the organism was cultivated for 4 days in 500 ml medium in a 2-liter flask without pH control. In the following experiments, the organism was cultured for 75 h in the jar fermentor.

Purification and crystallization Table 1 summarizes the purification procedure for obtaining crystalline serine hydroxymethyltransferase from the microbial source. The crystalline enzyme was homogeneous by the criteria of ultracentrifugation and SDS slab polyacrylamide gel electrophoresis. When assayed in the absence of pyridoxal-P, the crystalline enzyme preparation showed no activity and had only an absorption maximum at 280 nm. However, after incubating the enzyme with

Table 1. Summary of the Purification of Serine Hydroxymethyltransferase from Hyphomicrobium methylovorum

Procedure	Total protein (mg)	Specific activity[a] (units/mg)	Total activity[a] (units)	Yield (%)
Cell-free extract	58,800	-[b] (2)[d]	-[b]	-
Protamine treatment	56,100	0.08[c] (3)[d]	4703[c](124900)[d]	100[c] (100)[d]
$(NH_4)_2SO_4$ fractionation	40,350	0.12 (4)	4716(169900)	100 (98)
1st DEAE-cellulose	16,560	0.28 (7)	4694(166300)	99.8 (68)
2nd DEAE-cellulose	2,680	1.74 (46)	4680(115200)	99.5 (73)
Phenyl-Sepharose	622	6.13 (198)	3810(123400)	81.0 (73)
1st Sephadex G-100 superfine	202	11.4 (454)	2294 (91370)	48.8 (54)
2nd Sephadex G-100 superfine	184	11.3 (454)	2068 (91370)	44.0 (54)
Crystallization	184	11.3 (454)	2068 (91000)	44.0 (54)

a Enzyme activities were assayed by phenylserine degradation (c) and serine synthesis (d).
b The assay for the enzyme activity of phenylserine degradation did not work well in cell-free extracts as described previously (4).

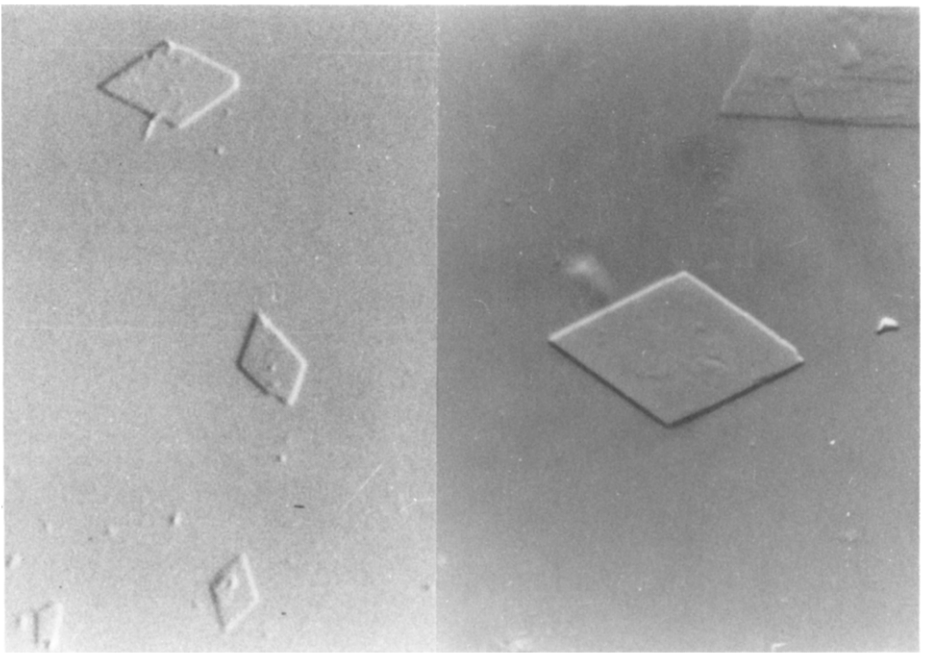

Fig. 2. Photomicrographs of the crystalline apo-serine hydroxymethyltransferase.

potassium phosphate buffer (pH 7.3) containing 0.1 mM pyridoxal-P, 10 μM Na_2EDTA and 10 μM 2-mercaptoethanol for 6 h at 30°C, the enzyme had activity and additional absorption maxima at 340 nm and 415 nm. Therefore, the crystalline enzyme was considered to be the apo form. Figure 2 shows the photomicrographs of the crystalline apo-serine hydroxymethyltransferase, which appeared as thin rombic plates. The overall purification achieved was around 134-fold with 44% yield when the activity was measured by phenylserine degradation, while it was 183-fold with 54% yield when the activity was measured by serine synthesis. The difference in the values of the purification and yield between the two assay methods may be attributed to their accuracy and sensitivity (10). Thus, the yield was improved about 1.5 times compared to the previous results (4).

Molecular properties of the crystalline enzyme, including the average molecular mass (90,000 daltons), subunit structure (homodimer), and isoelectric point were the same as those of the previously purified enzyme (4).

Amino acid composition The amino acid content was determined after 24-, 48-, and 72-h acid hydrolisis of the holoenzyme. The values are expressed

Table 2. Amino acid composition of serine hydroxymethyltransferase from H. methylovorum GM2

Amino acids	Residues/subunit
Asp	36[a]
Thr	14
Ser	15
Glu	32[a]
Gly	42
Ala	50
Cys	6
Val	28
Met	8
Ile	28
Leu	28
Tyr	6
Phe	20
Lys	25
His	12
Arg	19
Pro	12
Trp	3

a These values include both free and amidated residues.

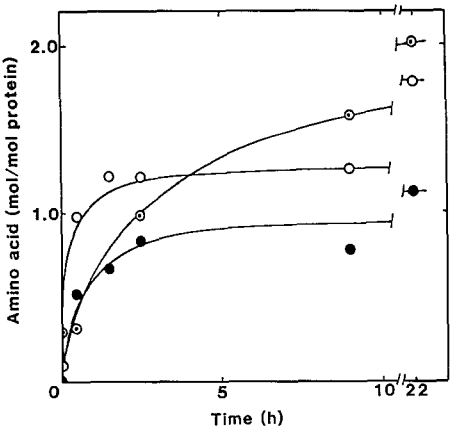

Fig. 3. Carboxy-terminal amino acid analysis. Serine hydroxymethyltransferase (2.8 mg) was incubated with carboxypeptidase Y at 25°C for 22 h. At various intervals, 30-μl aliquots were withdrawn. The released amino acids were: (○) tyrosine; (●) isoleucine; (⊙) alanine.

as an average of residues per subunit, assuming an average molecular mass of 45,000 daltons (4). Amino acid analysis of the enzyme from H. methylovorum shows that it had higher values per subunit for acidic amino acids, aspartic acid and glutamic acid, as well as neutral amino acids such as glycine, alanine, isoleucine and phenylalanine than that from rabbit liver (11), while the values for basic residues were nearly the same (Table 2). This may account for the lower pI (=5.5) of the H. methylovorum enzyme than that of rabbit liver (=7.2) as described previously (4).

The carboxyl-terminal amino acids released with carboxypeptidase Y was in turn tyrosine, alanine and isolelucine, suggesting the sequence -Ile-Ala-Tyr (Fig. 3).

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