A NEW AMIDINOHYDROLASE, METHYLGUANIDINE AMIDINOHYDROLASE FROM *ALCALIGENES* SP. N-42

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1. Introduction

Methylguanidine is known to accumulate in body fluids of uremic patients [1,2] and has been proved a uremic toxin [3]. It was found in several foods [4,5] and nitrosated under acidic conditions to give methylnitrosocyanamide with strong mutagenicity and carcinogenicity [6].

We attempted to isolate the microorganisms capable of producing methylguanidine-decomposing enzyme which may have a potential use for clinical assay and/or detoxification. Here, the purification and identification of a new enzyme, methylguanidine amidinohydrolase from *Alcaligenes* sp. N-42, which catalyzes specifically the hydrolysis of methylguanidine to yield methylamine and urea, is described.

2. Materials and methods

2.1. *Isolation and cultivation of the organism*

Alcaligenes sp. N-42 able to utilize methylguanidine as a sole source of carbon and nitrogen had been isolated from soil. The cultivation medium consisted of: glycerin, 10 g; methylguanidine sulfate, 1 g; $Na_2HPO_4 \cdot 7\ H_2O$, 1 g; $MgSO_4 \cdot 7\ H_2O$, 1 g; yeast extract, 0.5 g; $FeSO_4 \cdot 7\ H_2O$, 0.1 g; $MnCl_2 \cdot 4\ H_2O$, 0.1 g in 1 liter of distillated water. Initial pH was adjusted to 7.2. The organism was inoculated into 20 l of the medium in a 30 liter jar fermentor (L. E. Marubishi Co.) cultured at 30°C at 400 rev./min and ventilation of 2 liters of air/min, and harvested by centrifugation when methylguanidine disappeared from the medium.

2.2. *Assay of enzyme activity*

Two methods were used.

(A) The reaction mixture containing 50 μl enzyme solution and 0.45 ml 0.01% methylguanidine sulfate solution in 0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 10.0) was incubated for 10 min at 30°C and the reaction was stopped by the addition of 0.5 ml 0.4 M trichloroacetic acid. The extent of the reaction was quantified by measurement of methylguanidine and other guanidino compounds by high-performance liquid chromatography for the separation and detection of fluorescence reaction with 9,10-phenanthrenquinone [7].

(B) Methylamine formation was measured by the automated method with the trinitrobenzenesulfonate reaction utilized a discrete analyzer [8], AutoChemist model AC 60 (Pye Unicam, Cambridge) with a slight modification. Enzyme solution (100 μl) and 1 ml 0.01% methylguanidine sulfate solution in 0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 10.0), were automatedly transfered to a reaction tube and incubated at 37°C. After 5 min, enzyme reaction was stopped by addition of 2.5 ml 0.1 M borate buffer (pH 9.6).

An enzyme unit is defined as the activity required to catalyze the hydrolysis of 1 μmol methylguanidine/min at 30°C and pH 10.0.

2.3. *Other methods*

The concentration of protein was determined by the Lowry method [9]. The methylamine formation was confirmed by the high-performance liquid chromatography for the separation and detection method

of amines with *o*-phthalaldehyde [10]. Urea was measured according to the urease–indophenol method [11].

2.4. *Enzyme purification*

Wet cells (53 g) were disrupted by a Dyno-Mill (Willy A. Bachofen, Engineering Works, Basel), and solubilized with 2% Triton X-100 in 0.05 M $NaHCO_3$–Na_2CO_3 buffer (pH 10.0). The precipitated material was centrifuged down and discarded. In order to remove the nucleic acids, 4.8 ml 10% polyethyleneimine solution was added to the supernatant (483 ml) dropwise to 0.2% final conc. and some precipitated material was discarded by centrifugation. DEAE-cellulose (300 g) equilibrated with 0.01 M $NaHCO_3$–Na_2CO_3 buffer (pH 10.0) was added to the supernatant and stirred frequently. After 1 h the enzyme-adsorbed cellulose was separated by filtration. The cellulose was suspended in 0.05 M $NaHCO_3$–Na_2CO_3 buffer (pH 10.0) containing 0.5 M KCl, and stirred for 1 h and filtrated. The eluent (827 ml) was fractionated with $(NH_4)_2SO_4$ between 0–55% saturation. The resulting precipitate was collected by centrifugation, dissolved in 30 ml 0.05 M Tris–HCl buffer (pH 8.0) and dialyzed overnight against 2 liters of 0.01 M Tris–HCl buffer (pH 8.0). The enzyme solution was then clarified by centrifugation and chromatographed on a 2.5 × 30 cm column of DEAE–Sephacel equilibrated with 0.01 M Tris–HCl buffer (pH 8.0), using a linear gradient from 0.2–0.4 M KCl in the same buffer. The enzyme fraction (157 ml) was concentrated to 10 ml by ultrafiltration and applied to a 5 × 80 cm column of Sephadex G-200 equilibrated with 0.1 M KCl in 0.01 M Tris–HCl buffer (pH 8.0). The column was eluted with the same buffer at 35 ml/h. The active fraction (148 ml) was applied to a 1.5 × 30 cm column of hydroxylapatite equilibrated with 0.01 M phosphate buffer (pH 8.0). The enzyme was eluted from the column using a stepwise elution with 0.1 M phosphate buffer (pH 8.0).

2.5. *Materials*

Methylguanidine sulfate was prepared from methylamine and 2-methyl-2-thiopseudourea sulfate [12]. Ethylguanidine sulfate, *n*-propylguanidine sulfate and *n*-butylguanidine sulfate was prepared as above from ethylamine, *n*-propylamine and *n*-butylamine, respectively. Each product was purified by repeated crystallizations and identified by the above mentioned high-performance liquid chromatography for guanidino compounds, NMR and infrared. Guanidinoacetic acid, 3-guanidinopropionic acid, 4-guanidinobutyric acid, guanidinosuccinic acid, agmatine sulfate and urease were obtained from Sigma Chem. Co. (St. Louis, MO). DEAE–Sephacel and Sephadex G-200 were purchased from Pharmacia Fine Chem. (Uppsala). Hydroxylapatite was product of Bio-Rad Labs. (Richmond, MA).

All other chemicals were of analytical or reagent grade.

3. Results and discussion

The results of purification are summarized in table 1. The purified enzyme was homogeneous in polyacrylamide gel electrophoresis run at different polyacrylamide concentrations. The specific activity reached 102 units/mg protein. The overall yield of purification was 20.5%.

The enzyme catalyzed the hydrolysis of methyl-

Table 1
Purification of methylguanidine amidinohydrolase from *Alcaligenes* sp. N-42

Step	Total protein (mg)	Total activity (units)	Spec. act. (units/mg prot.)	Recovery (%)
Cell-free extract	2443	7800	3.2	100
Treatment with polyethyleneimine	2285	7800	3.4	100
DEAE-cellulose (batchwise)	1128	7700	6.8	98.4
Ammonium sulfate fractionation	678	6300	9.3	80.8
DEAE–Sephacel	54.3	3375	62.1	54.3
Sephadex G-200	40.7	2875	70.6	40.7
Hydroxylapatite	20.5	2090	102.0	20.5

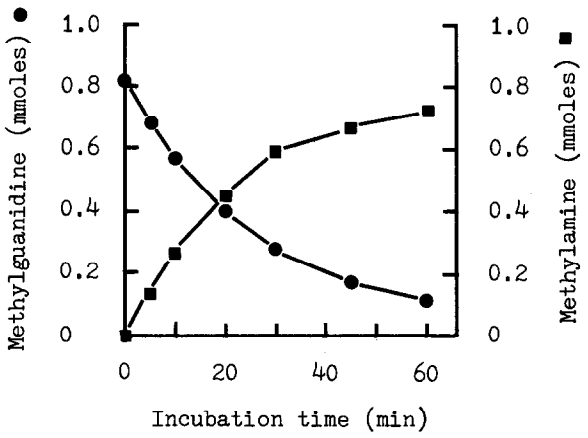

Fig.1. Stoichiometry of the reaction catalyzed by methylguanidine amidinohydrolase. The incubation mixture contained, in 2.0 ml: 0.2 mmol $NaHCO_3$–Na_2CO_3 buffer (pH 10.0); 0.82 mmol methylguanidine; 20 units enzyme. Incubation was at 30°C. Aliquots (0.1 ml) were removed at the indicated times and transfered to tubes containing 0.4 ml 0.1 M borate buffer (pH 9.6). Samples were analyzed for methylguanidine and methylamine.

guanidine to yield methylamine and urea. As shown in fig.1, the disappearance of methylguanidine and appearance of methylamine were parallel and stoichiometric. At completion of reaction, 1 mol methylamine and 1 mol urea were released/mol methylguanidine hydrolyzed. The equation for the reaction catalyzed by this enzyme is thus:

$$\text{Methylguanidine} + H_2O = \text{Methylamine} + \text{Urea}$$

Table 2
Substrate specificity of methylguanidine amidinohydrolase

Substrate	Relative rate (%)
Methylguanidine	100
Ethylguanidine	1.3
n-Propylguanidine	0.2
n-Butylguanidine	0.7
Guanidinoacetic acid	0
3-Guanidinopropionic acid	0
4-Guanidinobutyric acid	0
Agmatine	0.5
Guanidinosuccinic acid	0
Guanidine	0.3

The reaction mixture (1 ml) contained 90 μmol $NaHCO_3$–Na_2CO_3 buffer (pH 10.0) and enzyme (0.2 units). Compounds to be tested were added at 1 mg/ml and incubated at 30°C for 12 h, except that methylguanidine was incubated for 10 min. The activity was measured by the method A

A variety of compounds containing the guanidine group were tested with the enzyme, and the reaction mixtures were assayed for the disappearance of the guanidino compounds and appearance of the amines. None of guanidinoacetic acid, 3-guanidinopropionic acid, 4-guanidinobutyric acid and guanidinosuccinic acid was hydrolyzed. Ethylguanidine, *n*-propylguanidine, *n*-butylguanidine, agmatine and guanidine were slightly hydrolyzed <1.3% than observed with methylguanidine by the enzyme (table 2).

The present data shows that the purified methylguanidine-decomposing enzyme from *Alcaligenes* sp. N-42 exhibits a high order of substrate specificity for methylguanidine and has not hydrolyzed guanidino compounds with carboxyl group. Therefore, it is distinct from the known amidinohydrolases: guanidinoacetate amidinohydrolase (EC 3.5.3.2), 3-guanidinopropionate amidinohydrolase [13], 4-guanidinobutyrate amidinohydrolase (EC 3.5.3.7) and *N*-amidino-L-aspartate amidinohydrolase (EC 3.5.3.14). Consequently, this enzyme is a new type of amidinohydrolase and has been named methylguanidine amidinohydrolase. The specificity of the enzyme suggests that it may be useful as a means of detoxication and/or assay of methylguanidine in body fluids. Studies on the further characterization of the enzyme are in progress.

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