Kor. J. Appl. Microbiol. Biotechnol.
Vol. 19, No. 6, 610-613 (1991)

Some Properties of Xanthine Dehydrogenase from *Pseudomonas synxantha* A3

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Pseudomonas synxantha A3에서 분리한 Xanthine Dehydrogenase의 성질

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Aabstract — Some of the Kinetic properties of crystallic xanthine dehydrogenase form *Pseudomonas synxantha* A3 were studied. The enzyme activity was strongly inhibited by adenine, 8-azaadenine, 2-methyladenine, guanine, and 8-azaguanine, but not by caffeine, and the inhibitions by adenine and guanine were observed to be of noncompetitive type. The K_i values for adenine and guanine were 0.037 and 0.098 mM, respectively. Michaelis constants were found to be 0.33 and 0.06 mM for hypoxanthine and xanthine with NAD^+ as the second substrate, respectively, and 0.1 mM for NAD^+ with either hypoxanthine or xanthine as the second substrate.

In the preceding paper (1), the authors studied the purification, crystallization, and some properties of xanthine dehydrogenase (xanthine: NAD oxidoreductase EC 1.2.1.27) form *Pseudomonas synxantha* A3 grown on a medium containing adenine.

The xanthine-oxidizing enzymes (2-9) are known to oxidize various purines, and some of these enzymes oxidize aldehydes, utilizing various electron acceptors, such as NAD^+, $NADP^+$, ferricyanide, 2, 6-dichlorophenolindophenol, methylene blue, and tetrazolium derivatives, though the specificities differ depending on the enzyme's origin. Xanthine dehydrogenase from *P. synxantha* A3 (1) oxidizes hyposanthine, xanthine, and some purine analogues, and utilizes NAD^+, phenazine methosulfate, and 2, 6-dichlorophenolindophenol. Compared with xanthine dehydrogenase in *P. acidovorans* (9), the enzyme in *P. synxantha* A3 was of rather narrow specificity: that the former catalyzes the oxidation of xanthine, hypoxanthine, and purine, utilizing NAD^+, $NADP^+$, and oxygen as electron acceptors. From the results, xanthine dehydrogenase form *P. synxantha* A3 is different form the known xanthine-oxidizing enzymes.

As xanthine dehydrogenase from *P. synxantha* A3 (1) oxidizes hypoxanthine better than xanthine, and utilizes NAD^+, but not $NADP^+$, as an electron acceptor, it might be called hypoxanthine: NAD oxidoreductase.

This paper describes some of the kinetic properties of crystalline xanthine dehydrogenase from *P. synxantha* A3.

Key word: *Pseudomonas synxantha* A3, xanthine dehydrogenease, kinetics (hypoxanthine, xanthine, NAD^+)
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Materials and Methods

Chemicals

Nucleotides-related compounds were purchased

from Sigma Chemicals (St. Louis), except NAD^+ which was obtained from Oriental Yeast Co. Ltd. (Tokyo). Other chemicals were from Wako Pure Chemical Industries (Osaka), and were of certified reagent grade.

Cultivation

The organism was grown at 30℃ in a medium containing per liter: 10g meat extract, 10g peptone, 5g yeast extract, 1g adenine, 5g KH_2PO_4, 5g K_2HPO_4, 0.2g $MgSO_4 \cdot 7H_2O$, 0.1 mg $MnCl_2 \cdot 4H_2O$, 0.2 mg ferric ammonium citrate, 2 mg $CaCl_2$, and 10 mg sodium molybdate, pH 7.0. The cultivation was carried out in a 20-liter fermentor containing 15 liters of the medium described above under aeration for 40 hr. The cells were harvested by centrifugation, washed twice with 0.85% NaCl solution, and suspended in 100 mM potassium phosphate buffer containing 0.1 mM EDTA and 2 mM 2-mercaptoethanol, pH 8.2.

Enzyme prepration

Xanthine dehydrogenease was purified from the washed cells of *P. synxantha* A3 according to the methods descrbied previously (1).

Enzyme assay

Xanthine dehydrogenease activity was determined spectrophotometrically be determining absorption at 340 nm based on the reduction of NAD^+ accompanied by the dehydrogenation of hypoxanthine. The standard assay mixture was a total volume of 3.0 m*l* containing 1.2 μmol hypoxanthine, 0.6 μmol NAD^+, 150 μmol sodium pyrophosphate buffer, 0.3 μmol EDTA, and 6 μmol 2-mercaptoethanol, pH 8.5. The reaction was performed at 37℃ on a Hitachi model 124 spectrophotometer equipped with a thermostatically controlled cuvette holder and continous chart recorder. The molecular absorption coefficient of NADH under those conditions was 6.22×10^3 liter$\cdot M^{-1} \cdot cm^{-1}$. One unit of enzyme activity was defined as 1 μmol NAD reduced/min under the standard assay conditions.

Unless otherwise noted, all the materials and methods were the same as those described in the previous paper (1).

Results

Effect of purine analogues

A number of purine anlogues were tested for

Table 1. Effect of purine analogues on the xanthine dehydrogenase activity

Compound (0.1 mM)	Substrate	
	Hy. X. (R.A.)	X (R.A.)
None	100	100
Xanthine-N^3-oxide	74	76
8-Chloroxanthine	96	89
8-Azaxanthine	97	95
Theobromide	63	58
Theophylline	92	89
Dyphylline	100	100
Caffeine	100	100
Caffeine (1 mM)	100	100
Adenine	35	32
8-Azaadenine	10	13
2-Methyladenine	32	32
Editadenine[a)]	101	95
Guanine (0.15 mM)	9	15
8-Azaguanine	4	10
Inosine	61	105
Adenosine	69	58
Adenosine-N^1-oxide	104	100
2′-Deoxyadenosine	102	99
Formycin	102	102
Guanosine	102	97
Deoxyguanosine	90	86
6-Chloroguanosine	96	98
Uric acid	105	102
6-Methylpurine	96	102
6-Benzylpurine	72	73
2,8-Dithio-6-oxypurine	87	77
3′-Adenylic acid	100	100
Cytidine	100	96
Cytosine	81	81
5-Methylcytosine	91	89

[a)] = 2,3-dihydroxy-4-(9-adenyl)-butyric acid
Hy. X. = hypoxanthine, X. = xanthine, R.A. = relative activity
Reaction was at 37℃, in 50 mM sodium pyrophosphate buffer, pH 8.5. Incubations contained 0.5 mM NAD^+ and 0.2 mM substrates except that indicated compounds were added.

inhibition of the xanthine dehydrogenase activity. As shown in Table 1, many compounds caused some inhibition, but uric acid and caffeine, a methylpurine, had no effect, while theobromine and theophylline inhibited some extent. Adenine, 8-azaadenine, 2-methyladenine, guanine, and 8-azaguanine had a strong inhibitory effect at 0.1 mM.

Kinetics

The kinetics of NADH formation were investigated as a function of hypoxanthine or xanthine and NAD^+ concentrations. Results for the reaction of hypoxanthine with NAD^+ and xanthine with NAD^+ are shown in Fig. 1 and 2, respectively. The aparent Michaelis constants for the substrates were determined according to the method of Lineweaver and Burk (10). The Michaelis constant for hypoxanthine was 0.33 mM and for NAD^+ 0.1 mM. In the case of reaction with xanthine as substrate, the Michaelis constant for xanthine was estimated to be 0.06 mM and for NAD^+ 0.1 mM.

Inhibition by adenine and guanine

The effect of adenine and guanine on the enzyme reaction was studied. Lineweaver and Burk plots (10) for the enzyme reaction in the presence of these compounds demonstrated that adenine and guanine act as noncompetitive inhibitors (Fig. 3). The K_i values for adenine and guanine were 0.037 and 0.098 mM, respectively.

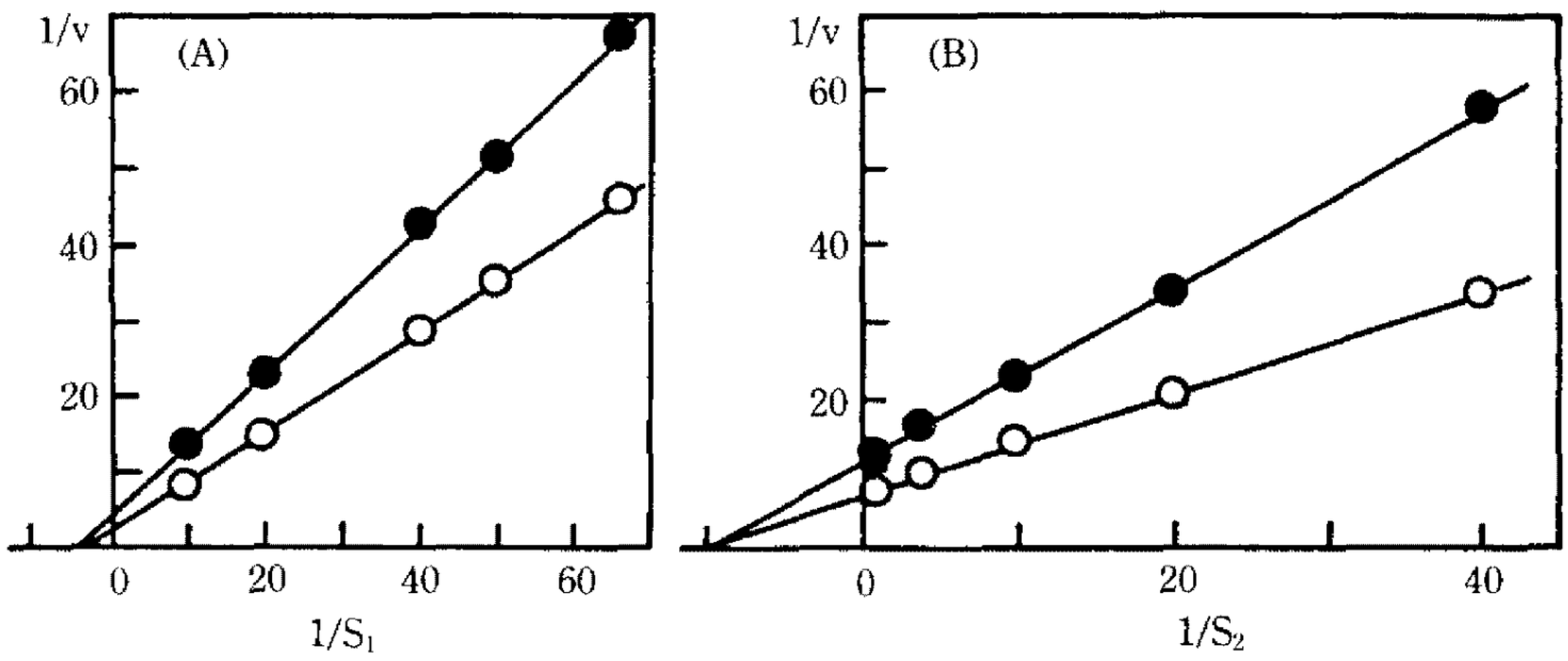

Fig. 1. Kinetics of the reaction between hypoxanthine and NAD^+ catalyzed by xanthine dehydrogenase.
Reaction was performed under standard conditions except that concentrations (represented in mM) of hypoxanthine (S_1) and NAD^+ (S_2) were varied as follows: (A) S_1 was varied between 0.015 and 0.1 mM with S_2 fixed to 0.5 mM (○) or 0.05 mM (●); (B) S_2 was varied between 0.025 and 1.0 mM with S_1 fixed to 0.1 mM (○) or 0.05 mM (●). Results were plotted in double reciprocal form.

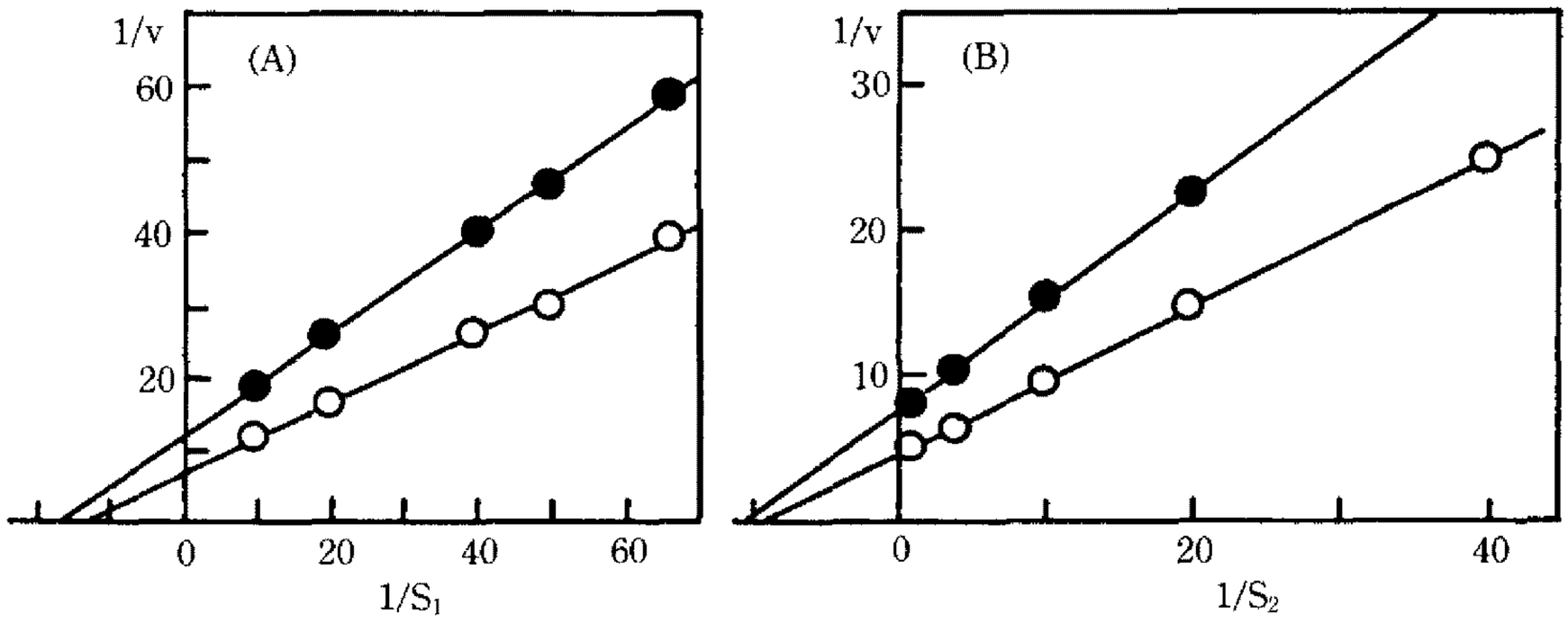

Fig. 2. Kineties of reaction between xanthine and NAD^+ catalyzed by xanthine dehydrogenase.
Reaction was performed under standard conditions except that concentration (represented in mM) of xantine (S_1) and NAD^+ (S_2) were varied as follows: (A) S_1 was varied between 0.015 and 0.1 mM with S_2 fixed to 0.5 mM (○) or 0.05 (●); (B) S_2 was varied between 0.025 and 1.0 mM with S_1 fixed to 0.1 mM (○) or 0.05 mM (●).

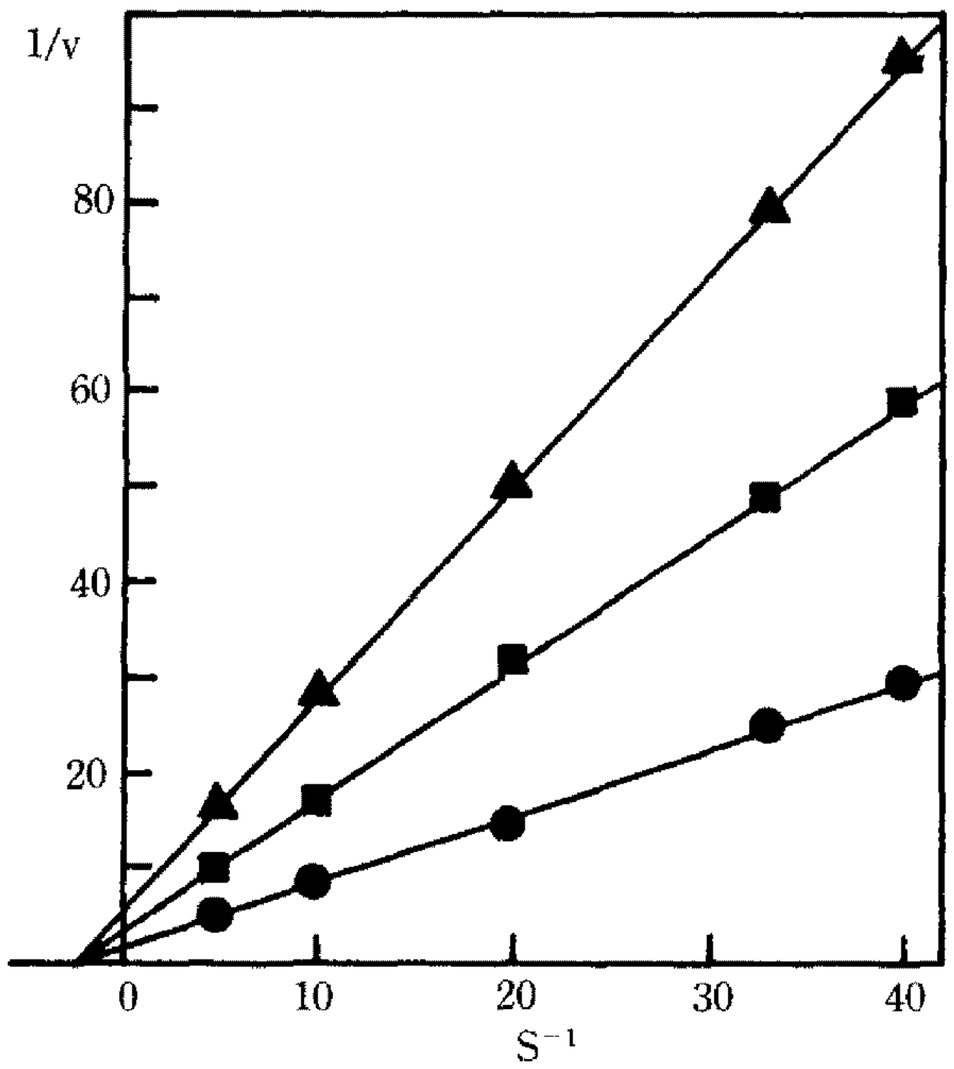

Fig. 3. Inhibition of xanthine dehydrogenase by adenine and guanine.
Conditions were identical with standard condition except that reaction was carried out in the presene of variously changed concentrations of substrate hypoxanthine (S, represented in mM) and, if added, 0.075 mM guanine or 0.1 mM adenine.
●, no added guanine and adenine; ■, added guanine; △, added adenine.

Discussion

The existence of xanthine dehydrogenase has been described from various sources (2-9). The crystallin xanthine dehydrogenase from *P. synxantha* A3 was inhibited by various purine analogues especially by adenine, 8-azaadenine, 2-methyadenine, guanine, and 8-azaguanine, but not by caffeine and urate. Xanthine dehydrogenease from *P. acidovorans* (9) was also inhibited by adenine and guanine, but not by urate, which was reported to be a potent inhibitor of xanthine dehydrogenase from insect (11), but it did not inhibited the enzyme from *P. acidovorans* and *P. synxantha* A3. The inhibition by adenine and guanine with respect to hypoxanthine was noncompetitive. In the case of the enzyme from *P. acidovorans*, the inhibition by adenine with respect to xanthine as substrate was also noncompetitive (9). The K_m values for hypoxanthine and xanthine were 0.33 and 0.06 mM, respectively, in *P. synxantha* A3, and 0.29 and 0.07 mM, respectively, in *P. acidovorans* (9). Thus, the K_m values of xanthine dehydrogenase from the two species of *Pseudomonas* showed the same order of magnitude. Though the substrate specificity of *P. synxantha* A3 (1) had been more narrow than that of *P. acidovorans*, some kinetic properties of *P. sunxantha* A3 studied in this paper were simular to those of *P. acidovorans*. There may be needed more detailed studies to compare the kinetic properties of the enzyme more clearly.

요 약

Pseudomonas synxantha A3로부터 정제된 결정화 효소를 사용하여 몇가지의 성질을 검토하였다. 본 효소에 대한 nucleoside 관련물질의 저해관계를 검토한 결과, 그 중에서 adenine, 8-azaadenine, 2-methyladeine, guanine, 8-azaguanine에 의해서 강한 저해를 받는 것으로 나타났으며, caffenine에 의해서는 저해를 받지 않았다. Adenine과 guanine은 비길항저해제로서, 그 저해상수(K_i)는 각각 0.037 mM과 0.098 mM이었다. 그리고 pH 8.5에 있어서 hypoxanthine과 NAD^+에 대한 K_m값은 각각 0.33 mM, 0.1 mM이며, 또한 xanthine과 NAD^+에 대한 K_m값은 각각 0.06 mM, 0.1 mM이었다.

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(Received Octoter 17, 1991)