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Purification and Characterization of 3-Ketovalidoxylamine A C-N Lyase Produced by *Stenotrophomonas maltrophilia*

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Abstract A soluble 3-ketovalidoxylamine A C-N lyase from *Stenotrophomonas maltrophilia* was purified to 367.5-fold from the crude enzyme, with a yield of 16.4% by column chromatography on High S IEX, Methyl HIC, High Q IEX, and Sephadex G 100. The molecular mass of the enzyme was estimated to be 34 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the enzyme was a neutral protein having an isoelectric point value at pH7.0. The optimal pH of 3-ketovalidoxylamine A C-N lyase was around 7.0. The enzyme was stable within a pH range of 7.0–10.5. The optimal temperature was found to be near 40°C, and the enzyme was sensitive to heat. The enzyme was completely inhibited by ethylenediaminetetraacetic acid, and it was reversed by Ca^{2+}. The product, *p*-nitroaniline, inhibited the enzyme activity significantly at low concentration. The enzyme has C-N lyase activity and C-O lyase activity, and need 3-keto groups. The apparent K_m value for *p*-nitrophenyl-3-ketovalidamine was 0.14 mM.

Keywords 3-Ketovalidoxylamine A C-N lyase · Valienamine · *p*-Nitrophenyl-3-ketovalidamine · *Stenotrophomonas maltrophilia*

Introduction

3-Ketovalidoxylamine A C-N lyase (EC 4.3.3.1) cleaving carbon–nitrogen bond belongs to the family of lyases, specifically amine lyases. It was first identified in *Flavobacterium saccharophilum*, which had been used in cleavage of validoxylamine A to valienamine, validamine, and unsaturated ketocyclitols [1]. It had been reported that two key enzymes, 3-ketovalidoxylamine A C-N lyase and glucoside 3-dehydrogenase (G3DH), participate in

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the cleavage of validoxylamine A by *F. saccharophilum* [2]. Another strain producing 3-ketovalidoxylamine A C-N lyase was *Stenotrophomonas maltrophilia* CCTCC M 204024, isolated previously for biotransformation of validamycin A to valienamine by our laboratory [3, 4]. Valienamine was a strong α-glucosidase inhibitor of honeybee α-glucosidase and pig sucrase in vitro with IC_{50} value of 5.2×10^{-5} and 5.3×10^{-5}M, respectively [5–7]. It could be applied to the development of new drugs and pesticides. Furthermore, valienamine can be used as starting materials for the production of some of the most important glucosidase inhibitors [8, 9]. 3-Ketovalidoxylamine A C-N lyase is greatly important for the enzymatic production of valienamine.

In our previous work, G3DH has been purified, and the characterization of this enzyme has been studied [10]. *N*-*p*-nitrophenyl-3-ketovalidamine, used as the substrate of 3-ketovalidoxylamine A C-N lyase, was prepared from *N*-*p*-nitrophenylvalidamine with free cells of *S. maltrophilia* [11]. To date, however, there is no published information about the purification and characterization of 3-ketovalidoxylamine A C-N lyase from other microorganisms except *F. saccharophilum*. In our previous experiments, the differences of validamycin A degradation by *F. saccharophilum* and *S. maltrophilia* have been observed [4]. This paper describes the purification and characterization of 3-ketovalidoxylamine A C-N lyase from *S. maltrophilia*.

Materials and Methods

Organism and Growth

S. maltrophilia CCTCC M 204024 was previously isolated from wheat soil near Hangzhou, China, by our laboratory and preserved at the China Center for Type Culture Collection (CCTCC, Wuhan, China). It was maintained at 4°C on nutrient agar slants with a composition (per liter) of 10 g validamycin A, 10 g $(NH_4)_2SO_4$, 5 g NaCl, 2 g K_2HPO_4, 0.2 g $MgSO_4$, and 20 g agar. The microorganism *S. maltrophilia* was cultured on the above-mentioned agar slant for 24 h at 30°C and was then inoculated into a 500-ml conical flask containing 100 ml of sterilized medium with a composition (per liter) of 5 g validamycin A, 5 g beef extract, 10 g peptone, 5 g NaCl, and 5 g K_2HPO_4 at 30°C for 24 h on an orbital shaker of 150 rpm. Cells were harvested by centrifugation at 15,000×*g* for 10 min and washed with 50 mM phosphate buffer (pH7.0, containing 0.8% NaCl) twice.

Reagents

Validamycin A was provided by QianJiang Biochem Co. Ltd., China. Valienamine was prepared according to the methods described [3]. *N*-*p*-nitrophenylvalidamine and *N*-*p*-nitrophenylvalienamine were prepared from validamine and valienamine according to Takeuchi's method [1]. *p*-Nitrophenyl-3-ketovalidamine and *p*-nitrophenyl-3-ketovalienamine were prepared according to previous method [11]. All other chemicals used were of A.R. grade.

Enzyme Assays and Protein Assay

3-Ketovalidoxylamine A C-N lyase activity was assayed with *p*-nitrophenyl-3-ketovalidamine as the substrate [11]. The reaction mixture consisted of 0.1 ml of 10 mM substrate, 0.3 ml of 100 mM Tris–HCl buffer (pH7.0), and 0.1 ml of enzyme solution, which was incubated at

40°C for 30 min. The enzyme products were analyzed by high-performance liquid chromatography (HPLC). HPLC was carried out with reversed C_{18} and acetonitrile–water (1:3, *v*/*v*) as the mobile phase. The products were determined by a UV absorption spectrophotometer at 398 nm. One unit of the 3-ketovalidoxylamine A C-N lyase activity was defined as the amount of enzyme that caused the production of 1 μmol of *p*-nitroaniline per minute at pH7.0, 40°C.

Protein quantitative analysis was determined by the method of Bradford [12] with bovine serum albumin as a standard.

Preparation of Cell-Free Extraction

The washed cells (20 g wet weight of cells were obtained from 2.8 L of culture) were suspended in 150 ml of 10 mM NaAc buffer (10% glycerol in the buffer, pH5.4) and ruptured with sonication (400 W) for 10 min with ice cooling. The suspension was subjected to 5-s sonic treatment at 5-s intervals. The clear supernatant resulting from centrifugation at 48,400×*g* for 1 h was used as the cell-free extraction.

3-Ketovalidoxylamine A C-N Lyase Purification

The purification process employed a Biologic Duo Flow system (Bio-Rad Laboratories), and all operations occurred at 4°C throughout the purification steps. The cell-free extraction was applied to a cation ion exchange column (High S IEX, 1.5×14 cm, Bio-Rad Laboratories), equilibrated with 20 mM NaAc buffer (10% glycerol in the buffer, pH5.4), and eluted with 120 ml of a NaCl concentration linear gradient (0–0.5 M) at a flow rate of 2 ml·min^{-1}. Active 3-ketovalidoxylamine A C-N lyase fractions were supplemented with solid $(NH_4)_2SO_4$ to 1.3 M and applied to a hydrophobic interaction chromatography column (Methyl HIC, 5 ml, Bio-Rad Laboratories), equilibrated with 50 mM phosphate buffer (pH7.0, 1.3 M $(NH_4)_2SO_4$ in the buffer). The enzyme was eluted with a step gradient (30 ml of 1.0 M $(NH_4)_2SO_4$, 30 ml of 0.8 M $(NH_4)_2SO_4$, and 20 ml of 0.5 M $(NH_4)_2SO_4$) in 50 mM phosphate buffer (pH7.0) at a flow rate of 1 ml·min^{-1}. The active fractions were combined and dialyzed against 50 mM Tris–HCl buffer (pH8.5) for 24 h. Then, the enzyme was applied to an anion ion exchange column (High Q IEX, 5 ml, Bio-Rad Laboratories), equilibrated with 50 mM Tris–HCl buffer (pH8.5). 3-Ketovalidoxylamine A C-N lyase which was bound to the column was eluted in step gradient (30 ml of 0.05 M NaCl, 30 ml of 0.1 M NaCl, and 20 ml of 0.15 M NaCl) in 50 mM Tris–HCl buffer (pH8.5) at a flow rate of 1 ml·min^{-1}. The active fractions were combined and concentrated by freeze-drying. The concentrated enzyme was applied to a column of Sephadex G 100 (1.5×68 cm), equilibrated with 50 mM phosphate buffer (pH7.0), and eluted with the same buffer at a flow rate of 0.4 ml·min^{-1}. The active fractions were combined and used as a purified enzyme sample.

SDS-PAGE Analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed using a Mini-gel system (Bio-Rad Laboratories) with a 5% acrylamide stacking gel (pH6.8) and 10% separating gel (pH8.8) [13]. The protein standards used were as follows: rabbit phosphorglase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; rabbit actin, 43.0 kDa; bovine carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 20.1 kDa; and hen egg white lysozyme, 14.4 kDa (Shanghai Shenggong Co. Ltd). The gel was stained with Coomassie brilliant blue R-250.

Isoelectric-focusing PAGE

The isoelectric point (pI) was estimated on the Phast-System of Pharmacia LKB using a precast gel, PhastGel IEF 3–10. pIs were determined using standard pI markers (Bio-Rad).

Effect of pH on the Activity of 3-Ketovalidoxylamine A C-N Lyase

The effect of pH on the activity of the purified enzyme was examined with 50 mM sodium acetate buffer (pH5.0–6.0), 50 mM Tris–HCl buffer (pH6.0–9.0), and 50 mM glycine–sodium hydroxide buffer (pH8.5–10.5). The effect of pH on the stability of enzyme was examined with the same buffer, which mixed with the enzyme at 4°C for 24 h. After incubating, the residual enzyme activities were determined at pH7.0 with standard assay conditions.

Effect of Temperature on the Activity of 3-Ketovalidoxylamine A C-N Lyase

The enzyme activity was measured at various temperatures (25, 30, 35, 40, 45, 50, and 55°C). The temperature stability of the purified enzyme was examined at pH7.0. Samples were incubated in a preheated water bath at temperatures of 45 and 55°C for times ranging from 0 to 150 min at 20-min intervals. Residual enzyme activity was determined with standard assay conditions.

Effect of Metal Ions on the Activity of 3-Ketovalidoxylamine A C-N Lyase

The effect of various metal ions (Ca^{2+}, Mg^{2+}, Ba^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Hg^{1+}, Al^{3+}, Li^{2+}, and Zn^{2+}) and the chelating agent ethylenediaminetetraacetic acid (EDTA) on the activity of purified enzyme was examined at a concentration of 4 mM.

Effect of Products and Substrate Analogs Concentration

The effect of products and analogs concentration on enzyme activity was examined using validamine or *p*-nitroaniline. The effect of substrate analogs concentration on enzyme activity was examined using *N*-*p*-nitrophenylvalidamine.

Substrate Specificity

The purified 3-ketovalidoxylamine A C-N lyase activity was determined for various substrates, including *N*-*p*-nitrophenylvalidamine, *N*-*p*-nitrophenylvalienamine, *p*-nitrophenyl-3-ketovalidamine, *p*-nitrophenyl-3-ketovalienamine, *p*-nitrophenyl-α-D-glucopyranoside, and *p*-nitrophenyl-β-D-glucopyranoside. The enzyme was also determined when purified G3DH and 2,6-dichlorophenolindophenol were added to *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-β-D-glucopyranoside, respectively.

Results and Discussion

Purification of 3-Ketovalidoxylamine A C-N Lyase and Molecular Properties

The 3-ketovalidoxylamine A C-N lyase from *S. maltrophilia* was purified in four steps. The High S IEX column gave three protein peaks in the eluted fraction. The second and biggest

peak with C-N lyase activity was collected and applied to the Methyl HIC column, which gave two major protein peaks in the eluted fraction. The second peak with C-N lyase activity was collected and applied to High Q IEX column, which gave three peaks. The middle minor peak with C-N lyase activity was obtained (Fig. 1). This part of the enzyme was collected and applied to a column of Sephadex G 100, which gave two peaks. The second major peak with C-N lyase was collected.

The results of the purification were summarized in Table 1. The enzyme was purified 367.5-fold from the crude enzyme, with a yield of 16.4% by these procedures. The specific activity of the purified enzyme was 13.94 U/mg. The enzyme was stable in storage at 4 °C with 10% glycerol for several weeks. The 3-ketovalidoxylamine A C-N lyase of *F. saccharophilum* was found in both membrane fraction (20%) and soluble fraction (80%) [1]. However, the enzyme purified in this study was a soluble enzyme. The enzyme activity of purified 3-ketovalidoxylamine A C-N lyase from *F. saccharophilum* was 644.1 U/mg protein. According to Takeuchi's method, 1 U of the lyase activity was defined per hour [1], so the enzyme activity was 10.74 U/mg protein, while that was defined per minute. Therefore, the lyase activity of *S. maltrophilia* was as much as that of *F. saccharophilum*.

The purified enzyme gave only one band on the SDS-PAGE with a molecular weight of 34,000 Da (Fig. 2), which was similar to that of *F. saccharophilum* with a molecular mass of 36 kDa [1]. Analytical isoelectric-focusing data showed that the enzyme was a neutral protein having a pI value at pH 7.0. The enzyme from *F. saccharophilum* was a basic protein having a pI at pH 10.5 [1]. That is, the enzyme purified in this study might be different from that of *F. saccharophilum* on amino acid composing. At this point, there was a possibility that the 3-ketovalidoxylamine A C-N lyase obtained in this work was of a novel type.

Properties of 3-Ketovalidoxylamine A C-N Lyase

The enzyme activity was affected by pH greatly, and the optimal pH was around 7.0 (Fig. 3). The enzyme was stable within a pH range of 7.0–10.5, and the activity significantly declined below pH 7.0. The residual enzyme activity was declined to 70% at pH 4.0 for 24 h. The optimal pH of 3-ketovalidoxylamine A C-N lyase from *F. saccharophilum* was 9.0 [1], whereas it was 7.0 from *S. maltrophilia* in this study.

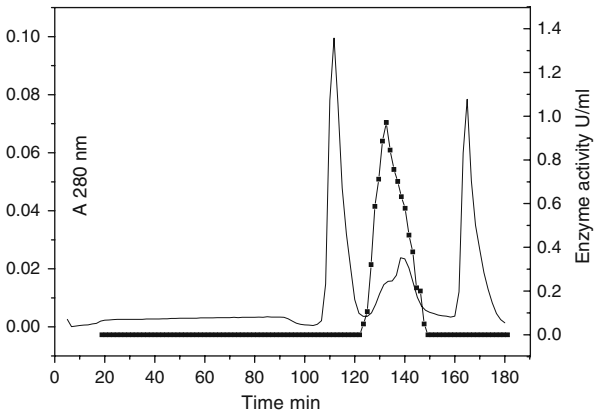

Fig. 1 Purification of 3-ketovalidoxylamine A C-N lyase on anion ion exchange column (High Q, IEX, 5 ml, Bio-Rad Laboratories). *Line* protein, *squares* enzyme activity

Table 1 Purification of 3-ketovalidoxylamine A C-N lyase.

	Total protein (mg)	Total activity (U)	Special activity (U/mg)	Fold	Yield (%)
Crude enzyme	807.81	30.62	0.038	1	1
High S IEX	250.29	22.48	0.090	2.4	73.4
Methyl HIC	103.31	19.02	0.18	4.9	62.1
High Q IEX	0.69	6.12	8.87	232.9	20.0
Sephadex G 100	0.36	5.02	13.94	367.5	16.4

The optimal temperature was near 40°C (Fig. 4a). It was similar to that from *F. saccharophilum* [1]. The enzyme activity decreased significantly at 45 and 55°C. Thus, the enzyme was sensitive to heat (Fig. 4b).

The effect of various metal ions and the chelating agent EDTA on the activity of purified enzyme was shown in Table 2. Ni^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Al^{3+}, Ba^{2+}, Hg^{1+}, and Cu^{2+} did not inhibit or promote enzyme activity at a concentration of 4 mM. The enzyme was completely inhibited by EDTA, and the EDTA inhibition was reversed by Ca^{2+}. Furthermore, the enzyme activity was promoted by Ca^{2+} and inhibited by Zn^{2+}. It needed Ca^{2+} for enzyme activity, so did that of *F. saccharophilum* [1, 14].

The effect of products and substrate analogs concentration on enzyme activity was shown in Fig. 5; validamine showed no significant effect on activity, whereas *p*-nitroaniline inhibited the enzyme activity significantly at low concentration. The substrate analogs, *N-p*-nitrophenylvalidamine, showed no effect on the enzyme activity. That is, the enzyme reaction might be applied at high substrate concentration.

The purified 3-ketovalidoxylamine A C-N lyase from *S. maltrophilia* showed limited substrate specificity. The enzyme had no activity on *N-p*-nitrophenylvalidamine and *N-p*-nitrophenylvalienamine. However, the enzyme had high activity on *p*-nitrophenyl-3-ketovalidamine and *p*-nitrophenyl-3-ketovalienamine. The enzyme activity was also

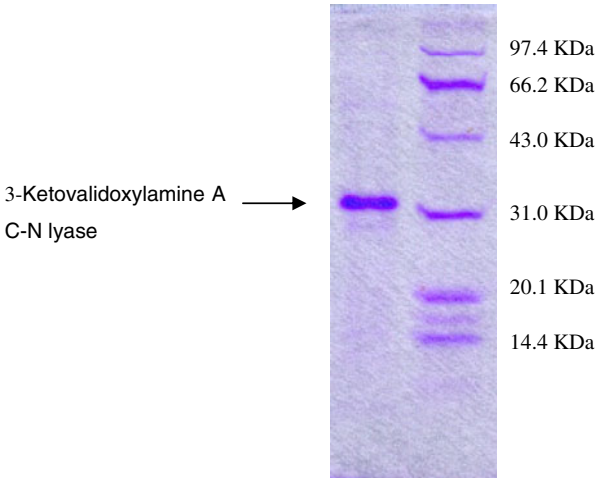

Fig. 2 SDS-PAGE analysis of 3-ketovalidoxylamine A C-N lyase from *S. maltrophilia*. *Left* sample of the enzyme from the final purification step. *Right* molecular mass marker proteins

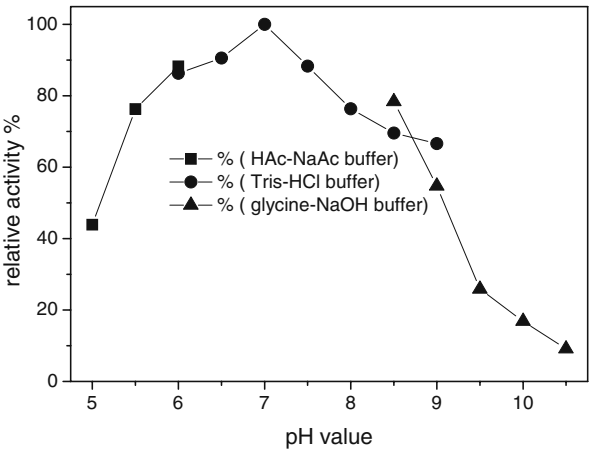

Fig. 3 Effect of pH on 3-ketovalidoxylamine A C-N lyase activity with 50 mM sodium acetate buffer (pH5.0–6.0), 50 mM Tris–HCl buffer (pH6.0–9.0), and 50 mM glycine–sodium hydroxide buffer (pH8.5–10.5)

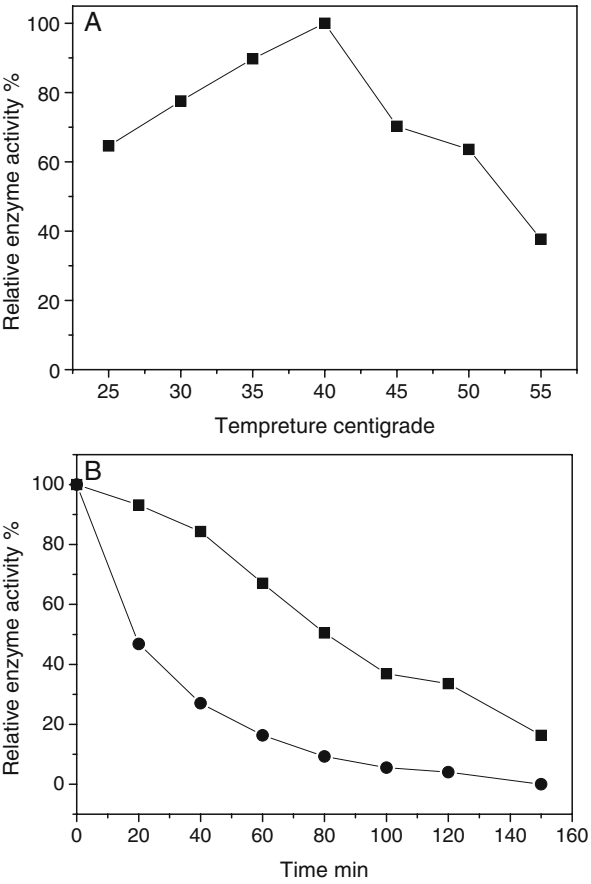

Fig. 4 Effect of temperature on 3-ketovalidoxylamine A C-N lyase activity. Note that the optimal temperature was near 40°C (**a**).The enzyme activity decreased significantly at 45°C (*squares*) and 55°C (*circles*) (**b**)

Table 2 Effect of metal ions on 3-ketovalidoxylamine A C-N lyase activity.

Chemicals (4 mM)	Relative activity (%)
Control	100
EDTA	0
$CaCl_2$	129.3
$CaCl_2$+ EDTA	99.0
$MgSO_4$	98.1
$MgSO_4$+ EDTA	3.8
$BaCl_2$	98.4
$NiCl_2$	99.3
$MnSO_4$	97.1
$CoCl_2$	100
$CuSO_4$	81.8
Hg_2Cl_2	90.0
$(Al)_2SO_4$	90.2
$LiCl_2$	74.0
$ZnSO_4$	6.8

measured on *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl β-D-glucopyranoside; no activity was detected on this two substrates. At the same time, when purified G3DH and 2,6-dichlorophenolindophenol were added to the reaction system, the enzyme produced *p*-nitrophenol. Thus, the enzyme has both C-N lyase activity and C-O lyase activity, and the 3-keto group was essential for the cleavage of the linkage by this enzyme, which was similar to that from *F. saccharophilum* [1].

Kinetic Studies

The apparent K_m value was determined by *p*-nitrophenyl-3-ketovalidamine. The kinetic parameter was determined from Lineweaver–Burk plots. The apparent K_m value was calculated to be 0.14 mM. It was similar to that from *F. saccharophilum* (0.24 mM) [1].

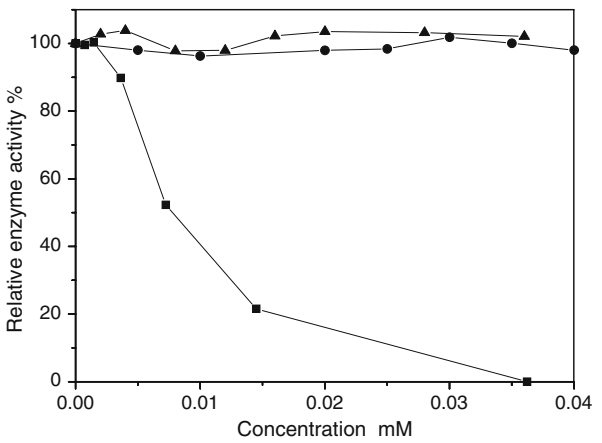

Fig. 5 Effect of validamine (*triangles*), *p*-nitroaniline (*squares*), and *N*-*p*-nitrophenylvalidamine (*circles*) on 3-ketovalidoxylamine A C-N lyase activity

Conclusions

The soluble 3-ketovalidoxylamine A C-N lyase from *S. maltrophilia* was purified in four steps to homogeneity, with a molecular mass of 34 kDa by SDS-PAGE. The properties of the enzyme showed that there were some differences compared with the enzyme from *F. saccharophilum*. Additional studies will be needed to analyze the amino acid sequence of the enzyme to obtain the gene of this enzyme.

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