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Purification and characterization of a low-temperature ammonia monooxygenase from heterotrophic nitrifier *Acinetobacter* sp. Y16

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ABSTRACT

Low-temperature ammonia monooxygenase (AMO) was purified from a heterotrophic nitrifier *Acinetobacter* sp. Y16 by anion-exchange and gel-filtration chromatography. The purified enzyme was a membrane-bound monomer with a molecular mass of approximately 31 kDa. It could catalyze the oxidation of ammonium without stabilizing agents *in vitro* at low temperature. Addition of $CuCl_2$ could stimulate AMO activity *in vitro*. The enzyme was stable in the temperature range of 4–15°C with less than 9% change in its activity. The optimal activity temperature was 15°C. Above 20°C, the enzyme completely lost its activity. The enzyme activity was stable when stored at 4°C for five days, at 10°C for two days, and at 15°C for one day. This study purified a highly pure AMO from a heterotrophic nitrifier *Acinetobacter* sp. for the first time.

Keywords: Purification; Ammonia monooxygenase; Heterotrophic nitrifier; *Acinetobacter* sp. Y16

1. Introduction

In China, some water sources have been seriously polluted by agricultural, municipal, and industrial waste, which causes ammonium accumulation in water sources [1]. The drinking water quality standard in China has a stringent requirement for ammonium contents in drinking water. Conventional drinking water treatment processes, coagulation, sedimentation, and filtration could not effectively remove ammonium from raw water when the ammonium contents in raw water exceeded $0.5\,mg\,L^{-1}$. To guarantee ammonium contents in drinking water in accordance with the drinking water quality standard ($<0.5\,mg\,L^{-1}$) promulgated by the Ministry of Health of China, chlorination is usually used for ammonium removal in drinking water production. However, the increase of chlorine content in water could generate halogenated by-products which are harmful to humans [2]. Advanced biological treatment technology has been developed and widely employed in drinking water treatment [3].

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Biologically activated carbon filtration by attaching heterotrophic nitrifying bacteria on activated carbon is the most efficient way to remove ammonium from drinking water [4]. Heterotrophic nitrifying bacteria can remove the ammonium from water by simultaneous nitrification and denitrification. However, the biodegradation efficiency remarkably decreases in winter due to high sensitivity of the bacteria to low temperature. Some studies exhibited that low temperature (<15˚C) sharply reduced the nitrification rate [5,6]. Ammonia monooxygenase (AMO) and hydroxylamine oxidase (HAO) are two important enzymes in nitrification process. They affect the nitrification rate. AMO catalyzes the oxidation of NH_4^+-N to NH_2OH and HAO catalyzes the oxidation of NH_2OH to NO_2^-. HAO has been purified from several nitrifiers, and its structure and function were well studied [7–10]. These HAOs are distinct from each other in molecular mass and enzyme structures. In contrast, less is known regarding AMO purification due to the instability of the enzyme in cell extracts. Until now, only one report has demonstrated the purification of an AMO complex with molecular mass of 38 and 46 kDa from *Paracoccus denitrificans* [11].

Acinetobacter sp. Y16 was a newly isolated heterotrophic nitrifier from Songhua River in winter. Previous study showed that the isolate had unusual ability of ammonium removal from raw water. It could remove lower concentrations of ammonium (0.2–5 mg L^{-1}) from raw water at low temperatures (2–15˚C). When the initial ammonium concentration in water was about 5 mg L^{-1}, the isolate could remove 30% of ammonium from water at 4˚C for 30 min with little nitrite and nitrate accumulation, more N_2 production, but no N_2O production. The isolate has potential applications in future ammonium removal from drinking water production in winter. HAO has been purified from the strain. It was entirely distinct from other reported HAOs in enzyme size and properties. In the present study, further research is needed to purify AMO from the strain and to investigate its properties. The study is helpful to unravel the mechanism of ammonia removal for the strain under low ammonium and low temperature conditions.

2. Materials and methods

2.1. Cultivation of organism

Acinetobacter sp. Y16, a heterotrophic nitrifier, was grown in liquid culture containing NH_4Cl 0.5 g/L, CH_3COONa 1.0 g/L, $MgSO_4{\cdot}7H_2O$ 0.05 g/L, K_2HPO_4 0.2 g/L, NaCl 0.12 g/L, $MnSO_4{\cdot}4H_2O$ 0.01 g/L, and $FeSO_4$ 0.01 g/L, pH 7.4. Fifty milliliters cultures were incubated in 250 mL conical flasks, shaken at 200 rpm at 4˚C for 5d. These cultures were used as an inoculum for expanding cultivation in a 500 mL conical flask. The expanding cultures were shaken at 200 rpm at 4˚C until the turbidity of bacteria $A_{600} = 0.7–1.0$.

2.2. Preparation of crude enzyme

A 2-liter culture was harvested at $A_{600} = 0.8$ by centrifugation at 5,000 × g at 4˚C for 30 min, washed and resuspended in 10 ml of 10 mM Tris–HCl (pH 7.5). The cells were broken by sonication at 4˚C. After centrifugation at 12 000 × g at 4˚C for 30 min, the particulate fraction was resuspended in 5 mL of 10 mM Tris–HCl (pH 7.5) buffer containing 1.0% dodecyl-β-D-maltoside for incubation at 4˚C in the dark for 1 h and centrifuged at 12,000 × g at 4˚C for 30 min. The upper aqueous was collected for the purification of ammonia monooxygenase.

2.3. Enzyme purification

Enzyme purification was performed with AKTA Purifier instrument. The crude enzyme extracts were loaded onto an anion-exchange column (HiTrap™ DEAE FF 1 mL, GE Healthcare) equilibrated with 0.05 M Tris–HCl (pH 7.5) + 0.02% dodecyl-β-D-maltoside and eluted with a linear gradient of 0 to 2 M NaCl in the same buffer. Each fraction was collected and analyzed for AMO activity. The active fractions were condensed by ultrafiltration and purified further using a gel-filtration column (Superdex™ 75 10/300GL, GE Healthcare). This column was eluted with 0.05 M phosphate + 0.15 M NaCl + 0.02% dodecyl-β-D-maltoside, pH 7.5. Each fraction was collected and the AMO activity was assayed. Active fractions were concentrated with TCA method. The purity of the purified AMO was determined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Enzyme assays

The AMO activity was assayed by measuring the change in oxygen uptake rate using ammonium as a substrate and a mixture of NADH, diaphorase, and duroquinone as the electron donor in the enzyme reaction. The reaction mixture (mL^{-1}) contained 5 mM $(NH4)_2SO_4$, 10 mM Tris–HCl (pH 7.5), 0.5 mM NADH, 1 unit diaphorase, 0.5 mM duroquinone, and 50 μL enzyme solution. The reaction was performed in a stoppered tube at 15˚C for 10 min and the oxygen uptake was measured. Each analysis was repeated three times.

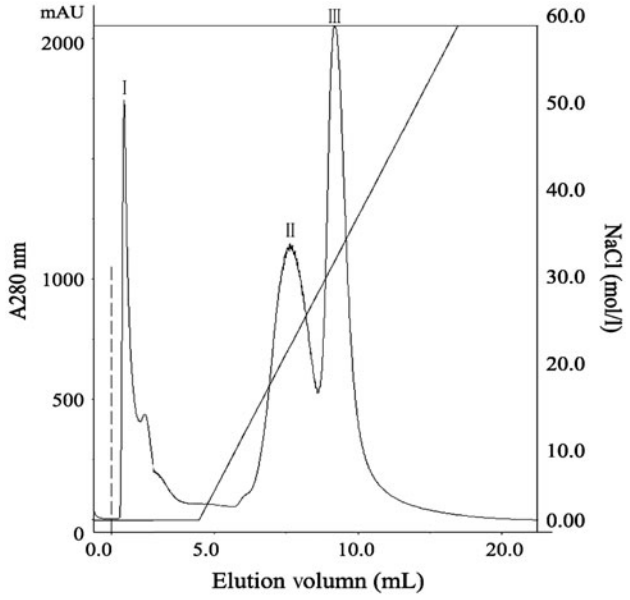

Fig. 1. Anion-exchange chromatography of the crude enzyme on a HiTrap™ DEAE FF column peak I: effusion eluate; peak II and III: elution eluate.

2.5. Effect of temperature and additives on AMO activity

Effect of temperature on the purified AMO activity was performed by changing the enzyme reaction temperature. The reaction mixtures were incubated at 4, 10, 15, 20, 30, 40, and 50°C for 10 min, respectively, and the O_2 uptake was measured.

To determine the impact of additives on the purified AMO, 100 μM $CuCl_2$ and 10 mg mL^{-1} BSA were added to the enzyme reaction mixture, respectively. The reaction mixture was incubated at 15°C for 10 min, and the O_2 uptake was measured.

For storage temperature experiments, the purified AMO without additives was kept at 4, 10, and 15°C for 1–7 days, respectively, and the AMO activity was assayed.

2.6. SDS-PAGE and molecular mass determination

After each purification step, the purity of AMO was determined by SDS-PAGE. SDS-PAGE was performed on 12% (w/v) polyacrylamide gel with mini protein gel equipment (Bio-Rad, USA). The samples were denatured by incubation at 100°C for 5 min in the protein loading buffer with SDS and β-mercaptoethanol (Sigma). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 for 3 h at room temperature, followed by acetic acid solution and methanol (4:1 v/v) destaining. A low molecular mass marker (from 14.3 to 97.2 kDa) was used to evaluate the molecular mass of the protein. The molecular mass of the purified AMO was measured by comparing the migration distance of the single protein band to the marker protein (Auoto Chemi System, USA). Protein concentration was determined using the method of Bradford. Bovine serum albumin was used as standard. Each analysis was repeated three times.

3. Results

3.1. Purification of AMO

The crude enzyme was fractionated using anion-exchange chromatography into one effusion peak I and two elution peaks II and III (Fig. 1). The AMO activity was detected in peak III which was eluted with 30 mM NaCl. The active fraction was subsequently purified by a gel-filtration chromatography. Only one peak appeared and exhibited the AMO activity (Fig. 2).

3.2. Activity analysis of AMO

The sonicated extract was separated into particulate and water soluble material by centrifugation. The AMO activity was mainly found in particulate fraction, less in water soluble fraction. After an anion-exchange and gel-filtration purification, the AMO activity increased from 2.4 nmol O_2 min^{-1} $mg^{-1}$to 18.1 nmol O_2 min^{-1} mg^{-1} and 23.2 nmol O_2 min^{-1} mg^{-1}, respectively. There was a 7.5-fold and a 9.7-fold activation of the enzyme following the anion-exchange and gel-filtration (Table 1). 47.4% of the total AMO activity from the crude enzyme was retained in purified enzyme during purification from crude

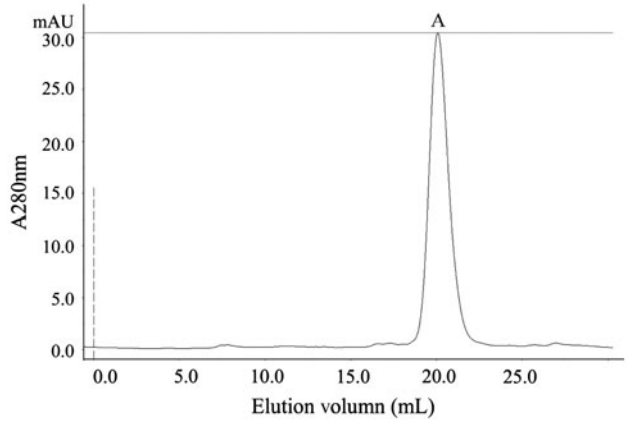

Fig. 2. Gel-filtration chromatography of the elution III fractions on a superdex™ 75 10/300 GL column.

Table 1
Purification of AMO from *Acinetobacter* sp. Y16

Purification step	Total protein (mg)	Total activity (nmol O_2 min^{-1})	Specific activity (nmol $O_2\,min^{-1}mg^{-1}$)	Purification fold
Sonicated extract	32.5 ± 0.12	9.7 ± 0.08	0.3 ± 0.16	
Crude enzyme	3.4 ± 0.18	8.2 ± 0.11	2.4 ± 0.13	
Anion exchange	0.3 ± 0.22	5.4 ± 0.18	18.1 ± 0.20	7.5
Gel filtration	0.2 ± 0.22	4.6 ± 0.17	23.2 ± 0.17	9.7

Note: Data are means ± SD of three replications.

Table 2
The activity of AMO at different reaction temperatures

Temperature (℃)	4	10	15	20	30	40	50
Specific activity (nmol $O_2min^{-1}mg^{-1}$)	21.2 ± 0.08	22.4 ± 0.12	23.2 ± 0.14	3.5 ± 0.13	0	0	0
Relative activity %	91.4	96.5	100	15.1	0	0	0

Note: Data are means ± SD of three replications.

enzyme to purified enzyme. It was estimated that AMO comprised of approximately 5.8% of the crude enzyme in a particulate soluble fraction.

The purified AMO activity fluctuated with variation of the enzyme reaction temperature. The enzyme was stable in the temperature ranges from 15℃ to 4℃, with less than 9% decrease in its activity. The optimal temperature of the enzyme activity was 15℃. When the reaction temperature exceeded 15℃ the enzyme activity decreased drastically. At 20℃, it was only 15.1% of AMO activity at 15℃. Above 20℃, the activity of AMO was completely lost (Table 2). The additive $CuCl_2$ increased the AMO activity by 14.7%, whereas BSA did not affect the AMO activity (Fig. 3). Different storage temperatures affected the AMO activity. The enzyme could maintain the activity for five days at 4℃, for two days at 10℃, and for oneday at 15℃ (Fig. 4).

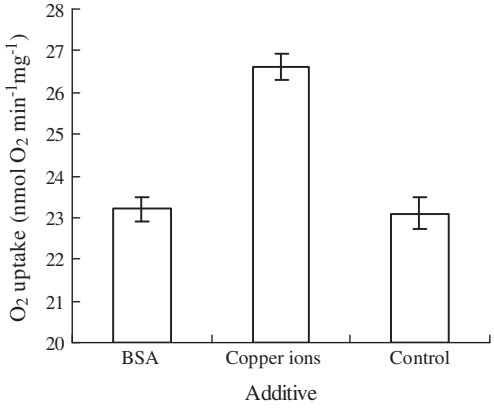

Fig. 3. Impact of additives on AMO activity.

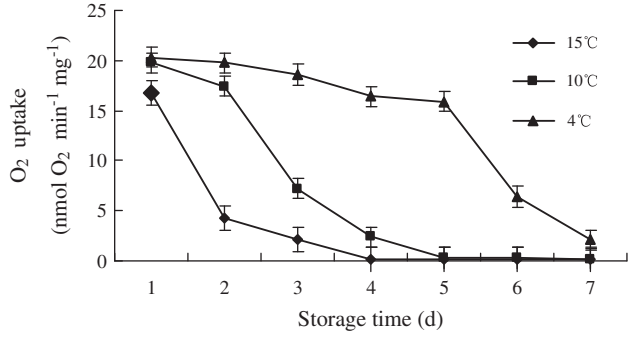

Fig. 4. Effect of storage temperature on AMO activity.

3.3. SDS-PAGE analysis of AMO

Fig. 5 shows the SDS-PAGE profiles of the protein with AMO activity obtained from crude enzyme, anion-exchange (peak III) and gel-filtration purification (peak A). The crude enzyme exhibited six protein bands in SDS-PAGE. The molecular mass was between 29 and 97.2 kDa. After ion-exchange chromatography, the protein bands of peak III decreased from six to three. Three protein bands were located between 43 and 29.0 kDa. After gel-filtration chromatography, peak A gave a single protein band in SDS-PAGE corresponding to a molecular mass of approximately 31 kDa. The results testified that AMO was successfully purified, and the purity was in accordance with the purpose of this study.

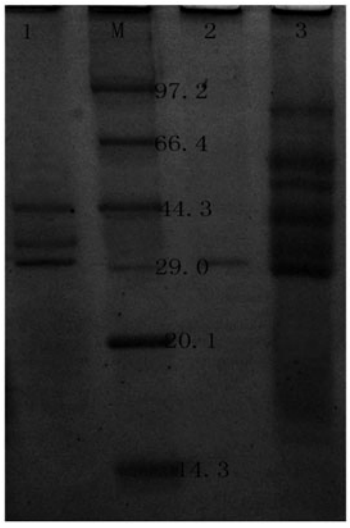

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of AMO from the purification process. Lane M: low molecular mass markers from TaKaRa Biotechnology (China); Lane 1: elution peak III; Lane 2: absorbed peak A; Lane 3: the crude AMO from the cell-free extract.

4. Discussion

Acinetobacter sp. Y16 was isolated from Songhua River in winter. Previous study showed that it grew rapidly at 4–15℃ and grew slowly at 20–37℃. CH_3COONa could be used as carbon source with NH_4^+, NO_2^-, or NO_3^- as sole nitrogen source. Less intermediate production (nitrite and nitrate) and terminal production (N_2) were detected when the bacterium *Acinetobacter* sp. Y16 degraded NH_4^+ to terminal production (N_2) (data not shown). Low-temperature HAO has been purified from the strain. It could catalyze the oxidation of NH_2OH to nitrite at low temperatures and entirely differed from the HAOs published (data not shown). AMO initiates the conversion of NH_4^+ to NH_2OH, and then HAO catalyzes the oxidization of NH_2OH to NO_2^- in biological nitrification. In this study, a monomer AMO was successfully purified for the first time from *Acinetobacter* sp. Y16 by an anion-exchange and gel-filtration chromatography. The AMO had the ability to oxidize NH_4^+ to NH_2OH at low temperature (4–15℃) *in vitro*. The present study, combined with previous studies indicated that the ammonia removal pathway of the strain at low temperature was $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^- \rightarrow N_2$. It was similar to that of heterotrophic nitrification process of *Providencia rettgeri* YL [12], which differed from *Alcaligenes faecalis* No. 4, *Alcaligenes faecalis* NR, and *Acinetobacter calcoaceticus* HNR ($NH_4^+ \rightarrow NH_2OH \rightarrow N_2O/N_2$) [13–15].

In autotrophic ammonia-oxidizing bacteria, AMO has not yet been isolated in a purified and active form although *in vitro* activity of cell lysates can be detected. Recently, it has been reported that AMO was a membrane-bound multiple subunit enzyme which consists of AMO-A, AMO-B, and AMO-C. A 27–30 kDa AMO-A was thought to be the catalytic active site for ammonia oxidization [16]. An active AMO has been achieved from *Paracoccus denitrificans*, a heterotrophic nitrifier [11]. The AMO was also a membrane-bound enzyme consisting of two subunits with molecular masses of 38 and 46 kDa. In the present study, the purified AMO from the strain Y16 exhibited a single protein band with the molecular mass of approximately 31 kDa on SDS-PAGE. It was similar to the molecular mass of AMO-A from *Nitrosomonas europaea*, but differed from the AMO from the heterotrophic nitrifier *Paracoccus denitrificans*. It has been reported that the detergent dodecyl-β-D-maltoside is able to remove the lipid and solubilize the AMO in the cell membrane at appropriate concentrations [11]. In this study, most AMO activities were found in a particular fraction solublized in the solution containing 1% dodecyl-β-D-maltoside after the cells were broken by sonication. The result showed that AMO from the strain Y16 was also a membrane-associated enzyme and could solubilize in 1.0% dodecyl-β-D-maltoside buffer.

Ammonia-dependent O_2 uptake measurement has been used in AMO activity assay in cell extracts from *Nitrosomonas europaea* and *Paracoccus denitrificans* [17,11]. AMO activity in cell extracts from *Nitrosomonas europaea* was unstable. *In vitro* assays of AMO activity required some additives, such as Mg^{2+}, spermine, bovine serum albumin, and Cu^{2+}. These additives could activate AMO in cell extracts [17]. The purified AMO from *Paracoccus denitrificans* was stable. The AMO activity could be detected without any additives, but Cu^{2+} could enhance its activity. The AMO purified from the strain Y 16 was similar to that from *Paracoccus denitrificans* in enzyme stability. It also maintained the activity without any additives. Cu^{2+} also increased the AMO activity, whereas BSA did not affect its activity. However, the AMO purified from the strain Y16 was special in maintaining the activity at 4℃ for five days without additives. However, previous studies showed that AMO activity was extremely labile and fresh extracts lost 50% activity within 2 h when stored at 4℃ [18]. BSA and copper ions could stabilize AMO activity for up to two or three days at 4℃ [17]. In this study, the higher purity and low temperature growth properties might be the reason for its stability at low temperature. The yield of the purified AMO appeared to be 5.8% of the crude protein. The results indicated that a

little AMO could be extracted from the strain Y16. We speculated that the purification process reduced the yield of AMO and most of AMO were consumed by their involvement in ammonia removal reaction during the strain growth.

To our knowledge, this is the first time to report that AMO has been purified from the species of genus *Acinetobacter*. AMO played a crucial role in the ammonia removal process at low temperatures. However, the primary structure of the purified AMO from Acinetobacter sp. Y16 is still unknown. It is also unclear why the AMO has activity at low temperatures and this issue should be addressed in the future.

Acknowledgements

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