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Novel affinity purification of xanthine oxidase from *Arthrobacter* M3

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ABSTRACT

An affinity protocol for purification of xanthine oxidase (XOD) from *Arthrobacter* M3 was developed. The isolation procedure consisted of only three steps, ammonium sulfate precipitation, affinity extraction to exclude the major impurities, and the final refining procedure with DEAE ion-exchange chromatography for removal of minor contaminants. In this affinity preparation, guanine, an analogue of xanthine, was chosen as the affinity ligand, and was coupled with Sepharose 4B through spacers composed of epichlorohydrin and ethylenediamine. Crude protein has been run through ammonium sulfate precipitation and the affinity column, 99.1% of proteins were removed. After DEAE ion-exchange chromatography, the purity of the refined XOD was 97.5% by Native-PAGE analysis. The activity recovery of purified XOD (36.1%) was almost higher than that of other methods reported. Reducing SDS-PAGE analysis showed that the purified XOD (one band in Native-PAGE analysis) showed two polypeptides with the molecular weights ~35 kDa and ~100 kDa, respectively. The desorption constant K_d and the theoretical maximum absorption Q_{max} on the affinity medium were 3.0 μg/ml and 2.2 mg/g medium in absorption analysis.

1. Introduction

Xanthine oxidase is a molybdo-flavin enzyme with a quaternary structure; each subunit contains molybdopterin, flavin adenine dinucleotide (FAD), and also iron-sulfur center [1]. In vivo it mainly catalyzed the oxidation of hypoxanthine to xanthine and xanthine to uric. This enzyme is widely used as a detection reagent for purines, superoxide dismutase, phosphates of blood serum [2], nucleotidase [3]. Moreover, this enzyme played a key role in cardiovascular diseases and innate immune, and could be employed as antimicrobial agents.

In previous reports, few articles were involved with the purification of xanthine oxidase. More protocols were focusing on the separation of xanthine dehydrogenase (XDH), which could be converted to XOD by oxidation [4], and these two enzymes were alternative forms from the same gene product. The purification of XOD/XDH is a challenging task, and the previous protocols usually contained several different steps such as heat treatment, salting-out, organic solvent precipitation, and chromatography. XDH from *Comamonas acidovorans* was purified through freeze-melt, heat treatment, ion-exchange chromatography on DE-52, gel filtration on Sephacryl S-300, hydrophobic chromatography on Phenyl Sepharose [5]. XDH from *Clostridium purinolyticum* was purified by cell rupture, ion-exchange chromatography on DEAE Sepharose, hydrophobic chromatography on Phenyl-Sepharose CL-6B, ion-exchange chromatography on SP-Sepharose and preparative CM-HPLC column [6]. XDH from *Eubacterium barkeri* was purified by ammonium sulfate, ion-exchange chromatography on DEAE Sephacel, ultrafiltration, and gel filtration on Sepharose CL 6B [7]. A rather efficient method was affinity chromatography, in which benzamidine was used as ligand for purification of XOD [8]. However, there is a need to develop a novel purification protocol for recovery of XOD/XDH on production scale, in few steps and meanwhile higher efficiency.

The present work describes the reformed purification of XOD from *Arthrobacter* M3, and this protocol consists of only three steps: ammonium sulfate precipitation, affinity chromatography, and DEAE ion-exchange chromatography for refining enzyme.

2. Materials and methods

2.1. Materials

The strain *Arthrobacter* M3 has been screened from soil and stored in our own laboratory. Guanine was obtained from Bio Basic INC. Canada. Sepharose 4B was purchased from Pharmacia Biotech, Sweden. Xanthine oxidase standard was obtained from Sinobio, China.

All the other reagents were of analytical grade from commercial supply.

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2.2. Preparation crude extract of Arthrobacter M3

Arthrobacter M3 was grown at 37 °C overnight in LB medium. Then the culture was transferred into 100 ml induction medium as previously described [9]. The cells were harvested by centrifugation at 4500 × *g* for 20 min at 4 °C and were re-suspended in 20 mM sodium phosphate (pH 7.0). The cells were disrupted by sonication and centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was then used for assay of enzyme activity. The protein concentration was determined by the Bradford method [10] using bovine serum albumin as the standard.

2.3. The preparation of the affinity medium

Sepharose 4B was first modified by epichlorohydrin, and then was linked to guanine (ligand) through spacers composed of ethylenediamine and epichlorohydrin by referring from Li et al. [11] and Xin et al. [12,13]. The information of ligand on the medium was obtained via electrospray ionization mass spectrometry (ESI-MS) according to the method reported [13].

2.4. Adsorption analysis

The adsorption capacity of this affinity medium was analyzed on the basis of the method reported [13]. Varying amount of xanthine oxidase solutions (2 ml) (at 0.3, 0.4, 0.5, 0.6, 0.8, 0.95, 1.1, 1.6, 2.4 mg), previously dialyzed against 20 mM sodium phosphate (pH 7.0), were mixed with 0.5 g of each affinity sorbent. The suspensions were shaken for 2 h at 25 °C to achieve system equilibrium, and then centrifuged (1500 × *g*, 5 min). The supernatants were subjected to activity analysis. Each experiment included a control to ensure that no loss of activity occurred under these conditions. The data were analyzed via the Scatchard method. Q_{max} (the theoretical maximum adsorption capacity) and K_d (the constant of desorption) were determined.

All the tests were repeated at least three times.

2.5. Condition for purification of xanthine oxidase

Arthrobacter M3 suspended in 20 mM sodium phosphate pH 7.0 was disrupted by sonication and centrifuged at 10,000 × *g* for 30 min at 4 °C. First, a saturated solution of ammonium sulfate was added to the supernatant to achieve 10% saturation. The supernatant was left at 4 °C with the ammonium sulfate for 30 min. The protein precipitate was then removed by centrifugation at 10,000 × *g* for 30 min at 4 °C. Second, a saturated solution of ammonium sulfate was continued to add to the supernatant until achieving 30% saturation. After 30 min, the protein precipitate was collected by centrifugation at 10,000 × *g* for 30 min, and then dissolved in 40 ml of 20 mM sodium phosphate (pH 7.0).

The equilibrated affinity column (25 mm × 80 mm) (~110 mg total proteins, ~2440 U) was loaded with that 40 ml supernatant at 2 ml/min and washed with equilibrating buffer until $A_{280\,nm}$ base line was achieved, and further eluted with 20 mM sodium phosphate/0.3 M NaCl (pH 7.0). 29 ml of elution (~3.6 mg proteins, ~1805 U) was collected and tested for activity and protein concentration.

Active fractions desalted and concentrated (~17.5 ml, contained ~3.4 mg proteins) were applied to the equilibrated DEAE-Sepharose CL 4B column (25 mm × 80 mm), The column was washed with equilibrating buffer until A280 nm base line was achieved, and then eluted with 20 mM sodium phosphate/0.5 M NaCl (pH 7.0). 27 ml of elution (~1.2 mg proteins, ~1255 U) was collected and tested for activity and protein concentration.

The concentrations of crude and purified enzymes were determined by the Bradford method using bovine serum albumin as a standard. The purified enzyme was further analyzed with Native-PAGE (10.0%) and SDS–PAGE (12.0%) under reducing conditions. The purification process was repeated for more than three times.

2.6. Assay of xanthine oxidase activity

Xanthine oxidase activity was assayed by quantifying H_2O_2 produced from the coupling reaction with horseradish peroxidase (0.01 mg/ml) and aminopyrine. One unit of xanthine oxidase activity was defined as the formation of 1 μmol H_2O_2 per min at 37 °C [14].

2.7. SDS–PAGE and native-PAGE analysis

Reducing SDS-PAGE (12.0%T) analysis was carried out on a Mini-protean II system from Bio-Rad (Hercules, CA, USA). For gel electrophoresis 10 μl samples were mixed with 10 μl loading buffer (2% SDS, 350 mM DTT, 25% (v/v) glycerol, 0.01% Bromophenol Blue in 62.4 mM Tris–HCl, and pH 6.8) and incubated at 95 °C for 5 min before loading. Proteins were separated on a 12.0% SDS polyacrylamide gel. The amount of protein loaded from lane 1–8 were 26.9 μg, 9.2 μg, 2 μg, 1.6 μg, 0.4 μg, 0.2 μg, 0.45 μg, 0.46 μg. Gels were stained with Coomassie Blue R, destained and imaged using Gelpro Analyser 3.0 software (Media Cybernetics, Inc.) for analysis of purity and molecular mass.

Native-PAGE (10.0%T) analysis was carried out on a Mini-protean II system from Bio-Rad (Hercules, CA, USA). For gel electrophoresis 10 μl samples (0.45 μg) were mixed with 10 μl loading buffer (25% (v/v) glycerol, 0.01% Bromophenol Blue in 62.4 mM Tris–HCl, and pH 6.8). Proteins were separated on a 10.0% Native polyacrylamide gel. One portion of gels was stained with Coomassie Blue R, and the other portion of gels were stained with solution for the detection of xanthine oxidase activity to do activity stain in situ in the native gel. All gels were destained and imaged using Gelpro Analyser 3.0 software (Media Cybernetics, Inc.) The protein purity on Native-PAGE was determined by densitometry analysis.

2.8. HPLC analysis

The purified protein samples were analyzed using a Knauer system (Berlin, German) composed of 2 K501 pumps and 1 K2501 UV-monitor. The samples were injected into a Vydac C18 column (Alltech Associates, Inc. IL, USA) (4.6 mm × 250 mm) at 1 ml/min and eluted with a gradient of acetonitrile/0.05% TFA from 10% to 60% in 20 min in 0.05% TFA.

2.9. Mass spectra of xanthine oxidase

The bands of XOD were excised from Native-PAGE (10.0%). The gels were destained by 200–400 μl 100 mmol/l NH_4HCO_3/30% acetonitrile. After lyophilization, 5 μl trypsin (2.5–10 ng/μl) was added at 37 °C overnight and then lyophilized. Peptide mass spectra were acquired in positive reflector mode on a 4800 Plus MALDI-TOF/TOF™ Analyzer (Applied Biosystems, Foster City, CA, USA) using 2 kV of acceleration voltage. The results were subsequently investigated with the Matrix Science website (http://www.matrixscience.com/), using "*Mascot Search → Peptide Mass Fingerprint*" to search for homologous proteins in the NCBInr (National Center for Biotechnology Information non-redundant) database.

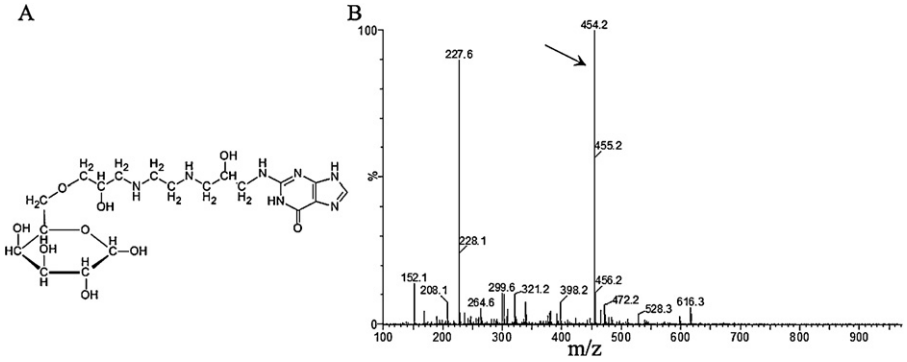


Fig. 1. The theoretical structure and ESI-MS analysis of affinity ligand. (A) Guanine was coupled with Sepharose 4B through spacers composed of epichlorohydrin and ethylenediamine with theoretical molecular formula $C_{19}H_{33}N_7O_9$ and molecular mass of 503.3. (B) With an ESI-MS cone voltage of 70 V. The principal peaks $[M+H]^+$ and $[M+2H]^{2+}$ were at 454.2 and 227.6.

3. Results

3.1. Affinity medium preparation and adsorption analysis

The affinity medium was synthesized through guanine was coupled with Sepharose 4B through spacers composed of epichlorohydrin and ethylenediamine. In order to confirm information of the affinity ligand, the medium was hydrolyzed with 6 M HCl and purified with ion-exchange chromatography. According to the principle of chemical reaction during the process of affinity ligand synthesis and hydrolyzation of the affinity medium, the theoretical ligand with a galactofuranose on affinity medium was shown in Fig. 1(A) with the molecular mass of 503.3. Parts of oxygen and hydrogen may be removed during hydrolyzation. Hence the molecular mass of the hydrolyzed affinity ligand was determined as 453.2 by ESI-MS. So the principal peaks $[M+H]^+$ and $[M+2H]^{2+}$ were at 454.2 and 227.6.

Equilibrium adsorption studies were employed to characterize the interaction of the enzyme and the affinity medium. The approach provides a relationship between the concentration of the enzyme in the solution and the amount of enzyme absorbed on affinity medium. According to the Scatchard method [13], the data should fit to:

$$Q = \frac{Q\,\max[C*]}{Kd + [C*]} \quad (1)$$

In Eq. (1), Q is the enzyme adsorption amount of the medium (μg/g), Q_{max} is the theoretical maximum absorption of xanthine oxidase to the medium (μg/g), $[C^*]$ is the concentration of xanthine oxidase in solution (μg/ml), and K_d is the desorption constant. Eq. (1) could be transformed to:

$$\frac{Q}{[C*]} = -\frac{Q}{Kd} + \frac{Q\,\max}{Kd}$$

According to the Scatchard method, a plot of Q and $Q/[C^*]$ should follow a straight line. The batch adsorption of xanthine oxidase on affinity medium in Fig. 2(A) and (B) indicated that the respective correlation coefficient R^2 was 0.991 and the model fit the data well. From Fig. 2(A) and (B), the desorption constant K_d was 3.0 μg/ml, and the theoretical maximum absorption Q_{max} was 2.2 mg/g medium.

3.2. Result of xanthine oxidase recovery

The supernatant of disrupted Arthrobacter M3 was first loaded onto the equilibrated affinity column and washed with 20 mM sodium phosphate (pH 7.0) until A280 nm base line was achieved, then eluted with 20 mM sodium phosphate/0.3 M NaCl (pH 7.0). As a result, two peaks having absorbance at 280 nm were obtained as shown in Fig. 3(A-1). Proteins in flow through peak at 0–49 min could not bind onto the affinity column. High xanthine oxidase activity was detected in peak eluted at 55–69 min. Finally, the active fractions eluted from affinity medium were applied to the equilibrated DEAE-Sepharose CL 4B column, and then the column was washed with 20 mM sodium phosphate (pH 7.0) until $A_{280\,nm}$ base line was achieved, and then eluted with 20 mM sodium phosphate/0.5 M NaCl (pH 7.0). High xanthine oxidase activity was detected in peak eluted at 53–68 min as shown in Fig. 3(A-2). During the whole process, ~1.2 mg of protein was obtained in

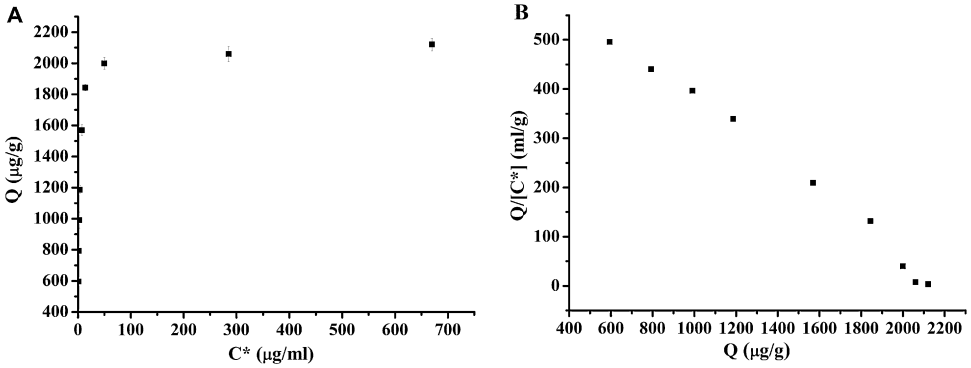


Fig. 2. Adsorption analysis of affinity medium. (A) Equilibrium adsorption of the xanthine oxidase on affinity medium in a batch system (pH 7.0, 25 °C). (B) Plot describing the equilibrium of the absorption on medium and the enzyme concentrations in liquid phase. The respective correlation coefficient was 0.991; the desorption constant K_d of medium was 3.0 μg/ml; the theoretical maximum absorption Q_{max} of medium was 2.2 mg/g medium.

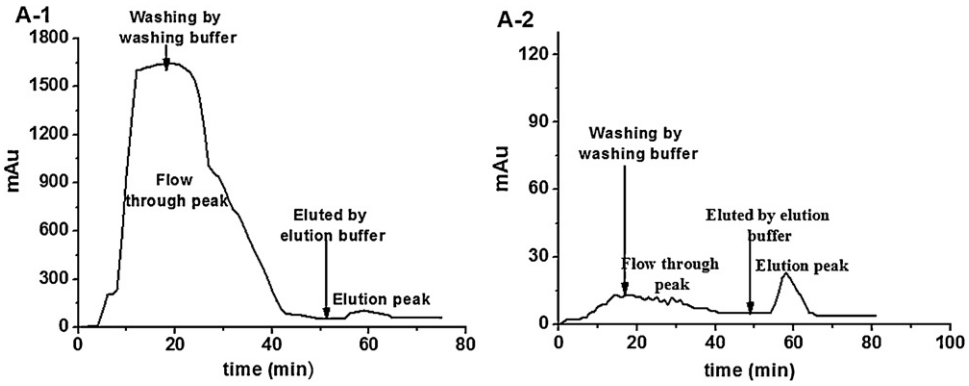


Fig. 3. Elution profiles of purified enzyme. (A-1) The ammonium sulphate extract was applied on affinity column, ~3.6 mg of enzyme was obtained in elution peak, and the specific activity was 498 U/mg. (A-2) The affinity eluted solution was further purified by DEAE-Sepharose CL 4B medium, ~1.2 mg of xanthine oxidase was obtained in elution peak, and the specific activity was 1033 U/mg.

Table 1
The recovery of purification for xanthine oxidase.

Step	Protein		Activity			
	mg	Yield (%)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude sample	403	100	3486	8.6	100	1
30% ammonium sulfate precipitation	110	27.3	2440	22.2	70.0	2.7
Affinity medium	3.6	0.9	1805	498	51.8	58
DEAE-CL 4B	1.2	0.3	1255	1033	36.1	120

elution peak with a yield of 0.3% starting with ~403 mg of protein (Table 1). Compared with the other methods (Table 2), as 19% of Xiang et al. [5], 3.2% of Self et al. [6], 5.7% of McManaman et al. [8], 38% of Thomas et al. [7], the xanthine oxidase activity recovery of this whole purification process (36.1%, Table 1) is almost higher.

3.3. Criteria of homogeneity

Reducing SDS-PAGE (12.0%) analysis showed that the xanthine oxidase had two heterogonous polypeptides with the mass of ~35 kDa and ~100 kDa respectively in Fig. 4(A-1). The protein purity of enzyme purified was up to 96% according to HPLC analysis in Fig. 4(A-3). And the specific activity of this enzyme was 1033 U/mg.

The only one purified protein band on Native-PAGE gel was hydrolyzed into peptide fragments by trypsin, and conducted to PMF analysis with MALDI–TOF MS. The resulting MS data were investigated with the "Matrix Science online service" to search for homologous proteins. The 10 peptide fragments from XOD were identical with segments of xanthine dehydrogenase (molybdenum binding subunit apoprotein) from *Arthrobacter* sp. FB24 (score 361, error 80 ppm) (Fig. 5).

4. Discussion

Compared with the previous methods reported, only three steps involved with guanine affinity medium successfully purified xanthine oxidase in this work. Xanthine oxidase is a molybdo-flavin enzyme, and its molybdenum center plays a role of oxidative hydroxylation on the acceptor carbon atom of aromatic ring of the substrate as xanthine and hypoxanthine [15]. Guanine was chosen as a potential affinity ligand based on its structural similarity to the XOD substrates, and was chemically coupled with amino-Sepharose through spacers as epichlorohydrin and ethylenediamine to prepare an immobilized affinity medium. During our experiments, another affinity medium, in which guanine (ligand) and amino-sepharose were linked through different spacers composed of epichlorohydrin and cyanuric chloride, had less than half of adsorption capacity than affinity medium in this work (data not shown). This may be the steric hindrance effect of cyanuric chloride during the combination of molybdenum binding sites and guanine ligand.

In this work, the crude enzyme extracted from *Arthrobacter* M3 contained some proteins having similar structure with XOD, and the result of reducing SDS–PAGE analysis also showed that the elution fractions of affinity medium still contained a small portion of contaminants. To obtain highly pured xanthine oxidase, a final step of DEAE-Sepharose CL 4B chromatography was applied, and the

Table 2
Comparison of purification methods for xanthine oxidase.

Purification	Activity recovery (%)	Specific activity (U/mg)	Purification factors	Source
1. Method one [5] (A, B, C, D, E)	19	50	108	*Comamonas acidovoran*
2. Method two [6] (B, E, F, G)	3.2	–	–	*Clostridium purinolyticum*
3. Method three [7] (H, B, I, C)	38	164	39	*Eubacterium barkeri*
4. Method four [8] (A, H, J)	5.7	2785	199	Rat liver
5. This work (H, J, B)	36.1	1033	120	*Arthrobacter* M3

A, heat treatment; B, DEAE chromatography; C, gel filtration chromatography; D, Cibacron blue agrose chromatography; E, phenyl sepharose chromatography; F, SP-sepharose chromatography; G, preparative CM-HPLC chromatography; H, ammonium sulfate precipitation; I, ultrafiltration; J, affinity chromatography.

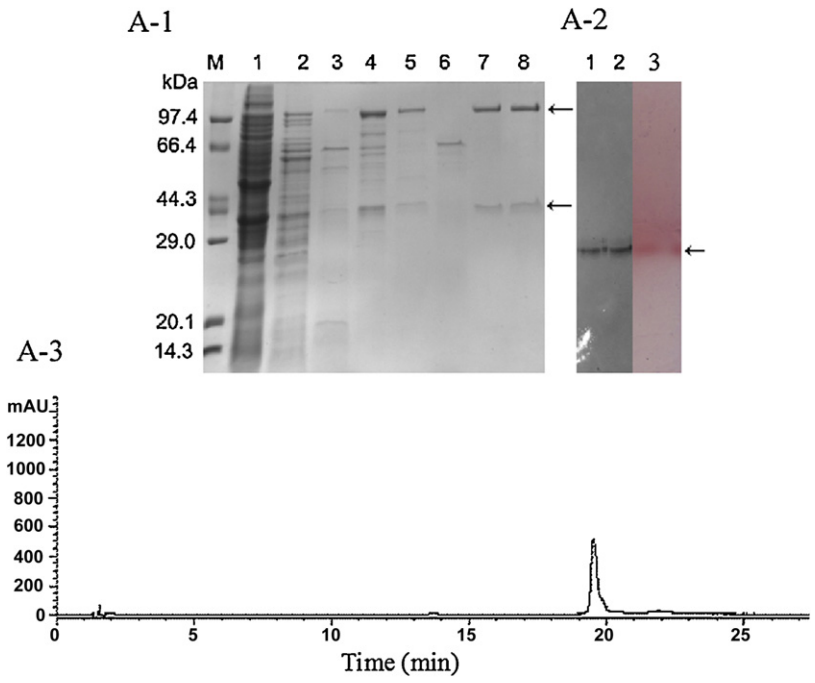
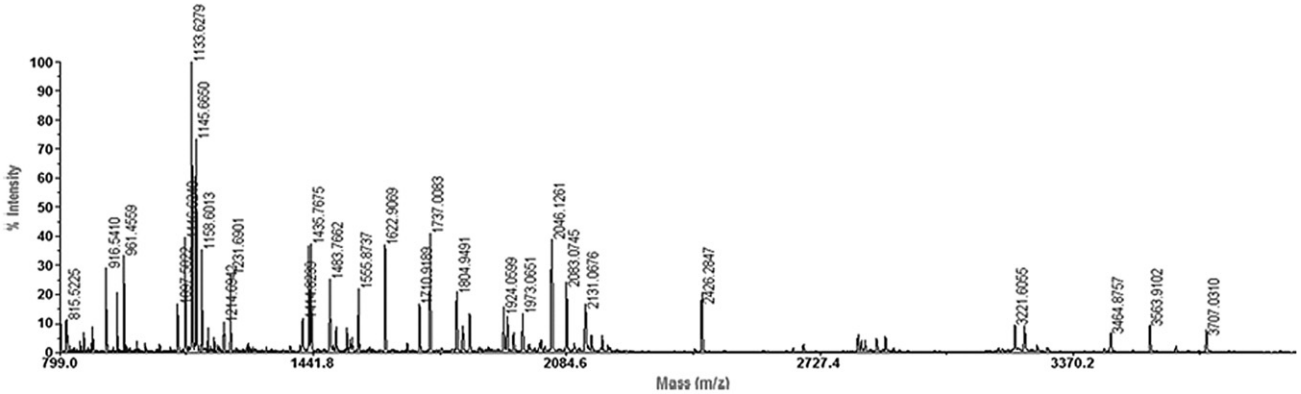


Fig. 4. Analysis of purified enzyme. (A-1) Reducing SDS-PAGE (12.0%) analysis showed that the xanthine oxidase had two polypeptides with the molecular weights of ~35 kDa and ~100 kDa respectively. Lane marker was molecular marker; Lane 1 was total protein extract from *Arthrobacter* M3; Lane 2 was 30% saturation ammonium sulfate; Lane 3 was the flow-through fraction of affinity medium; Lanes 4 and 5 were two elution fractions of affinity medium containing xanthine oxidase; Lane 6 was the flow-through fraction of DEAE-Sepharose CL 4B medium; Lanes 7 and 8 were two elution fractions of DEAE-Sepharose CL 4B medium containing xanthine oxidase. (A-2) Native-PAGE (10.0%) analysis for the xanthine oxidase purified by DEAE-Sepharose CL 4B medium, and the result showed that the purity of xanthine oxidase was about 97.5%. Lanes 1 and 2 were stained by Coomassie Blue R; Lane 3 was stained by the detection solution for xanthine oxidase activity (A-3) HPLC analysis of purified enzyme on Vydac C18 column showed that the purity was about 96%.



Matched peptides were underlined

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1   MANDTTRAIE INGVQAEAAP RPGQCLRTFL REQGNFGVKK GCDGGDCGAC TVHVDGTPVH
61  SCIYPAVRAE GHSVTTVEGL AGTCGTAGAL HPMQQQFLDR QGFQCGFCTA GMVMTAATFD
121 EEQKENLPRN LKGNLCRCTG YRAIEDAVCG QEGHPDPRQP GSGIAGEGQP EPKPGQLGDD
181 VPAPASLAVV TGKARYTLDV PAEELQGLLH LKLLRSPHAH ARVVSIDSGA ALQVPGVVAV
241 LTHEDAPAQL FSTAQHELYT DDPDDTRVLD NVVRFIGQKV AAVVAESVAA AEAGVRALTV
301 EYEVLDAVFT PEDAMRPGAP AIHGEKDAVT ARISRPGQNV VAELHSELGS VAAGFAEADF
361  IHEQTYRTQR VQHTALETHA AIASVDGEGR LQVRTSSQVP FLVRRTLSRV FDIPEEQIRV
421 VAGRVGGGFG GKQEVLTEDV VALAAMKLKR PVQLEFTRTE QFTAATTRHP FTIHLKAGAR
481 RDGRVTALQL DVLTNTGAYG NHAPGVMFHG CGESLGVYKC ANKKVDAHAV YTNTVPSGAF
541 RGYGLSQMIF AIESGIDELA TGIGMDPLEF RRRNMVLEGD DMLSTHPEPE EDVHYGSYGL
601 DQCTRLVQGA LARGLERYRA AGLDDLGPDW VTGEGTALSM IDTVPPRGHF AHSKLRLLPD
661 GTYAADVGTA EFGNGTTTVH AQLAATALST TAGRITVRQS DTDLVQHDTG AFGSAGTVVA
721  GKATLAAAEE LAIRIRAFAA GIRQVQSSGC VLEDDAVVCE GTRVPLTEIF DAAEAAGVEL
781 ATEGHWGGTP RSVAFNVQGF RVAVNTGTGE LKILQSVQAA DAGVVVNPRQ CRGQIEGGIA
841 QALGAALYEE VKVDAGGKVT TDILRQYHIP TFADVPRSEV YFAETSDKLG PLGAKSMSES
901 PFNPVAPALA NAIRNATGVR FAELPIARDK IYLGLKEAGV ASRFESANGL*
```

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result T
814.4781	814.5284	0.0503	62	268	274	VLDNVVR					Mascot
817.4778	817.4966	0.0188	23	859	865	VTTDILR					Mascot
916.525	916.541	0.016	17	921	928	FAELPIAR					Mascot
1133.6313	1133.6283	-0.003	-3	395	404	TSSQVPFLVR					Mascot
1133.6313	1133.6283	-0.003	-3	395	404	TSSQVPFLVR	47	93.052			Mascot
1145.6426	1145.665	0.0224	20	450	458	RPVQLEFTR					Mascot
1145.6426	1145.665	0.0224	20	450	458	RPVQLEFTR	36	14.919			Mascot
1736.9655	1737.0068	0.0413	24	813	829	ILQSVQAADAGVVVNPR					Mascot
1736.9655	1737.0068	0.0413	24	813	829	ILQSVQAADAGVVVNPR	140	100			Mascot
1804.8977	1804.9471	0.0494	27	525	541	VDAHAVYTNTVPSGAFR					Mascot
1804.8977	1804.9471	0.0494	27	525	541	VDAHAVYTNTVPSGAFR	105	100			Mascot
1932.9927	1933.0522	0.0595	31	524	541	KVDAHAVYTNTVPSGAFR					Mascot
1971.9957	1971.9871	-0.0086	-4	896	914	SMSESPFNPVAPALANIAR					Mascot
1971.9957	1972.1543	0.1586	80	896	914	SMSESPFNPVAPALANIAR					Mascot
1987.9906	1988.0392	0.0486	24	896	914	SMSESPFNPVAPALANIAR			Oxidation (M)[2]		Mascot
2870.3877	2870.426	0.0383	13	620	647	AAGLDDLGPDWVTGEGTALSMIDTVPPR			Oxidation (M)[21]		Mascot

Fig. 5. The PMF analysis. PMF data were investigated with the Matrix Science website to search for homologous proteins. The 10 peptide fragments from the purified protein were identical with segments of xanthine dehydrogenase (molybdenum binding subunit apoprotein) from *Arthrobacter* sp. FB24 (score 361, error 80 ppm).

examine results from HPLC analysis showed that the purity of this enzyme was up to 96% in Fig. 4(A-3).

For comparison of this work and the previous reported methods, different purification steps, activity yields, and specific activity were listed, which might reflect the obvious advantages such as fewer steps, better activity recoveries, and higher purity from our work. Coupled with time-saving operation and accessible reagent, this novel affinity purification protocol is of great potential for industrial application.

Furthermore, the related research about the other characterization of xanthine oxidase, such as the kinetic parameters (K_m and V_{max}), the molecular mass, and isoelectric point et al. is in progress.

Acknowledgments

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