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Purification and Characterization of Trimethylamine-*N*-Oxide Demethylase from Jumbo Squid (*Dosidicus gigas*)

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Trimethylamine-*N*-oxide demethylase (TMAOase) was purified from Jumbo squid (*Dosidicus gigas*) and characterized in detail herein. The TMAOase was extracted from squid with 20 mM Tris−acetate buffer (pH 7.0) containing 1.0 M NaCl, followed by acid treatment and heat treatment. Then it was purified by deithylaminoethyl−cellulose and Sephacryl S-300 chromatography, subsequently resulting in an 839-fold purification. The molecular mass of the TMAOase was defined to be 17.5 kDa. The optimum pH of the purified TMAOase was 7.0, and its optimum temperature was confirmed to be 55 °C. The TMAOase was stable to heat treatment up to 50 °C and stable at pH 7.0−9.0. Reducing agents such as DTT, Na_2SO_3, and NADH were effective at activating TMAOase, and ethylenediaminetetraacetic acid, as well as Mg^{2+} and Ca^{2+}, could also enhance the activity of TMAOase remarkably, whereas the TMAOase could be significantly inhibited by tea polyphenol, phytic acid and acetic acid. In addition, the TMAOase converted TMAO to dimethylamine and formaldehyde stoichiometrically with a K_m of 26.2 mM.

KEYWORDS: Characterization; jumbo squid; purification; trimethylamine-*N*-oxide demethylase (TMAOase)

INTRODUCTION

Trimethylamine-*N*-oxide demethylase (TMAOase) is capable of catalyzing the conversion of trimethylamine-*N*-oxide (TMAO) to dimethylamine (DMA) and formaldehyde (FA) (*1*). Many studies have shown that TMAOase is mainly concentrated in the internal organs and red muscle (*1*, *2*). Benjakul (2004) studied the distribution of TMAOase in various internal organs of lizardfish (*Saurida micropectoralis*) (*3*), and among the internal organs tested, the kidney contained the highest TMAOase activity, followed by spleen, bile sac, intestine, and liver, subsequently. Moreover, the TMAOase purified from kidney showed a higher activity at pH 7.0 than at pH 5.0, and the molecular mass of this TMAOase was estimated to be 128 kDa. Rehbein and Schreiber (1984) reported that TMAOase was mostly localized in kidney and spleen but rare in the muscle of fishes (*2*). However, other reports showed TMAOase was found in muscle of some fish species, such as red hake (*4*, *5*) and walleye pollack (*6*). Kimura (2003) found the TMAOase purified from walleye pollack muscle was a thermostable protein, which remained active after heating at 80 °C for 30 min (*7*). Moreover, TMAOase was also presumed to exist in the mantle of squid (*8*, *9*).

Jumbo squid is an important aquatic species in the world. This species is undervalued because its muscle is too large and too soft. However, it can be profitable if its muscle is used as protein base of thermostable gel for the manufacture of squid analogues or other products. It contains a large amount of TMAO in the muscle (15−70 mg/kg), which is a natural osmolyte and present in most marine species. TMAO can be broken down through three pathways. One is heating: over 90% of TMAO was converted to trimethylamine (TMA) and DMA after heating for 1 h at 200 °C (*10*). Another one is via a nonenzymic pathway: during frozen storage, TMAO is degraded to FA and DMA or TMA by this pathway (*11*). The last one is TMAOase-involved enzymatic pathway: TMAOase catalyzes the conversion of TMAO to DMA and FA. FA is harmful, and its production draws more and more attention. In addition, TMAOase also leads to a loss in protein solubility, which is associated with increased cross linking and aggregation of proteins (*3*). Notably, the production of FA is the most important cause, which can induce structural changes of proteins as shown by a remarkable increase in surface hydrophobicity, resulting in a decrease in the gel-forming ability of aquatic muscle (*12*−*14*). It was reported that the gel-forming ability of lizardfish muscle decreased rapidly during the post-harvest handing, which

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was believed to be associated with the formation of FA to a great extent (*15*). It is well known that squid (*Dosidicus gigas*) has poor gel-forming ability. While there is no report about TMAOase from jumbo squid till now, the aims of the present study are to purify TMAOase from squid muscle using a new method and characterize the purified TMAOase in detail.

MATERIALS AND METHODS

Chemicals. TMAO was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). High-molecular-weight standards were purchased from Sigma Chemical Co. (St. Louis, MO). DE52 was obtained from Whatman (Maidstone, UK), and Sephacryl S-300 was from Anersham Pharmacia (Uppsala, Sweden).

Purification of the TMAOase. Jumbo squid mantle obtained from the Peru coast was homogenized with 4-fold of 20 mM Tris−acetate (pH 7.0) containing 1 M NaCl, followed by the addition of 1 M HCl to adjust the pH to 4.5. Next the homogenate was centrifuged at 10 000 × *g* for 30 min. The supernatant obtained was neutralized and heated at 80 °C for 20 min and then centrifuged again. The supernatant was dialyzed against 20 mM Tris−acetate and then purified by successive chromatographies on DE52 and Sephacryl S-300 subsequently. Briefly, the crude enzyme was applied to a DE52 column (2.6 × 30 cm) equilibrated with 20 mM Tris−acetate containing 5 mM Ca^{2+} and then eluted by a liner NaCl gradient of 0−0.5 M at flow rate of 30 mL/h. The fractions with high TMAOase activity were pooled and applied to a Sephacryl S-300 column (1.5 × 100 cm), equilibrated, and eluted with the same loading buffer mentioned above. The molecular mass of purified TMAOase was determined both by Sephacryl S-300 and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) method (*16*).

TMAOase Assay. TMAOase activity was determined using TMAO as substrate in the presence of selected cofactors. The reaction mixture consisting of 100 mM Tris−acetate (pH 7.0), 20 mM TMAO, 2 mM cysteine, 2 mM ascorbate, and 0.2 mM $FeCl_2$ was incubated at 50 °C for precisely 60 min, and 1 mL of 10% trichloroacetic acid (TCA) was added to terminate the reaction. The reaction mixture was then centrifuged at 8 000 × *g* for 15 min, and the supernatant was subjected to DMA determination. The blank was run in the same manner, except that no enzyme was added. The blank was subtracted from the data to get rid of the nonenzymatical production of DMA and TMA. One unit (U) of TMAOase activity was expressed as 1 μmol DMA production per minute.

DMA and FA Determination. DMA was determined by the copper−dithiocarbamate method as described by Dyer and Mounsey (*17*). Briefly, 1 mL of copper−ammonia reagent was added to 2 mL of sample and mixed thoroughly, followed by addition of 4 mL of 5% CS_2−toluene solution and a 2-min incubation at 50 °C. Then 400 mL of 30% acetic acid was added, and the toluene layer was transferred to a tube containing 0.5−1.0 g anhydrous Na_2SO_4 to remove water. Next the absorbance was measured at 440 nm, and the DMA content was calculated from a standard curve. FA was determined using acetylacetone reagent according to the method of Nash (*18*). Briefly, 3 mL of acetylacetone reagent was added to the sample (3 mL) and mixed thoroughly. The reaction mixture was incubated at 60 °C for 15 min and cooled in running water. Then the absorbance was measured at 412 nm, and the FA content was calculated from a standard curve.

Characterization of the Purified TMAOase. *Effect of Temperature on the Enzyme Activity.* For the temperature profile study, the enzyme activity was assayed at different temperatures (20, 30, 40, 50, 60, 70, 80, 90, and 100 °C) for 30 min under the optimum pH. The crude enzyme or the purified TMAOase were added into the assay mixture (2.5 mL) to initiate the reaction. The effect of temperature on the stability of the crude or the purified TMAOase was tested by incubating the enzyme at different temperatures described above for 30 min in temperature controlled water bath. Thereafter, the treated samples were acutely cooled in ice water, followed by measurement of residual activity as described above.

Effect of pH on the TMAOase Activity. TMAOase activity was assayed over a pH range of 5.0−11.0 (100 mM Tris−acetate buffer for pH 5.0−6.5, 100 mM Tris−HCl buffer for pH 6.5−9.0 and 100 mM Gly-NaOH for pH 10.0−11.0) at optimum temperature for 30 min. Next the pH stability of the TMAOase was examined in various buffers over a pH range from 5.0 to 11.0. The purified TMAOase was put into different pH buffer and kept at 4 °C for 30 min. Then the reaction mixture was added and the residual enzyme activity was measured as described above.

Effects of Chemicals on the TMAOase Activity. The effects of ethylenediaminetetraacetic acid (EDTA), reductants, and metal ions on the activity of TMAOase were determined as follows: the TMAOase with 10 mM EDTA, different reductants (1 mM DTT, 10 mM Na_2SO_3, and 0.4 mM NADH), and metal salts (10 mM $CaCl_2$ and $MgCl_2$), respectively, was incubated for 30 min at 4 °C, then the relative activity was determined under standard assay conditions. The purified TMAOase was incubated with different enzyme inhibitors for 30 min at 4 °C to investigate the effects of inhibitors on the activity of TMAOase. The inhibitors utilized were as follows: tea polyphenol, phytic acid, and acetic acid at concentrations of 1 and 10 mM, respectively. The residual enzyme activities were determined under standard assay conditions.

Kinetic Studies. TMAO at different concentrations was added into standard assay mixture (pH 7.0), and the reaction was conducted for 20 min at 50 °C after addition of the purified TMAOase. DMA was determined as a reaction product. All of the determinations were repeated three times, and the kinetic parameters including V_{max} and K_m were evaluated by plotting the data on a Lineweaver−Burk double-reciprocal graph (*19*).

Statistics. All the experiments were carried out three times and each in four replicates. Student's *t*-test and analysis of variance (ANOVA) were performed using Statview.

Table 1. Purification Scheme of the TMAOase from Squid

steps	protein (mg)	protease activity (U)	specific activity (U/mg)	purification fold	yield (%)
myofibillar fraction	4510	848.7	0.19	1	100
acid treatment	286	684	2.39	12.6	80.6
heat treatment	80.4	545.1	6.78	35.7	64.2
DE52	8.1	426	52.6	276	50.2
S-300	2.3	135.7	159	839	16.0

RESULTS AND DISCUSSION

Purification of TMAOase from Squid Muscle. The purification of TMAOase from squid muscle was summarized in **Table 1**. Acidification without detergent treatment was used to remove other proteins, leading to a higher purity of TMAOase (*7*). The TMAOase activity was recovered to be 80.6% yield. The specific activity was increased by 12.6-fold to be 2.39 U/mg. Then the extraction solution was neutralized to pH 7.0 with 0.1 M NaOH, followed by heat treatment. Approximately 64.2% of the total enzyme activity remained, while 72.1% of protein was removed. This step can improve the yield of enzyme and simplify purification steps. The TMAOase was recovered in the supernatant solution and dialyzed overnight against 20 mM Tris−acetate (pH 7.0) containing 5 mM Ca^{2+}, which was proved to play a crucial role in stabilizing enzyme activity. The crude enzyme extract was purified using a deithylaminoethyl (DEAE)−cellulose column next. After elution with linear gradient of 0−0.5 M NaCl in loading buffer, one activity peak was eluted by 0.12−0.18 M NaCl (**Figure 1A**). The active fractions were concentrated and then applied to a Sephacryl S-300 column, and a single symmetric protein peak with high TMAOase activity was obtained (**Figure 1B**). A purification fold of 839 with activity yield of 16% was obtained in the final step. The purified TMAOase from jumbo squid muscle migrated as a single band with molecular mass of 17.5 kDa by SDS−PAGE analysis, and it was further confirmed to be 17.7 kDa by Sephacryl S-300, which is different from other reports

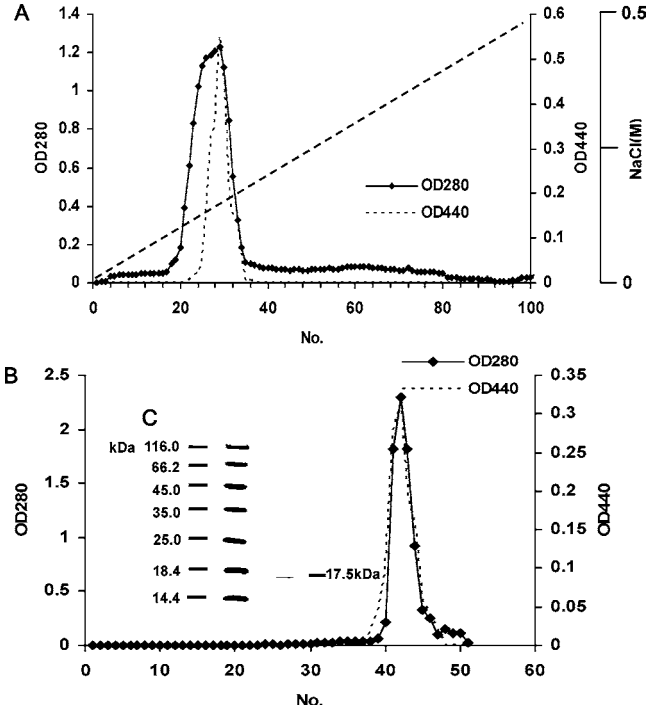

Figure 1. Purification of the TMAOase from jumbo squid. **A**, ion exchange chromatography on DE 52; **B**, gel filtration on Sephacryl S-300; **C**, SDS–PAGE of the purified TMAOase.

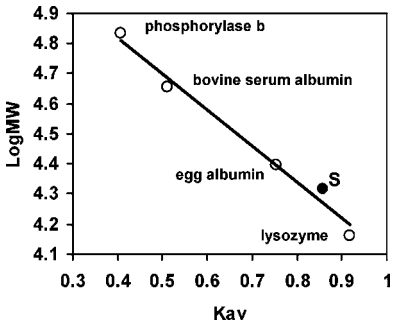

Figure 2. Calibration curve for determination of the molecular mass of TMAOase by Sephacryl S-300 chromatography. S, the purified TMAOase.

(**Figures 1C** and **2**). Benjakul (2003) has partially purified the TMAOase from lizardfish kidney by 82-fold with a yield of 65.4% using acidification and DEAE−cellulose chromatography, and the molecular mass of the enzyme was estimated to be 128 kDa (*15*). Kimura (2000b) obtained 13260-fold purification and 13% yield TMAOase from Alaska Pollack muscle by Sephacryl S-300 with molecular mass of 400 kDa (*6*).

Temperature and pH Profiles of TMAOase. Crude and purified TMAOase from jumbo squid muscle share different optimal temperature. As shown in **Figure 3A**, the optimal temperature of crude and purified TMAOase was 70 and 55 °C, respectively. The effect of temperature on TMAOase stabilization was shown in **Figure 3B**. Both the crude and purified TMAOase was fairly stable up to 50 °C after a 30-min incubation. Interestingly, the crude enzyme could remain about 90% activity up to 100 °C for 30 min. However, a sharp decrease was observed in the enzyme activity of the purified TMAOase at temperature above 50 °C, and it became nearly inactivated at 80 °C. The temperature profile of TMAOase differed greatly between different aquatic species. It was reported that TMAOase from lizardfish kidney was stable for 10 min when heated to 50 °C, and no activity was found after heat

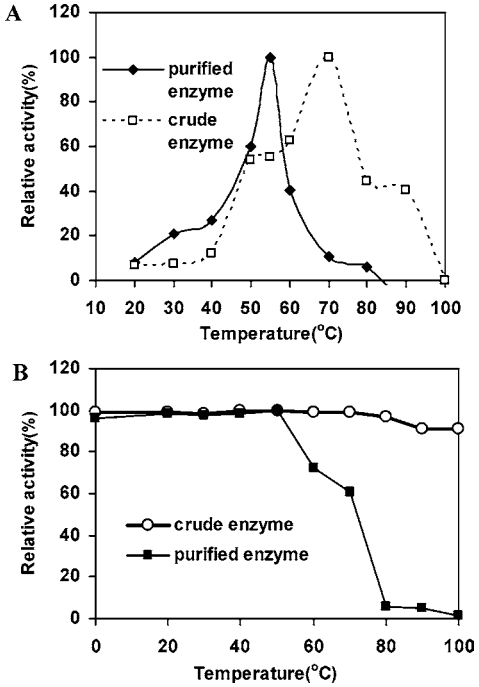

Figure 3. Temperature profiles of the crude and purified TMAOase. **A**, Effect of temperature on the activity of the crude and purified TMAOase; the TMAOase activity was measured at different temperature (20–100 °C); **B**, thermal stability of the crude and purified TMAOase. The thermal stability was determined by incubating the TMAOase at temperature range of 20–100 °C for 30 min, and the percentage of residual activity was estimated under standard assay conditions.

treatment at 70 °C (*15*). Kimura et al. (2000a) found that the TMAOase in myofibrillar fraction of walleye pollock was stable up to 25 °C and that most of activity disappeared with heat treatment at temperature higher than 50 °C (*6*). However, the purified TMAOase from walleye pollack muscle was a thermostable protein, which kept activated even after heating at 80 °C for 30 min (*7*). TMAOase from red hake muscle was reported to be stable for 10 min at 50 °C but mostly destroyed at 60 °C (*5*). No loss was found in the activity of TMAOase from silver hake muscle after heated to 60 °C, but complete inactivation occurred when heated at 80 °C (*20*). Data in the present paper showed that the TMAOase purified from jumbo squid had relatively high optimal temperature and good thermal stability, which would cause potential harm by producing a large amount of harmful DMA and FA during the heating process.

The optimal pH for the purified TMAOase was determined with various pH buffers. As shown in **Figure 4A**, the optimal pH of TMAOase was pH 7.0, similar to the enzymes in walleye pollack and red hake muscle (*4*, *6*). Rehbein and Schreiber reported that TMAOase isolated from cod internal organs including kidney, spleen, and pyloric caeca had optimum pH of 4.5−5.0 (*2*), manifesting that their existing difference in pH profile of TMAOase obtained from different aquatic species. The difference in optimal pH seemed to be attributed to particular environmental pH in vivo. In addition, the TMAOase purified from squid was stable over a broad pH range of 5.0−10.0, retaining over 60% of its original activity after a 30-min incubation at 4 °C, particularly retaining over 90% activity at pH 7.0−9.0 (**Figure 4B**). Under extreme acidic or alkaline pH, the conformation of enzyme may be changed due to charge repulsion and a decrease in electrostatic bonds, which may subsequently cause the enzyme to unfold to minimize the free energy change (*21*). Therefore some treatment such as acidifica-

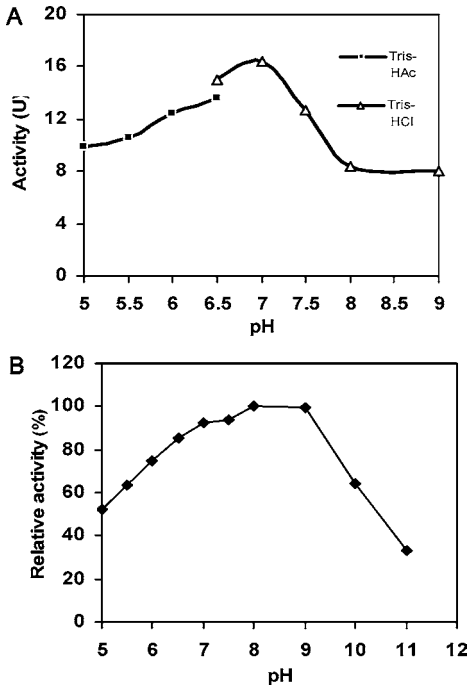

Figure 4. pH profiles of the TMAOase. **A**, Effect of pH on the TMAOase activity, the TMAOase activity was measured in various buffers over a pH range of 5.0–11.0; **B**, pH stability of the TMAOase. The pH stability was determined by incubating the TMAOase in buffers of different pH for 30 min at 4 °C, and the percentage of residual enzyme activity was determined under standard assay conditions.

Table 2. Effect of Additives on the Activity of TMAOase

additives	concentration (mM)	relative activity (%)
Control		10.1
$FeCl_2$ + Cys + Vc	0.2 mM $FeCl_2$	100
$FeSO_4$ + Cys+ Vc	0.2 mM $FeSO_4$	100
$FeCl_2$ + Cys	2 mM Cys	78.9
$FeCl_2$ + Vc	2 mM Vc	38.3
Cys + Vc		6.25
EDTA + $FeCl_2$ + Cys + Vc	10 mM EDTA	194.2
Na_2SO_3 + $FeCl_2$ + Cys + Vc	10 mM Na_2SO_3	197.4
NADH + $FeCl_2$ + Cys + Vc	0.4 mM NADH	141.7
DTT + $FeCl_2$ + Cys + Vc	1 mM DTT	117.9
$CaCl_2$ + $FeCl_2$ + Cys + Vc	10 mM $CaCl_2$	120.5
$MgCl_2$ + $FeCl_2$ + Cys + Vc	10 mM $MgCl_2$	128.8

tion and basification may be helpful to decrease the activity of TMAOase and reduce the production of harmful FA.

Effects of Chemicals on the Activity of TMAOase. TMAOase activities in the absence or presence of different cofactors, including ascorbate, cysteine, and $FeCl_2$, were shown in **Table 2**. The highest activity was observed in the presence of all cofactors. Only negligible activity was found in the presence of both cysteine and ascorbate. It was observed that cysteine exhibited a higher potential in improving TMAOase activity than ascorbate in the presence of $FeCl_2$. The TMAOase activity was increased to 38.3 and 78.9% in the presence of $FeCl_2$−ascorbate or $FeCl_2$–cysteine, respectively. The $FeSO_4$ could activate TMAOase similarly with $FeCl_2$ in the presence of ascorbate and cysteine. As shown in **Table 2**, the TMAOase activity increased about 2-fold by 10 mM EDTA. It was reported that EDTA can contact and concentrate Fe^{2+} to the activity center of enzyme and subsequently improve the TMAO demethylating activity. Reducing agents including Na_2SO_3, NADH, and DTT significantly elevated the TMAOase activity to different extent as external ferric iron reductants. Reducing

Table 3. Effect of Inhibitors on the Activity of TMAOase

inhibitors	concentration (mM)	inhibition (%)
Control		0
tea polyphenol	1	95.6
	10	98.6
phytic acid	1	75.4
	10	95.6
acetic acid	1	78.3
	10	97.1

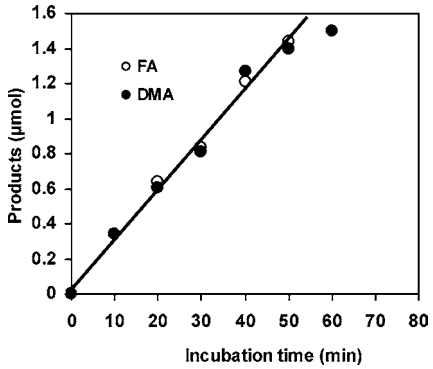

Figure 5. Stoichiometerical production of DMA and FA catalyzed by TMAOase. During incubation, the products of DMA and FA were examined under standard assay conditions and calculated from a standard curve.

agents were required to maintain iron function through a cyclic mechanism between the ferrous and ferric states (*4*, *6*). Metal irons of Mg^{2+} and Ca^{2+} were capable of keeping TMAOase at high activity. Ca^{2+} and Mg^{2+} were generally believed to play crucial roles in stabilizing the structure of enzyme and improve enzyme activity efficiently (*22*).

The TMAOase activity was inhibited remarkably by 1 mM tea polyphenol with an inhibition of 95.6%, as shown in **Table 3**; phytic acid and acetic acid at 1 mM also exerted significant inhibitions (over 70%). Notably, all the three inhibitors at 10 mM exerted great inhibitions of nearly 100% on TMAOase activity. Therefore these inhibitors can be well applied in the handling process of squid to decrease the activity of TMAOase and subsequently reduce the production of harmful FA. There are investigations about inhibition of polyphenol on activities of some enzymes such as acetylcholinesterase and matrix proteases (*23*, *24*); however, this is the first report about influence of polyphenol on TMAOase.

Catalysis and Kinetic Analysis of TMAOase. During incubation, the TMAOase activity was assayed in standard reaction. DMA and FA were stoichiometrically produced, and the result demonstrated a demethylase reaction (**Figure 5**). It is well known that TMAO is demethylated enzymatically to DMA and FA (*25*). FA can react with proteins, leading to conformational changes, insolubilization, loss of extractablity, as well as the toughening of fish muscle (*26*). It has been reported that the contamination of TMAOase from lizardfiah kidney into muscle possibly caused the deterioration of muscle proteins induced by FA formation. Therefore, proper process should be taken into consideration to inhibit the TMAOase activity and minimize the muscle deterioration caused by FA in aquatic species.

A Lineweaver−Burk polt of TMAOase from squid muscle was shown in **Figure 6**. The K_m value of TMAOase was about 26.2 mM in an assay system containing 2 mM ascorbate, 2 mM cysteine, and 0.2 mM $FeCl_2$. The K_m of TMAOase from red hake muscle for TMAO was 2.7 mM. The K_m of TMAOase from walleye pollack muscle was reported to be 30 mM for a

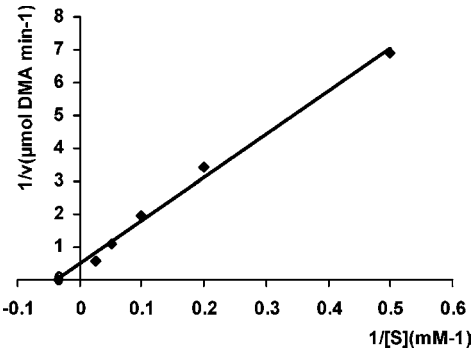

Figure 6. Kinetic analysis of TMAOase. The purified TMAOase was added into standard assay mixture containing TMAO at different concentrations and the reaction was run at 50 °C for 20 min. The Lineweaver–Burk polt of TMAOase from Jumbo squid mantle was shown.

reaction catalyzed aerobically by iron (0.2 mM), ascorbate (20 mM), and cysteine (20 mM) (*4*, *6*). Therefore, the affinity of TMAOase from squid to the substrate of TMAO was approximately as high as TMAOase from Alaska pollack muscle but was lower than that from red hake muscle.

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