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Full Length Article

Evaluation of Genes Encoding 4-*N*-Trimethylaminobutyraldehyde Dehydrogenase and 4-*N*-Trimethylamino-1-butanol Dehydrogenase from *Pseudomonas* sp. 13CM

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Abstract

Quaternary ammonium compounds (QACs) found in many microbial environments, which have different biological functions. Recently, we obtained two enzymes, 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABaldehyde-DH) and 4-*N*-trimethylamino-1-butanol dehydrogenase from *Pseudomonas* sp. 13CM those oxidizes QACs, respectively, 4-*N*-trimethylaminobutyraldehyde (TMABaldehyde) and 4-*N*-trimethylamino-1-butanol. TMABaldehyde-DH also mediates the biosynthesis of carnitine. In this study, the genes encoding both enzymes were sequenced and the TMABaldehyde-DH gene over-expressed in *Escherichia coli*. Their primary structures showed, respectively 93.95% and 87.0% positional identity with aldehyde dehydrogenase and iron containing alcohol dehydrogenase of *Pseudomonas putida* GB-1. Characterization of purified recombinant TMABaldehyde-DH, confirmed the enzyme had essentially the same properties as that of TMABaldehyde-DH purified from cell-free extract of *Pseudomonas* sp. 13CM. It is indicated that the function of aldehyde dehydrogenase and iron containing alcohol dehydrogenase of *Pseudomonas putida* GB-1, function as oxidization of QACs. © 2013 Friends Science Publishers

Keywords: Quaternary ammonium compounds; Carnitine biosynthetic pathway; 4-*N*-trimethylaminobutyraldehyde-DH; *Pseudomonas* sp. 13CM

Introduction

Naturally occurring quaternary ammonium compounds including choline, glycine betaine and carnitine, found in many microbial environments, which have different biological functions. The microorganisms using the choline as a sole source of carbon and nitrogen and their respective degradation pathway have been well studied (Nagasawa *et al*., 1976; Ikuta *et al*., 1977; Mori *et al*., 1980; Abee *et al*., 1990; Boch *et al*., 1994, Mori *et al*., 1992, 2002). Much attention has been paid to the osmoprotective role of glycine betaine in a number of diverse microbial systems (Smith *et al*., 1988; Roberts, 2005; Annamalai and Venkitanarayanan, 2009). A wide ranges of genes, encode the enzymes involved in degradation of organic compounds have been cloned (Cheong and Oriel, 2000; An *et al*., 2001; Ibrahim, 2003; Comlekcioglu *et al*., 2010). Recently, we identified a microorganism from soil as *Pseudomonas* sp. 13CM, grown on 4-*N*-trimethylamino-1-butanol (TMA-Butanol), and the enzyme 4-*N*-trimethylamino-1-butanol dehydrogenase (TMA-Butanol-DH) purified to apparent homogeneity. The isolated enzyme converts TMA-Butanol (considerable structural resemblance to choline as a theme) into trimethylamino butyraldehyde (TMABaldehyde) (Hassan *et al*., 2007). Additionally, 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABaldehyde-DH), isolated from the same organism, oxidized TMABaldehyde to yield γ-butyrobetaine (Hassan *et al*., 2008). Consistent with these observations, postulated a complete pathway of TMA-Butanol degradation.

TMABaldehyde-DH also responsible for the reactions of the biosynthetic route of L-carnitine (Hulse *et al*., 1978) that serves either as a nutrient, such as a carbon and nitrogen source (Kleber, 1997) or as an osmoprotectant (Robert *et al*., 2000). The enzyme, TMABaldehyde-DH has been also purified from bovine liver (Hulse and Henderson, 1980) and rat liver (Vaz *et al*., 2000). To improve the yield of the TMABaldehyde-DH from *Pseudomonas* sp. 13CM, we applied the general cloning technique. The present study deals with the sequencing of the genes encoding TMABaldehyde-DH and TMA-Butanol-DH from *Pseudomonas* sp. 13CM and enzymological properties of recombinant TMABaldehyde-DH, over-expressed in *Escherichia coli.*

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Materials and Methods

Materials, Bacterial Strains and Plasmids

NAD^+ and $NADP^+$ were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). TMA-Butanol iodide and TMABaldehyde iodide were prepared from, respectively, 4-dimethylamino-1-butanol and 4-aminobutyraldehyde dimethylacetal (Tokyo Kasei Kogyo Co. Ltd., Japan), according to the method described by Hassan *et al*. (2007). 4-dimethylaminobutyraldehyde dimethyl acetal was hydrolyzed by 0.1 M HCl overnight at room temperature to produce 4-dimethylaminobutyraldehyde (DMABaldehyde). All other chemicals and materials were of the highest purity grade. *Pseudomonas* sp. 13CM strain was used as the donor strain for the TMABaldehyde-DH and TMA-Butanol-DH gene (Hassan *et al*., 2007). Plasmid vector pET-24b (+) (Novagen, Madison, USA) was used for expression. *E. coli* JM109 and *E. coli* BL21 (DE3) cells were used as the host strain for general cloning and gene expression, respectively.

Sequence the Genes Encoding TMABaldehyde-DH and TMA-Butanol-DH

Choromosomal DNA was prepared from *Pseudomonas* sp. 13CM using the method of Saito and Miura (1963). The primers aldeNF, aldeR, alcoNF, alcoNR, and alcoR (Table 1) were designed on the basis of N-terminal amino acid sequences of TMABaldehyde-DH and TMA-Butanol-DH from *Pseudomonas* sp. 13CM (Hassan *et al*., 2007, 2008) and from *Pseudomonas putida*-GB-1 genes (locus ID: B0KJD3, B0KJD2). The coding region was amplified using standard PCR method. PCR products were sequenced using the dideoxy chain-termination method (Sanger *et al*., 1977). The inverse PCR primers alde2F, alde2R, alco3F and alco3R (Table 1) were designed based on inverted repeats border sequences. The inverse PCR was performed to determine the nucleotide sequence of the regions upstream and downstream of the TMABaldehyde-DH and TMA-Butanol-DH gene (Ochman *et al*., 1988).

Construction of Expression Vector for TMABaldehyde-DH

The T7 promoter over-expression system of *E. coli* was used for TMABaldehyde-DH production. A DNA fragment containing the structural gene encoding TMABaldehyde-DH of *Pseudomonas* sp. 13CM was amplified by PCR using the primer pair 13CM_aldeF and 13CM_aldeR and thereby incorporating the 5´-NdeI and 3´-HindIII restriction sites (Table 1). The PCR product was digested with the appropriate restriction enzymes, alongside the relevant vectors; gel purified and then ligated using T4 DNA ligase. Resulting ligation mixture was then transformed into the *E. coli* JM109 cells. Colonies were screened for recombinant using standard plasmid DNA isolation techniques and retransformed into *E. coli* BL21 (DE3) for small-scale protein expression tests.

Over-expression and Purification of Recombinant TMABaldehyde-DH

Expression of recombinant TMABaldehyde-DH was induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 25ºC. After 16 h of cultivation, cells were harvested by centrifugation at 14000 x g for 20 min at 4ºC and washed twice with 0.85% KCl solution, stored at -20ºC until protein purification. Purification of recombinant TMABaldehyde-DH was performed by hydrophobic chromatography using Phenyl-Toyopearl spin column. For this, cells were resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT and disrupted at 4 ºC by sonic treatment and centrifuged. To the cell free extract, same volume of 0.5 M of ammonium sulfate solution in the 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT was added. The resulting solution was loaded onto a Phenyl-Toyopearl spin column equilibrated with the same buffer which contained 0.25 M of ammonium sulfate and desalted through repeated concentration or dilution against low salt buffer (50 mM potassium phosphate buffer, pH 7.5 containing 1 mM DTT). The protein concentration was determined using the Lowry method (Lowry, 1951) with bovine serum albumin as the standard or by the absorbance at 280 nm, where an $E^{1\%}_{1cm}$ value of 10.0 was used. Specific activity was defined as units of enzyme activity per mg protein.

Enzyme Activity Assay

The routine assay of TMABaldehyde-DH was performed at 30ºC by monitoring the increase in absorbance at 340 nm. The reaction mixture (1.5 mL) in a cuvette contained 150 mM glycine-NaOH buffer (pH 9.5), 3.0 mM of NAD^+ and an appropriate amount of the enzyme. The enzyme reaction was initiated by the addition of the TMABaldehyde iodide at a final concentration of 0.8 mM. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the production of 1 µmol of NADH per min under the assay conditions. A molar absorption coefficient 6,200 M^{-1} cm^{-1} for NADH was used in the calculation.

Polyacrylamide Gel Electrophoresis

Native PAGE was performed using 10% gels at pH 8.8 and that gels were run at 4ºC. The protein was stained with Coomasie brilliant blue R-250 or checked for enzyme activity at room temperature. For the activity staining, immediately after electrophoresis, gels were incubated at room temperature for 15 min and placed in the reaction mixture which contained 150 mM of glycine-NaOH buffer (pH 9.5), 0.064 mM of 1-methoxy phenazine methosulfate, 0.24 mM of nitroblue tetrazolium, 0.12 mM of TMABaldehyde, and 3 mM of NAD^+. SDS–PAGE was performed following the method of Laemmli (1970) at room temperature using the mini slab size 5–20% gradient polyacrylamide gels purchased from Atto (Tokyo, Japan).

Table 1: PCR primer sequences used for sequencing of the *Pseudomonas* sp. 13CM TMABaldehyde-DH and TMA-Butanol-DH genes and expression vector construction

Primer	Direction	Sequence (5´-3´)	Tm
aldeNF	Forward	CCSCARCTSAGRGAYGCSGCSTAYTGG	68.1
aldeR	Reverse	AGSACSGGSCCGAAGATYTCYTC	61.4
alcoNF	Forward	ATGATYGAYAAYCTSTCSCCSCT	56.5
alcoNR	Reverse	AGSGGSGASAGRTTRTCRATCA	54.5
alcoR	Reverse	TCSGCSCCSGTSCCSGCSGTSGT	69.3
alde2F	Forward	CCTTCTGGTCAGCCGACCTCGG	75.0
alde2R	Reverse	GTCCACGTCAGGCTCACGGC	72.8
alco3F	Forward	CCTGGTGGCCCGGCAGAC	73.8
alco3R	Reverse	TGCCACTCGCGGAACGTCG	75.3
[a]13CM_aldeF	Forward	GAGGGGATGCATATGCCGCAACTCAG	64.3
[b]13CM_aldeR	Reverse	ACAGGGGGGAAGCTTGTCAATCACG	62.9

[a]NdeI site (underlined); [b]HindIII site (underlined)

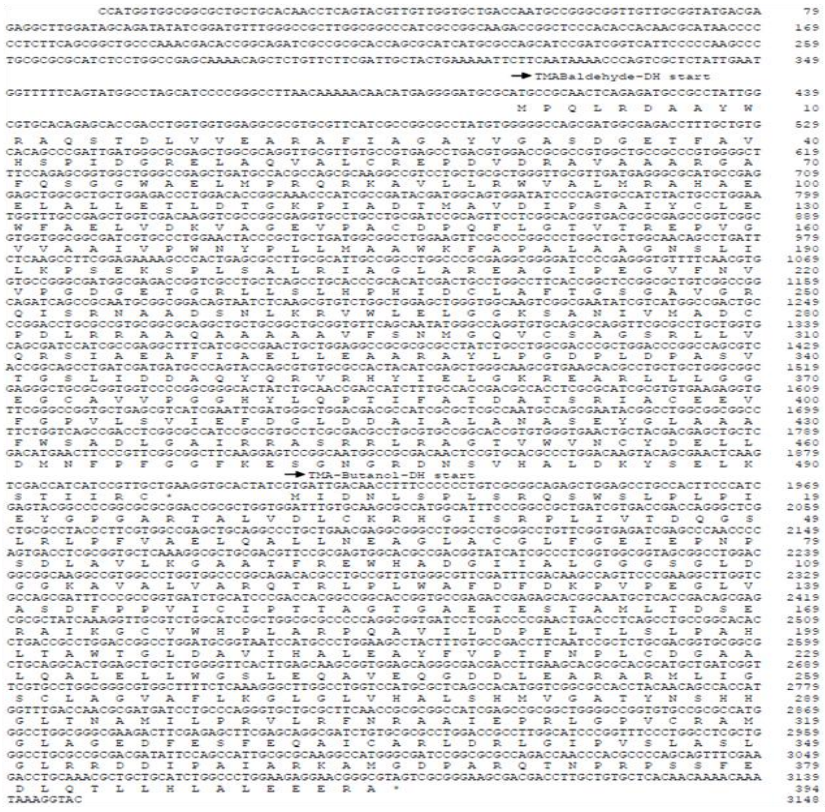

Fig. 1: Nucleotide and deduced amino acid sequences of the TMABaldehyde-DH and TMA-Butanol-DH genes. The deduced amino acid sequenced is shown under the nucleotide sequence. The numbers to the right of the sequence stands for nucleotide and amino acid positions. Asterisks represents the translational stop codon

The purified enzyme samples were prepared for SDS–PAGE by mixing with an equal volume of 2x EzApply sample buffer (Atto, Tokyo, Japan) and boiled for 5 min before use. Gels were stained with Coomasie brilliant blue R-250.

Measurement of Molecular Mass

The molecular mass of purified enzyme was estimated by SDS–PAGE and gel filtration on a TSK-gel G3000SW column (0.78 × 30 cm) (Tosoh Corp., Tokyo, Japan) equilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT at a flow rate of 0.5 mL min^{-1}. Approximate 42 µg of protein was loaded. The fraction (0.5 mL each) was quantified by absorption 280 nm and by assaying the enzymatic activity.

Results

Amino acid Sequence of TMABaldehyde-DH and TMA-Butanol-DH

The N-terminal amino acid sequences of TMABaldehyde-

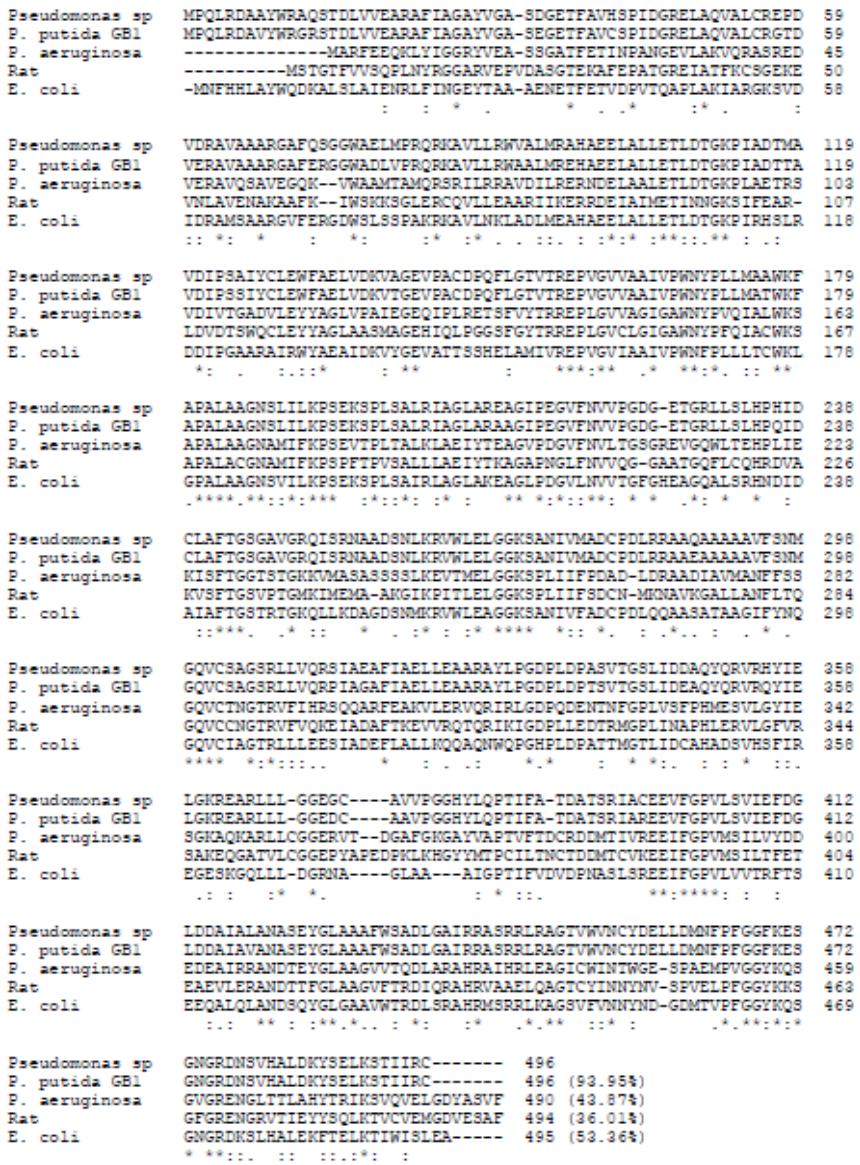

Fig. 2: Amino acid sequences of recombinant TMABaldehyde-DH and similar polypeptides. The sequences are from *Pseudomonas* sp. 13CM TMABaldehyde-DH (top line), *P. putida* GB1 (B0KJD3), *P. aeruginosa* betaine aldehyde dehydrogenase (PA5373), rat TMABaldehyde-DH (AF170918) and *E. coli* K-12 γ-glutamyl-γ-aminobutyraldehyde dehydrogenase (P23883). Numerals show quantities of amino acid residues, starting from the initial codon

DH and TMA-Butanol-DH of *Pseudomonas* sp. 13CM had the similarity with, respectively, aldehyde dehydrogenase (B0KJD3) and iron containing alcohol dehydrogenase (B0KJD2) of *Pseudomonas putida* GB-1. Therefore, we assumed that both DHs obtained were the homologous with the enzymes of *Pseudomonas putida* GB-1. Using the inverse PCR technique, we obtained gene containing two Open Reading Frames (ORFs) coding for a 496 and 394 amino acids polypeptide chain for TMABaldehyde-DH and TMA-Butanol-DH, resepectively (Fig. 1). The deduced primary structure from ORF1 and ORF2 were completely matched with the N-terminus from *Pseudomonas* sp. 13 CM, respectively TMAbaldehyde-DH and TMA-Butanol-DH, analyzed previously (Hassan *et al*., 2007, 2008) by Edman degradation. The deduced amino acid sequences from *Pseudomonas* sp. 13CM were compared with other protein sequences. Database analysis revealed that the primary structure of protein encoded in ORF1 and ORF2 showed high similarity with aldehyde dehydrogenase and iron containing alcohol dehydrogenase of *P. putida* GB-1 (Fig. 2). ORF1 (TMABaldehyde-DH), which encodes 496 amino acids also shares identity with betaine aldehyde dehydrgenase of *Pseudomonas aeruginosa*, TMABaldehyde-DH of rat liver (Fig. 2). Interestingly, γ-glutamyl-γ-aminobutyraldehyde dehydrogenase, which is responsible for oxidation of γ-glutamyl-γ-

aminobutyraldehyde into γ-glutamyl-γ-aminobutyrate in putrescene utilization pathway of *E. coli* K-12 also showed a considerable positional identity to TMABaldehyde-DH of *Pseudomonas* sp. 13CM (Fig. 2).

Over-expression and Purification of Recombinant TMABaldehyde-DH

To clarify the function and role of *Pseudomonas* sp. 13CM TMABaldehyde-DH, we constructed the expression vector for production of TMABaldehyde-DH as described in Materials and Methods section. Upon induction with IPTG, recombinant TMABaldehyde-DH was expressed at 25ºC. The expressed enzyme was partially purified by hydrophobic chromatography using Phenyl-Toyopearl spin column giving a preparation with a specific activity at 30.97 unit mg^{-1}. The purified recombinant TMABaldehyde-DH appeared as single protein band on native PAGE and the enzyme activity was detected at the same position on the gel, additionally, moved as a single protein band with SDS–PAGE at around 55 kDa (Fig. 3). The molecular mass of the native enzyme was estimated by gel filtration on a TSK-Gel G3000SW column. The enzyme was eluted at a molecular mass of about 160 kDa (Fig. 4).

Substrate Specificity and Kinetic Assay

The recombinant TMABaldehyde-DH was very specific for NAD^+ and did not react with $NADP^+$. The enzyme oxidized TMABaldehyde iodide and DMABaldehyde iodide. No reduction reaction was observed. The enzyme showed no activity towards 4-aminobutyraldehyde, betainealdehyde, acetaldehyde, propionaldehyde, butyraldehyde, isovaleraldehyde and pivaleraldehyde. The apparent K_m values for TMABaldehyde iodide, DMABaldehyde iodide and NAD^+ were calculated to be 0.12, 0.07, and 0.15 mM, respectively.

Accession Numbers

The entire nucleotide sequence data reported in this article encoding the TMABaldehyde-DH and TMA-Butanol-DH genes have been deposited in the DDBJ database under the accession numbers AB741624 and AB741625, respectively.

Discussion

We disclosed an enzymatic reduction system composed of both TMA-Butanol-DH and TMABaldehyde-DH for the degradation of choline like structure 4-*N*-trimethylaminobutanol within the same organism, *Pseudomonas* sp. 13CM (Hassan *et al*., 2007, 2008). Recently, the microorganisms, degrading homocholine as the only source of carbon and nitrogen have been isolated in our laboratory and proposed a possible degradation pathway (Mohamed Ahmed *et al*., 2009a, b and 2010). The genes encoding the enzymes TMABaldehyde-DH and TMA-

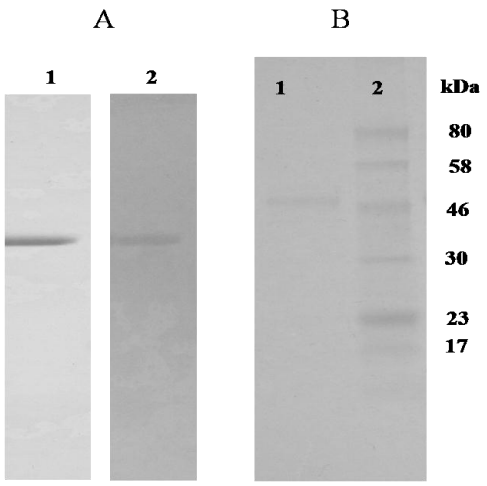

Fig. 3: Native PAGE and SDS–PAGE of purified recombinant *Pseudomonas* sp. 13CM TMABaldehyde-DH. A, Native PAGE: Gels were stained for protein with CBB (1) and enzyme activity with the TMABaldehyde (2). B, SDS–PAGE: Lane 1, purified recombinant TMABaldehyde-DH; lane 2, molecular weight markers (color plus prestained protein marker, New England Biolabs)

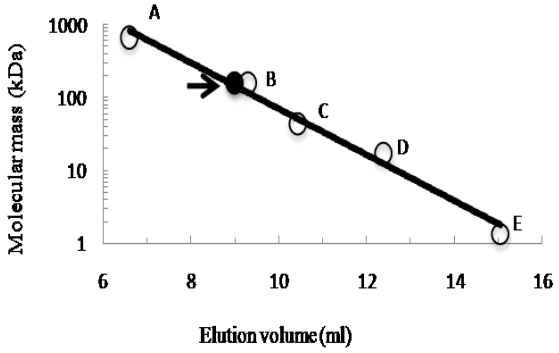

Fig. 4: Molecular weight determination of recombinant TMABaldehyde-DH. The molecular mass of recombinant TMABaldehyde-DH was estimated by HPLC from a semilogarithmic plot of molecular mass against retention time with each standard protein: A, bovine thyroglobulin (670 kDa); B, bovine γ-globulin (158 kDa); C, chicken ovalbumin (44 kDa); D, horse myoglobin (17 kDa); E, vitamin B12 (1.35 kDa)

Butanol-DH of *Pseudomonas* sp. 13CM remained unknown. In this study, we sequenced the genes and constructed the recombinant TMABaldehyde-DH, first report of bacterial enzymes; mediate the L-carnitine biosynthetic reactions. The specific activity of 30.97 unit mg^{-1} in the present preparation showed the activity more than two times of TMABaldehyde-DH isolated from the cells of *Pseudomonas* sp. 13CM, 12.4 unit mg^{-1} (Hassan *et al*., 2008). It also higher than those for TMABaldehyde-DH

from *Bos taurus* (Hulse and Henderson, 1980) and *Rattus norvegicus* (Vaz *et al*., 2000), respectively, 5.1 unit mg^{-1} and 0.77 unit mg^{-1}. The deduced amino acid sequence of TMABaldehyde-DH and TMA-Butanol-DH were respectively, similar to those of aldehyde dehydrogenase and alcohol dehydrogenase superfamily proteins. In particular, both of the enzymes have high similarity to proteins from *P. putida* GB-1(B0KJD3, B0KJD2) (Fig. 2). It uncovers the facts that the function of both enzymes of *Pseudomonas putida* GB-1, function as oxidization of quaternary ammonium compounds.

The deduced amino acid sequences (Fig. 1) and calculated molecular mass of TMABaldehyde-DH (53030.91 Da) and TMA-Butanol-DH (42090.94 Da), are in good agreement with that of the partial amino acid sequences and electrophoresis data described by Hassan *et al*. (2007, 2008). In addition, the present preparation of TMABaldehyde-DH, moved as a single protein band with SDS–PAGE at around 55 kDa (Fig. 3), which is in accordance with the theoretical monomeric molecular mass of the TMABaldehyde-DH. The molecular mass of the over-expressed TMABaldehyde-DH of *Pseudomonas* sp. 13CM is found to be 160 kDa by gel filtration (Fig. 4) suggesting that the protein existed in trimer in solution under native condition, which is similar to the molecular mass of TMABaldehyde-DH of *B. taurus* (Hulse and Henderson, 1980) and the same to the predicted molecular mass of TMABaldehyde-DH from *R. norvegicus* (Vaz *et al*., 2000).

In full agreement with Hassan *et al*. (2008), the recombinant enzyme showed oxidative activity toward only TMABaldehyde and no aldehyde produced by the reversible reaction. The recombinant protein in this study gave a K_m value of 0.12 mM for TMABaldehyde is appeared to be higher than the K_m values for TMABaldehyde-DHs from *Pseudomonas* sp. 13CM (Hassan *et al*., 2008), *B. taurus* (Hulse and Henderson, 1980), *R. norvegicus* (Vaz *et al*., 2000), respectively, 7.4, 4.2 and 1.4 μM. Both NAD^+ and $NADP^+$ can be used as coenzyme in *R. norvegicus* (Vaz *et al*., 2000), but the enzyme of *B. taurus* (Hulse and Henderson, 1980) and the enzymes purified from *Pseudomonas* sp. 13CM were highly specific only for NAD^+.

In conclusion, molecular cloning with *Pseudomonas* sp. 13CM as a gene donor led to the production of a large quantity of the enzyme in a recombinant strain. Characterization of degradative genes, may explore to evaluate the microbial populations optimal for biodegradation and bioremediation technologies. Currently, further investigation is in progress to incorporate novel enzyme-cofactor interactions.

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