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An enzyme possessing both glutathione-dependent formaldehyde dehydrogenase and S-nitrosoglutathione reductase from *Antrodia camphorata*

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ABSTRACT

Glutathione-dependent formaldehyde dehydrogenase (GFD or GSH-FDH) plays important roles in formaldehyde detoxification and antioxidation. A gene encoding GFD from *Antrodia camphorata* was identified based on sequence homology. The deduced amino acid sequence of 378 amino acid residues is conserved among the reported GFDs. To characterise the Ac-GFD, the coding region was subcloned into a vector pET-20b(+) and transformed into *Escherichia coli*. The recombinant GFD was expressed and purified by Ni^{2+}-nitrilotriacetic acid Sepharose. This purified enzyme showed a single band on a 10% SDS–PAGE. The enzyme retained 50% GFD activity after heating at 50 °C for 5 min. The enzyme is bifunctional. In addition to the GFD activity, it also functions as an effective S-nitrosoglutathione reductase (GSNOR) presumably to safeguard against nitrosative stress. The K_m values for S-hydroxymethylglutathione and S-nitrosoglutathione were 1.20 and 0.28 mM, respectively.

1. Introduction

Antrodia camphorata (*A. camphorata*) is a medicinal fungus found only in the forests of Taiwan which uses *Cinnamomum kanehirai* as its host. The fruiting bodies of *A. camphorata* have been used for health food, drug intoxication, and liver cancer, among others, in folk medicine for years. Recently, we established EST (expressed sequence tag) from fruiting bodies of *A. camphorata* in order to search active components for possible health food applications. We have cloned and characterised a superoxide dismutase (Liau, Wen, Shaw, & Lin, 2007), a 2-Cys peroxiredoxin (Huang, Ken, Huang, & Lin, 2007), a 1-Cys peroxiredoxin (Wen, Huang, Juang, & Lin, 2007) and a catalase (Ken, Chen, Chang, & Lin, 2008) based on the established EST from *A. camphorata*. Here, we report the cloning and characterisation of a bifunctional enzyme, glutathione-dependent formaldehyde dehydrogenase (GFD) and S-nitroglutathione reductase (GSNOR) from *A. camphorata*.

Mammalian GFD is an alcohol dehydrogenase 3 (ADH 3) which uses S-hydroxymethylglutathione (HMGSH) as the main substrate (Hoog, Hedberg, Stromberg, & Svensson, 2001). GFD detoxifies formaldehyde thus preventing its lethal interactions with proteins, nucleic acids, and other cell constitutes (Barber & Donohue, 1998). Plants can also metabolize formaldehyde through the action of a GFD (Fliegmann & Sandermann, 1997). A connection between ADH3 and nitric oxide (NO) metabolism was established by Liu et al. (2001) and Liu et al. (2004) through studies of yeast and mouse ADH3 knockouts lacking GSNOR activity which showed increased S-nitrosylated proteins. NO, a signaling molecule involved in many functions, can act by S-nitrosylation of proteins which in term alter the activities of enzymes, structural proteins, or transcription factors (Godoy, Gonzàlez-Duarte, & Albalat, 2006; Hess, Matsumoto, Kim, Marshall, & Stamler, 2005). Recently, Godoy et al. (2006) reported that purified cephalochordate ADH3 exhibited not only formaldehyde dehydrogenase activity but also GSNOR activity. There are no reports in mushroom that ADH3 possesses GSNOR activity.

The Ac-GFD first reported here possesses both GFD and GSNOR activities which are believed to be responsible for formaldehyde detoxification and fighting against nitrosative stress. The properties and kinetic studies of this Ac-GFD should help in understanding its functions. Thus, the coding region of the Ac-GFD gene was introduced into an *Escherichia coli* expression system, the active enzyme was purified and its properties and kinetic studies were investigated.

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2. Materials and methods

2.1. Antrodia camphorata

Fruiting bodies of *A. camphorata* which naturally grew in the hay of *C. kanehirai* were obtained from Asian Biotec. Company (Taiwan).

2.2. Total RNA preparation and cDNA synthesis

Total RNA was prepared from fresh fruiting bodies (wet weight 5 g) using TRIzol reagent (GIBCO, Frederick, MD). The total RNA (20 μg) was obtained. Three microgram of the total RNA was used for cDNA synthesis using a ZAP-cDNA kit from Stratagene (La Jolla, CA).

2.3. Isolation of Ac-GFD cDNA

We have previously established an EST database from fruiting bodies of *A. camphorata* and sequenced all clones with insert size greater than 0.4 kb (data not shown). The identity of a GFD cDNA clone was assigned by comparing the inferred amino acid sequence in various databases using the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.4. Subcloning of Ac-GFD cDNA

The coding region of the Ac-GFD cDNA was amplified using two gene-specific primers. The 5′ upstream primer contains *Nde*I recognition site (5′ CAT ATG TCC ACA GTA GGA AAA CC 3′) and the 3′ downstream primer contains *Not*I recognition site (5′ GCG GCC GCA GAC ATG TCG ACG ACA CAG C 3′). Using 0.1 μg of *A. camphorata* cDNA as a template, and 10 pmole of each 5′ upstream and 3′ downstream primers, a 1.1 kb fragment was amplified by PCR. The fragment was ligated into pCR4-Topo and transformed into *E. coli*. Plasmid DNA was isolated from the clone and digested with *Nde*I and *Not*I. The digestion products were separated on a 1% agarose gel. The 1.1 kb insert DNA was gel purified and subcloned into *Nde*I and *Not*I sites of pET-20b(+) vector (Novagen). The recombinant DNA was then transformed into *E. coli* BL21(DE)pLysS. The recombinant protein was overexpressed in *E. coli* and its function checked by enzyme activity assay.

2.5. Expression and purification of the recombinant Ac-GFD

The transformed *E. coli* containing the Ac-GFD was grown at 37 °C in 200 ml of Luria Bertani medium containing 50 μg/ml ampicillin until A_{600} reached 0.9. Protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The culture was incubated at 25 °C for an additional 24 h under 80 rpm, and then the bacterial cells were harvested by centrifugation. The cells were harvested and soluble proteins extracted with glass beads as described before (Ken, Hsiung, Huang, Juang, & Lin, 2005). The recombinant Ac-GFD was purified by Ni-NTA affinity chromatography as per the manufacturer's instruction (Qiagen). The purified enzyme (6 ml) was dialyzed against 200 ml PBS containing 1% glycerol at 4 °C for 4 h. Fresh PBS (phosphate buffered saline) containing 1% glycerol was changed once during dialysis. The dialyzed sample was used directly for analysis.

2.6. Protein concentration measurement

Protein concentration was determined by a Bio-Rad Protein Assay Kit (Richmond, CA) using bovine serum albumin as a reference standard.

2.7. Molecular mass determination by ESI Q-TOF

The purified recombinant Ac-GFD (0.4 mg/ml) was prepared in 0.03 × PBS containing 5 mM imidazole and 2% glycerol. The sample (3 μl) was used for molecular mass determination using an ESI Q-TOF mass spectrometry (Micromass, Manchester, England).

2.8. GFD activity assay

Assays for GFD activity with HMGSH as a substrate were according to Uotila and Koivusalo (1981) with some modifications. The reaction mixture contained 0.23 μg Ac-GFD, 100 mM potassium phosphate buffer (pH 8.0), 1 mM HMGSH [GSH and formaldehyde (1:1) mM were premixed at 25 °C for 5 min to generate HMGSH], and 2 mM NAD^+ in a final volume of 100 μl. Enzyme activity was measured at 340 nm by the time-dependent reduction of NAD^+ at room temperature in a spectrophotometer (Genesys 20, Thermo Spectronic, Madison, USA).

2.9. GSNOR activity assay

Assays for GSNOR activity with GSNO as a substrate were according to Godoy et al. (2006) with some modifications. The reaction mixture contained 0.1 μg Ac-GFD, 100 mM potassium phosphate buffer (pH 8.0), 0.3 mM GSNO, and 0.2 mM NADH in a final volume of 100 μl. Enzyme activity was measured at 340 nm by the time-dependent oxidation of NADH and reduction of GSNO at room temperature in a spectrophotometer (Genesys 20, Thermo Spectronic, Madison, USA).

2.10. Kinetic studies

GFD activities were tested at 25 °C by monitoring the production of NADH at A_{340} for formaldehyde oxidation. The GFD activity was measured at pH 8.0 in 0.1 M potassium phosphate, with HMGSH (formed by mixing formaldehyde and glutathione) and NAD^+. The kinetic properties of GFD (0.23 μg) in a total volume of 100 μl were determined using different concentrations of HMGSH (0.05–4 mM) with the fixed amount of 2 mM NAD^+ or using different concentrations of NAD^+ (0.05–8 mM) with the fixed amount of 1 mM HMGSH. The K_m, V_{max} and K_{cat} were calculated from Lineweaver–Burk plots.

GSNOR activities were tested at 25 °C by monitoring the consumption of NADH and GSNO ($\varepsilon_{340} = 7.06\ mM^{-1}\ cm^{-1}$) at A_{340} for GSNO (Sigma) reduction. The GSNOR activity was measured at pH 8.0 in 0.1 M potassium phosphate, with GSNO and NADH. The kinetic properties of Ac-GFD (0.1 μg) in a total volume of 100 μl were determined using different concentrations of GSNO (0.025–1.6 mM) with the fixed amount of 0.2 mM NADH or using different concentrations of NADH (0.025–0.8 mM) with the fixed amount of 0.3 mM GSNO. The K_m, V_{max} and K_{cat} were calculated from Lineweaver–Burk plots.

2.11. Enzyme characterisation

The enzyme sample was tested for activity under various conditions. The Ac-GFD samples were treated as follows: (1) *Thermal effect*. Enzyme sample was heated to 50 °C for 2, 4, 8, or 16 min. (2) *pH effect*. Enzyme sample was adjusted to desired pH by adding a half volume of buffer with different pHs: 0.2 M citrate buffer (pH 2.2 or 5.4), 0.2 M Tris–HCl buffer (pH 7.8 or 9.0) or 0.2 M glycine-NaOH buffer (pH 10.4 or 11.2). Each sample was incubated at 37 °C for 1 h. (3) *SDS effect*. SDS, a protein denaturing reagent, was added to the enzyme sample to the levels of 0.5%, 1%, or 2% and incubated at 37 °C for 1 h. (4) *Imidazole effect*. During protein purification, the Ac-GFD enzyme was eluted with imidazole. There-

fore, the effect of imidazole on the enzyme activity was examined. Imidazole was added to the enzyme sample to the levels of 0.2, 0.4, or 0.8 M and incubated at 37 °C for 1 h. (5) *Proteolytic susceptibility.* The enzyme was incubated with one-tenth its weight of trypsin or

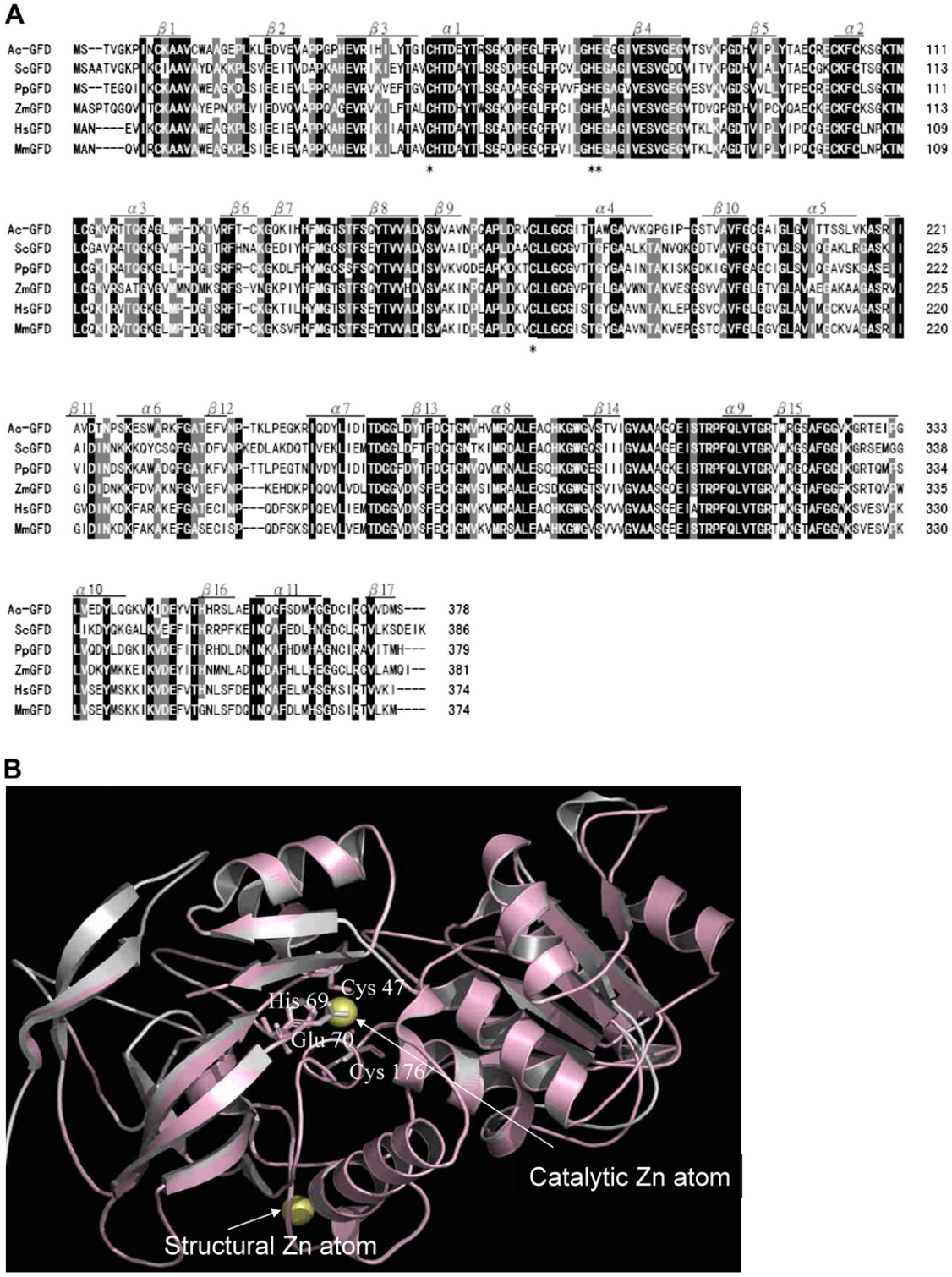

Fig. 1. Optimal alignment of the amino acid sequences of GFD from various sources and homology structure prediction. (A) Sequence alignment: Ac-GFD (this study), ScGFD (*Saccharomyces cerevisiae*), PpGFD (*Pichia pastoris*), ZmGFD (*Zea mays*), HsGFD (*Homo sapiens*), and MmGFD (*Mus musculus*). Identical amino acids in all sequences are shaded black, conservative replacements are shaded gray. Protein secondary structure was predicted by SWISS-MODEL program and represented as α helices and β strands. Asterisk denoted the amino acids coordinate catalytic site binding. (B) Three-dimensional homology model of Ac-GFD. The model was created by SWISS-MODEL based on the known X-ray structure of human GFD. They were superimposed with the SYBYL 7.3 program. Superimposition of Ac-GFD (pink) and human GFD (gray) was shown by using protein solid ribbon. The four residues coordinating active site Zn (Cys47, His69, Glu70, Cys176) are conserved as they are present in all reported GFD sequences. Upper yellow ball represented the catalytic Zn atom, and lower yellow ball represented the structural Zn atom. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chymotrypsin at pH 8.0, 37 °C for a period of 10, 20, or 40 min. In the chymotrypsin digestion, $CaCl_2$ was added to 5 mM. Aliquots were removed at various time intervals for analysis. After each treatment, half of the sample was electrophoresed onto a 10% native gel to determine the changes in protein levels and mobility. The other half was assayed for GFD activity.

2.12. GFD and GSNOR detection by activity staining on a native gel

Triplicate samples containing the Ac-GFD enzyme were electrophoresed on a 10% native gel for 2.5 h at 100 V. The triplicate lanes were sliced into three strips. One strip was stained for GFD activity, the other for GSNOR activity as previously described (Godoy et al., 2006), and the third for protein staining. For GFD activity staining, the gel was incubated in 70 mM potassium phosphate buffer (pH 8.0), 0.5 M potassium chloride, 4.8 mM formaldehyde and 1 mM reduced GSH at 25 °C for 5 min. After the 5 min incubation, NAD^+, nitroblue tetrazolium and phenazine methosulfate were added to a final concentration of 1.2 mM, 0.4 mg/ml and 0.03 mg/ml, respectively. The gel was incubated at 37 °C with gentle shaking for 1 h, followed by rinsing with water to stop the reaction. The gel became deep purple at the position of GFD. The GFD band catalysed HMGSH (generated by mixing formaldehyde and GSH) in the presence of NAD^+ to produce NADH, NADH consequently reduced nitroblue tetrazolium to form purple color. For GSNOR activity staining, the gel was incubated in 0.1 M potassium phosphate buffer (pH 8.0) with 2 mM NADH for 15 min in an ice-bath. Excess buffer was drained and the gel was covered with filter paper strip soaked in 3 mM GSNO (Godoy et al., 2006). After 15 min, the filter paper was removed and the gel was exposed under ultraviolet light. GSNOR converted the GSNO to NO using NADH as a reducing agent. The location of GSNOR on gel was observed by the disappearance of the NADH fluorescence under ultraviolet light. The area and intensity of the bands were measured by a computing densitometer (Molecular Dynamics, Modesto, CA).

2.13. Computational analysis of Ac-GFD

The BLASTP program was used to search homologous protein sequences in the nonredundant database (NRDB) at the National Center for Biotechnology Information, National Institutes of Health (http://www.ncbi.nlm.nih.gov/). Multiple alignments were constructed using ClustalW2 program. Structural modeling was carried out by using the SWISS-MODEL program (Arnold, Bordoli, Kopp, & Schwede, 2006) (http://swissmodel.expasy.org/SWISS-MODEL.html) to create the 3-D homology model based on the known X-ray structure of human glutathione-dependent formaldehyde dehydrogenase (PDB ID 1MC5). The modeling data was then superimposed by SYBYL 7.3 program (TRIPOS Associates, Inc. It was kindly provided by Dr. Nady Shaw [National Changhua University of Education, Taiwan]).

3. Results

3.1. Cloning and characterisation of a cDNA encoding Ac-GFD

Approximately 40,000 *A. camphorata* cDNA clones were sequenced. Nucleotide sequences and the inferred amino acid sequences of these clones were compared to the sequences in various nucleic acid and protein data banks using appropriate BLAST programs (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). A putative GFD cDNA clone was identified by sequence homology to the published GFDs. This Ac-GFD cDNA (1373 bp, EMBL accession no. DQ396859) encodes a protein of 378 amino acid residues with calculated molecular mass of 40315 Da. Fig. 1a shows the amino acid sequence alignment of the putative GFD with GFD from several other sources. The Ac-GFD shared 63% identity with ScGFD (*Saccharomyces cerevisiae*, accession no. X68020), 65% identity with GFD from Pp (*Pichia pastoris*, accession no. AF066054), 61% identity with ZmGFD (*Zea mays*, accession no. Y11029), 62% identity with HsGFD (*Homo sapiens*, accession no. M30471), and 62% identity with MmGFD (*Mus musculus*, accession no. M84147). The high identity indicates this cDNA belongs to GFD.

3.2. Predicted structure of recombinant Ac-GFD

A structural model of Ac-GFD based on the known X-ray structure of human GFD (PDB ID 1MC5) was created (Fig. 1B) using SWISS-MODEL and SYBYL 7.3 programs. The amino acid sequences of Ac-GFD and the human enzyme share 62% identity. The secondary structure was predicted by the same program and represented as α helices and β strands (Fig. 1A). The structure model was superimposed to the relative domains of the human GFD with a root mean square (rms) deviation of 0.44 Å. Structure studies have showed that the active site zinc of human GFD can move back and forth during the catalytic cycle (Sanghani, Davis, Zhai, & Robinson, 2006), and the active site zinc is coordinated with Cys44, His66, Glu67, and Cys173 (Sanghani, Robinson, Bosron, & Hurley, 2002). In this Ac-GFD 3-D homology model, the active site zinc also showed potential coordination with the corresponding conserved Cys47, His69, Glu70, and Cys176 residues. Other residues in human GFD involved in the active site are Arg114, Lys283, and Gln111; the corresponding residues in Ac-GFD show conservation at Arg117 and Lys286 with exception at Gly114. The difference at this active site residue and other residues may lead to different kinetic data for the two enzymes. Human GFD has lower Km values for GSNO and HMGSH (see Table 1).

3.3. Expression and purification of the recombinant Ac-GFD

The coding region of Ac-GFD (1.1 kb) was amplified by PCR and subcloned into an expression vector, pET-20b(+), as described in the Section 2. Positive clones were verified by DNA sequence analysis. The recombinant Ac-GFD protein was expressed, and the proteins were analysed by a 10% SDS–PAGE in the presence of a reducing agent without boiling (Fig. 2). The recombinant Ac-GFD was expressed as a 6His-tagged fusion protein and was purified

Table 1
Kinetic characterisation of Ac-GFD and other published ADH3

		A. camphorata[a]	*S. cerevisiae*[a]	Human[1a]	Human[2a]	Rat
GSNO	Km (mM)	0.28	0.15	0.027	0.027	0.028
	kcat (min^{-1})	14300	52600	2400	12000	2640
	kcat/km	51100	350000	90000	444400	94300
NADH	Km (mM)	0.21	0.13	0.008	0.03	0.024
	kcat (min^{-1})	17300		2700		
	kcat/km	82400		340000		
HMGSH	Km (mM)	1.20	0.02	0.002	0.0014	
	kcat (min^{-1})	3000	3100	115	320	
	kcat/km	2500	155000	58000	229000	
NAD	Km (mM)	1.31	0.045	0.007	0.007	
	kcat (min^{-1})	2800		90		
	kcat/km	2140		13000		

Values are from this work (*A. camphorata* GFD) or from the literature (*S. cerevisiae* [Fernandez, Biosca, & Pares, 2003]; Human[1] [Hedberg, Griffiths, Nilsson, & Höög, 2003]; Human[2] [Fernandez et al., 2003] and Rat [Jensen, Belka, & DuBois, 1998]).
[a] These values are from recombinant expressed enzymes.

by affinity chromatography with nickel chelating Sepharose. A band with molecular mass of ~40 kDa was detected in Ni-NTA eluted fractions by SDS–PAGE (Fig. 2, lane 3). An ESI Q-TOF of Ac-GFD confirms the presence of only one protein band (monomer) under the conditions of 0.03 × PBS containing 5 mM imidazole and 2% glycerol. This indicates that the enzyme is monomeric in nature. The Ni-NTA eluted fractions that contained pure protein were pooled and characterised further. The yield of the purified 6His-tagged Ac-GFD was 220 μg from 200 ml of culture.

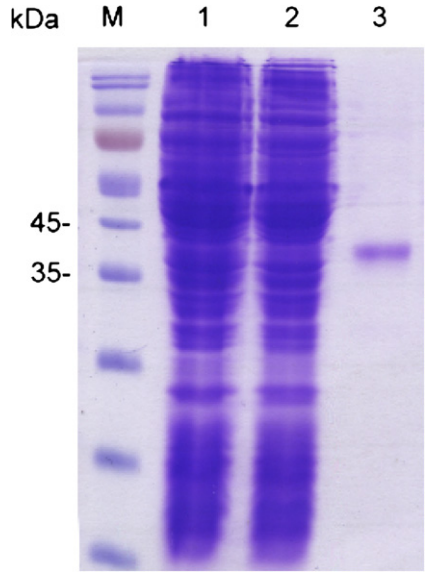

Fig. 2. Coomassie blue-stained SDS–polyacrylamide gel showing the purification of 6His-tagged Ac-GFD expressed in *E. coli*. Fifteen microlitre of each fraction was loaded into each lane of a 10% SDS–PAGE. Lane 1, crude extract from *E. coli*. expressing Ac-GFD; 2, flow-through proteins from the Ni-NTA column; 3, purified Ac-GFD eluted from Ni-NTA column. Molecular masses (in kDa) of standards are shown at left.

3.4. HMGSH and GSNO activities of Ac-GFD

As shown in Fig. 3A–D, the Lineweaver–Burk plot of the velocity ($1/V$) against 1/HMGSH gave the K_m = 1.20 mM, V_{max} = 164 μM/min, and K_{cat} = 3000 min^{-1}. The velocity ($1/V$) against 1/NAD^+ gave the K_m = 1.31 mM, V_{max} = 156 μM/min, and K_{cat} = 2800 min^{-1} for the Ac-GFD, respectively. The Lineweaver–Burk plot of the velocity ($1/V$) against 1/GSNO gave the K_m = 0.28 mM, V_{max} = 345 μM/min, and K_{cat} = 14,300 min^{-1}. The velocity ($1/V$) against 1/NADH gave the K_m = 0.21 mM, V_{max} = 417 μM/min, and K_{cat} = 17,300 min^{-1} for the Ac-GFD, respectively. These data are summarized in Table 1 along with kinetic data of ADH3 from other sources.

3.5. Characterisation of the purified Ac-GFD

To examine the thermal stability of the enzyme activity, the purified Ac-GFD was heat-treated as described in the Section 2 and then analysed by a 10% native gel or activity assay. The activity appeared to be somewhat heat stable. Approximately 60% of the Ac-GFD activity was retained at 50 °C for 5 min (Fig. 4). The Ac-GFD activity was stable in alkaline pH ranging from 7.8 to 11.2 (Fig. 5A). The enzyme appeared to be sensitive to SDS, the activity

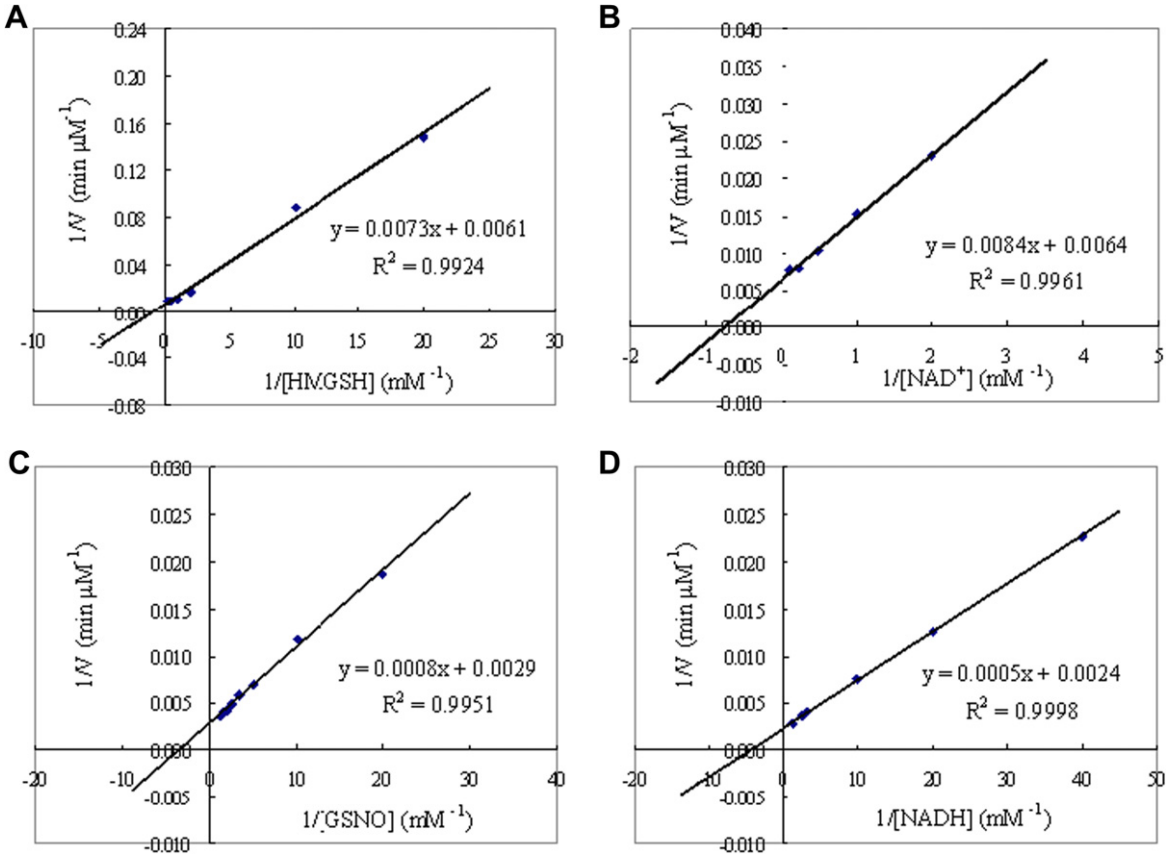

Fig. 3. Double-reciprocal plot of varying HMGSH (A), NAD^+(B), GSNO (C), and NADH (D) on Ac-GFD activity. The initial rate of the enzymatic reaction was measured at 2 mM NAD^+ with the HMGSH concentration varied from 0.05 mM to 4 mM (A), at 1 mM HMGSH with the NAD^+ concentration varied from 0.05 mM to 8 mM (B), at 0.2 mM NADH with the GSNO concentration varied from 0.025 mM to 1.6 mM (C), at 0.3 mM GSNO with the NADH concentration varied from 0.025 mM to 0.8 mM.

was lost even under 0.5% SDS (Fig. 5B). The enzyme retained about 70% activity under 0.8 M imidazole (Fig. 5C). The enzyme activity was not affected after 40 min incubation at 37 °C with one-tenth its weight of chymotrypsin (Fig. 5D). The enzyme showed activity after 40 min of incubation at 37 °C with trypsin (data not shown). In this protease digestion experiment, 1-Cys peroxiredoxin was

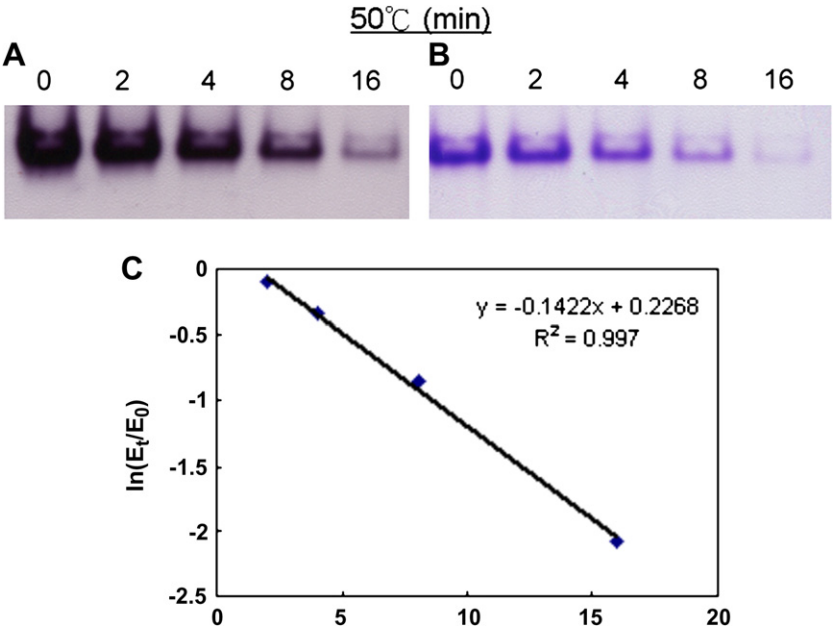

Fig. 4. Effect of temperature on the purified *Ac-GFD*. The enzyme sample was heated at 50 °C for various time intervals. Aliquots of the sample were taken at 0, 2, 4, 8 or 16 min then separated by a 10% native-PAGE. (A) Enzyme activity staining (2 μg protein/lane). (B) Staining for protein (2 μg protein/lane) as described in Section 2. (C) Plot of thermal inactivation kinetics. E_0 and E_t are original activity and residual activity after being heated for different time intervals. Data are means of three experiments.

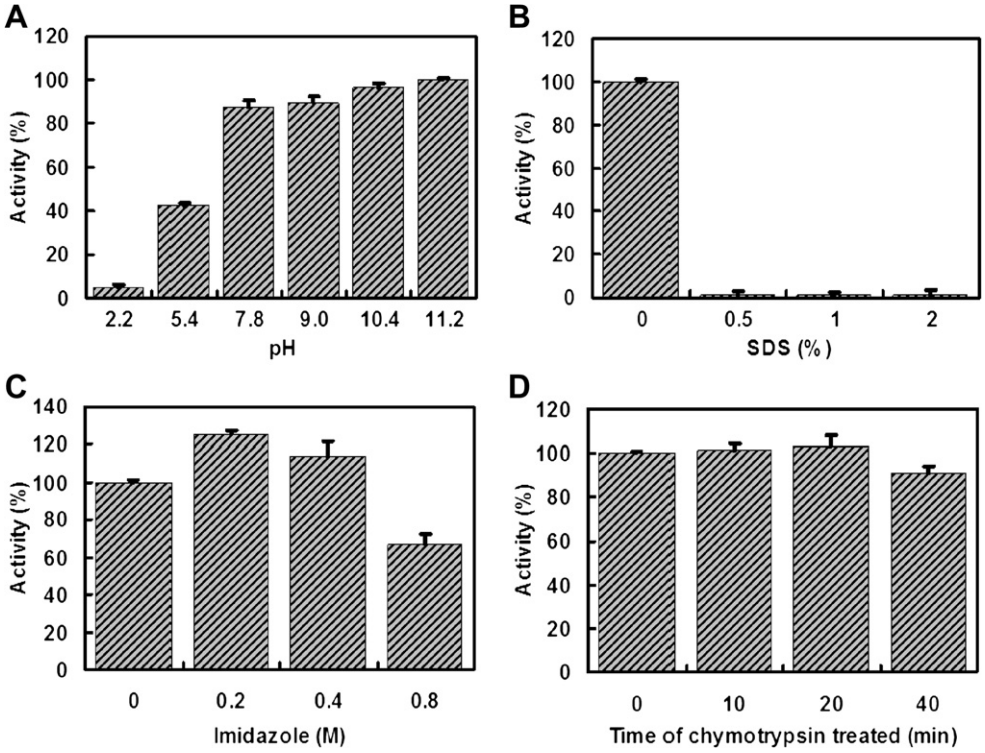

Fig. 5. Effect of pH, SDS, imidazole and chymotrypsin on the purified Ac-GFD. (A) The enzyme samples were incubated with different pH buffer at 37 °C for 1 h and then assayed for GFD activity (2 μg protein/reaction). (B) The enzyme samples were incubated with various concentration of SDS at 37 °C for 1 h and then checked for activity (2 μg protein/reaction). (C) The enzyme samples were incubated with various concentration of imidazole at 37 °C for 1 h and then checked for GFD activity (2 μg protein/reaction). (D) The enzyme samples were incubated with chymotrypsin at 37 °C for various time and then checked for GFD activity (2 μg protein/reaction). Data are means of three experiments.

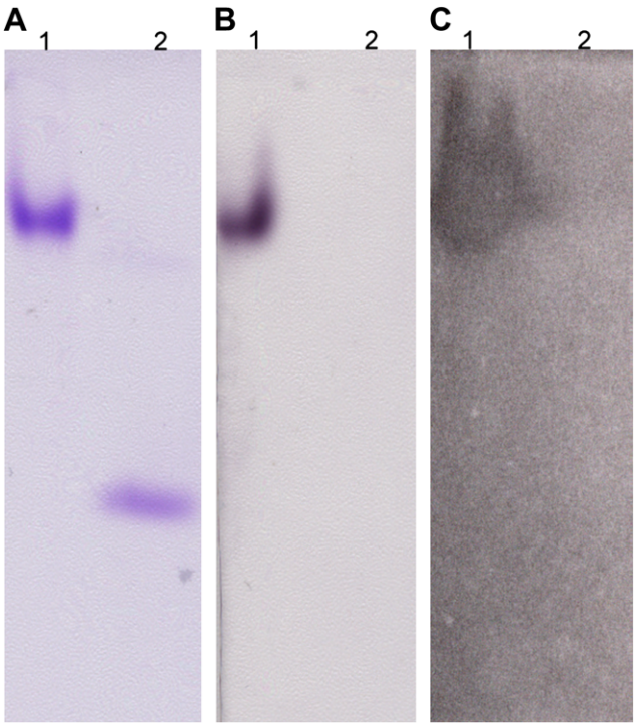

Fig. 6. Ac-GFD possessing GSNOR activity on gel. Triplet samples containing Ac-GFD (lane 1) and BSA (lane 2) as control were electrophoresed on a 10% native gel for 2.5 h at 100 V. The gel was sliced into three strips. One part was stained for GFD activity (B), the other for GSNOR activity (C), and another for Coomassie blue staining (A) as previously described.

used as a positive control. The peroxiredoxin was degraded within 20 min of treatment with either trypsin or chymotrypsin (results not shown).

To test GSNO reductase staining activity of the Ac-GFD as described in the Section 2, the gel was exposed to ultraviolet light to observe the disappearance of the NADH fluorescence (Fig. 6C). The result demonstrates that the Ac-GFD possessing GSNO reductase activity as detected on the gel.

4. Discussion

Alcohol dehydrogenase family (ADH, EC1.1.1.1) consists of six classes (ADH1–ADH6) which differ in substrate specificities (Fliegmann et al., 1997; Hoog et al., 2001). The ADH1, a classical liver form, involves in several metabolic pathways such as oxidation of ethanol and bile acid metabolism. ADH2 enzymes are divided into two subgroups, one group consisting of the human enzyme and the other consisting of the rodent form. ADH3 is the ancestral ADH form and uses HMGSH as the main substrate. ADH4 is responsible for the metabolism of ingested ethanol in the stomach. ADH5 and ADH6 are less defined and their substrate is unknown. The entire ADH system is responsible for detoxifying alcohols and aldehydes. Based on the sequence homology and the detected GFD activity, the Ac-GFD cDNA appears to belong to ADH3.

Sanghani, Robinson, Bennett-Lovsey, Hurley, and Bosron (2003) reported that the active site of the human GFD apoenzyme is coordinated to Cys44, His66, and Cys173. In the GDH-NAD(H) binary complex study, Glu67 is added to the coordination of the active site. Anion binding comprised of residues Gln111, Arg114 and Lys283 at the base of the active site. The importance of Arg114 in the binding of HMGSH was evident from decreased binding of the substrates to an Arg114 to Ala mutant (Engeland et al., 1993). Almost all the above referred residues involving in the active site can also be found in this Ac-GFD (Cys47, His69, Cys176, Glu70, Arg117, Lys286) except Gln111(human) instead of Gly114(Ac-GFD). From the point of sequence evolution, it is reasonable to assume that the Ac-GFD also possesses GSNOR activity, our results confirm this point. In 2001, GFD was reported to be the only enzyme in mammalian cells to possess S-denitrosating activity (Liu et al., 2001). In 2002, it was first reported that plant GFD is GSNOR (Sakamotoa, Uedab, & Morikawaa, 2002). In 2006, GSNOR activity of amphioxus ADH3 was reported (Godoy et al., 2006). In this study, we found that Ac-GFD possesses GSNOR activity which is first reported in mushroom.

The Ac-GFD appeared to be resistant to both trypsin and chymotrypsin. The Ac-GFD, as shown in Fig. 1, contains 35 potential trypsin cleavage sites and 24 potential chymotrypsin-high specificity (C-term to [FYW], not before P) cleavage sites. The results suggested that this enzyme may have a rigid structure and the potential cleavage sites were not accessible by the proteases under the reaction conditions. The result is in agreement with the report that native proteins are more resistant to trypsin than the denature proteins (Haurowitz, Tunca, Schwerin, & Göksu, 1945).

The enzyme was characterised on its effect by heat, the influence of SDS, imidazole or proteases sensitivity. Heat and imidazole effects were tested because the information may be useful for developing enzyme purification protocols. Protease tests would be useful in understanding the effect of the digestive enzymes to the Ac-GFD and its suitability as health food.

The fruiting body of *A. camphorata* is well known in Taiwan as a folk medicine for treating cancer and inflammation. Very little is known about the mode of actions of its biological effects. As a step toward understanding the effects, we cloned the enzyme Ac-GFD from *A. camphorata* expressed in *E. coli* and characterised. This enzyme appears to be stable under various conditions. The potential of the enzyme to be served as health food can now be investigated.

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