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ORIGINAL PAPER

Cloning and expression of mitochondrial malate dehydrogenase of *Clonorchis sinensis*

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Abstract The NAD-dependent mitochondrial malate dehydrogenase (mMDH, EC1.1.1.37) plays pivotal roles in tricarboxylic acid and is crucial for the survival and pathogenecity of parasites. A cDNA, which was identified by high throughput sequencing from the cDNA library constructed from adult *Clonorchis sinensis*, encoded a putative peptide of 341 amino acids with more than 50% identity with mMDHs from other organisms. The mMDH was expressed in *Escherichia coli* as the recombinant protein with a GST tag and purified by glutathione–Sepharose 4B column. The recombinant mMDH showed MDH activity of 63.6 U/mg, without lactate dehydrogenase activity and NADPH selectivity. The kinetic constants of recombinant mMDH were determined.

Introduction

Human clonorchiasis, caused by the infection of Chinese liver fluke (*Clonorchis sinensis*), is endemic in Korea, Japan, Southern China, and other Southeast Asian countries. It is currently estimated that more than about 35 million people are infected with clonorchiasis globally, of whom 15 million people are in China (Lun et al. 2005). Clonorchiasis becomes increasingly common in developed countries and non-endemic regions including North America and west Europe as a result of increased migration (Lin et al. 1987). Although clonorchiasis is not common in Western countries, people can be infected with liver fluke by eating frozen, dried, or pickled fresh-water fish imported from epidemic areas.

Some researches have revealed that schistosomes, an anaerobic parasite, utilize exogenous glucose for energy generation through glycolytic pathway. These parasites can ingest as much glucose as 26% of their dry body weight per hour (Bueding and Fisher 1982; Thompson et al. 1984). Adult *C. sinensis* is also an anaerobic trematode and also takes up as much external glucose as schistosome does. It takes up 1.13 mg glucose per wet weight per hour (Han et al. 1961). *C. sinensis* transports the external glucose in muscular tissues, in which it breaks glucose down through glycolysis to supply energy and produce lactic acid as the major end product or intermediate products needed for its physiological metabolism (Kang et al. 1969).

Malate dehydrogenases (MDHs) are ubiquitous enzymes that exist in at least two isozymic forms in all eukaryotes. Malate dehydrogenases catalyze the interconversion of malate to oxaloacetate. The mitochondrial MDH (mMDH) is a central component of the citric acid cycle and the aspartate–malate shuttle (Musrati et al. 1998). The reaction that mMDH converts malate to oxaloacetate produces two important products: NADH, a necessary reducing equivalent used in respiration, and oxaloacetate, the substrate for the condensation with acetyl CoA which begins the cycle anew (Anderson et al. 1998).

Nucleotide sequence data reported in this paper are available in the GenBank under the accession number AY605670.

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The glycolytic enzymes including mMDH are recognized as crucial molecules for trematode survival and have been targeted for vaccine development (Hong et al. 2000) and for drug screening (Chan and Sim 2004). On the other hand, tracing the history of MDH isoenzymes in eukaryotic evolution is handicapped by the lack of sequences from early branching eukaryotes (Sogin 1991). In this study, we have constructed the cDNA library of *C. sinensis* and identified the cDNA encoding mMDH. The recombinant *C. sinensis* mMDH (Cs-mMDH) was expressed in *Escherichia coli* and was purified for enzyme kinetic studies.

Materials and methods

Collection of adult *C. sinensis* worms

The metacercariae of *C. sinensis* were collected from freshwater fish by artificial digestion (digestive juice put up by adding 2 g protease and 7 ml hydrochloric acid to 993 ml distilled water) and then fed to rabbits with a gastric tube. Seven weeks after infection, the rabbits were anaesthetize by ether and killed to collect *C. sinensis* adult worms from the bile ducts. The adults were stored in liquid nitrogen immediately.

Construction of *C. sinensis* cDNA library

The total RNA was extracted from adults of *C. sinensis* using Trizol reagent (Life Technologies, USA). The mRNA was purified by oligotex mRNA Maxi kit (Clontech, USA) and used for the synthesis of double strand cDNA by the SMART™ polymerase chain reaction (PCR) cDNA synthesis kit (Clontech). The synthesized cDNA was digested with *Sfi*I, followed by size fractionation with Sepharose CL-2B column. The DNA fragments containing *Sfi*I A (5′-GGCCATTATGGCC-3′) and *Sfi*I B (5′-GGCCGCC TCGGCC-3′) recognition sites were cloned into *Eco*RI and *Not*I sites of pBluescript II SK$^{(+)}$. cDNA library was constructed by following the SMART™ PCR cDNA library construction kit protocol (Clontech). The cDNA inserts were sequenced on ABI PRISM™377 DNA sequencer (Perkin-Elmer, USA) using the BigDye Terminator Cycle sequencing Kit and BigDye Primer Cycle Sequencing Kit (Perkin-Elmer) with −21M13 primer. Each part of the insert was sequenced at least three times bi-directionally.

Bioinformation analysis of mMDH gene

Subsequent editing and assembly of all the sequences were performed by wu-blast program (2.0a19MP version, Washington University) and phrap (version 0.990329). DNA and protein sequence comparisons were carried out using BLAST2.2.9 at NCBI (http://www.ncbi.nlm.nih.gov/blast). The predicted amino acid sequences were compared against the profile entries in PROSITE to predict the potential functional motifs (http://www.expasy.org/pfscan). Other sequence analyses were performed by genDoc software (http://www.psc.edu/biomed/gendoc/).

Expression and purification of recombinant mMDH from *E. coli*

The Cs-mMDH coding region was generated by PCR with Pfu polymerase using a 5′-primer (5′-CGC GGA TCC ATG TTT TCA CGC GCT GCT C-3′) and 3′-primer (5′-CAG CTC GAG TTA CTG GGG TTT GAA TGT G-3′). The pair of PCR primers was designed based on the 5′ and 3′ sequences of the Cs-mMDH ORF and incorporated the *Bam*HI and *Xho*I restriction sites (underlined in the primer sequences) at the 5′ and 3′ primers, respectively. Polymerase chain reaction product was purified and digested by *Bam*HI and *Xho*I and then cloned into pGEX-4T-1 vector double-digested by *Bam*HI and *Xho*I enzymes for the expression of GST-tagged fusion recombinant Cs-mMDH protein. The recombinant construct (pGEX-Cs-mMDH) was transformed into *E. coli* DH5α and verified by sequencing analysis.

An *E. coli* DH5α clone harboring pGEX-Cs-mMDH plasmid was inoculated in LB medium, cultured at 37°C to early log phase, and induced by IPTG at 20°C for 5 h. The bacteria were collected by centrifugation and then sonicated. The GST-tagged recombinant Cs-mMDH protein was purified by glutathione–Sepharose 4B column (Amersham, England). The fusion protein bounded to the glutathione–Sepharose 4B column was digested by thrombin at 20°C for over 16 h. The cleaved Cs-mMDH was recovered from the column. Protein concentration was determined by the method of Bradford (1976), with BSA as standard.

SDS-PAGE and Western-blot analysis

Bacterial lysates and purified protein were subjected to 12% SDS-PAGE and visualized by Coomassie brilliant blue G-250 (Laemmli 1970). After SDS-PAGE, the proteins were electroblotted onto a cellulosenitrate sheet (Schleicher & Schuell, Germany). The sheet was blocked and then incubated with monoclonal donkey anti-mouse-GST antibody (Amersham) at 30°C for 2 h. The sheet was then incubated with HRP-conjugated-sheep anti-donkey antibody (Amersham) at 30°C for 2 h. Immunoreactive proteins were detected and visualized by DAB (Amresco, USA).

Enzymatic assay of mMDH

Enzymatic assay of mMDH was performed at 25°C according the method of Wise et al. (1997). In each case,

the change in absorbance at 340 nm because of the oxidation or reduction of NADH was measured on UV-260 recording spectrophotometer (Shimadzu, Kyoto, Japan). For the determination of kinetic parameters, initial velocities were obtained by varying one substrate concentration and the second substrate in fixed concentration. A unit of enzyme activity was defined as 1 μmol of coenzyme oxidized or reduced per minute. NADH concentration were calculated from absorbance measurements using an NADH extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} (McComb et al. 1976). Experiment was performed as above using NADPH as the cofactor of the Cs-mMDH.

Lactate dehydrogenase (LDH) activity of Cs-mMDH was measured following the method of Gomez et al. (1997).

Malate, OAA, NADH, NAD, NADPH, pyruvate, and L-lactate were purchased from Sigma. The kinetic constants were determined with a computer program fitting the data to a hyperbola by applying the Gauss–Newton algorithm (Fraser and Suzuki 1973).

Result

Cloning and bioinformatic analysis of *C. sinensis* mMDH gene

The *C. sinensis* adult worm cDNA library was constructed and sequenced. The total 8,900 expressed sequence tag

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   1                                                    GGACACTCCTCATTTTTGGGAAGGACTGCGTA
  33 ATGTTTTCACGCGCTGCTCTCTCTGTTCGACATTTCACCGGATCGAAATGTTTTTCGACATCGGCAAAAAGCCTA
      M  F  S  R  A  A  L  S  V  R  H  F  T  G  S  K  C  F  S  T  S  A  K  S  L
 108 AGGAAGGTTGCTCTGCTTGGAGCTAGTGGCGGTATTGGTCAGCCAACCGCGCTACTTCTTAAGCAATCCCCTCTT
      R  K  V  A  L  L  G  A  S  G  G  I  G  Q  P  T  A  L  L  L  K  Q  S  P  L
 183 GTTTCTCATCTTGCATTGTATGATATCGCTCATGTAAAAGGTGTAGCGGCTGACCTAAGCCATATCGAAACCAAA
      V  S  H  L  A  L  Y  D  I  A  H  V  K  G  V  A  A  D  L  S  H  I  E  T  K
 258 GCTCGTGTCACCGGGCATGATGGTCCAGCGCAACTGGCCGAATGTCTCACTGGTGCTGAGGTCGTGATAATCCCA
      A  R  V  T  G  H  D  G  P  A  Q  L  A  E  C  L  T  G  A  E  V  V  I  I  P
 333 GCCGGAGTTCCGCGCAAACCGGGTATGACACGTGATGACCTGTTCAACACCAACGCGTCCATCGTAGCACAGTTG
      A  G  V  P  R  K  P  G  M  T  R  D  D  L  F  N  T  N  A  S  I  V  A  Q  L
 408 GTTCACGCCTGTGCATTGAACTGTCCCAAGGCGATGATCTGTATAGTCACGAATCCCGTGAATAGCACTGTCCCC
      V  H  A  C  A  L  N  C  P  K  A  M  I  C  I  V  T  N  P  V  N  S  T  V  P
 483 ATTGCAGCTGAGATAATGAAGCGCCACGGTGTGTTCGATCCGCTACGTCTTTTCGGTGTAACTACGTTGGACATC
      I  A  A  E  I  M  K  R  H  G  V  F  D  P  L  R  L  F  G  V  T  T  L  D  I
 558 ATCCGGTCAAATACATTCATAGCCGAAGCCAAAGGTCTGGATGTCCAGAAAGTTTCATGCCCTGTGATCGGCGGG
      I  R  S  N  T  F  I  A  E  A  K  G  L  D  V  Q  K  V  S  C  P  V  I  G  G
 633 CATTCTGGAATCACCATATTGCCTGTGATTTCCCAATGCTCCCCGACAGTCTCGTTCCCCCAGAACGAACGGGAA
      K  S  G  I  T  I  L  P  V  I  S  Q  C  S  P  T  V  S  F  P  Q  N  E  R  E
 708 CAGTTGACCAGTCGAATTCAGAATGCCGGAACCGAGGTTGTCGAAGCCAAAGCTGGAGCAGGTTCAGCCACACTG
      Q  L  T  S  R  I  Q  N  A  G  T  E  V  V  E  A  K  A  G  A  G  S  A  T  L
 783 TCTATGGCCTACGCCGGTGTCCGCTTCGCCACCTCGCTCATGGAGGCGATGAGTGGACGACAGGGAGTGGTGGAG
      S  M  A  Y  A  G  V  R  F  A  T  S  L  M  E  A  M  S  G  R  Q  G  V  V  E
 858 TGTACATTTGTCCACGGTGAGGTCTCCGAATGCGAGTTCTTCGCAGCTCCAATCGCACTCGGGGTGAACGGAGTG
      C  T  F  V  H  G  E  V  S  E  C  E  F  F  A  A  P  I  A  L  G  V  N  G  V
 933 GAAAAGAACATGGGGATCGGCAAGCTGAACGAATATGAGATACAACTTCTCCAAAAGCTTATTCCAGAATTGCAA
      E  K  N  M  G  I  G  K  L  N  E  Y  E  I  Q  L  L  Q  K  L  I  P  E  L  Q
1008 AAGAACATCAAGAGGGGCAAGGAATTTGCCGCCACATTCAAACCCCAGTAAACCGGTTATCCACGATCCACCCAC
      K  N  I  K  R  G  K  E  F  A  A  T  F  K  P  Q  *
1083 GCGCACTTTCTGTGGACGTGTTTTCGTTTGTCATATGCGTATAAATATAGACTAAAGTATCCGACATTTATCCAA
1158 TCCCCACAGCTGTCGTAATGATTACATGAGATTCATCGAGCATTTTCGTTTCTGATTTCTCAGCTGTCGTTGCTG
1233 CGATTTCCACTCTGCTGCTACCAATACAATCGATAAGAATTCGCCATGGACGAGCGGTTCTTTGGCACTGTGCTT
1308 CGACTATCGTGTCAGGTTTTTGTTACCACCATCGTGTTCTGTCAAATGACTCTTGCGCATACTACGGCACTGTGT
1338 GCATTTTCATACTGTCACTTTTTTGCCGCGTGTGTTCTGTTTCGAATCGCCAATATATGGTGGAAGAAATTTCTN
1458 TTCTGTGACCGACCGCAA
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Fig. 1 Nucleotide and deduced amino acid sequence of *C. sinensis* mMDH. The nucleotide sequence of the 1,475 bp cDNA is shown on the *top line* and its predicted amino acid sequence was shown *below* in a *single letter code*. The ORF extended from nucleotide 33 to 1,058 and encoded a protein of 341 amino acids. *Numbers on the left* refer to the first nucleotide on each line. An *asterisk* indicated the terminator in the protein sequence

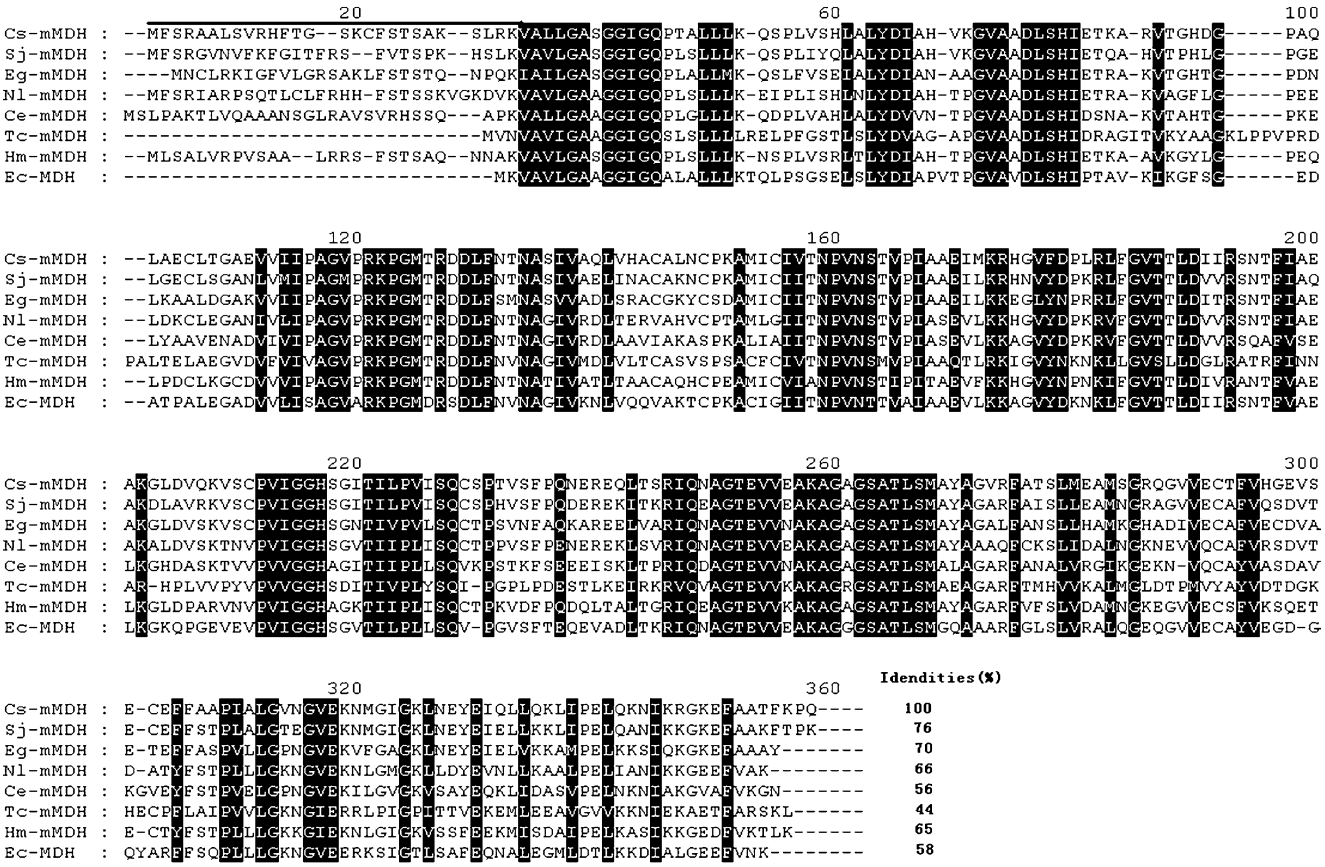

Fig. 2 Alignment of the predicted amino acid sequence of the cloned putative Cs-mMDH to other MDH sequences. *Cs-mMDH* mMDH of *C. sinensis* (AY605670), *Sj-mMDH* mMDH of *S. japonicum* (AAW27425), *Eg-mMDH* mMDH of *Echinococcus granulosus* (CAF18421), *Nl-mMDH* mMDH of *Nucella lapillus* (AAF27650), *Ce-mMDH* mMDH of *Caenorhabitis elegans* (O02640), *Tc-mMDH* mMDH of *T. cruzi* (AF051893), *Hm-mMDH* mMDH of human (CR536548), *Ec-MDH* MDH of *E. coli* (BAB37532). The *single line* over the sequence indicates the presequence of mitochondrial MDH. The identities of the MDHs sequences with the sequence of Cs-mMDH were listed in the *last*. The alignment was produced using GeneDoc software

(EST) clones randomly selected were sequenced and merged into 1,775 UniGenes, the unigene for MDH originated from six ESTs. The entire cDNA was 1,475-bp long containing an ORF of 1,026 bp (from nucleotide 33 to 1,058; Fig. 1), which encodes a 36.129-kDa protein with an isoelectric point of 8.83.

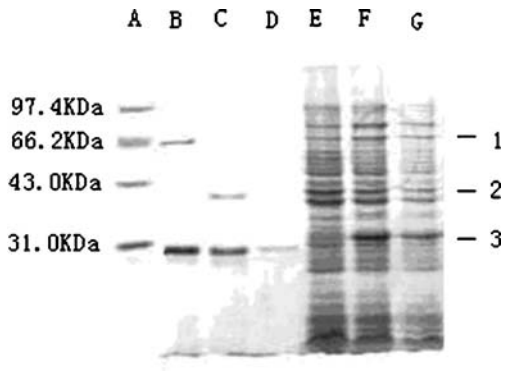

Fig. 3 SDS-PAGE of expression and purified Cs-mMDH. *A* protein marker, *B* recombinant Cs-mMDH purified by glutathione–Sepharose column, *C* recombinant Cs-mMDH cleaven by thrombin, *D* GST, *E* uninduced *E. coli* with pGEX-Cs-mMDH, *F* induced *E. coli* with pGEX-Cs-mMDH, *G* induced *E. coli* with plasmid pGEX-4T-1, *1* position of recombinant GST-Cs-mMDH, *2* position of Cs-mMDH without GST tag, *3* position of GST

The Cs-mMDH cDNA sequence was analyzed with the Blast program against the GenBank database. The alignment of the Cs-mMDH with the other homologous MDH genes was shown in Fig. 2. The highest identity (76%) was observed with mMDH of *Schistosoma japonicum* (trematode). It had moderate identity with MDH of *E. coli* (identity 58%). There is a presequence in every mMDH, and Cs-mMDH has a 27 amino acid mitochondrial presequence. The Cs-mMDH has an active site "VTTLDIIRSNTF", in which the D and R are the active site residues. From alignment, it can be seen that this consensus active site pattern exists in all MDH (Fig. 2).

Expression of mMDH in *E. coli*

To confirm the function of the cloned Cs-mMDH gene, it was expressed in *E. coli* as described in the "Materials and methods". A recombinant pGEX-4T-1 plasmid containing Cs-mMDH cDNA was sequenced and revealed that the

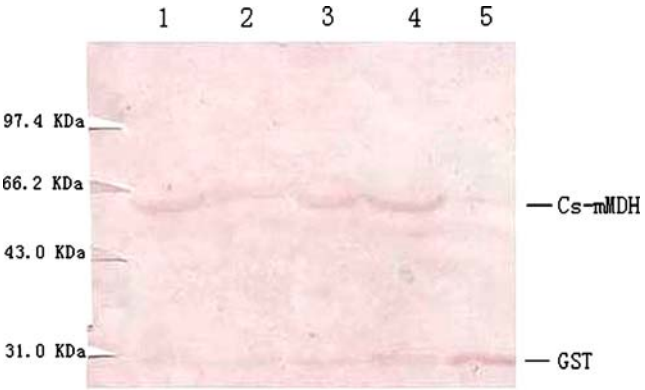

Fig. 4 Western blot of recombinant Cs-mMDH. *Lanes 1–3* 2–4-h-induced *E. coli* with pGEX-Cs-mMDH, *lane 4* purified Cs-mMDH by glutathione–Sepharose column, *lane 5* induced *E. coli* with pGEX-4T-1

ORF of Cs-mMDH was in frame to the tag of the vector. The recombinant Cs-mMDH was expressed in *E. coli* as a fusion protein with a GST tag at the N-terminal. Purified recombinant Cs-mMDH protein was obtained by affinity column chromatography. Thrombin was used to cleave GST from recombinant Cs-mMDH protein in glutathione–Sepharose 4B column, and the purified Cs-mMDH without GST tag was obtained. Gel electrophoresis (Fig. 3) of extracts revealed that the purified Cs-mMDH with GST tag showed a single band of 62 kDa when run under denaturing condition in SDS-PAGE. The cleaved Cs-mMDH showed a band in 36 kDa. Figure 4 showed the result of Western-blot analysis. Anti-GST antibody detected a 62-kDa protein in the IPTG-induced *E. coli* with pGEX-Cs-mMDH recombinant plasmid and the column purified sample. The antibody also detected a 26-kDa protein in *E. coli* harboring the parent plasmid, pGEX-4T-1.

Enzyme activity and kinetic characterization of recombinant Cs-mMDH

The recombinant Cs-mMDH demonstrated the MDH enzymatic activity with the specific activity of 63.6 U/mg. The kinetic constants for both substrates in the forward and reverse directions were determined through initial velocity studies. Table 1 shows the apparent Km and Vmax values and the Vmax/Km ratios obtained for both directions of reaction. The enzyme was inhibited when the concentration of OAA is more than 300 μM. The activity was inhibited at 37% when the concentration of OAA reached 1 mM. The enzyme did not show any substrate inhibition by malate, up to 20 mM.

The recombinant Cs-mMDH did not show any enzymatic activity when NADPH was used as a substitute for NADH in the assay even with the NADPH concentration higher than 20 mM or with the enzyme concentration ten times higher. The enzyme did not show any LDH activity even the enzyme concentration was ten times higher than that in MDH enzymatic assay.

Discussion

cDNA library of *C. sinensis* adult worms was constructed. Through high throughput sequencing, the cDNA of *C. sinensis* mMDH was identified. Although the mitochondrial presequences of all mMDH showed very low similarity (Fig. 2), Cs-mMDH showed a high degree of homology to mMDHs of other organisms. Perhaps *C. sinensis* and humans have experienced a long period of co-evolution as the parasitism developed, so the Cs-mMDH cDNA has higher identity (65%) with the mMDH of humans than the mMDH of *Trypanosoma cruzi* (44%) or the MDH of *E. coli* (58%).

The recombinant Cs-mMDH was expressed in *E. coli*. When purified by affinity column chromatography, the Cs-mMDH showed a single band on SDS-PAGE gel with the molecular weight about 62 kDa. When cleaved by thrombin, only 36-kDa protein was appeared. It revealed that the recombinant protein was a fusion protein with a 26-kDa tag. Western-blot analysis showed that the expression level reached the maximum from 2 to 4 h.

The recombinant Cs-mMDH has MDH enzymatic activity. The Cs-mMDH exhibited substrate inhibition by OAA at concentration above 300 μM in the direction of reduction of OAA. This substrate inhibition at high concentration of OAA is a common feature of MDH (van Kuijk and Stams 1996; Hunter et al. 2000). However, malate did not exhibit substrate inhibition to Cs-mMDH. So, the kinetic constants were determined under the OAA

Table 1 Apparent Km and Vmax values for the recombinant Cs-mMDH

Substrate	Co-substrate	Apparent Km (mM)	Vmax (U mg^{-1})	Vmax/Km
Malate	NAD	1.3000±0.750	26.2±1.4	20.1
NAD	Malate	0.0760±0.008	40.24±2.9	529.5
OAA	NADH	0.0082±0.001	72.8±5.9	8,878
NADH	OAA	0.0276±0.007	109.05±12	3,967

The kinetic constants are the means of 4–5 experimental results. The apparent Km and Vmax were determined as described in the "Materials and methods".

concentration of 300 μM. The maximum rate of OAA reduction by NADH proceeded 3-fold more rapidly than that of malate oxidation by NAD. The more relevant parameter, however, is Vmax/Km, reflecting the rate of reaction at low physiological substrate concentrations. Comparing Vmax/Km for the nonnucleotide substrate, the NADH-dependent reduction of OAA was favored by a factor of about 443-fold over the oxidation of malate. It suggests that the Cs-mMDH catalyzes the reduction of OAA more efficiently than the oxidation of malate. *C. sinensis* adult produces most of the energy by the route of gluconeogensis, because glucose is absent in bile; therefore, the reduction of oxaloacetate into malate by mMDH is taking place in the mitochondrion.

Wu found that the cMDH sequence of *Trichomonas vaginalis* has high similarity with the sequences of NADP-specific chloroplast MDH of *Flaveria trinervia* and *Zea mays* (Wu et al. 1999). Van Kuijk even discovered that the MDH in bacterium strain MPOB has a low activity with NADP and NADPH as cofactor, although the MDH shows selectivity for the cofactor NAD and NADH (van Kuijk and Stams 1996). But this recombinant Cs-mMDH was NADH-specific and had no MDH activity when NADPH was the cofactor.

Malate dehydrogenases present extensive structural similarities with LDH, and they are considered derived from a common ancestral gene. So, it is believed that LDH, cytosolic MDH, and mMDH probably have similar catalytic mechanisms (Birktoft et al. 1982). In *Mycoplasma genitalium*, the same protein is believed to have both MDH and LDH activity (Cordwell et al. 1997). Because of the high similarity between MDH and LDH, it is easy to convert the LDH into active MDH (Wu et al. 1999) or produce LDH from MDH (Boerncke et al. 1995) by mutation. But this Cs-mMDH did not show any LDH activity.

Glycolytic enzymes, such as glyceraldehydes 3-phosphate dehydrogenase, triose phosphate isomerase, and phosphoglycerate kinase (PGK), were identified as vaccine candidates, because glycolytic enzymes are recognized as crucial molecules for trematode survival. The sequence of *Schistosoma mansoni* PGK shared 61.2% similarity with human PGK. But an affinity-purified antibody using a recombinant *S. mansoni* PGK from sera of humans naturally infected with *S. mansoni* did not cross-react with PGKs from human and rabbit (Hong et al. 2000). For vaccine development, Cs-mMDH has 65% sequence similarity with human mMDH; further immunological research will be done to confirm whether the antibody induced by Cs-mMDH has cross-reacted with human mMDH. The superimposition of the model obtained for MDH of *Plasmodium falciparum* over porcine cytosolic MDH revealed significant conformational differences in the active site loop and other solvent-exposed regions; these observation suggested a large scope for the use of some of the domains of malarial MDHs as potential drug targets (Chan and Sim 2004). Further efforts will be made to obtain crystals of Cs-mMDH and determine three-dimensional structure by X-ray crystallography.

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