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ORIGINAL ARTICLE

# Enhancement of menadione stress tolerance in yeast by accumulation of hypotaurine and taurine: co-expression of cDNA clones, from *Cyprinus carpio*, for cysteine dioxygenase and cysteine sulfinate decarboxylase in *Saccharomyces cerevisiae*

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**Abstract** Taurine is known to function as a protectant against various stresses in animal cells. In order to utilize taurine as a compatible solute for stress tolerance of yeast, isolation of cDNA clones for genes encoding enzymes involved in biosynthesis of taurine was attempted. Two types of cDNA clones corresponding to genes encoding cysteine dioxygenase (CDO1 and CDO2) and a cDNA clone for cysteine sulfinate decarboxylase (CSD) were isolated from *Cyprinus carpio.* Deduced amino acid sequences of the two CDOs and that of CSD showed high similarity to those of CDOs and those of CSDs from other organisms, respectively. The coding regions of *CDO1, CDO2,* and *CSD* were subcloned into an expression vector, pESC-TRP, for *Saccharomyces cerevisiae.* Furthermore, to enhance the efficiency of synthesis of taurine in *S. cerevisiae*, a *CDO–CSD* fusion was designed and expressed. Expression of CDO and CSD proteins, or the CDO–CSD fusion protein was confirmed by Western blot analysis. HPLC analysis showed that the expression of the proteins led to enhancement of the accumulation level of hypotaurine, a precursor of taurine, rather than taurine. The yeast cells expressing corresponding genes showed tolerance to oxidative stress induced by menadione, but not to freezing–thawing stress.

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**Keywords** *Cyprinus carpio* · Hypotaurine · *Saccharomyces cerevisiae* · Stress tolerance · Taurine

**Abbreviations**

| | |
|---|---|
| CDO | Cysteine dioxygenase |
| CSD | Cysteine sulfinate decarboxylase |
| DIG | Digoxygenin |
| NBT | Nitro blue tetrazolium |

## Introduction

Taurine, a $\beta$-amino acid with a sulfonic group instead of a carboxyl group, is synthesized in the liver and some parts of the brain in mammals (Hayes 1985; Dominy et al. 2004) and is suggested to function as an osmoprotectant, antioxidant, and membrane stabilizer (Schaffer et al. 2003). Taurine has also been reported to exist in fish and shellfish, probably working as an osmoprotectant (Takeuchi et al. 2000). The mechanisms of the osmoprotective or antioxidative functions of taurine are yet to be fully elucidated, however, taurine is expected to function as a protectant in organisms against several stresses. On the other hand, freezing stress accompanies osmotic stress caused by extracellular freezing and oxidative stress during freezing and thawing process (Mazur 1970; Kendall and McKersie 1989). Furthermore, taurine is effectively used in frozen storage of bull sperm, presumably due to its protection against oxidative stress induced by freezing (Chen et al. 1993).

In contrast, bacterial and plant cells have been considered to lack taurine (Huxtable 1992), implying that taurine may not be a compatible solute for these organisms. However, cactus pears, a source of commercial fruit juice, have been found to

contain detectable levels of taurine (Stintzing et al. 2001), though physiological roles of taurine in cactus pears are yet to be investigated. Therefore, based on the antistress effects recognized in animal cells, taurine could also play similar roles in plant cells, when introduced into the cells. If found to be true, taurine supplementation would provide plants with elevated levels of freezing and/or oxidative stress tolerance.

We have investigated the effects of taurine, using proline as a comparison, on stress tolerance of eukaryotic cells using yeast as a model organism (Honjoh et al. 2007). It was shown that addition of taurine to the culture medium at a concentration range of 0.2 to 0.6 M increased the tolerances to freezing stress and oxidative stress based on hydrogen peroxide in a dose-dependent manner. Although yeast cells accumulated lower amounts of taurine than that of proline, based on the intracellular amounts, the taurine-introduced yeast showed almost the same levels of freezing and oxidative stress tolerance as those of proline-introduced yeast, suggesting that taurine could play an effective antistress role as well as proline in the model eukaryote. However, addition of taurine at high concentration to medium induced osmotic stress and led to expression of stress-responsive genes in yeast. To assess the effect of taurine on stress tolerance, it is important to exclude osmotic stress condition.

The pathway for biosynthesis of taurine has been well understood in mammalian cells (Bagley et al. 1995). Cysteine dioxygenase (CDO) and cysteine sulfinate decarboxylase (CSD) are regarded as being important in the pathway. CDO catalyzes the reaction from cysteine to cysteine sulfinic acid, and CSD converts the cysteine sulfinic acid to hypotaurine, a precursor of taurine. Although an enzyme catalyzing the reaction from hypotaurine to taurine has been assumed to exist, no corresponding gene has been identified in any organism. Therefore, hypotaurine is believed to be autoxidized to taurine.

The present study was aimed at evaluating the antistress functions of taurine that is intracellularly synthesized in yeast. Isolation of cDNA clones encoding CDO and CSD from a bony fish, the common carp (*Cyprinus carpio*), and expression of the genes in *Saccharomyces cerevisiae* suggest that these key enzymes derived from the vertebrate can work to produce taurine in yeast and confer freezing tolerance and/or oxidative stress tolerance based on menadione, which is known as an inducer of reactive oxygen species intracellularly (Hassan and Fridovich 1979).

## Materials and methods

### Materials

Common carp *C. carpio* (L.) were purchased from a fish farm in Kumamoto Prefecture, Japan, and acclimated for 2 weeks to laboratory conditions at 22°C in a 60 l aquarium containing recirculating well water. During acclimation, fish were fed with commercial pellets.

The yeast strain used in this study was *S. cerevisiae* YPH500 (*MATα ura3-52 lys2-801*$^{amber}$ *ade2-101*$^{ochre}$ *trp1-Δ63 his3-Δ200 leu2-Δ1*; Stratagene, La Jolla, CA, USA). Yeast cells were cultured in YPD medium that contained 1% yeast extract, 2% peptone, 2% dextrose (Sherman et al. 1986) or synthetic raffinose lacking tryptophan (SR-trp) medium, which contained 2% raffinose and 0.67% yeast nitrogen base (Difco, Detroit, MI, USA), and was supplemented with adenine, aminobenzoic acid, inositol, uracil, and all the amino acids except for tryptophan. For the expression of the gene(s), galactose was added to the SR-trp medium to a final concentration of 2%. Furthermore, cysteine was also added to the medium at a final concentration of 2 mM as a substrate for taurine biosynthesis.

### RNA preparation and cDNA library construction

RNA preparation and construction of cDNA library was done as described by Nakao et al. (1998). Total RNA was isolated from carp hepatopancreas using TriZol reagent (Invitrogen, Tokyo, Japan). Poly(A)$^+$RNA (5 μg) purified with an oligo-dT spin column (Amersham Bioscience, Uppsala, Sweden) were subjected to synthesis of double-stranded cDNA using cDNA Synthesis Module (Amersham Bioscience), followed by ligation with *Eco*RI adaptor and with λZAPII vector (Stratagene). After in vitro packaging with GigaPack Gold II, the library was amplified once before use.

### PCR amplification of partial fragments of cDNA for CDO and cysteine sulfinate decarboxylase

For PCR amplification of partial fragments of cDNA for CDO and CSD, degenerated primers were designed based on the conserved amino acid sequences from humans and rats (Hosokawa et al. 1990; McCann et al. 1994; Park et al. 2002; Reymond et al. 1996). Primers used for amplification of a partial fragment of *CDO* were *CDO-forward* (5′-(A/C)AA (A/G)TT (C/G/T)GA (C/T)CA (A/G)TA CAG GTA (C/T)AC-3′) and *CDO-reverse* (5′-GAA TCA TT(A/G) AT(A/G) TA(A/G) GCA CAC TGG-3′). Primers used for amplification of a partial fragment of *CSD* were *CSD-forward* (5′-A(C/T)T C(A/T)G T(G/T)G C(C/G/T)T GGA A(C/T)C C(A/C)C AC-3′) and *CSD-rev3* (5′-AC(A/G) TC(A/G) TAG AA(C/T) TT(A/G) TCC TGC TG-3′). PCR amplification of the cDNA fragments for *CDO* and *CSD* was conducted on a PCR Thermal Cycler (model MP: Takara Biomedicals, Kyoto, Japan) using KOD plus DNA polymerase (Toyobo, Tokyo, Japan). Addition of adenine to the amplified PCR products was performed using Taq DNA polymerase (Sigma, St Louis, MO, USA) and the adenylated DNA was

then ligated into a pGEM-T easy vector (Promega, Madison, WI, USA). Nucleotide sequences of the recombinant plasmids were determined using a Pharmacia ALF express DNA sequencer (Amersham Bioscience). The inserts were used as probes for library screening.

The sequences obtained were compared with those of nucleotides in the databases, using the BLAST program (Altschul et al. 1990) on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/).

### Screening of a cDNA library

Plaques ($1 \times 10^6$) of carp hepatopancreas cDNA library were transferred onto Hybond-N+ membranes (Amersham Bioscience). After baking at 80°C for 2 h, the membranes were prehybridized for 2 h at 65°C in a solution containing 5× SSC (1× SSC contains 15 mM sodium citrate and 150 mM sodium chloride), 0.1% sodium *N*-lauroylsarcosinate, 0.02% SDS, and 1% DIG (Digoxigenin) blocking reagent, followed by hybridization with DIG-labeled DNA probes at 65°C for 16 h. After washing with 0.1× SSC containing 0.1% SDS at 65°C for 30 min, colorimetric detection was performed using X-phosphate and NBT in the DIG DNA detection kit (Roche Diagnostics K.K., Tokyo, Japan) as described in the manufacturer's instructions. Positive plaques were converted to plasmid using the ExAssist helper phage (Stratagene).

### 5′-RACE and 3′-RACE

To obtain the 5′-end and 3′-end of *CSD*, 5′-RACE (Rapid amplification of 5′-cDNA ends) and 3′-RACE were carried out using SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). To amplify the 5′-end, forward primer (5′-TCC TGC TGG AAT AAG TAG GTT GCC TTG G-3′) and nested reverse primer (5′-GGC ACT GTG ACA GTG CAT TAA CAG-3′) were constructed. To amplify the 3′-end, forward primer (5′-CTG GAA CCC ACA CAA AAT GCT TCT AGC G-3′) and nested forward primer (5′-GCA GTG CTC TGT GGT TTT GTT CAG-3′) were constructed. Other primers, which were attached to the kit, were also used. KOD plus DNA polymerase (Toyobo) was used for PCR. After PCR, 3′-ends of the PCR fragment was adenylated as described above and subcloned into a pGEM-T easy vector. The sequences of the 5′ parts and 3′ parts of the *CSD* cDNA were then determined.

### Construction of expression plasmids in *S. cerevisiae*

To express the *CDO* and *CSD* genes in yeast, the cDNA for the *CDO* gene was first modified to create restriction sites adjacent to the start and stop codons. Furthermore, the stop codon was modified to fuse the *CDO* gene with the region encoding *c-myc*, which was the original sequence of a pESC-TRP vector (Stratagene). For this purpose, two oligonucleotides, CDOfor*Bam*HI (5′-CGC GGA TCC ATG GAG CAC ACC GAG CTC-3′) and CDOrev*Sal*I (5′-CGA CGT CGA CGT TGT TCT CCT GTG AAA CG-3′), were synthesized as primers. The open reading frame of *CDO* was amplified by PCR using KOD plus DNA polymerase (Toyobo), and the amplified fragment was digested with a combination of *Bam*HI and *Sal*I. Subsequently, the digested fragment was subcloned into the *Bam*HI–*Sal*I-digested pESC-TRP vector. The resultant plasmids were designated pESC-TRP/*CDO1-c-myc* and pESC-TRP/*CDO2-c-myc*. The *CSD* gene was also amplified with the primers, CSDfor*Eco*RI (5′-CCG GAA TTC TCA CAA TGA GCT CAT CTA AAG-3′) and CSDrev*Cla*I (5′-CCA TCG ATA CCA AGT CCT TCC CTA GGT C-3′). The stop codon was modified to fuse the *CSD* gene with the region encoding FLAG, which was the original sequence of a pESC-TRP vector. The amplified fragment was digested with a combination of *Eco*RI and *Cla*I. The digested fragment was subcloned into the *Eco*RI–*Cla*I-digested pESC-TRP/*CDO1-c-myc* and *Eco*RI-*Cla*I-digested pESC-TRP/*CDO2-c-myc*. The obtained constructs were designated as pESC-TRP/*CDO1-c-myc*/*CSD-FLAG* and pESC-TRP/*CDO2-c-myc*/*CSD-FLAG*. For convenience, pESC-TRP/*CDO1-c-myc*/*CSD-FLAG* and pESC-TRP/*CDO2-c-myc*/*CSD-FLAG* were designated pESC/*CDO1*/*CSD* and pESC/*CDO2*/*CSD*, respectively (Fig. 1a).

For expression of the *CDO–CSD* fusion genes, pESC-TRP/*CDO1–CSD* and pESC-TRP/*CDO2–CSD* were constructed by using the region encoding c-Myc polypeptide as a linker. The two primers, CSDfor*Xho*I (5′-GAC CTC GAG ATG AGC TCA TCT AAA GAA TAT ATG-3′) and CSDrev*Nhe*I (5′-GAG GCT AGC CTA CAA GTC CTT CCC TAG G-3′), were designed for the *CSD* gene to be fused with downstream of the *CDO1-c-myc* or *CDO2-c-myc* regions. The *CSD* region was amplified by PCR using KOD-plus-DNA polymerase (Toyobo) and then digested with a combination of *Xho*I and *Nhe*I. The digest was subcloned into the *Xho*I–*Nhe*I-digested pESC-TRP/*CDO1-c-myc* and pESC-TRP/*CDO2-c-myc*. The obtained constructs were designated as pESC/*CDO1–CSD* and pESC/*CDO1–CSD* (Fig. 1b).

To propagate the recombinant plasmids, *E. coli* DH5 was used as a host.

### Expression of objective genes in *S. cerevisiae* and immunoblot analysis

To express the objective genes (*CDO* and *CSD*), the resultant plasmids were introduced into *S. cerevisiae* YPH500 by the lithium acetate method (Ito et al. 1983).

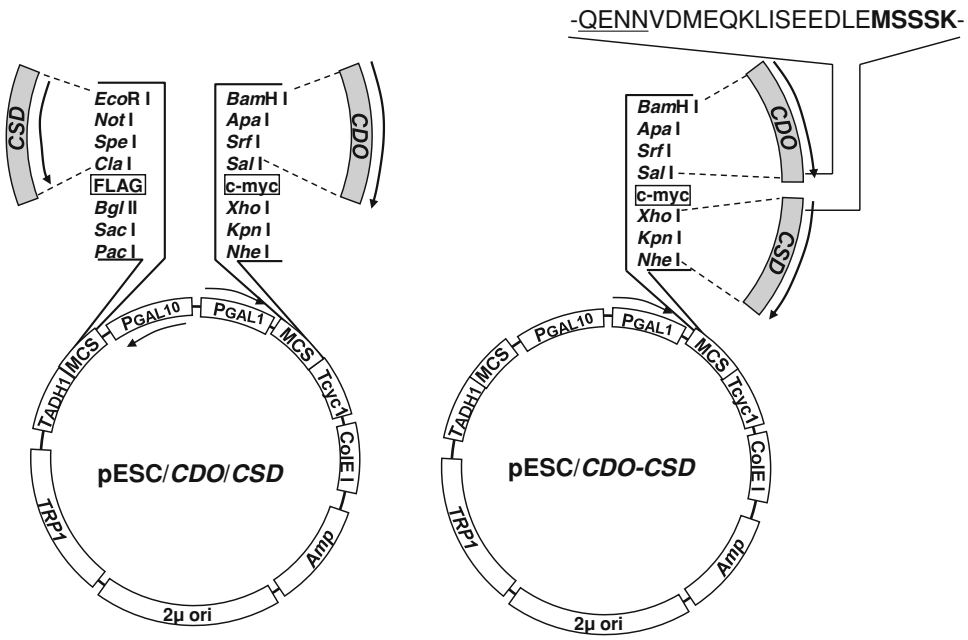


**Fig. 1** Structure of plasmids used for expression of *CDO*, *CSD*, and *CDO–CSD* in *S. cerevisiae*

Transformants were screened on SR-trp agar plates and confirmed by the method of Hoffman and Winston (1987).

After confirmation of the transformation, individual transformed yeast cells were grown with shaking at 30°C overnight. The overnight cultures in SR-trp medium were diluted into SR-trp/galactose medium to express the objective genes. Following incubation with shaking at 30°C for 24 h, the yeast cells were harvested by centrifugation at 13,000×*g* for 1 min and suspended in 20 mM Tris–HCl buffer (pH 7.5) that contained 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was homogenized with glass beads of 0.5 mm in diameter on a Vortex mixer. The homogenates were centrifuged at 20,000×*g* at 4°C for 30 min to harvest cellular extracts as the supernatants.

The supernatants were subjected to SDS-PAGE on a 12.5% gel (Laemmli 1970), and the separated polypeptides were electroblotted onto a nitrocellulose membrane (Towbin et al. 1979). The membrane was blocked with TTBS [100 mM Tris–HCl buffer (pH 7.5), 0.9% (w/v) NaCl, and 0.1% (w/v) Tween 20] containing 3% (w/v) skim milk. After blocking, to confirm the expression of CDO or CDO–CSD fusion proteins, the membrane was incubated with anti-(c-myc)-peroxidase (Sigma) in TTBS containing 3% skim milk. Following washing, the membrane was used to detect peroxidase activity. To detect expressed CSD, the membrane was incubated with anti-FLAG M2 monoclonal antibody (Sigma) in TTBS containing 3% skim milk. After washing, the membrane was incubated with goat anti-mouse IgG peroxidase conjugate (Dainihonseiyaku, Osaka, Japan), followed by detection of the peroxidase activity in 100 mM Tris–HCl buffer (pH 7.5) containing 0.8 mg/ml diaminobenzidine, 0.4 mg/ml $NiCl_2$, and 0.008% $H_2O_2$.

## Extraction of amino acids

For analysis of free amino acids, the yeast cells were harvested by centrifugation at 560×*g* for 5 min. The collected cells were washed three times with water and lyophilized. The lyophilized samples were suspended in 200 μl of 0.5 μmol/ml of methionine sulfone, which was used as an internal standard, and vortexed with an equal volume of 0.5 mm glass beads at maximum speed for 1 min. Homogenization of the cells with a vortex mixer was repeated 10 times. After disruption with the vortex mixer, the sample concentration was adjusted to 0.5 mg dry-weight of yeast cells per 100 μl by adding the methionine sulfone solution. The samples were then boiled for 10 min and centrifuged at 20,000×*g* for 20 min. The supernatants were lyophilized and then used for derivatization of amino acids with phenylisothiocyanate (PITC). To determine hypotaurine contents, an aliquot of half the supernatants was treated with $H_2O_2$ at a final concentration of 0.3% to convert hypotaurine to taurine, prior to the amino acid analysis. This conversion was confirmed to quantitatively yield taurine from hypotaurine in a preliminary experiment (data not shown).

## Derivatization of amino acids with PITC

Derivatization of free amino acids with PITC was done according to the method of Bidlingmeyer et al. (1984). Amino acid standards or the lyophilized samples were treated with 70 μl of a mixture of ethanol–triethylamine–water (1:1:1, v/v) and dried under vacuum. Thus, the derivatization reagent consisted of 70 μl of a mixture of

ethanol–triethylamine–water (7:1:1, v/v) and 5 µl of PITC. The dried sample was dissolved in the reagent, vortexed for 30 s, and left to react for 10 min at room temperature. Excess reagent, triethylamine, and other volatile products were then removed by evaporation under vacuum. Derivatized samples were dissolved in a mixture of 5 mM sodium phosphate buffer (pH 7.5)-acetonitrile (93:7, v/v) based on the cell concentration of yeast that had been used for preparation of amino acid analysis.

### Amino acid analysis

Amino acid analysis was done according to the method of Bidlingmeyer et al. (1984). The amino acid standards were obtained as type H (Wako, Tokyo, Japan) and supplemented with hypotaurine (Wako), cysteine sulfinic acid (Sigma), or taurine (Nacalai Tesque, Kyoto, Japan). Free amino acids were separated on a Pico-Tag column (3.9 mm i.d. × 300 mm; Waters, Milford, MA, USA.) with a Shimadzu HPLC system (Model LC-9A, Shimadzu, Kyoto, Japan). HPLC analysis was done according to the manual attached to the Pico-Tag column.

### Freezing tolerance test

Cells of *S. cerevisiae* were pre-cultured in SR-trp medium with shaking at 130 rpm at 30°C for 24 h.

Pre-cultured cells (from 2 to 8 µl) were inoculated into 5 ml of SR-trp medium containing 2% galactose and 2 mM cysteine, with shaking at 30°C for 24 h. The cultured cells, whose $OD_{600}$ were 0.5–0.7, were used after 24-h induction. The cells were harvested by centrifugation at 560×*g* for 10 min, washed three times with 0.9% NaCl solution, and the concentration of the cell suspension was adjusted to an $OD_{600}$ of 0.1 (approximately $3 \times 10^6$ cells/ml). The suspensions (100 µl) in eppendorf tubes were cooled in a deep freezer at −80°C for 2 min for ice formation. Then, the samples were transferred to another freezer and kept at −20°C for 24 h. The frozen samples were then thawed in a bath kept at 25°C and diluted with a 0.9% NaCl solution and spread onto YPD agar plates. After 2 days of incubation at 30°C, the colonies were counted. The relative survival rate was determined by comparison of frozen–thawed cells to unfrozen cells. The number of colonies that form on the plates after freezing and thawing is expressed as a percentage of the number of colonies that form on agar plates without freezing.

### Oxidative stress tolerance test using menadione

As described in the section on freezing tolerance test, yeast cells were cultured in 5 ml of SR-trp medium containing 2% galactose and 2 mM cysteine, with shaking at 30°C for 24 h, and harvested by centrifugation at 560×*g* for 5 min. The collected cells were washed twice with 100 mM potassium phosphate buffer (pH 7.5) and suspended in 1 ml of the same buffer. The concentration of the cell suspension was adjusted to an $OD_{600}$ of 0.1 in a test tube by dilution with buffer to obtain a final volume of 2 ml of the cell suspension. Oxidative stress tolerance tests were carried out using menadione. Menadione dissolved in ethanol was added to the test tube at a final concentration of 200 µM. The samples were incubated at 30°C with shaking at 130 rpm for 1 h. The samples were serially diluted and spread onto YPD agar plates. After 2 days of incubation at 30°C, the colonies were counted. The relative survival rate was determined by comparing stressed cells to unstressed control cells.

### Fluorescence microscopic detection of superoxides

To detect intracellular superoxides generated by menadione, the yeast cells, which were subjected to the oxidative stress described above, were treated with 2 µM dihydroethidium (Molecular Probes, Eugene, OR, USA). After 1-h treatment, the cells were centrifuged at 560×*g* for 5 min. The collected cells were washed with 100 mM potassium phosphate buffer (pH 7.5). After removing the buffer, the cells suspended in 5 µl of the buffer were mounted onto a slide glass. Fluorescence inside the cells was visualized using a fluorescence microscope ECRIPSE E600 (Nikon, Tokyo, Japan) with excitation wavelength at 510–550 nm and emission fluorescence >590 nm. The number of cells emitting fluorescence was compared between transformed and control cells to judge the ability of scavenging superoxides by hypotaurine and/or taurine.

### Statistical analysis

Statistical analysis was performed using Excel 2004 (Microsoft, USA) with the add-in software Statcel 2 (Yanai 2004).

## Results

### Cloning of cDNAs for CDO and CSD from *C. carpio*.

PCR with primers designed for *CDO* led to the amplification of a 240-bp fragment from carp hepatopancreas cDNA. The fragment was sequenced and confirmed to be a part of cDNA for *CDO*. The fragment was used as a probe to isolate a corresponding cDNA clone from the library, resulting in the isolation of five positive clones for *CDO*. Nucleotide sequences of the clones were determined and categorized into two groups. The representative clones of the respective groups were designated *CDO1* (DDBJ/

EMBL/GenBank accession number AB220583) and *CDO2* (accession number AB220584). The length of *CDO1* and *CDO2* were 1,216 and 1,102 bp, respectively. Both clones contained the open reading frame coding for 201 amino acid residues with a calculated molecular mass of about 23.5 kDa. Deduced amino acid sequences of the two *CDO* clones differed in 15 residues. A homology search showed sequence similarity of predicted proteins of *CDO1* and *CDO2* to those of *CDO* from other organisms (Fig. 2).

To isolate cDNA corresponding to the gene encoding CSD from *C. carpio*, PCR amplification was carried out. The amplified DNA fragment was 140-bp long, as expected, and showed sequence similarity to *CSD* from other organisms. As library screening was not successful, 5′- and 3′-RACE methods were performed to obtain a full-length cDNA sequence for *CSD*. The result of each experiment showed the amplification of about 1,300- and 1,600-bp fragments with 5′- and 3′-RACE, respectively, which were assembled into a 2,567-bp long cDNA for *CSD* (accession number AB220585) containing the entire coding region. Deduced carp CSD is composed of 500 amino acid residues with predicted molecular mass of 56.2 kDa and the deduced amino acid sequence shows a striking similarity to those of CSDs from other organisms (Fig. 3).

## Expression of CDO, CSD, and CDO–CSD in *S. cerevisiae*

To express *CDO* and *CSD* in *S. cerevisiae*, the four kinds of transformants carrying pESC/*CDO1*/*CSD,* pESC/*CDO2*/*CSD*, pESC/*CDO1–CSD*, or pESC/*CDO2–CSD* were constructed. Coding regions of individual genes were amplified by PCR, digested with appropriate restriction enzymes, and subcloned into pre-digested pESC (pESC-TRP) vector (Fig. 1). As a control strain, a transformant carrying pESC was also constructed. The expression of the objective genes were induced by addition of galactose to the medium and then confirmed by immunoblot analysis (Fig. 4). In the plasmids, pESC/*CDO1*/*CSD* and pESC/*CDO2*/*CSD,* the coding region of *CDO* was followed by that of *c-myc*, and the coding region of *CSD* was followed by that of *FLAG*. To detect respective proteins, anti-(c-myc)-peroxidase and anti-FLAG M2 monoclonal antibody were used. In the case of CDO-c-myc protein, two bands of size 26.5 and 28 kDa were detected (Fig. 4a). Similar doublet band has recently been reported for mammalian CDOs to likely be attributable to a heterogeneity dependent on their cofactor, ferrous ion (Dominy et al. 2008). Thus, detected bands were thought derived from objective proteins. On the other hand, the CSD–FLAG fusion protein was detected as a single band of 56 kDa, in fair agreement with the size expected from the deduced primary structure (Fig. 4b).

To enhance the accumulation level of taurine, efficient synthesis of taurine is absolutely necessary. Expression of fusion proteins, which are involved in the synthesis, is one method for efficient synthesis. For this purpose, the coding regions of *CDO* and *CSD* were fused via a *c-myc* region coding linker polypeptide consisting of 14 amino acids (-VDMEQKLISEEDLE-). Thus, anti-(c-myc)-peroxidase was used to detect CDO–CSD fusion protein. The size of the detected band was about 75 kDa (Fig. 4c). The detected size was smaller than the expected size of the CDO–CSD fusion protein (82.1 kDa). However, the detected band was probably derived from the fusion protein because transformed yeast cells were confirmed to produce taurine as shown later (Table 1).

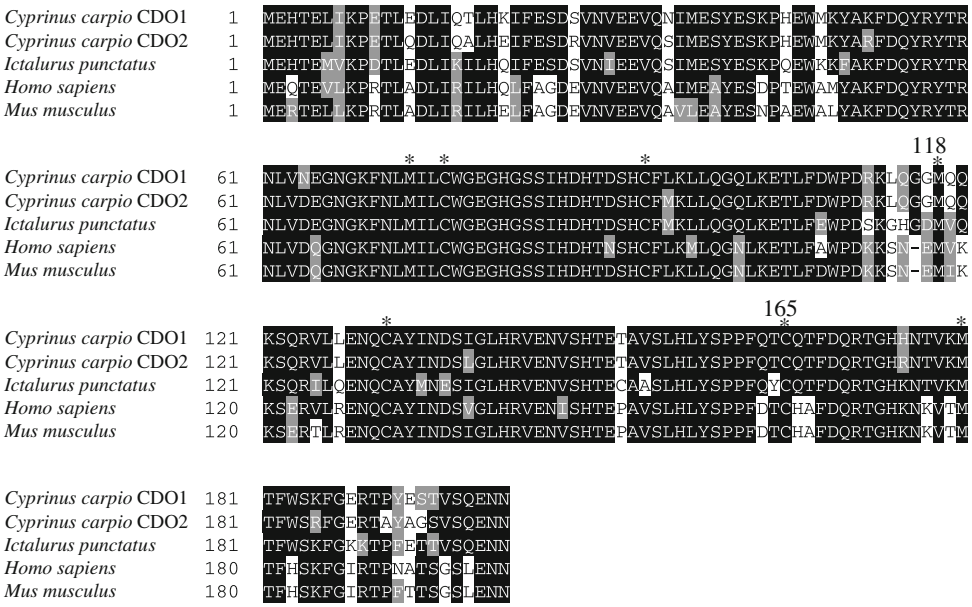


**Fig. 2** Alignment of deduced amino acid sequences of CDO. Identical amino acids and similar amino acids are indicated with *black* backgrounds and are shaded, respectively. *Dashes* indicate gaps. *Asterisks* show highly conserved methionine and cysteine residues. Accession numbers for the sequences are as follows: *Ictalurus punctatus* (deduced amino acid sequence from cDNA registered as BM438396), *Homo sapiens* (AAH24241), *Mus musculus* (XP_123217)

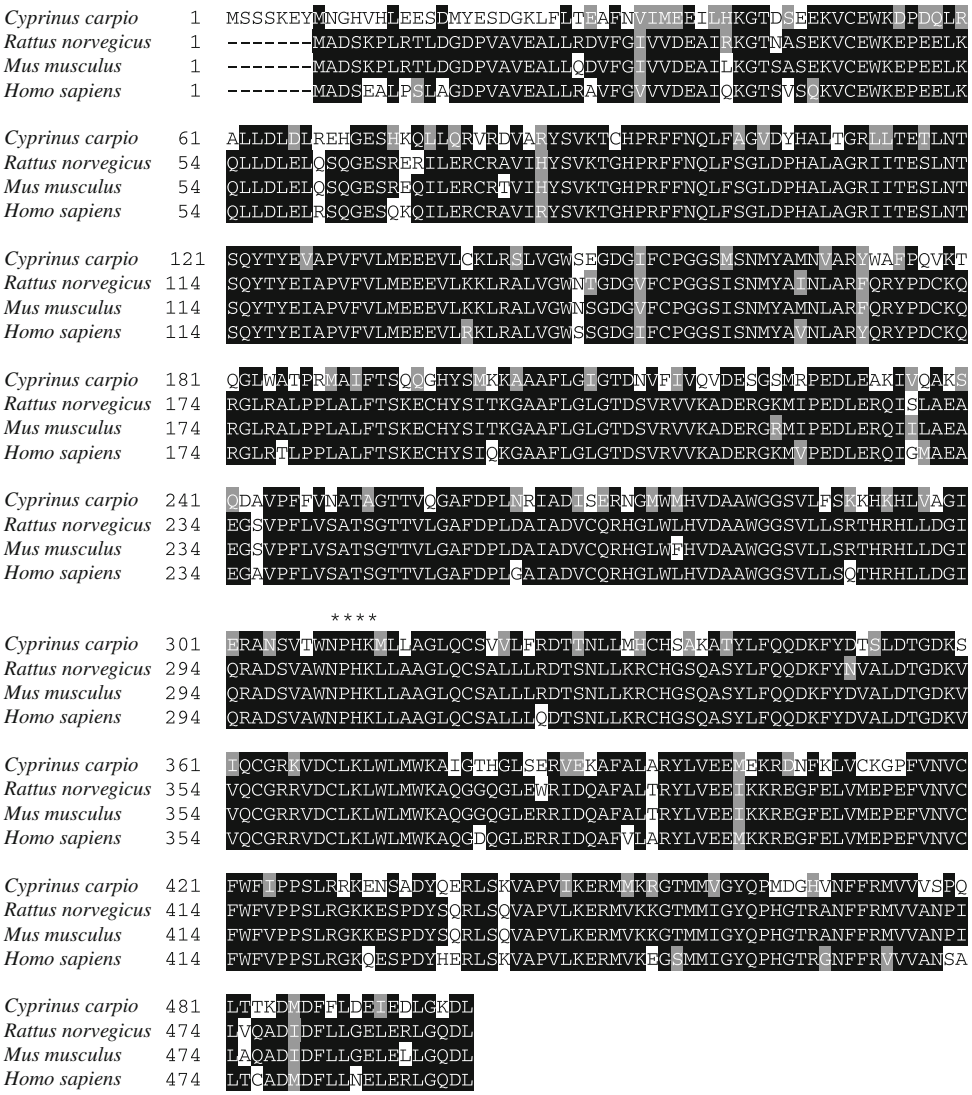


**Fig. 3** Alignment of deduced amino acid sequences of CSD. Identical amino acids and similar amino acids are indicated with *black* backgrounds and are shaded, respectively. *Dashes* indicate gaps. *Asterisks* show predicted binding sites of 5′-pyridoxal phosphate. Accession numbers for the sequences are as follows: *Rattus norvegicus* (NP_068518), *Mus musculus* (NP_659191), *Homo sapiens* (Q9Y600). Asterisks show a putative binding region for a cofactor of CSD

## Hypotaurine and taurine accumulation

The accumulation levels of taurine in yeast cells were determined by HPLC analysis (Table 1). In each recombinant, the apparent taurine value after $H_2O_2$-treatment of the cell extract was higher than that of untreated extract, indicating that substantial amounts of hypotaurine remained unoxidized to taurine in the cells. When a comparison was done between the two isoforms of CDO, accumulation levels of the total products (hypotaurine and taurine) of the enzymes introduced into the yeast were higher for CDO1 than CDO2, both in the case of expression as a separate protein from CSD (*CDO1*/*CSD*; 45.5 ± 4.5 μmol/g dry-weight, *CDO2*/*CSD*; 43.4 ± 6.8 μmol/g dry-weight) and in the case of expression as a fusion protein with CSD (*CDO1–CSD*; 85.3 ± 15.2 μmol/g dry-weight, *CDO2–CSD*; 67.4 ± 11.9 μmol/g dry-weight). In addition, the expression of CDO–CSD fusion protein yielded higher amounts of the products than the enzymes expressed as separate proteins. Although the reason was unclear, the taurine levels in the cells expressing *CDO1* were higher than those in the cells expressing *CDO2*. Thus, further experiments were done using yeast cells carrying pESC/*CDO1*/*CSD* or pESC/*CDO1–CSD*.

## Stress tolerance of transformed yeast

To investigate effects of taurine on freezing tolerance and oxidative stress tolerance induced by menadione treatment, the viability of yeast cells carrying pESC, pESC/*CDO1*/*CSD*, or pESC/*CDO1–CSD* was investigated. As Fig. 5 shows, the freezing tolerance of the yeast cells carrying pESC/*CDO1*/*CSD* or pESC/*CDO1–CSD* was relatively higher than that of control yeast cells with a vector (pESC), but not showing any significant differences. On the other

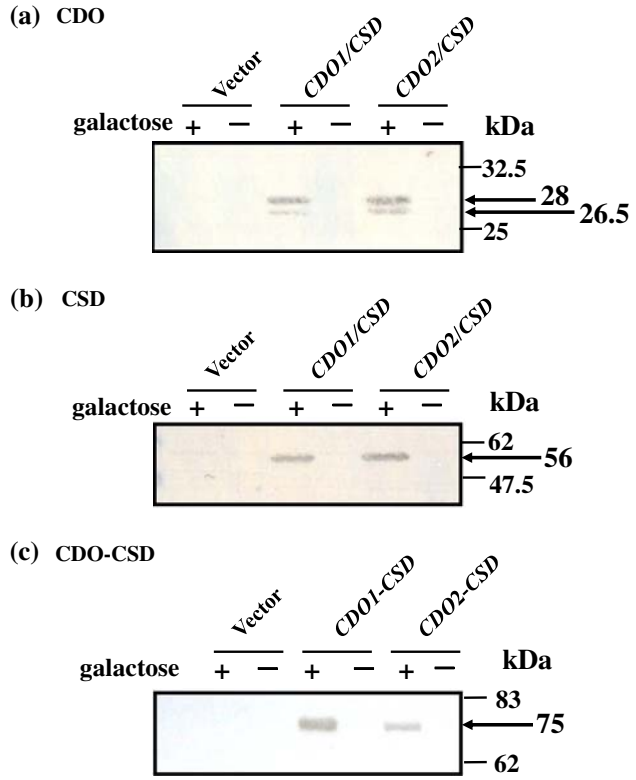


**Fig. 4** Immunoblot analysis of the CDO (**a**), CSD (**b**) and CDO–CSD (**c**) proteins. Equal amounts of soluble proteins (5 μg) were separated on 12.5% SDS-PAGE and immunoblot detection was done as described in "Materials and Methods". The *arrows* indicate the detected bands

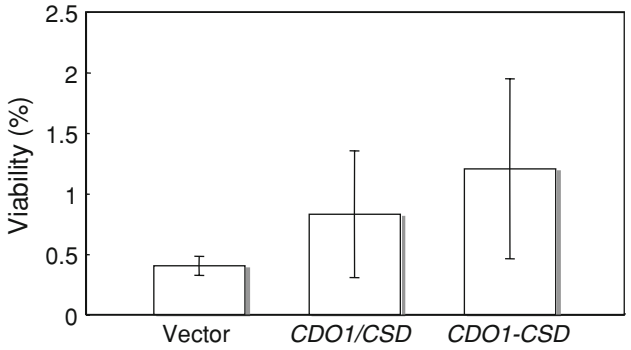


**Fig. 5** Freezing tolerance of transformed yeast. Results represent the mean ± SD from three independent experiments

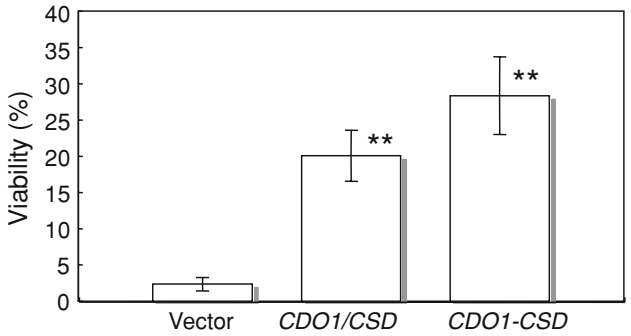


**Fig. 6** Menadione-induced oxidative stress tolerance of transformed yeast. Results represent the mean ± SD from three independent experiments. *Asterisks* indicate significant differences at $P < 0.01$, compared to cells transformed with vector

**Table 1** Taurine and hypotaurine contents

| Plasmids | Taurine content (μmol/g dry-weight) | | Hypotaurine content in yeast cells[a] (μmol/g dry-weight) |
|---|---|---|---|
| | Untreated | $H_2O_2$ treated | |
| pESC | ND[b] | ND | – |
| pESC/*CDO1/CSD* | 17.0 ± 7.6[c] | 45.5 ± 4.5 | 29.5 ± 8.9 |
| pESC/*CDO2/CSD* | 14.4 ± 6.3 | 43.4 ± 6.8 | 29.5 ± 9.7 |
| pESC/*CDO1–CSD* | 21.6 ± 8.9 | 85.3 ± 15.2 | 64.6 ± 20.2 |
| pESC/*CDO2–CSD* | 18.1 ± 8.4 | 67.4 ± 11.9 | 49.7 ± 19.0 |

[a] Taurine contents, which remained as hypotaurine in yeast, were calculated by following formula: Taurine content remained as hypotaurine = ($H_2O_2$ treated taurine amounts) − (untreated taurine amounts)

[b] Not detected

[c] Values are the means ± SD obtained from three independent experiments

hand, the transformed yeasts showed significantly higher tolerance than control yeast cells against oxidative stress induced by menadione (Fig. 6). Although viability of control yeast cells with vector was about 2.5% after exposure to menadione, yeast cells transformed with pESC/*CDO1/CSD* and pESC/*CDO1–CSD* showed about 20 and 28% viability, respectively. It is likely that expression of the corresponding genes led to the enhancement of oxidative stress tolerance of yeast.

### Detection of superoxides by fluorescence microscopy

The intracellular state of the yeast cells subjected to oxidative stress induced by menadione was investigated using fluorescence microscopy. Dihydroethidium was used to detect superoxides. As Fig. 7 shows, treatment with menadione led to intracellular accumulation of superoxides. In particular, the cells expressing taurine biosynthetic enzymes showed less fluorescence intensity than the control cells, suggesting less accumulation of superoxides.

## Discussion

For the biosynthesis of taurine, a possible pathway via cysteine, cysteine sulfinic acid, and hypotaurine is known (Yokoyama et al. 2001). To date, genes encoding CDO, which catalyzes a reaction from cysteine to cysteine

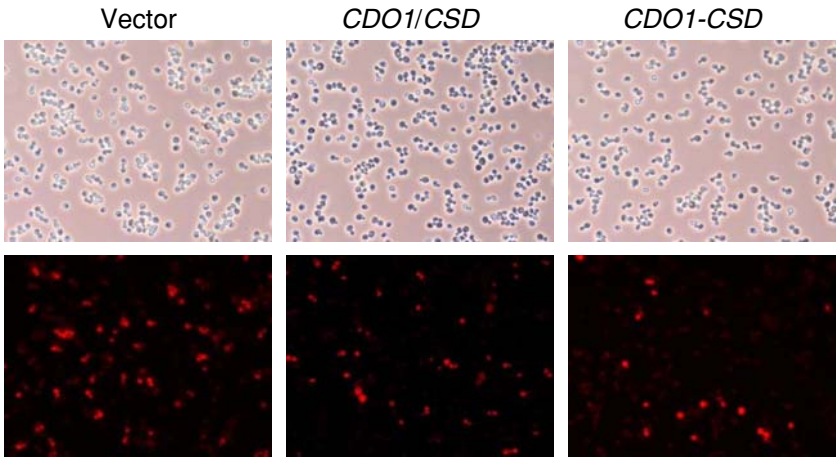


**Fig. 7** Fluorescence microscopy of yeast cells stained with dihydroethidium. Upper photos were taken with phase contrast microscopy and lower photos were taken with fluorescence microscopy

sulfinic acid, and CSD, which catalyzes a reaction from cysteine sulfinic acid to hypotaurine, have been isolated from *Homo sapiens, Rattus norvegicus* and so on (Hosokawa et al. 1990; McCann et al. 1994; Park et al. 2002; Reymond et al. 1996). Taurine is found in fish and shellfish, suggesting the presence of the taurine synthetic pathway in lower vertebrates and invertebrates. Since some fish and shellfish species are rich in taurine, it is expected that these animals possess the corresponding enzymes with high specific activity. Hence, in the present study, we tried to isolate the cDNA clones corresponding to genes encoding CDO and CSD from *C. carpio*.

The deduced amino acid sequences of two isolated *CDO*s (*CDO1* and *CDO2*) from carp showed high similarity to those of *CDO*s from other organisms (Fig. 2). CDO proteins were reported to contain $Fe^{2+}$, coordinated with methionine and cysteine residues, which play an important role in CDO activity (McCann et al. 1994). In the deduced amino acid sequences of *CDO1* and *CDO2*, there were six or seven methionine residues and four cysteine residues conserved among the sequences of CDOs from several organisms (Fig. 2). The hydropathy plots of CDO1 and CDO2 drawn by the method of Kyte and Doolittle (1982) suggest that $Met^{118}$ and $Cys^{165}$ are located in hydrophilic regions (data not shown). Therefore, these two residues could be involved in the coordination of $Fe^{2+}$ at the surface of the CDO molecule.

There is a 15 amino acid difference between sequences of CDO1 and CDO2 (Fig. 2). Nevertheless, both CDO isoforms showed no significant difference in the activity (177 and 148 nmol cysteine sulfinic acid/min mg-protein for CDO1 and CDO2, respectively; data not shown) in a preliminary assay performed as described by Bagley et al. (1995) using the CDOs overexpressed in yeast cells carrying pESC/*CDO1*/*CSD* and pESC/*CDO2*/*CSD*, respectively. On the other hand, the cell extract with pESC did not show any activity for CDO. Thus, both the carp CDO proteins were confirmed to be expressed with enzyme activity.

The deduced amino acid sequence of carp *CSD* showed close similarity to those of CSDs from rats and humans (Park et al. 2002; Reymond et al. 1996), as shown in Fig. 3. The NPHK sequence motif (Kaisakia et al. 1995), which has been proposed as a binding site for its cofactor, pyridoxal 5′-phosphate, is conserved in the carp CSD sequence.

CSD activity of the transformed yeast cell extract was also confirmed in a preliminary experiment. The activity of the overexpressed recombinant CSD, assayed by the method of Daniels and Stipanuk (1982), was 414 and 322 nmol hypotaurine/min mg-protein in yeast cells carrying pESC/*CDO1*/*CSD* and pESC/*CDO2*/*CSD*, respectively (data not shown). Thus, it is also certain that carp CSD is fully functional to catalyze the synthesis of hypotaurine in yeast.

A preliminary analysis of taurine contents showed poor production of taurine in yeast, when *CDO* and *CSD* genes were introduced and expressed as separate proteins. Thus, to enhance the accumulation levels of taurine, fusion genes of *CDO* and *CSD* was constructed. Some reports regarding fusion proteins include a fusion of $\beta$-galactosidase and galactokinase, a fusion of $\beta$-galactosidase and galactodehydrogenase (Bulow and Mosbach 1991), and a fusion of trehalose 6-phosphate synthase and trehalose 6-phosphate phosphatase (Seo et al. 2000) to enhance the efficiency of two enzymatic reactions. In the present paper, a similar trial was performed using the c-myc region as a linker peptide (14 amino acids) between CDO and CSD proteins (Fig. 1). The expression of the fusion gene led to an increase in the accumulation levels of the sum of taurine and hypotaurine in yeast (Table 1). Based on the levels of taurine and hypotaurine, the efficiency of the reaction of the CDO–CSD fusion protein was better than those of the two enzymes expressed as separate proteins, suggesting that the linker peptide does not inhibit the enzyme activities.

As Table 1 shows, we detected a considerable amount of hypotaurine synthesized in the transformed yeast, indicating that the deduced proteins of *CDO* and *CSD* genes cloned from carp are fully functional in the cell. In our previous study (Honjoh et al. 2007), culturing yeast in taurine containing medium led to the enhancement of stress tolerance by accumulating high amounts (66.7–126 μmol/g dry cell weight) of taurine in yeast. In the present paper, although biosynthesized hypotaurine were 43.4–85.3 μmol/g dry cell weight, amounts of autoxidized taurine were 14.4–21.6 μmol/g dry cell weight (Table 1). Conversion of hypotaurine to taurine is widely believed to be autoxidation process, however, the present data suggest that the autoxidation is rate-limiting for accumulation of taurine in the transformed yeast cells and that the conversion is possibly catalyzed by an enzyme, which could be named as hypotaurine dehydrogenase (Perry et al. 1979) and whose gene has not been cloned from any organisms to our knowledge.

As another approach to further enhance the accumulation of hypotaurine and/or taurine, we tried to increase the concentration of cysteine, a substrate for CDO, in the SR-trp medium for yeast in preliminary experiments. However, addition of over 2 mM cysteine to the medium led to lowering the growth rate of wild-type yeast (data not shown). This growth lag was thought to be based on intracellular toxic effects of cysteine on living organisms (Stipanuk et al. 2009). In human, high concentration levels of cysteine are reported to be related with several serious diseases (Heafield et al. 1990). One of main functions of CDO in animals seems to oxidize cysteine to maintain the concentration of intracellular free cysteine within a proper range, leading to cysteine homeostasis (Stipanuk et al. 2009). In the present paper, excess addition of cysteine to the medium led to the growth lag of wild-type yeast. Thus, limited addition of cysteine at the concentration of 2 mM to the medium might limit high accumulation of hypotaurine and/or taurine.

As Fig. 5 shows, freezing tolerances of the yeast cells, with pESC/*CDO1*/*CSD* or pESC/*CDO1*–*CSD*, expressing taurine biosynthetic enzymes were higher than that of the control cells, although not showing any significant differences. As mentioned above, although parts of hypotaurine were autoxidized to taurine, the level of autoxidation from hypotaurine to taurine would not be enough to enhance freezing tolerance of yeast. Furthermore, as hypotaurine has not been reported to function as a cryoprotectant to our knowledge, it could not enhance the tolerance of transformed yeast cells.

In our preliminary experiments, exposure of yeast cells to hydrogen peroxide did not show any significant differences in tolerance to hydrogen peroxide between cells expressing taurine biosynthetic enzymes and control cells (data not shown). To investigate effect of accumulated hypotaurine and/or taurine in the yeast cells, menadione was used as generator of reactive oxygen species in the yeast cells. As Fig. 6 shows, yeast cells accumulating hypotaurine and/or taurine showed significant improvement of tolerance of oxidative stress induced by menadione. Hypotaurine is known for its ability to scavenge hydroxyl radical and for its antioxidant activity (Shi et al. 1997). Thus, in yeast cells, synthesized hypotaurine might play a role in oxidative stress tolerance but not in freezing tolerance. Alleviation of oxidative stress by accumulation of hypotaurine and/or taurine was also supported with the observation of fluorescence using dihydroethidium (Fig. 7). The reduction of the levels of superoxides was likely to be parallel with the accumulation levels of hypotaurine and/or taurine in the yeast cells, leading to enhancement of oxidative stress tolerance.

In conclusion, although our primary purpose of enhancement of freezing tolerance of yeast was not accomplished, we showed that hypotaurine could function as protectant against menadione-induced intracellular oxidative stress in yeast by introduction of vertebrate genes responsible for taurine synthesis.

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