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Cloning, biochemical characterization, and phylogenetic analysis of a novel isopentenyl diphosphate isomerase gene from *Lactarius volemus*

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ABSTRACT

Lactarius volemus is a mushroom species that produces beneficial organic compounds. A large number of these compounds are isoprenoids. Isopenteyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are essential for isoprenoid biosynthesis. The reversible isomerization of IPP to DMAPP is catalyzed by isopentenyl diphosphate isomerase (IDI, EC 5.3.3.2). However, there is no information on IDI from mushrooms. In this study, we report a novel cDNA encoding IDI from L. volemus (LvIDI). LvIDI was composed of 253 amino acid residues with a molecular weight of 28.8 kDa and anisoelectric point of 4.99. The kinetic assay results indicated that the K_m was 0.39 μM and V_{max} was 0.034 μmol mg^{-1} min^{-1}. Moreover, LvIDI was classified into Basidiomycota in the phylogenetic analysis.

Key words: cloning, enzyme assay, isopentenyl diphosphate isomerase, *Lactarius volemus*, phylogenic analysis.

INTRODUCTION

Mushrooms possess many beneficial effects, for example, twenty species of mushroom are effective against cancer [1]. Mushrooms contain many organic compounds, for example, proteins, fats, ash, glycosides, alkaloids, volatile oils, tocopherols, phenolics, flavonoids, carotenoids, folates, ascorbic acid enzymes, and organic acids [1]. Carotenoid and tocopherol are classified into isoprenoids.

Isoprenoids are the most diverse family of chemicals and consist of more than 23,000 compounds [2]. The isoprenoid biosynthetic pathway is involved in the synthesis of several important metabolites, such as terpenoid, carotenoid, dolicol, and rubber [3] [4]. All of these compounds are synthesized by the condensation of the isomeric 5-carbon molecules isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) [2]. Isopentenyl diphosphate isomerase (IDI) (EC 5.3.3.2), which catalyzes the isomerization of IPP to DMAPP, is a key enzyme for producing isoprenoid compounds; therefore, it is important to study IDI. Recently, genes encoding IDI have been cloned from various organisms such as humans, *Saccharomyces cerevisiae*, and *Escherichia coli*. *Lactarius volemus*, a mushroom species, is classified into the phylum Basidiomycota, which comprises almost 23,000 species [6]. However, there are no data on IDI from Basidiomycota.

In the present study, the IDI gene from *L. volemus* (LvIDI) was cloned and functionally characterized. Additionally, we carried out phylogenetic analysis of IDIs of other fungi species.

EXPERIMENTAL SECTION

Amplification of the core domain of LvIDI

The fruiting body of *L. volemus* was collected from Fukushima in Japan in August. The sample was immediately dipped in 4 M guanidine thiocyanate (GTC) and stored at -80℃. Total RNA was extracted from the fruiting body of *L. volemus* by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method [7]. Single-strand cDNA was

synthesized from 1 μg of total RNA with an oligo(dT)$_{16}$ primer, according to the manufacturer's protocol (Roche Applied Science). The single-strand cDNA was used for degenerate PCR. The primers for degenerate PCR were designed according to previous reports on IDI genes from fungi. The primer sets for cloning the core cDNA fragment of LvIDI are as follows: LvIDI-ds1 and LvIDI-da1 for the first PCR, LvIDI-ds2 and LvIDI-da2 for the second PCR, and LvIDI-ds2 and LvIDI-da3 for the third PCR (Table 1). PCR was carried out using HybriPol DNA Polymerase (BIOLINE) as follows: 95℃ for 5 min; 35 cycles of 95℃ for 30 s, Tm-2℃ for 30 s, 72℃ for 1 min; and 72℃ for 3 min. The PCR fragments were sub-cloned with the pGEM T-easy vector (Promega, USA) and sequenced with the ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems). The core fragments were used to design gene-specific primers for cloning the full-length cDNA of LvIDI by rapid amplification of cDNA ends (RACE).

Cloning of the full-length cDNA of LvIDI

The single-stranded cDNA for 3'-RACE (rapid amplification of cDNA ends) PCR was synthesized from 20 □g of total RNA with oligo(dT)$_{16}$ adapter primer (GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TT) according to the manufacturer's protocol (Roche Applied Science). The first, second, and third 3'-RACE PCR amplifications were carried out with LvIDI3-adp (GGC CAC GCG TCG ACT AGT AC) and specific primers as follows: LvIDI3-1 for the first PCR, LvIDI3-2 for the second PCR, and LvIDI3-3 for the third PCR (Table 1). The single-stranded cDNA was used for 3'-RACE PCR and was amplified using the same conditions as those used for the degenerate PCR.

Single-strand cDNAs for 5'-RACE PCR were synthesized with the GeneRacer™ Kit (Invitrogen Life Technologies). 5'-RACE PCR was performed with following primers: LvIDI5-1 (Table1) and the GeneRacer™ 5' primer for the first PCR; LvIDI5-2 (Table 1) and GeneRacer™ 5' Nested Primer for the second PCR. MightyAmp® DNA Polymerase Ver.2 (TaKaRa) was used for the PCR amplification. The first and nested PCR procedures were carried out as follows: first denaturation at 98℃ for 2 min and 35 cycles of amplification (95℃ for 10 sec, 55℃ for 15 sec, and 68℃ for 1 min).

Table 1. Sequence of primer used for PCR cloning

Name	Sequence (5'>3')
Degenerate	
LvIDI-ds1	TGY CAY CTN AWN GAR AAN ATH
LvIDI-da1	NGC RAT NAR YTC RTS
LvIDI-ds2	GAG AAG ATY CAN TTY CCN
LvIDI-da2	DAK RTA RTC IAY YTC RTG
LvIDI-da3	RTC RAT CTC RTG CTC NCC CCA
3' RACE	
LvIDI3-1	CAT GTT GCT CAC ACC CAC TCG ACG
LvIDI3-2	TGA GAA GGA TCA GCT GGG AGT AC
LvIDI3-3	CTG ACG CGG ATA CAC TAT CTC GCA C
5'RACE	
LvIDI5-1	GTG TTC GAG CTT CCG TGA C
LvIDI5-2	TCC TCC TCA AAG TCG TCG AG

Construction of vector for expression of LvIDI

The full-length cDNA of LvIDI was cloned and amplified with KOD -Plus- Neo DNA polymerase (TOYOBO LIFE SCIENCE) and the primer pair LvIDI-full-f (CGG GGT ACC ATG GCC ACT ACT ACC GTG ACC) and LvIDI-full-r (ATA AAG CTT TTA CAT CTT CAC TAC CTT GGA TCC), and then introduced into the pCold Ⅰ vector (TaKaRa) through the *Kpn* Ⅰ and *Hind* Ⅲ restriction site, namely pColdI/LvIDI. The construct was transformed into BL21 competent *E. coli* (BioLabs), and transformations were selected by ampicillin (50 μg/ml).

Expression and purification of LvIDI

A single colony transformed with LvIDI was cultured in LB/ampicillin medium (3 ml) overnight at 37℃. The cultures were transferred to fresh LB/ampicillin medium (300 ml), which was incubated at 37℃ until the OD_{600} was 0.5 and immediately cooled down at 15℃ for 30 min (Cold-shock system, TaKaRa). Then, IPTG was added at a final concentration of 0.1 mM and incubated at 15℃ for 36 h. Cells were harvested by centrifugation at 5,000 rpm for 10 min. After suspending in Tris-HCl buffer (pH 8.0), cells were broken by ultrasonic waves. The suspension was centrifuged at 15,000 rpm for 20 min., and the supernatant was transferred.

We used His GraviTrap (GE Healthcare) to purify the recombinant protein and eluted it with the elution buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 500 mM imidazole). To remove needless salt, the eluted protein was desalted with a PD-10 column (GE Healthcare) and 20 mM Tris-HCl (pH 8.0).

Enzyme assay

Enzyme assay was carried out with purified 2.5 ng/μl LvIDI, 50 mM Tris-HCl (pH 7.0), 50 mM 2-mercaptethanol, 5 mM $MgCl_2$ and 25 μM [1-^{14}C]IPP (11000 dpm). All assays were performed in triplicate at 25℃ for 10 min [8][9][10]. After incubation, the reaction mixture was treated with 1 M HCl at 37℃ and extracted by diethyl ether. The extracts were measured with a liquid scintillation counter. The effects of temperature (0 to 60℃), pH (4 to 9), Mg^{2+} (0.1-25 mM), and Mn^{2+} (0.0050-10 mM) were graduated.

Phylogenetic analysis

Several DNA sequences of IDI from fungi were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/). The evolutionary history was inferred using the neighbor-joining method [11]. The optimal tree with the sum of branch length = 1.80575107 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [12] and are in the units of the number of amino acid substitutions per site. The analysis involved 13 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 217 positions in the final dataset. Evolutionary analysis was conducted with MEGA5 [13].

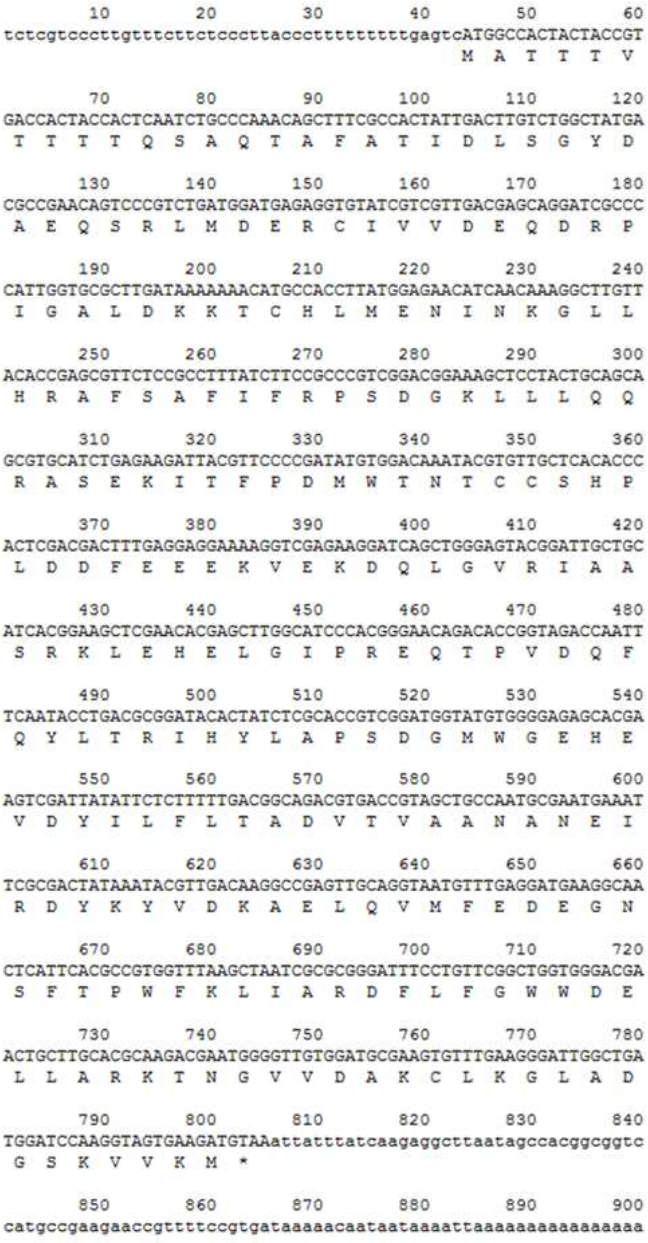

Figure 1. The full-length cDNA sequence and the translated amino acid sequence of LvIDI. The open reading frame is typed in capital letters. The stop codon is marked with an asterisk, the 5'- and 3'-untranslated regions are shown in small letters

RESULTS AND DISCUSSION

Molecular Cloning of the full-length cDNA of LvIDI

The 240 bp product was amplified by degenerate RT-PCR. LvIDI showed similarity to IDI from another organism by NCBI/BLAST. The identified DNA sequence was used to design specific primers for 5'-and 3'-RACE PCR. 5'-and 3'-RACE PCR were amplified 411 bp and 593 bp DNA sequences respectively. Full-length cDNA of LvIDI was 900 bp and contained an ORF of 759 bp encoding a polypeptide of 253 amino acids, flanked by 43 bp 5'-untranslated region and 98 bp 3'- untranslated region with a poly (A) tail of 18 bp (Fig. 1). The translated protein had 253 amino acids, a molecular weight of 28.8 kDa, and an isoelectric point of 4.99 [14].

LvIDI was compared with other IDI proteins by NCBI/BLAST. The result showed that LvIDI exhibited high similarity with IDI1 from other species: *X. dendrorhous*: O42641 (identities 64%, Positives 73%); *S. pombe* (identities 61%, positives 69%) [22]; *R. norvegicus* (identities 54%, positives 71%) [23]; *B. taurus* (identities 56%, positives 69%) [24]; *M. auratus* (identities 54%, positives 69%) [23]; *M. musculus* (identities 54%, positives 70%) [25]; *M. fascicularis* (identities 55%, positives 69%); *H. sapiens* (identities 55%, positives 69%) [26] (Fig. 2). The phylogenic analysis showed that IDIs are divided into Basidiomycota, Ascomycota, and Zygomycota phylum (Fig. 3).

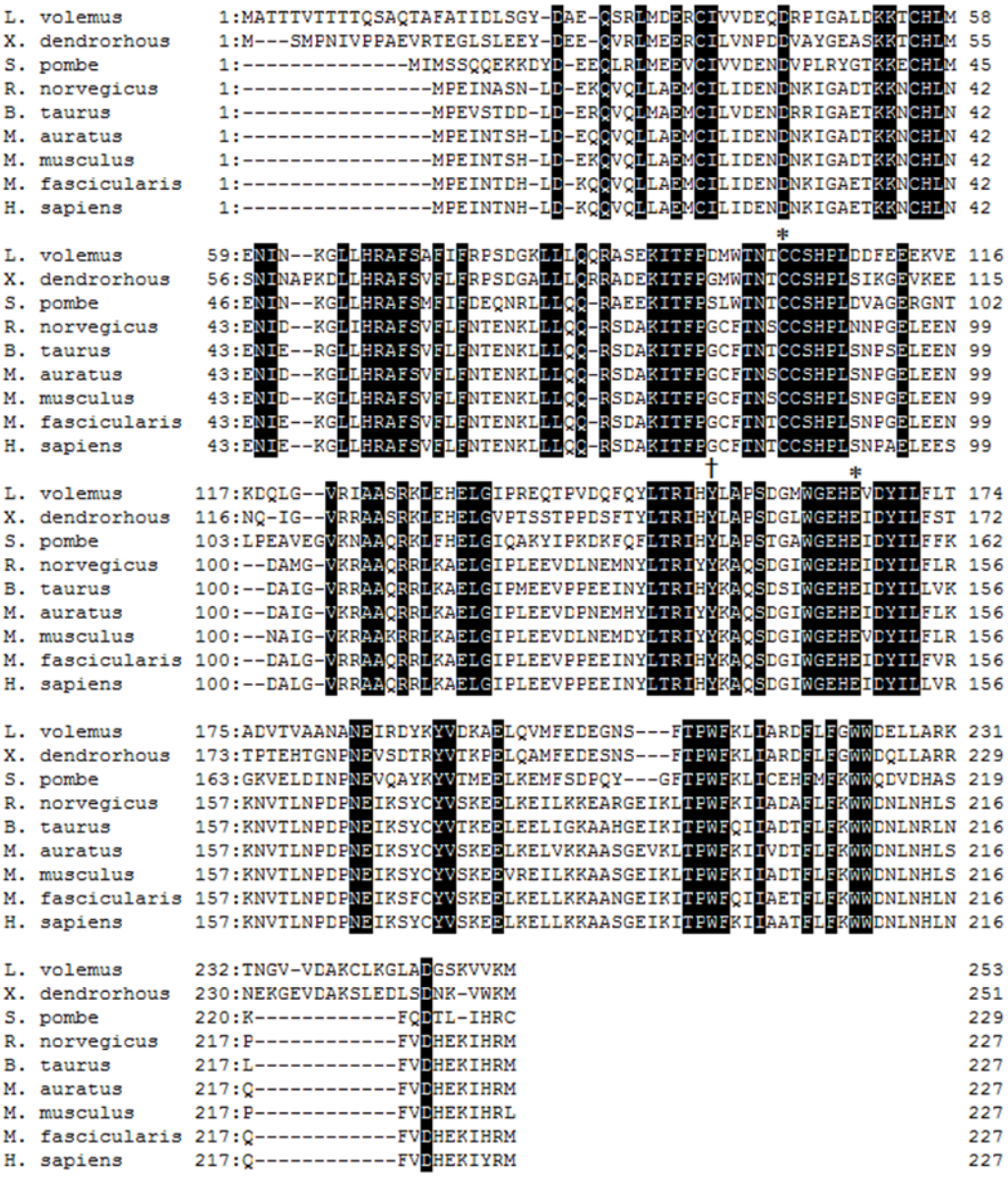

Figure 2. The multiple sequence alignments of IDIs

The conserved amino acid residues are shown in white with black background. The conserved amino acid residues of LvIDI are marked with asterisk (Cys[102] and Glu[166]) and dagger (Tyr[154]). X. dendrorhous: O42641; S. pombe: Q10132; R. norvegicus: O35760; B. Taurus: Q1LZ95; M. auratus:O35586; M. musculus:P58044; M. fascicularis: Q4R4W5; H. sapiens: Q13907.

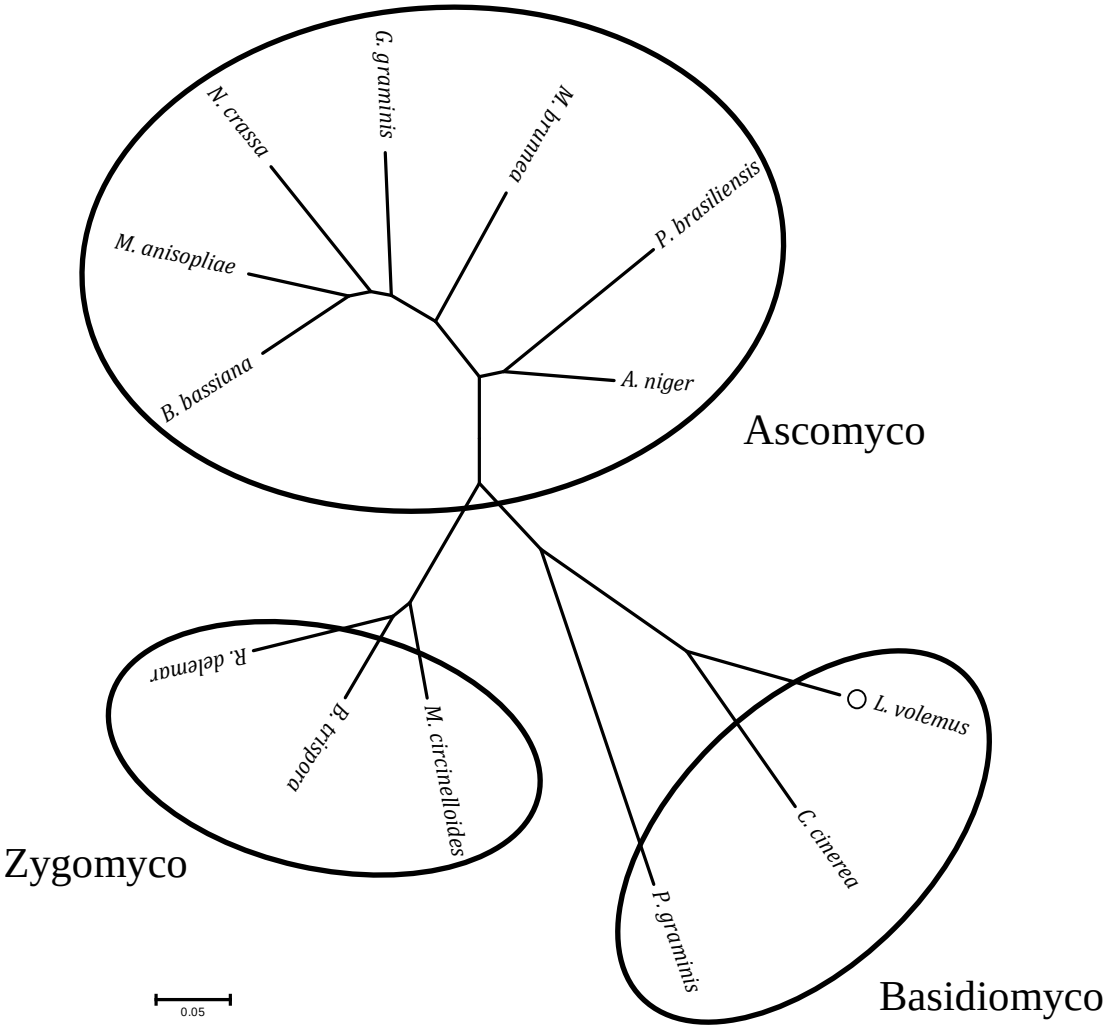

Figure 3. The phylogenetic analysis of the IDI proteins from Fungi

The mark of ○ is L. volemus in this paper. The accession number of the aligned sequences are as follows; A. niger: XP_001392874; B. bassiana: EJP65722; G. graminis: EJT77765; M. brunnea: EKD18288; M. anisopliae: EFY98343; N. crassa: XP_961969; P. brasiliensis: EEH18413; B. rispora: AFR51716; M. circinelloides: CAP17174; R. delemar: EIE88881; C. cinerea: XP_001828702; P. graminis: XP_003334611.

Expression, purification and Enzyme assay of LvIDI

The expression of purified protein was checked by SDS-PAGE (Fig. 4). The concentration of the LvIDI was determined by Bradford method. Enzyme activity was increased at metal concentrations ranging from 0.10 to25 mM for Mg^{2+} (Fig. 5A) and 0.0050 to 10 mM for Mn^{2+} (Fig. 5B). Interestingly, a higher concentration of Mg^{2+} and Mn^{2+} inhibited the enzyme activity. The most stable pH was 7.0 (data not shown), and the most stable temperature was 35℃ (Fig. 6) for the enzyme assay. The kinetic properties of LvIDI were analyzed using the Lineweaver-Burk plot (Fig. 7). The K_m, Vmas, K_{cat} and K_{cat} /K_m were 0.39 μM, 0.034 μmol mg^{-1} min^{-1}, 0.39 s^{-1} and 1.0×10^6 M^{-1} • s^{-1}, respectively. Each parameter was compared with other species (Table 2) [10] [15] [16].

Table 2. Comparison of kinetic parameters for IDI between various species

Species	V_{max} (μmol mg^{-1} min^{-1})	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (M^{-1} s^{-1})	reference
L. volemus	0.034	0.39	0.39	1.0×10^6	This study
E. coli	0.97	7.9	0.33	4.2×10^4	[10]
H. sapiens	4.1	33	1.8	5.5×10^4	[15]
S. cerevisiae	-	46	8	1.9×10^5	[16]

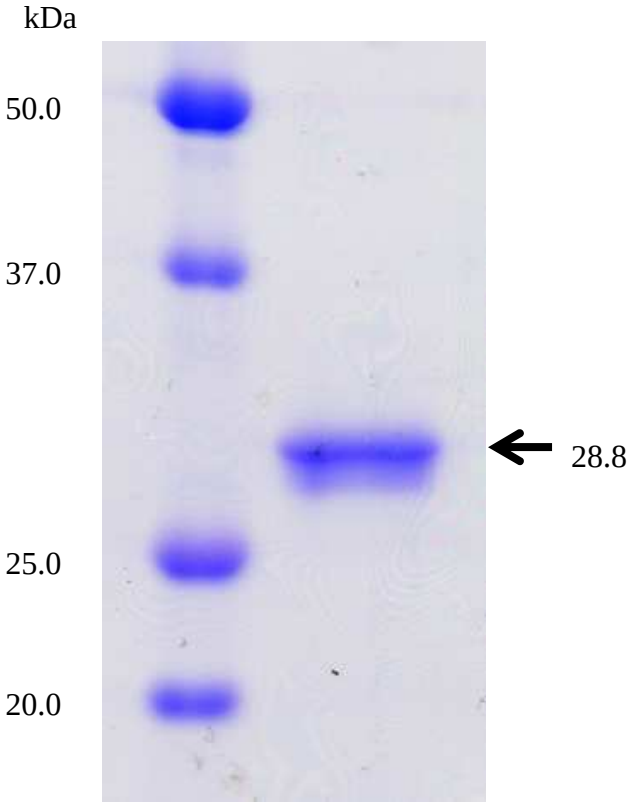

Figure 4. SDS-PAGE of LvIDI protein purified by Ni-NTA column, and desalted with PD-10 column. Lane 1: molecular mass standards (BIO-RAD), Lane 2: LvIDI protein purified and desalted

Generally, the concentrations of Mg^{2+} and Mn^{2+} affect enzyme activity [17] [18]. In this study, increasing in the Mg^{2+} concentration lowered the enzyme activity. The enzyme activity of LvIDI was highest when the concentration of Mg^{2+} was 1 mM.

However, when using $MnCl_2$, the enzyme activity was significantly decreased. In general, two type of IDI is known; IDI1 and IDI2. Amino acid sequences are different in both IDI. NADPH of FMN is essential for IDI2 to catalase isomerization [19] [20]. In this assay, LvIDI catalyzed the isomerization between IPP and DMAPP without cofactor. It is suggested that LvIDI is IDI1. Interestingly, LvIDI showed differing behavior compared to IDI2 obtained from *M. thermautotrophicus* [21] with respect to temperature, pH, and $MgCl_2$. It seems that the difference of activity is caused by existence or nonexistence of cofactor binding site. Kinetics parameters were similar to other species. We suggest that the isomerization of IPP and DMAPP is not the rate-limiting reaction in isoprenoid biosynthesis of *L. volemus*.

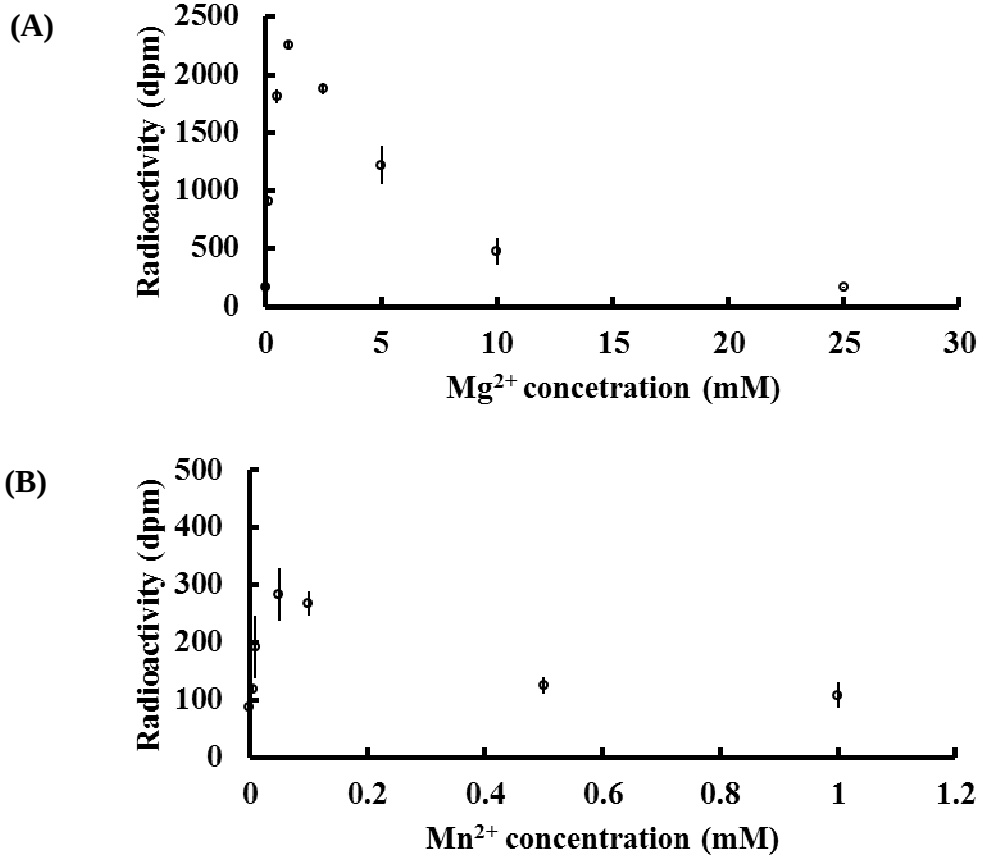

Figure 5. The effect of Mg^{2+} (A) and Mn^{2+} (B) for LvIDI activity

The concentrations of Mg^{2+} were 0.10, 0.50, 1.0, 2.5, 5.0, 10 and 25 mM. Those of Mn^{2+} were 0.0050, 0.010, 0.050, 0.10, 0.50, 1.0, 2.5, 5.0 and 10 mM. Error bars were showed standard deviation (S. E.).

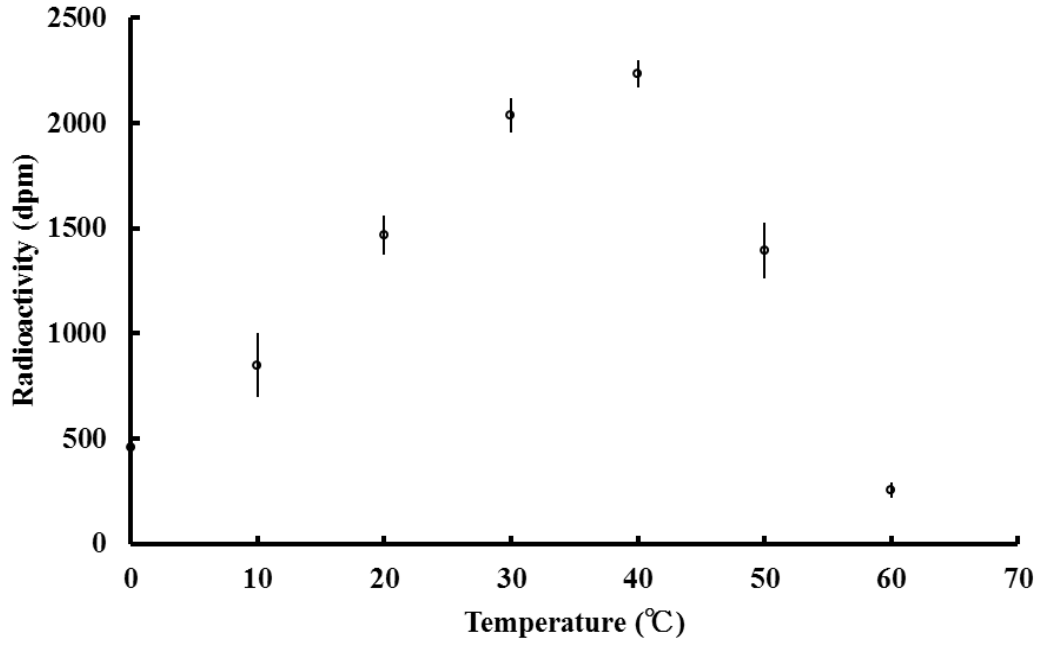

Figure 6. The effect of temperature for LvIDI activity

Enzyme assays were performed at 0 to 60 ℃ every 10 ℃.

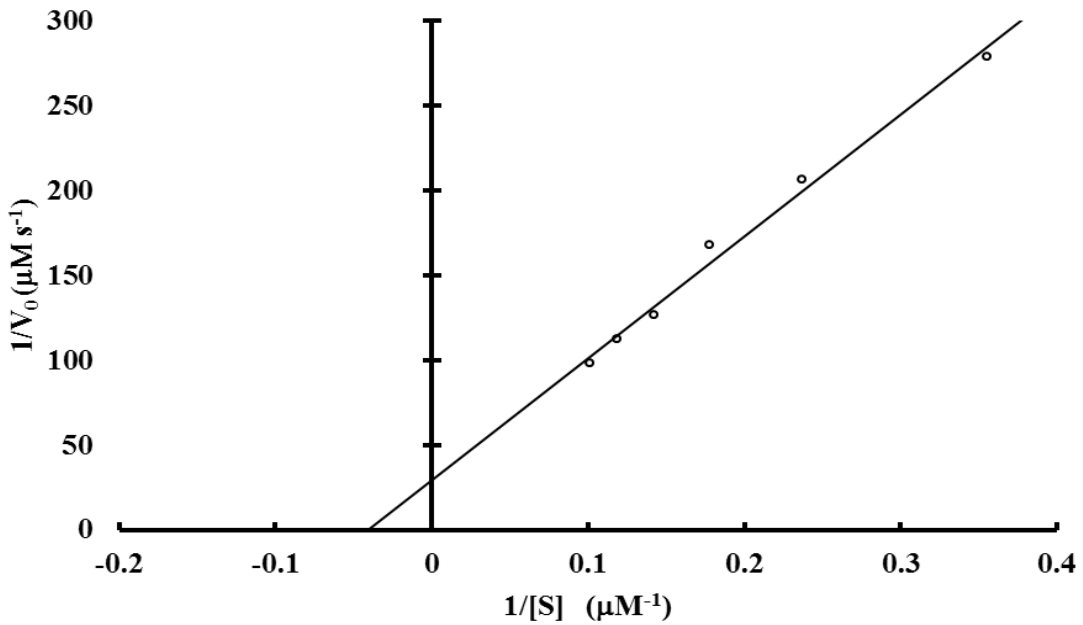

Figure 7. Lineweaver-Burk plot of LvIDI
The concentrations of [1-^{14}C]IPP were 1.4, 2.8, 4.2, 5.6, 7.0, 8.4 and 9.8 mM.

The multiple alignments of LvIDI and other IDIs showed high similarity in the catalytic regions [27] [28] [29]. Two conserved amino acid residues, cysteine, and glutamic acid, were found in LvIDI and other aligned species [18] [30]. It is known that the tyrosine is important for the stability of IDI [9] [30]. In LvIDI, tyrosine was observed upstream of glutamic acid.

As a result of the analysis utilizing the WoLFPSORT program (http://wolfpsort.org/), it was suggested that LvIDI is localized in the cytosol or peroxisome. Generally, the localization of peroxisomal matrix proteins is regulated by peroxisomal targeting signal (PTS), and two types of PTS have been well known [31]. The PTS1 is located at the extreme C-terminus of the protein, and it has consensus sequence: (S/A/C)(K/H/R)(L/M). The PTS2 resides at the N-terminus of a protein has the consensus sequence: (R/K) (L/V/I)X5(H/Q)(L/A) [32]. The PTS1 was observed in the LvIDI and other IDIs, which are similar to LvIDI (Table 3). The LvIDI gene did not have consensus PST2 motif, however, it is known that PST2 is not necessary for peroxisomes targeting [33]. Thus, we suggest that LvIDI might be cytosolic or peroxisomal protein. Moreover, it is suggested that the isoprenoid compounds that are important for the organism, and the precursor of identical organic matter like anti-cancer, are produced at cytosol or peroxisome of *L. volemus*.

Table 3. C- or N-terminus amino acid residue of IDIs
Consensus sequence of PTS1 and PTS2 were shown by iatric.

Organism	*Sequence of C-terminus*	*Sequence of N-terminus*
L. volemus	*KGLADGSKVVKM*	*SGYDAEQSRL*
X. dendrorhous	*LEDLSDNKVWKM*	*EEYDEEQVRL*
S. pombe	*ASKFQDTLIHRC*	*KDYDEEQLRL*
R. norvegicus	*SPFVDHEKIHRM*	*SNLDEKQVQL*
B. taurus	*NLFVDHEKIHRM*	*DDLDERQVQL*
M. auratus	*SQFVDHEKIHRM*	*SHLDEQQVQL*
M. musculus	*SPFVDHEKIHRL*	*SHLDEKQVQL*
M. fascicularis	*NQFVDHEKIHRM*	*DHLDKQQVQL*
H. sapiens	*NQFVDHEKIYRM*	*HHLDKQQVQL*

CONCLUSION

A novel cDNA encoding IDI was cloned from *L. volemus*. IDI catalyzes the revisable conversion of IPP and DMAPP, which are the essential precursors for isoprenoids. Functionally characterization of LvIDI and phylogenetic analysis of IDIs of other fungi species will be helpful to understanding the biosynthesis mechanism of isoprenoid. It was revealed that LvIDI belongs to the Basidiomycota phylum group in phylogenetic analysis. Recently, a new insight into the evolutionary relationship among subgroups of fungal amylolytic enzymes and fungal evolutionary adaptation to ecological conditions was provided by the investigation of the relationships between phylogenomic analysis and putative starch binding domains [34]. The phylogenetic analysis of IDI genes and putative PTS domains will provide more support for fungal evolutionary adaptation to ecological conditions. Such an

investigation might promote the understanding of ecological conditions for mycorrhizal fungi which are difficult to grow under artificial cultivation conditions. Since *L. volemus* is a kind of mycorrhizal fungus, it is an interesting Basidiomycota species. Therefore, our findings might aid in the cloning and cultivation of *L. volemus* or other mycorrhizal fungi.

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